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REVIEW

Vitamin D deficiency and hepatitis viruses-associated liver diseases: A literature review

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Abstract

The secosteroid hormone vitamin D has, in addition to its effects in bone metabolism also functions in the modulation of immune responses against infectious agents and in inhibiting tumorigenesis. Thus, deficiency of vitamin D is associated with several malignancies, but also with a plethora of infectious diseases. Among other communicable diseases, vitamin D deficiency is involved in the pathogenesis of chronic liver diseases caused by hepatitis B and C viruses (HBV, HCV) and high prevalence of vitamin D deficiency with serum levels below 20 mg/mL in patients with HBV and HCV infection are found worldwide. Several studies have assessed the effects of vitamin D supplementation on the sustained virological response (SVR) to interferon (IFN) plus ribavirin (RBV) therapy in HBV and HCV infection. In these studies, inconsistent results were reported. This review addresses general aspects of vitamin D deficiency and, in particular, the significance of vitamin D hypovitaminosis in the outcome of HBV- and HCV-related chronic liver diseases. Furthermore,

current literature was reviewed in order to understand the effects of vitamin D supplementation in combination with IFN-based therapy on the virological response in HBV and HCV infected patients.

Key words: Vitamin D; Vitamin D deficiency; Chronic liver disease; Hepatitis B virus infection; Hepatitis C virus infection; Liver cirrhosis; Hepatocellular carcinoma; Sustained virological response; Vitamin D supplementation

Core tip: Vitamin D deficiency is common and associated with chronic liver diseases. Several studies have ascribed a strong association of vitamin D insufficiency with unfavorable clinical courses and progression of liver disease in hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. However, any causal relation is so far not fully understood. In addition, there are inconsistent results with regard to the impact of vitamin D supplementation on the virological response to IFN-based therapy; this applies particularly to HCV infections. The present review addresses general aspects of vitamin D deficiency and focuses on its association with HBV and HCV infection. Furthermore, the effects of vitamin D supplementation in combination with IFN-based therapy on the virological response in HBV and HCV infected patients are reviewed.

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INTRODUCTION

Vitamin D deficiency affects almost one billion people globally[1]. Further to its crucial role in bone metabolism by supporting enteric absorption of calcium, magnesium, phosphate, iron and zinc, vitamin D has important non-skeletal effects which are involved in many biological processes. In addition to insufficient sun exposure, seasonality, place of residence, diet and the extent of skin pigmentation, which all affect vitamin D bioavailability, hepatitis B and C, the major causes of liver cirrhosis (LC) and hepatocellular carcinoma (HCC), may also contribute to vitamin D deficiency. Low vitamin D serum levels are associated with many human diseases[2,3] and frequently observed in chronic liver diseases; vitamin D constraints contribute to disease progression in chronic hepatitis B[4,5], chronic hepatitis C[6,7], but also to non-alcoholic fatty liver disease (NAFLD)[8-10]. Protective properties of vitamin D in preventing HBV and HCV replication and in retarding clinical progression of HBV/HCV-related liver diseases have been reported[11-14].

The prevalence of vitamin D insufficiency in patients with HBV and HCV infection covers the broad range from 16% up to 100%[5,15,16]. Several studies have demonstrated a strong association between vitamin D insufficiency and the clinical outcome and disease progression of HBV and HCV infections. This applies in particular to the onset of LC. However, the causal relation and applying pathophysiological mechanisms are not fully understood. Although increasing numbers of studies describe the influence of vitamin D deficiency on either the outcome of HBV/HCV-related liver disease or on the virological response to interferon (IFN)/ribavirin (RBV) treatment, the findings are still inconsistent[6,17-23] (Ref. 19: ClinicalTrials.gov; identifier NCT01277601). Conflicting observations and conclusions apply also to several randomized clinical trials in which the effects of vitamin D supplementation were evaluated[24-29].

Here, we address general aspects of vitamin D deficiency and, in particular, focus on its association with HBV and HCV-related chronic liver disease. We also review the effects of vitamin D supplementation in combination with IFN-based therapy on the virological response in HBV and HCV infected patients.

LITERATURE SEARCH

A systematic literature search was conducted using PubMed, MEDLINE and ClinicalTrials.gov (identifiers given where applicable). Search terms used in various combinations were "vitamin D", "vitamin D deficiency", "hepatitis B virus infection", "hepatitis C virus infection", "chronic liver disease", "liver cirrhosis" and "hepatocellular carcinoma". We did not restrict the search to a certain period of time. Thus, articles written in English and published in peer-reviewed journals describing associations of vitamin D deficiency with clinical outcomes or the effects of vitamin D in combination with IFN-based therapy on the virological response in HBV and HCV infected patients were included. Abstracts, letters and posters presented in conferences were not considered.

VITAMIN D: METABOLISM AND FUNCTION

Vitamin D was first identified as a prohormone early in the 20th century. It is a fat-soluble secosteroid and regulates skeletal and non-skeletal functions[30]. Adequate vitamin D levels are required for bone growth and remodeling of osseous structures by osteoblasts and osteoclasts, thus protecting from osteoporosis[30,31]. Vitamin D promotes the absorption of calcium, magnesium, phosphate, iron and zinc from the gut and maintains essential serum calcium and phosphate concentrations to warrant normal bone mineralization and to prevent hypocalcaemia.

Since the discovery of the vitamin D receptor (VDR)

the non-skeletal functions of vitamin D have gained attention. VDR is a member of the nuclear receptor family of transcription factors and is expressed on more than 35 types of solid tissues[32], but also on macrophages as well as on T and B cells[33,34]. Vitamin D is involved in physiological processes through VDR activation, including the regulation of immune responses, cell growth and cell differentiation[35,36]. Therefore, vitamin D is considered a powerful modulator of pathophysiological mechanisms in several infectious diseases, cancers and metabolic disorders[6,37-39].

Vitamin D occurs as vitamin D3 (25(OH)D_3; cholecalciferol) and vitamin D2 (25(OH)D2; ergocalciferol). More than 90% of vitamin D3, the prevailing form of vitamin D, are produced in the skin by means of sunlight exposure, while the remainder is retrieved from dietary components[3,30]. Vitamin D2 does not depend on sunlight and only minute amounts of vitamin D2 are derived from plants[40]. Both vitamin D3 and D2 are inert. To become biologically active they need to be sequentially converted to their intermediate metabolite [calcidiol, 25(OH)D] and the final active form [calcitriol, 1,25$(OH)_2$D] by hydroxylation in the liver and the kidney[1]. Hydroxylation of vitamin D is a process that introduces a hydroxyl group (-OH) into vitamin D2 and D3 in the liver to form 25-hydroxyvitamin D [25(OH)D]. The metabolites are further hydroxylated in the kidney to produce the active form calcitriol. The active form circulates as a hormone in the blood stream to regulate the concentrations of calcium and phosphate and to promote healthy growth and remodeling of bones[41].

Precise quantification of calcitriol is problematic due to its short half-life and the serum concentrations that are 1000 times less compared to those of 25(OH)D. In contrast, 25(OH)D has a half-life of approximately three weeks, making it an appropriate and largely reliable indicator of the individual vitamin D status[1,42].

An appropriate duration of exposure to ultraviolet B (UVB) radiation is crucial in cutaneous vitamin D production[1,43], and a strong correlation exists between vitamin D serum levels, UVB exposure and geographical residence[1,44,45]. As latitudes increase, disposable amounts of vitamin D decrease[44]. At latitudes > 37°N and < 37°S, sunlight does not sufficiently induce vitamin D synthesis in the skin, in particular during the winter months[46]. Latitude and UVB exposure are, however, not exclusive indicators of vitamin D deficiency. Other factors are age, nutritional components and skin pigmentation as well as certain chronic pathological conditions[1,43,45].

VITAMIN D DEFICIENCY

A standard definition of vitamin D deficiency does not exist. Formerly, the vitamin D status was assessed empirically, *e.g.*, through overt diagnoses of childhood rickets and osteomalacia in adults[47,48]. Today, the recognition of deficiency relies on quantification of vitamin D serum levels, representing the current supply rather than functional activity and, thus, not sufficiently supporting a standard definition of vitamin D deficiency.

Serum 25(OH)D levels are inversely correlated with parathyroid hormone (PTH) levels. Low levels of vitamin D stimulate PTH production and, consequently, PTH may be considered a surrogate marker in the diagnosis of vitamin D deficiency. However, high vitamin D levels do not always lead to decreased PTH levels. If vitamin D values are above approximately 30 ng/mL, serum PTH levels will be at a low steady level[49,50]. Thus, current and widely accepted definitions of vitamin D levels include deficiency (< 20 ng/mL), insufficiency (20-30 ng/mL), and sufficiency (> 30 ng/mL)[1].

Vitamin D deficiency is associated with a wide spectrum of diseases including not only bone disorders, but also several autoimmune and infectious diseases, asthma and malignancies as well as psychiatric conditions[1,51,52]. Vitamin D inadequacy involves both deficiency and insufficiency and constitutes an underestimated health factor in many populations[53]. In developed countries, vitamin D deficiency is very common, with almost half of the population affected[1]. Moreover, global assessment of the vitamin D status in postmenopausal women with osteoporosis showed that 24% had severe deficiency (< 10 ng/mL), with the highest prevalences reported in central and southern Europe[42]. A similar trend was reported in a cross-sectional, observational study conducted at 61 sites across the United States, indicating that 52% and 18% among 1536 postmenopausal women receiving osteoporosis treatment had 25(OH)D levels of less than 30 ng/mL and 20 ng/mL[49], respectively.

Vitamin D deficiency is common in western and northern countries, but also in Africa and Asia[4,54-58]. Serum levels in Asian populations were assessed in three large cross-sectional studies in China (n = 3262)[56], South Korea (n = 6925)[55], and in Thailand (n = 2641)[54]. These studies defined deficiency as levels of < 20 ng/mL and indicated highest prevalences of deficiency in China (69%) and in South Korea (males 47%; females 65%)[55]. In contrast, a significantly lower prevalence of deficiency of 6% only was observed in Thailand[54]. This results most likely from its geographical location close to the equator. In Vietnam, recent studies with, however, smaller sample sizes found that vitamin D deficiency prevalences range from 16 to 63%[4,59,60].

Vitamin D deficiency in African populations may be attributed to the skin pigmentation, traditional full-length clothing, and the occurrence of infectious diseases (tuberculosis, HIV/AIDS, malaria) which are associated with deficiency[61-65]. A cross-sectional analysis of adults in a National Health and Nutrition Examination Survey (n = 8415) conducted in the United States reported that vitamin D insufficiency among African Americans was as high as 81%, but only 28% in individuals of European descent[66]. Other studies also consistently indicate that vitamin D deficiency is more prevalent in immigrants from Africa to the United States and to Europe[67,68]. These reports underline that skin pigmentation is an

important factor in reducing vitamin D production.

Sub-Saharan Africa and several parts of Asia bear a heavy burden of communicable diseases, which may affect the vitamin D status. Several studies investigated the causal effect of vitamin D deficiency on the severity and progression of infectious diseases, in particular of tuberculosis[69-71] and respiratory tract infections[72-74]. Recently, vitamin D deficiency has also been implicated in susceptibility to viral hepatitis and the severity and progression of viral hepatitis-associated chronic liver diseases[4,12,75-77].

VITAMIN D DEFICIENCY IN CHRONIC HEPATITIS B AND C

Whether low vitamin D levels are the cause or the result of certain diseases, including chronic viral liver diseases, is not clear. Based on 290 prospective and intervention studies, a systematic review has recently concluded that vitamin D deficiency might be a result and a biological marker of deteriorating health, driving 25(OH)D to low concentrations, rather than a cause of disease[2]. Vitamin D deficiency may contribute to liver damage through increased inflammation and fibrosis[6,39]. Other studies have shown that vitamin D deficiency is clearly associated with unfavorable clinical outcomes and accelerated progression of chronic liver diseases due to viral hepatitis, alcohol consumption and NAFLD[4,8,10,12,60,78-82]. Although vitamin D is associated with NAFLD, a recent study showed that vitamin D insufficiency was not associated with the presence of NAFLD[83]. Relationship between vitamin D deficiency and the pathogenesis of NAFLD has been systematically reviewed[10], and that vitamin D could be used as a supplement in the management of NAFLD. However, clinical trials concluded that vitamin D supplementation has a less impact on the NAFLD pathogenesis such as hepatic fat, injury, and hepatic steatosis[84,85]. Notably, vitamin D deficiency may also contribute to reduced antiviral responses in IFN/RBV treatment of hepatitis B and C[6,19,28,86]. Comparable studies with regard to more recent treatment regimens such as IFN-free and direct-acting antiviral agents are not available so far.

Worldwide, approximately 257 and 130-150 million people are affected by chronic hepatitis B and C, respectively, making it a significant cause of viral infection-related fatality[87,88]. A high prevalence of vitamin D deficiency occurs in almost all chronic liver diseases and their progression, irrespective of their etiology[9,19,78]. Based on results of studies on vitamin D insufficiency and deficiency in chronic hepatitis B and C, serum vitamin D levels of < 20 ng/mL range from 16%-100%[5,15,16] (Tables 1 and 2). Although high prevalences of vitamin D insufficiency/deficiency are observed both in healthy populations and in patients with viral hepatitis, significantly higher rates of deficiency were found in hepatitis patients compared to controls in several studies[4,6,80].

Vitamin D deficiency and chronic hepatitis B

So far, most studies on associations of HBV-related liver diseases with vitamin D deficiency were cross-sectional studies (Table 1). In such study designs, any fluctuation of vitamin D levels over the course of HBV infection cannot be assessed and a causative association of vitamin D levels with HBV-related liver diseases cannot reliably be established.

Vitamin D is significantly associated with virus replication in chronic HBV infection. Recently, several studies have shown that insufficient vitamin D levels most likely fail to suppress HBV replication and contribute to poor clinical courses[4,11,12,89]. Vitamin D levels are positively correlated with albumin levels and platelet counts and, inversely, with ALT levels during the active phase of hepatitis B[19,39,78]. Serum levels of < 10 ng/mL can be predictive for low serum albumin levels and the severity of chronic liver disease[22,75]. However, other studies have reported that vitamin D insufficiency was not correlated with liver function parameters, possibly due to the fact that vitamin D levels depend also on the composition of study cohorts and the study designs[4,11]. Liver disease progression in patients with chronic hepatitis B appears also to be influenced crucially by distinct viral factors, in particular by the infecting HBV genotypes. Genotypes C and B are the major causes of chronic hepatitis B and subsequent LC and HCC in East Asia[90-92]. Recent studies indicate that patients infected with genotype B had a higher prevalence of vitamin D insufficiency than those infected by the C genotype[23,93].

To the best of our knowledge, there are only two studies which have investigated the association of baseline vitamin D levels with sustained virological response (SVR) to nucleoside/nucleotide analogues (NUC) or IFNα in addition to treatment with NUC in chronic hepatitis B. It was shown that the baseline levels (cutoff value: 30 ng/mL) can predict the virological response at week 104 (67% in the insufficiency group *vs* 82% in the sufficient group, $P < 0.001$) in patients with chronic hepatitis B treated with NUC[29]. Chan *et al*[19], however, concluded, inconsistent with the findings given in Ref. 29, that baseline vitamin D levels are not associated with more favorable treatment outcomes in patients treated with either tenofovir disoproxil fumarate (TDF) plus Peg-IFNα or TDF or Peg-IFNα monotherapy[19]. Further prospective studies assessing associations of baseline vitamin D levels and treatment outcomes in chronic hepatitis B, particularly in the IFNα-based therapy, are worth to be conducted.

Association of vitamin D deficiency with SVR to antiviral therapy in chronic hepatitis C patients

In several studies the role of the vitamin D status as well as the effects of vitamin D supplementation on the efficacy of IFNα plus RBV in the treatment of chronic hepatitis C have been investigated (Table 2). Most studies showed high prevalences of vitamin D deficiency and significant associations of low baseline

Table 1 Representative studies on vitamin D deficiency in chronic hepatitis B virus patients

Study population Diagnosis Sample size (*n*)	Study design	Length of follow-up	Vitamin D cutoff (ng/mL)	(%)	Main results	Ref.
China CHB (*n* = 560)	Multicenter, randomized, controlled	104 wk from initiation of antiviral treatment	< 20: deficiency < 30: insufficiency ⩾ 30: sufficiency	21 55 24	Vitamin D insufficiency highly prevalent in treatment-naïve patients with chronic hepatitis B Baseline levels predict virologic response at week 104 after treatment initiation (67% in the insufficiency group *vs* 82% in the sufficient group)	Yu *et al*[29], 2017
China CHB (*n* = 133)	Cross-sectional	NA	< 14: severe deficiency ⩾ 14: deficiency < 30 sufficiency	27 66 7	Vitamin D deficiency significantly associated with HBV genotype B	Zhu *et al*[93], 2016
Vietnam CHB (*n* = 165) LC (*n* = 127) HCC (*n* = 108)	Cross-sectional	NA	< 10: severe deficiency < 20: deficiency < 30: insufficiency ⩾ 30: sufficiency	10.4 41.5 32.4 15.7	Vitamin D insufficiency frequent among HBV patients Reduced vitamin D levels significantly associated with clinical progression of LC Vitamin D levels and HBV DNA loads strongly and inversely correlated	Hoan *et al*[4], 2016
China CHB (*n* = 115) LC (*n* = 115) HC (*n* = 115)	Cross-sectional	NA	< 10: deficiency < 20: insufficiency ⩾ 20: sufficiency	83 17 0	Vitamin D levels significantly lower in LC compared to CHB and HC groups ($P < 0.001$) Child-Pugh score independently associated with vitamin D deficiency (cutoff < 10 ng/mL)	Zhao *et al*[5], 2016
South Korea CHB (*n* = 110) Other CLD (*n* = 97)	Cross-sectional	NA	< 10: deficiency < 20: insufficiency ⩾ 20: sufficiency	34.8 45.4 19.8	Vitamin D deficiency independently associated with advanced liver fibrosis	Ko *et al*[154], 2016
Iran CHB (*n* = 84)	Cross-sectional	NA	< 10: deficiency < 20: insufficiency ⩾ 20: sufficiency	17.9 34.5 47.6	No significant association of vitamin D levels in treated and treatment-naïve patients	Sali *et al*[155], 2016
Multicenter in Europe, Asia and North America CHB (*n* = 737)	Randomized, open-label, active-controlled clinical trial	48 wk of TDF + PegIFN 16 wk of TDF + PegIFN followed by 32 wk of TDF 48 wk PegIFN or 120 wk of TDF	< 20: deficiency < 30: insufficiency ⩾ 30: sufficiency	58 35 7	Reduced vitamin D levels highly prevalent among untreated CHB patients Low baseline levels of vitamin D associated with high HBV DNA loads, abnormal ALT at week 48 independent of treatment groups Baseline vitamin D levels not associated with treatment outcomes	Chan *et al*[19], 2015
China CHB (*n* = 426)	Cross-sectional	NA	< 32 insufficiency ⩾ 32 sufficiency	82 18	Vitamin D deficiency common among patients with CHB and associated with adverse clinical outcomes	Wong *et al*[78], 2015
China CHB (*n* = 242)	Cross-sectional	NA	< 20: deficiency < 30: insufficiency ⩾ 30: sufficiency	8.7 31.4 60	Higher prevalence of vitamin D insufficiency in HBV genotype B patients than in genotype C patients Vitamin D levels not associated with HBV DNA levels or the stage of fibrosis in CHB patients	Yu *et al*[23], 2015
China CHB (*n* = 133)	Cross-sectional	NA	< 14: deficiency < 30: insufficiency ⩾ 30: sufficiency	27 66.2 6.8	Higher prevalence of vitamin D insufficiency in HBV genotype B patients than in genotype C patients Vitamin D levels not associated with other clinical parameters	Zhu *et al*[93], 2016
Germany CHB (*n* = 203)	Cross-sectional	NA	< 10: deficiency < 20: insufficiency ⩾ 20: sufficiency	34 47 19	HBV DNA viral loads as strong predictor of low vitamin D levels in CHB patients	Farnik *et al*[12], 2013

Egypt OBI (n = 16) CHB (n = 52)	Cross-sectional	NA	< 10: deficiency < 30: insufficiency ⩾ 30: sufficiency	OBI: 12.5 CHB: 40.4 OBI: 62.5 CHB: 59.6 OBI: 25 CHB: 0	Vitamin D levels significantly higher in OBI than in CHB patients Serum level of vitamin D inversely correlated with HBV DNA loads	Mashaly *et al*[156], 2016
China CHB (n = 128)	Cross-sectional	NA	< 10: deficiency < 20: insufficiency ⩾ 20: sufficiency	13.3 61.7 25	Vitamin D levels negatively correlated with HBV DNA loads Effective antiviral therapy might increase the level of vitamin D in CHB patients	Chen *et al*[11], 2015
Iran CHB (n = 173)	Cross-sectional	NA	< 10: deficiency < 20: insufficiency ⩾ 20: sufficiency	58 39 3	Vitamin D levels inversely correlated with HBV DNA levels	Mohamadkhani *et al*[89], 2015

CHB: Chronic hepatitis B; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; CLD: Chronic liver disease; OBI: Occult hepatitis B infection; NA: Not applicable.

levels of 25(OH)D at the time of antiviral therapy initiation and lower odds of achieving SVR, which is defined as undetectable serum HCV RNA level at 24 wk after cessation of treatment. However, other studies reported rather inconsistent and partly contradicting results[7,16,20,94], possibly due to heterogeneity in patient inclusion criteria (HCV infection or HIV/HCV coinfection, ethnicity) and characteristics of vitamin D assessment (seasonality, cutoff values, laboratory methods)[86].

To date, five meta-analyses have described an association of baseline vitamin D levels with SVR[28,86,95-97] (Table 2). One study showed a significant association of SVR with vitamin D deficiency. Low odds of achieving SVR rates were found in patients with vitamin D levels of < 20 ng/mL compared to patients with levels of ⩾ 20 ng/mL (OR = 0.5, 95%CI: 0.3-0.9)[86]. A similar result was found in another study, which reported high rates of SVR in HCV patients with vitamin D levels of ⩾ 30 ng/mL (OR = 1.6; 95%CI: 1.1-2.2) and in patients supplemented with vitamin D (OR = 4.6; 95%CI: 1.7-12.6), regardless of viral genotypes[28]. In contrast, Kitson *et al*[95] reported that baseline 25(OH)D levels were not associated with SVR in Peg-IFN/RBV treatment, also regardless of the viral genotype involved[95]. The main differences in these meta-analyses are the study designs and patient selection strategies, as, for instance, studies involving patients with HCV/HIV coinfections were excluded in the third meta-analysis, but included in the other meta-analyses.

When looking at the effect of vitamin D supplementation as an adjuvant to IFNα/RBV therapy for treatment of chronic HCV infections, some evidence indicates that vitamin D supplementation improves the SVR (Table 2). SVR rates in patients supplemented with vitamin D depend on the infecting HCV genotypes, and range from 54%-86% for HCV genotype 1 (18.5% and 42% in the non-supplemented control groups)[17,98] up to 95% for HCV genotype 2 and 3 infections (77% in the non-supplemented control group)[21]. A meta-analysis including eleven studies reported high odds of SVR (OR = 4.6, 95%CI: 1.7-12.6) in vitamin D supplemented groups compared to non-supplemented patient groups, regardless of genotypes[28]. A retrospective study in Italy has assessed the effect of supportive vitamin D treatment in combination with antiviral therapy (IFNα plus RBV) in recurrent HCV infections of patients who had undergone liver transplantation. Vitamin D supplementation could increase SVR rates significantly[98]. In contrast, other studies showed inconsistent results for the HCV genotypes 4 and 1[15]. Randomized prospective studies with small sample sizes and lacking a placebo-controlled arm challenge the application of vitamin D as an adjuvant substance in order to enhance SVR[15,17,21,26]. Although vitamin D may be relevant in the treatment of chronic hepatitis C, further randomized, placebo-armed studies are required in order to confirm whether vitamin D supplementation in fact improves the SVR in combination with IFN in HCV infections.

VITAMIN D AND VIRAL HEPATITIS-RELATED LIVER CIRRHOSIS

In an assessment of liver cirrhosis (LC) mortality in 187 countries during the period from 1980 to 2010, global fatalities increased from approximately 676000 in 1980 to more than 1 million in 2010, accounting for approximately 2% of all causes of death[99]. There is growing evidence that vitamin D deficiency is associated with progression of LC caused by various etiologies, mainly by HBV and HCV infection, but also by alcoholic and NAFLD[4,10,39,75,100-104]. Vitamin D deficiency reflects also hepatic dysfunction and is associated with mortality in patients with LC, regardless of underlying causes[102,104].

The association of vitamin D with LC has been more intensively discussed in chronic hepatitis C and in NAFLD patients, rather than in chronic hepatitis B. A recent meta-analysis included seven studies in order to assess vitamin D serum levels and advanced liver

Table 2 Representative studies regarding vitamin D status in chronic hepatitis C virus patients

Study population Diagnosis Sample size (*n*) Study objective	Study design	Length of follow-up	Vitamin D cutoff (ng/mL)	(%)	Main results	Ref.
Italy CHC (*n* = 197) HCV genotype 1 controls (*n* = 49)	Cross-sectional	NA	< 30: deficiency ⩾ 30: sufficiency	73 27	Low vitamin D linked to severe fibrosis and low SVR in IFN-based treatment	Petta *et al*[6], 2010
United States CHC (*n* = 218) LC (*n* = 123) Non-LC (*n* = 95)	Prospective	12 wk after cessation of antiviral therapy SVR12: defined as a viral load undetectable or below the level of detection at week 12 after cessation of antiviral treatment	< 20: deficiency < 30: insufficiency ⩾ 30: sufficiency	43 33 24	Vitamin D deficiency associated with HCV–related LC and with hepatic function No significant association between SVR12 and serum vitamin D levels at baseline	Backstedt *et al*[18], 2017
Switzerland CHC (*n* = 269) HCV genotypes 1-4	Case-control	NA	< 30: deficiency ⩾ 30: sufficiency	74 26	No significant association between SVR and serum vitamin D levels irrespective of genotypes	Lange *et al*[20], 2012
Spain CHC genotypes 1-4 (*n* = 182)	Cross-sectional	NA	< 20: deficiency < 30: insufficiency ⩾ 30: sufficiency	36 41 23	Vitamin D deficiency not related to biochemical and virological variables or to the stage of fibrosis stage	Ladero *et al*[157], 2013
Northern Italy CHC (*n* = 211) HCV genotypes 1-5	Prospective	24 wk after cessation of antiviral therapy SVR24: defined as a viral load undetectable or below the level of detection at week 24 after cessation of antiviral treatment	< 20: deficiency ⩾ 20: sufficiency	46.4 53.6	SVR24 rates to IFNα therapy were 50%, 61%, and 69% in CHC patients with baseline vitamin D levels of ⩽ 10 ng/mL, 10-20 ng/mL, and > 20 ng/mL, respectively	Bitetto *et al*[25], 2011
Multicenter study, United States Cases (histological progression or clinical decompensation; (*n* = 129), controls (*n* = 129)	Nested case-control study	Over 4 yr	At baseline: cases: 44.8 controls, 44.0	Not stated	No difference in vitamin D levels in patients with and without progression of HCV-associated liver disease	Corey *et al*[158], 2012
Multicenter study, Japan CHC (*n* = 247) HCV genotype 1b	Case-control	NA	< 20: deficiency < 30: insufficiency ⩾ 30: sufficiency At baseline: 22(6–64)	Not stated	NS5A Y93H and L31M resistance-associated variants associated with vitamin D deficiency	Okubo *et al*[159], 2016
Multicenter study, France HCV-HIV coinfection (*n* = 189)	Cross-sectional	NA	< 30: deficiency ⩾ 30: sufficiency	85 15	Low serum vitamin D levels correlated with liver fibrosis as assessed by FibroTest No association between SVR rate to IFN-based therapy and baseline vitamin D levels	Terrier *et al*[7], 2011
Japan CHC (*n* = 619)	Cross-sectional	NA	< 20: deficiency < 30: insufficiency ⩾ 30: sufficiency	47 36.7 16.3	Vitamin D levels influenced by gender, age, hemoglobin level, albumin and seasonality	Atsukawa *et al*[160], 2015
Egypt CHC (*n* = 70) controls (*n* = 50)	Cross-sectional	NA	At baseline Cases: 18.6 Control: 56	NA	Vitamin D decreased in HCV patients	Reda *et al*.[161], 2015
Australia CHC (*n* = 274) HCV genotype 1	Case-control	NA	< 20: deficiency < 30: insufficiency ⩾ 30: sufficiency	16 48 36	Baseline vitamin D levels not associated with SVR or fibrosis stage in HCV genotype 1 but deficiency associated with high activity	Kitson *et al*[16], 2013
Japan CHC (*n* = 177)	Prospective	24 wk after cessation of antiviral therapy	Not stated	NA	SVR24 rates: 65% in patients with vitamin D levels > 18 ng/mL *vs* 38.5% in patients with vitamin D levels of < 18 ng/mL	Atsukawa *et al*[162], 2014

Egypt CHC (n = 101) HCV genotype 4 Vitamin D supplementation group (n = 50), controls (n = 51)	Randomized prospective	Until 72 wk from start of antiviral therapy SVR was assessed at week 72 from initiation of antiviral treatment	< 20: deficiency ⩾ 20: insufficiency ⩾ 30: sufficiency	95 5 0	No impact of vitamin D supplementation on SVR in HCV genotype 4 patients No correlation between vitamin D levels and stage of liver fibrosis	Esmat *et al*[15], 2015
Israel CHC (n = 72) HCV genotype 1 Vitamin D supplementation group (n = 36), controls (n = 36)	Randomized prospective	24 wk after cessation of antiviral treatment	< 10: severe deficiency < 20: insufficiency ⩾ 20: sufficiency	21 59 20	Addition of vitamin D to Peg-IFNα/RBV therapy improves SVR24 (86% *vs* 42%)	Abu-Mouch *et al*[17], 2011
Israel CHC (n = 50) HCV genotype 2 and 3 Vitamin D supplementation group (n = 20), controls (n = 30)	Randomized prospective	24 wk after cessation of antiviral treatment	< 12: deficiency < 32: insufficiency ⩾ 32: sufficiency	26 54 20	Addition of vitamin D to IFNα/RBV therapy improves SVR24 (95% in treated group *vs* 77% in controls)	Nimer *et al*[21], 2012
France CHC (n = 516) HCV genotype 1	Randomized controlled	Until 72 wk from initiation of antiviral therapy	Not stated	Not stated	No impact of vitamin D levels on efficacy of antiviral therapy in naïve genotype 1 HCV patients	Belle *et al*[24], 2017
Egypt CHC (n = 66) Vitamin D group (n = 20) controls (n = 30)	Randomized prospective	24 wk after cessation of antiviral treatment	< 12: deficiency < 32: insufficiency ⩾ 32: sufficiency	33.3 43.3 23.4	Addition of vitamin D to conventional Peg-IFNα/RBV therapy improved SVR24	Eltayeb *et al*[26], 2015
Germany CHC (n = 468) HCV genotypes 1-3	Retrospective	24 wk after cessation of antiviral treatment	< 30: deficiency ⩾ 30: sufficiency	66 34	Vitamin D deficiency correlated with SVR in HCV genotype 2 and 3 patients (50% *vs* 81%: SVR24 for patients with and without severe vitamin D deficiency)	Lange *et al*[80], 2011
Taiwan CHC (n = 132) HCV genotype 1-2	Retrospective	SVR was assessed at week 48 (HCV genotype 1) and at week 24 (HCV genotype 2) from initiation of antiviral treatment	Not stated	Not stated	Vitamin D can suppress HCV replication in hepatic cell lines Vitamin D serum levels associated with both SRV and RVR to Peg-IFNα based therapy	Jee-Fu *et al*[13], 2017
Germany CHC (n = 398) HCV genotype 1	Retrospective	SVR was assessed at week 24 from initiation of antiviral treatment	At baseline 18.7 (3-84.3)	Not stated	Addition of vitamin D to Peg-IFNα/RBV therapy for treatment-naïve patients with chronic HCV genotype 1: no significant association with SVR	Grammatikos *et al*[94], 2014
Austria HCV-HIV coinfection (n = 65)	Retrospective	24 wk after cessation of antiviral treatment	< 10: deficiency < 30: insufficiency ⩾ 30: sufficiency	57 23 20	Low vitamin D levels may impair virological response to Peg-IFNα/RBV therapy, especially in difficult-to-treat patients	Mandorfer *et al*[163], 2013
Italy CHC (n = 42) Vitamin D supplementation group (n = 15) controls (n = 27)	Retrospective	SVR was assessed at week 48 from initiation of antiviral treatment	< 10: severe deficiency < 20: insufficiency ⩾ 20: sufficiency	Not stated	Vitamin D supplementation improves SVR rate following Peg-IFNα/RBV therapy (54% in vitamin D group *vs* 18.5% in control group)	Bitetto *et al*[98], 2011a
Multicenter study, United States CHC (n = 1292) HCV genotype 1	Retrospective	24 wk after cessation of antiviral treatment	< 12: severe deficiency < 20: insufficiency ⩾ 20: sufficiency	19 48 33	Higher vitamin D levels not associated with SVR in Peg-IFNα/RBV therapy	Loftfield *et al*[27], 2016
Meta-analysis To assess vitamin D levels related to ALF and/or SVR (n = 3755) (11 studies for SVR, 7 studies for ALF)	Meta-analysis	NA	< 10: severe deficiency < 20: deficiency < 30: insufficiency ⩾ 30: sufficiency	Not stated	Low vitamin D levels related to ALF Low vitamin D levels at baseline in CHC patients were associated with a higher likelihood of having ALF and lower odds of achieving SVR	Garcia-Alvarez *et al*[86], 2014

Meta-analysis To clarify any association between baseline vitamin D levels and SVR (n = 2605) (11 studies)	Meta-analysis	NA	Not stated	NA	Baseline vitamin D levels not associated with SVR in Peg-IFNα/RBV therapy, regardless of genotype Effect of vitamin D supplementation on SVR to be determined	Kitson *et al*[95], 2014
Meta-analysis To assess the association of vitamin D levels with the severity of liver fibrosis in CHC (n = 8321) (6 studies)	Meta-analysis	NA	Not stated	NA	Lower serum vitamin D is a risk factor for severity of liver fibrosis in chronic HCV patients.	Luo *et al*[97], 2014
Meta-analysis To evaluate the association between vitamin D levels and SVR in CHC (n = 1575) (8 observational and 3 interventional studies)	Meta-analysis	NA	At baseline 17-43 ng/mL	NA	High SVR rates observed in patients with vitamin D levels > 30 ng/mL High SVR rates observed in CHC patients supplemented with vitamin D, regardless of genotype	Villar *et al*[28], 2013
Meta-analysis To access the association between vitamin D supplementation and SVR rate to PEG-IFN/RBV in CHC (n = 548) (7 studies)	Meta-analysis	NA	NA	NA	Vitamin D supplementation significantly increased SVR rates to Peg-IFNα/RBV at 24 wk	Kim *et al*[96], 2017

CHC: Chronic hepatitis C; LC: Liver cirrhosis; ALF: Acute liver fairlure; IFNα: Interferon alpha; RBV: Ribavirin; Peg-IFN: Pegylated interferon; SVR: Sustained virolagical response; RVR: Rapid virological response; NA: Not applicable.

fibrosis in patients with chronic hepatitis C. Low vitamin D levels were related to advanced fibrosis, with two cutoff values set of either 10 ng/mL (OR = 2.5, 95%CI: 1.2-4.7) or 30 ng/mL (OR = 2.2, 95%CI: 1.2-4.0)[86]. With regard to chronic hepatitis B, two studies have recently reported a strong and inverse correlation of serum vitamin D levels with progression of LC[45]. Abnormal vitamin D metabolism in LC was described almost four decades ago[105,106]. It was mainly attributed to impaired hydroxylation resulting from impaired liver function[100]. In LC patients, vitamin D deficiency is also caused by dietary lacks, malabsorption and decreased hepatic production of vitamin D binding protein[75,107,108].

Vitamin D is involved in inhibition of inflammation and liver fibrosis, substantiated by the observation that VDR knockout mice spontaneously develop hepatic fibrosis[77,109]. The function of vitamin D in mesenchymal multipotent cells is to decrease expression of collagen and profibrotic factors [transforming growth factor beta 1 (TGFβ1) and serpin family E member 1 (SERPINE1)][110], suggesting vitamin D supplementation as preventive and supportive treatment in LC[110]. Furthermore, vitamin D directly inhibits the proliferation and profibrotic phenotype of hepatic stellate cells and reduces thioacetamide-induced liver fibrosis in an animal model[109]. There are several lines of evidence to support an inverse association of vitamin D levels with liver fibrosis induced by chronic viral hepatitis[4,100,111,112]. More specifically, a high expression of hepatic Toll-like receptors (TLR2 and TLR4) can result in the production of tumor necrosis factor alpha (TNFα) in chronic hepatitis C[113]. This cytokine is shown to modulate fibrosis[114,115]. In this context, vitamin D might elicit an anti-inflammatory mechanism by downregulating the expression of TLR2 and TLR4 molecules. Recent *in-vivo* studies have documented on the reduced production of TNFα by monocytes, macrophages and myeloid dendritic cells treated with vitamin D[116,117]. Corroborating the findings, a yet another study show that circulating vitamin D levels inversely correlate with TLR2 and TLR4 expression[118].

Fibrotic conditions appear to be reversible and even curable[119,120] when interventions are initiated at early stages[121]. Several observations have underlined the importance of vitamin D supplementation in the treatment of chronic liver diseases. However, so far there have been no randomized prospective trials to assess the role of vitamin D supplementation in the treatment of LC.

VITAMIN D DEFICIENCY AND HEPATITIS-RELATED HEPATOCELLULAR CARCINOMA

Both incidences and mortality rates of certain cancers are higher in northern latitudes, where sunlight exposure is rather scarce[122,123]. Sound epidemiologic studies have shown that vitamin D deficiency is associated with an increased risk of colon, breast, prostrate, and ovarian cancers[124-130]. Not much information is, however, available on an association

of serum vitamin D levels with either the risk or the incidence and mortality rates of HCC caused by chronic viral hepatitis.

In a recent cross-sectional study from Vietnam a high prevalence of vitamin D deficiency was observed in HBV-related HCC patients compared to healthy individuals, and vitamin D deficiency was associated with unfavorable courses of the disease[4]. In chronic hepatitis C, distinct single nucleotide polymorphisms in genes related to the vitamin D signaling pathway, including cytochrome P450 family 2 subfamily R member 1 [*CYP2R1,* encoding the liver 25-hydroxylase (rs1993116, rs10741657)], 7-dehydrocholesterol reductase [*DHCR7,* encoding the 7-dehydrocholesterol reductase (rs7944926, rs12785878)] were investigated and an association between the human genotypes and reduced 25(OH)D3 serum levels in the development of HCV-related HCC was observed[131]. Another study indicated that vitamin D might be a potential biomarker for the development of HCC in patients with chronic hepatitis C[132]. In addition, a large prospective cohort study examined the association between serum vitamin D levels and the incidence of liver cancer among 520000 participants in ten European countries[76]. During more than 10 years of follow-up, a total of 204 HCC cases, mostly due to HBV and HCV infection, were identified. Serum levels of 25(OH)D were inversely associated with the risk of HCC. This finding was in agreement with another prospective study showing that lower serum 25(OH)D3 concentrations in 200 HCC patients, also caused largely by HBV and HCV infection, were associated with poor outcomes and end stages of HCC, classified according to the BCLC (Barcelona Clinic Liver Cancer) staging system and the Cancer of the Liver Italian Program (CLIP) score[133]. Overall survival rates of HCC patients with serum 25(OH)D3 levels of ≤ 10 ng/mL were significantly lower than those of patients with serum levels > 10 ng/mL. In addition, the levels were independently associated with the overall survival in a multivariate analysis[133]. Apparently, vitamin D deficiency is associated with tumor progression and a poor prognosis in HCC patients. Although the results suggest this role of vitamin D in HCC, it remains to be determined further whether the association holds and is causal.

VITAMIN D AND ITS ANALOGUES IN HCC PREVENTION

Vitamin D has numerous additional functions in the prevention of cancer due to its antiproliferative, pro-apoptotic, differentiating, antiangiogenic and antiinvasive properties[134-136]. Several *in vitro* and *in vivo* studies have suggested that vitamin D inhibits growth of HCC cell lines and effectively suppresses DNA damage[137-139]. Data from several preclinical studies have assigned an important role of vitamin D in prevention and treatment of certain malignancies[135,140,141]. Furthermore, in a randomized clinical trial (ClinicalTrials.gov; identifier NCT00352170) vitamin D and calcium supplementation have substantially reduced the risk of cancer[142]. These observations have raised increasing awareness of ensuring adequate vitamin D levels in order to reduce the risk of neoplasms.

The vitamin D analogues paricalcitol, doxercalciferol and tacalcitol have meanwhile been approved for application in patients with osteoporosis and psoriasis[143] and analogues of vitamin D receptor activators such as maxacalcitol (OCT), 16-ene analogs, 19-nor analogs, LG190119 have been tested in preclinical studies on diabetes, several cancers (*e.g.*, leukemia, colon, breast, prostate, pancreatic cancer)[144-146]. With regard to HCC, the vitamin D analogue seocalcitol, which has proven effects in animal models of cancer[147-149] has been investigated in patients with inoperable HCC in a phase Ⅱ clinical trial (ClinicalTrials.gov; identifier NCT00051532)[150]. Seocalcitol may be effective in the treatment of HCC, especially in early stages when prolonged treatment can be instituted. In addition, seocalcitol is 50-200 times more effective in inhibiting proliferation and differentiation of human cancer cell lines than natural vitamin D3[151].

In a phase 1 clinical trial the safety of high doses of vitamin D administered in lipiodol and directly injected into the hepatic artery of 8 patients with refractory HCC was evaluated[152]. Lipiodol is an oily substance consisting of polyunsaturated esters enriched in iodine used as a vector for chemoembolization or internal radiotherapy in unresectable HCCs[153]. Although this study was not specifically designed as a pilot study of vitamin D efficacy in HCC, the results showed a certain stabilization of tumor marker levels, suggesting some efficacy of vitamin D[152]. Another clinical trial (ClinicalTrials.gov; identifier NCT01575717) is currently performed to assess the effect of two different doses of vitamin D3 (2000 IU *vs* 4000 IU) on serum 25OHD levels in HCC patients on liver transplant lists. Nevertheless, so far, there are no approved vitamin D analogues available for supportive HCC treatment.

CONCLUSION

Vitamin D deficiency is very common and frequently observed in HBV- and HCV-associated chronic liver diseases. It negatively affects the clinical courses and promotes progression of liver diseases, but causal relations are still not fully understood. Several lines support that sufficient vitamin D levels play an important role during antiviral treatment of HBV and HCV infections. However, the effect of vitamin D supplementation in combination with IFN-RBV based therapy on virological responses is still unclear. Various non-skeletal effects of vitamin D, including antiinflammatory, antifibrotic and antitumor properties have emphasized an association of vitamin D deficiency with unfavorable liver disease outcomes, in particular, liver cirrhosis. There is currently no approved

recommendation for vitamin D supplementation and vitamin D analogues as supportive adjuvant treatment regimes in viral hepatitis and related chronic disorders. Further randomized, placebo-armed studies need to be performed in order to confirm whether supplementation of vitamin D or vitamin D analogues improve SVRs in combination with specific antiviral treatment strategies in HBV or HCV infections.

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ORIGINAL ARTICLE

Basic Study

Diet switch and omega-3 hydroxy-fatty acids display differential hepatoprotective effects in an obesity/nonalcoholic fatty liver disease model in mice

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Author contributions: Rodriguez-Echevarria R and Armendariz-Borunda J designed the study; Armendariz-Borunda J and Macias-Barragan J contributed with data analysis, direction and guidance; Rodriguez-Echevarria R and Parra-Vargas M developed the methodology, collected the data and performed data analysis; Davila-Rodriguez JR, and Amezcua-Galvez E performed alpha-SMA immunohistochemistry; Rodriguez-Echevarria R and Armendariz-Borunda J wrote the manuscript.

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Abstract

AIM

To study the effect of 18-hydroxy-eicosapentaenoic acid (18-HEPE) and 17-hydroxy-docosahexaenoic acid (17-HDHA) in a murine model of obesity/nonalcoholic fatty liver disease.

METHODS

C57BL/6 mice were fed with standard chow diet (CD) or high-fat, fructose-enriched diet (HFD) for 16 wk. Then, three groups were treated for 14 d with either, diet switch (HFD for CD), 18-HEPE, or 17-HDHA. Weight

and fasting glucose were recorded on a weekly basis. Insulin tolerance test was performed at the end of treatment. Histological analysis (HE and Masson's trichrome stain) and determination of serum insulin, glucagon, glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic polypeptide, adiponectin and resistin were carried out as well as liver proteins by western blot.

RESULTS

Mice treated with hydroxy-fatty acids 18-HEPE and 17-HDHA displayed no weight loss or improved insulin sensitivity. However, these mice groups showed a significant amelioration on serum GLP-1, adiponectin and resistin levels. Also, a significant reduction on inflammatory infiltrate was observed at both portal and lobular zones. Furthermore, up-regulation of PPARα/γ protein levels was observed in liver tissue and it was associated with decreased levels of NF-κB also determined by western blot analysis. On the other hand, diet switch regimen resulted in a marked improvement in most parameters including: weight loss, increased insulin sensitivity, decreased steatosis, restored levels of insulin, glucagon, leptin, adiponectin and resistin. However, no significant changes were observed regarding inflammatory infiltrate in this last group.

CONCLUSION

18-HEPE and 17-HDHA differentially exert hepatoprotective effects through up-regulation of nuclear receptors PPARα/γ and amelioration of serum adipokines profile.

Key words: Nonalcoholic fatty liver disease; Polyunsaturated fatty acids; 18-hydroxy-eicosapentaenoic acid; 17-hydroxy-docosahexaenoic acid; Obesity

Core tip: Our study aimed to prove the efficacy of hydroxy-fatty acids 18-hydroxy-eicosapentaenoic acid and 17-hydroxy-docosahexaenoic acid (17-HDHA) in an obesity/nonalcoholic fatty liver disease (NAFLD) model in mice. We determined the effect of these molecules on histological morphology as well as in protein levels of key nuclear receptors and serum hormones, incretins and adipokines as these parameters are altered in NAFLD. We reported an effect by these hydroxy-fatty acids on the most relevant target proteins involved in this pathological process (PPARα/γ). Also, we demonstrated that diet switch regimen is a selective treatment control as most NAFLD markers and histological alterations were ameliorated by this intervention.

Rodriguez-Echevarria R, Macias-Barragan J, Parra-Vargas M, Davila-Rodriguez JR, Amezcua-Galvez E, Armendariz-Borunda J. Diet switch and omega-3 hydroxy-fatty acids display differential hepatoprotective effects in an obesity/nonalcoholic fatty liver disease model in mice. *World J Gastroenterol* 2018; 24(4): 461-474 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i4/461.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i4.461

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has become a major chronic liver condition over the last decades[1]. It comprises a wide range of morphological alterations ranging from simple steatosis to an inflammatory state known as nonalcoholic steatohepatitis. Should the inflammation persist throughout the years, it could potentially lead to advanced established fibrosis and ultimately become a form of end-stage liver disease which includes cirrhosis and hepatocellular carcinoma[2]. Notably, NAFLD shows a high growth rate in the Americas, and it is thought to derive mainly from modern lifestyle habits featuring low physical activity and chronic exposure to high-fat, high-fructose diet[3]. Those mentioned factors have dramatically increased the prevalence of obesity and metabolic syndrome along with its comorbidities: dyslipidemia, insulin resistance, and hypertension[4].

Although the typical fat vesicles in the liver can be originated by *de novo* lipogenesis from an excess of dietary substrates, in the case of NAFLD it is largely the result of a hypertrophied insulin-resistant white adipose tissue. Such event leads to hyperlipidemia in which the released fatty acids reach the liver where they can be esterified and stored within hepatocytes[5]. Remarkably, it has been proposed that NAFLD might be endorsed by a constant vicious cycle operating insulin resistance and progressing fatty liver as both conditions frequently coexist[6]. Furthermore, insulin resistance is primarily triggered by low-grade chronic inflammation. In NASH, just as in many other pathological conditions, persistence of inflammatory cell infiltration (in this case white adipose tissue and liver) is a remarkable feature[7].

Currently, the first line treatment for NAFLD remains weight loss and overall lifestyle modification including physical activity and healthy diet[8]. However, given the complexity of obesity treatment, in many cases it leads to an elevated number of unsuccessful attempts. These facts have prompted a tremendous need for alternative strategies. In this regard, several drugs have been proposed in the clinical scenario over the last years such as pioglitazone, vitamin E, liraglutide, sitagliptine, elafibranor, obeticholic acid, and pentoxifylline just to name a few. Additionally, a large pipeline of preclinical studies are under way[9]. They are generally intended to target major features of NAFLD either separately or combined (lipid accumulation, oxidative stress, inflammation, and fibrosis).

Diet-wise, a low intake of saturated fat and fructose

from soft drinks has been part of NAFLD treatment[3,10]. However, much attention has been paid to the anti-inflammatory and lipid-lowering properties of other types of fats such as ω 3 polyunsaturated fatty acids (ω 3 PUFA), which have long been investigated and showed positive impact on cardiovascular and hepatic alterations as well as in overall health[11-13]. Actions exhibited by these fatty acids became a niche in lipid research in the late 1970s after a study conducted in a Greenland Inuit population[14]. Furthermore, there is a wide family of 3 PUFA-derived compounds mainly produced by enzymatic oxidation routes on eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that are thought to exert more potent actions compared to their non-oxidized versions. In this regard, 18-hydroxy-eicosapentaenoic acid (18-HEPE) and 17-hydroxy-docosahexaenoic acid (17-HDHA) have been reported to possess a high affinity for nuclear receptors such as peroxisome proliferator-activated receptors α and γ (PPARα and PPARγ) which in turn orchestrate key processes on lipid metabolism and inflammation[15,16]. In fact, the effects of 18-HEPE and 17-HDHA as well as other hydroxy-fatty acids have been tested on several metabolic and chronic inflammation models at dosages within the nanomolar range and showed protective effects[17-19]. Finally, we conducted this study with the aim to assess the effect of 18-HEPE and 17-HDHA in an obesity/NAFLD model in C57BL/6 mice.

MATERIALS AND METHODS

Animals and diets

This research protocol was approved by the CUCS Research Committee from Universidad de Guadalajara. Also, it was carried out in accordance with the National Institutes of Health guide for care and use of laboratory animals. five-week old male C57BL/6NHsd mice were purchased from Harlan (Mexico City) and were fed with standard chow for 1 wk to stabilize their metabolism. The animals were group-housed in polycarbonate cages in a moderated environment and temperature at 22 ± 1 ℃ and a 12 h light/dark cycle. Mice were randomly allocated to cages and fed with either standard chow diet (CD) or high-fat, fructose-enriched diet (HFD) ad libitum for 16 wk to induce obesity and NAFLD. CD group received standard diet Prolab RHM 2500 5P14* (12% of calories from fat) and had free access to pure water, whereas HFD group received Testdiet 58V8 diet (45% of calories from fat) and had free access to high fructose-enriched water at a concentration of 42 g/L (ratios at 55% fructose and 45% sucrose). Treatment of the following began on week 17th and it lasted 14 days: intraperitoneal administration of either 18-HEPE or 17-HDHA every 24 h (Cayman Chemicals; CAS 141110-17-0 and CAS 90780-52-2) in 100 μL 0.9% saline with 2% ethanol as vehicle. Additionally, one HFD-fed group underwent diet switch for chow diet and pure water plus vehicle as a third type of control. At the end of the treatment (18th week) mice were euthanized with tiletamine-zolazepam (15 mg/kg body weight), blood was extracted by cardiac puncture and centrifuged for serum separation, whereas liver tissue was collected and kept at -70 ℃ for further molecular analysis and fixed in 4% paraformaldehyde for histological analysis.

Weight, glucose and insulin tolerance test

Animals were weighed on a weekly basis systematically at 9:00-10:00 during the entire protocol. They were five-hour fasted prior blood glucose determination (One Touch Ultra, LifeScan Inc., Wayne, PA, United States). Additionally, to assess insulin sensitivity, all mice underwent an insulin tolerance test (ITT) by the end of the 18th week. Mice were short-fasted for 5 h, basal blood glucose was determined and shortly after this, 100 μl saline solution containing a standardized dose of 0.025 IU of human-recombinant short-acting insulin (Humulin R, Lilly, Indianapolis, IN, United States) was intraperitoneally administered in every animal. Blood glucose measurement was repeated thereafter at 30 min and 60 min. An additional solution of dextrose in sterile water was ready to use in case an animal might be at risk of death by the hypoglycemic effect of short-acting insulin. Once the protocol was finished, all animals were given free access to food and water. No animal losses occurred during the ITT.

Serum biomarkers

Blood was allowed to clot during 20 min at room temperature and then centrifuged at 1500 × *g* for 10 min in a refrigerated centrifuge. Insulin, glucagon, leptin, ghrelin, glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), adiponectin and resistin were measured in mouse serum by multiplex detection immunoassay (Bio-Plex Pro Diabetes Assay #171F7001M, Bio-Rad Laboratories, Inc., Hercules, CA, United States) according to manufacturer instructions.

Histological analysis

Morphological and extracellular matrix deposition assessment was carried out in liver tissue, which was harvested and immersed in a fixation solution (4% paraformaldehyde and 0.1 mol/L PBS at pH 7.4). Afterwards, tissues were embedded in paraffin wax. Serial block (5 μm) sections were subjected to hematoxylin-eosin (HE) and Masson Trichrome staining according to standard procedures. An independent pathologist performed histology grading based on NAS (NASH Activity Score)[20]. All parameters like hepatocyte ballooning, lobular and portal inflammation were scored 0-3. Fibrosis was determined by morphometrical analysis (ImagePro, Rockville, MD, United States).

Western blot analysis

Liver protein was extracted as follows: total protein was extracted in lysis buffer containing 1 mol/L Tris/HCl pH 7.4, 1% triton X-100, 10% glycerol, 137 mmol/L NaCl, 0.5 mmol/L EDTA, and protease inhibitors

(Complete Protease Inhibitor Cocktail, Sigma-Aldrich Corp., Si. Louis, MO, United States; NaF and Sodium Orthovanadate). Homogenate lysates were centrifuged at 12000 *g* for 30 min at 4 ℃. Briefly, aliquots from each sample containing 30 g of total protein quantified by the Bradford protein quantification assay were resuspended in SDS-containing Laemmli sample buffer, heated for 5 min at 95 ℃, and separated through 10% SDS-PAGE under reducing conditions (2-mercaptoethanol). Proteins were electro-blotted overnight at 4 ℃ onto PVDF membranes and the efficiency of the transfer was confirmed by Ponceau staining. Thereafter, membranes were blocked 1 h at room temperature in Tris-buffered saline (20 mmol/L Tris/HCl pH 7.5 and 0.5 mol/L NaCl) containing 0.1% (v/v) Tween 20 (0.1% T-TBS) and 5% (w/v) nonfat dry milk. Blots were washed three times for 5 min each with 0.1% T-TBS and subsequently incubated for 2 h at room temperature with primary mouse/rabbit polyclonal antibodies: anti-PPARα ab8934 (1:1000), anti-LXRα ab3585 (1:300), anti-CPT1A ab128568 (1:1000), anti-ACOX1 ab59964 (1:1000), anti-SREBP1 ab3259 (1:1000), anti-PPARγ ab19481 (1:1000) (Abcam, Cambridge, MA, United States), and anti-NF-κB #8242 (1:1000) (Cell Signaling, Danvers, MA, United States) in 0.05% T-TBS containing 1% BSA. After washing the blots three times for 5 min each with 0.1% T-TBS, the membranes were incubated for 1 h at room temperature with a peroxidase-linked anti-mouse/rabbit antibody (1:16000) in 0.01% T-TBS. To normalize against a loading control, all membranes were stripped and reblotted with anti-β-actin (1:5000). The obtained bands were visualized by chemiluminescence (BM Chemiluminescence Western Blotting substrate POD, Sigma-Aldrich Corp., Si. Louis, MO, United States) kit and quantified using ChemiDoc MP Imaging System with Image Lab software (Bio-Rad Laboratories, Inc, Carlsbad, CA, United States).

Alfa-mouse smooth muscle actin (SMA) immunohistochemistry

Liver biopsies were subjected to react with anti-mouse smooth muscle actin (α-SMA) antibody, which was obtained from Boehringer (Mannheim, Germany). Briefly, histological-processed liver sections were deparaffinized and endogenous activity of peroxidase was quenched with a solution 0.03% H_2O_2 in methanol. Tissue was incubated with a 1/100 dilution of a monoclonal goat anti-mouse α-SMA antibody. Anti-goat peroxidase-labeled secondary antibody was revealed with diaminobenzidine and tissue was counterstained with Harris's hematoxylin

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Groups were compared using Mann-Whitney *U* test for quantitative data and Fisher's exact test for qualitative data. All analyzes were performed using Statistical Program for Social Sciences (SPSS v20.0) for Windows medical pack (Chicago, IL, United States). Statistical significance was determined at $P < 0.05$.

RESULTS

Weight and fasting glucose

As shown in Figure 1A and B, all HFD-derived groups showed significant increase in weight from the sixth week onwards, whereas fasting glucose alterations appeared sooner at the second week. Diet switch (HFD + DS) reduced body weight and restored fasting glucose levels (32.8 ± 1.3 g and 111.3 ± 1.3 mg/dL respectively) compared to HFD group (35.8 ± 0.9 g and 159.1 ± 3.4 mg/dL). Administration of both 18-HEPE and 17-HDHA showed no differences in weight *vs* HFD (36.3 ± 0.5 g and 37.3 ± 1.1 g). Regarding fasting glucose, we observed higher levels in the HFD+18-HEPE group (191.5 ± 5.0 mg/dL) while HFD+17-HDHA group showed a similar value to HFD group.

Insulin tolerance test

Blood glucose values were plotted (mg/dL) and area under the curve (AUC) was calculated based on these data (Figure 1C and 1D). The lowest AUC value was observed in CD (60.6 ± 19 Arbitrary Units, AU) while the highest values in the graphic are observed in the HFD group (113.8 ± 23 AU), as well as in both HFD + 18-HEPE and HFD + 17-HDHA groups (118.7 ± 37 and 98 ± 29 AU). HFD + DS was the only group that displayed improved insulin sensitivity (68.9 ± 21 AU).

Daily energy intake

As shown in Table 1, analysis of daily energy intake was divided into two phases: prior and during treatment. First, we observed a significant difference between CD and all HFD-derived groups. While CD showed a mean daily consumption of 11.9 ± 0.3 kcal, HFD, HFD + DS, HFD + 18-HEPE, and HFD + 17-HDHA showed higher energy intake values (13.1.0 ± 0.2, 13.0 ± 0.3, 13.1 ± 0.3, and 13.0 ± 0.2 kcal respectively). Further, during treatment phase, values in CD, HFD, HFD + 18-HEPE, and HFD + 17-HDHA groups remained unaltered. However, we observed a significant decrease in daily energy intake in the HFD + DS group (10.7 ± 0.4).

Glucose homeostasis hormones

Insulin and glucagon (Figure 2A and B) were significantly increased in HFD, HFD + 18-HEPE and HFD + 17-HDHA groups (insulin: 2736 ± 119, 4138 ± 351, and 2889 ±1242 pg/mL; glucagon: 397 ± 29, 477 ± 38, and 422 ± 10 pg/mL respectively) compared to CD group (insulin: 1105 ± 142; glucagon: 162 ± 24 pg/mL). In fact, insulin levels in HFD + 18-HEPE group were significantly higher compared to HFD group. On the other hand, HFD + DS group displayed lower levels

Table 1 Comparison of daily energy intake between phases

		CD (n = 6)	HFD (n = 6)	HFD+DS (n = 6)	HFD + 18-HEPE (n = 6)	HFD + 17-HDHA (n = 6)
Prior treatment	Daily energy intake (kcal)	11.9 ± 0.3	13.1 ± 0.2[a]	13.0 ± 0.3[a]	13.1 ± 0.3[a]	13.0 ± 0.2[a]
During treatment	Daily energy intake (kcal)	11.9 ± 0.2	13.1 ± 0.3[a]	10.7 ± 0.4[ac]	13.0 ± 0.2[a]	13.1 ±0.2[a]

Values represent mean ± SEM. Mice per group are indicated in parentheses. [a]P < 0.05 *vs* CD, [c]P < 0.05 *vs* HFD by Mann Whitney *U* test.

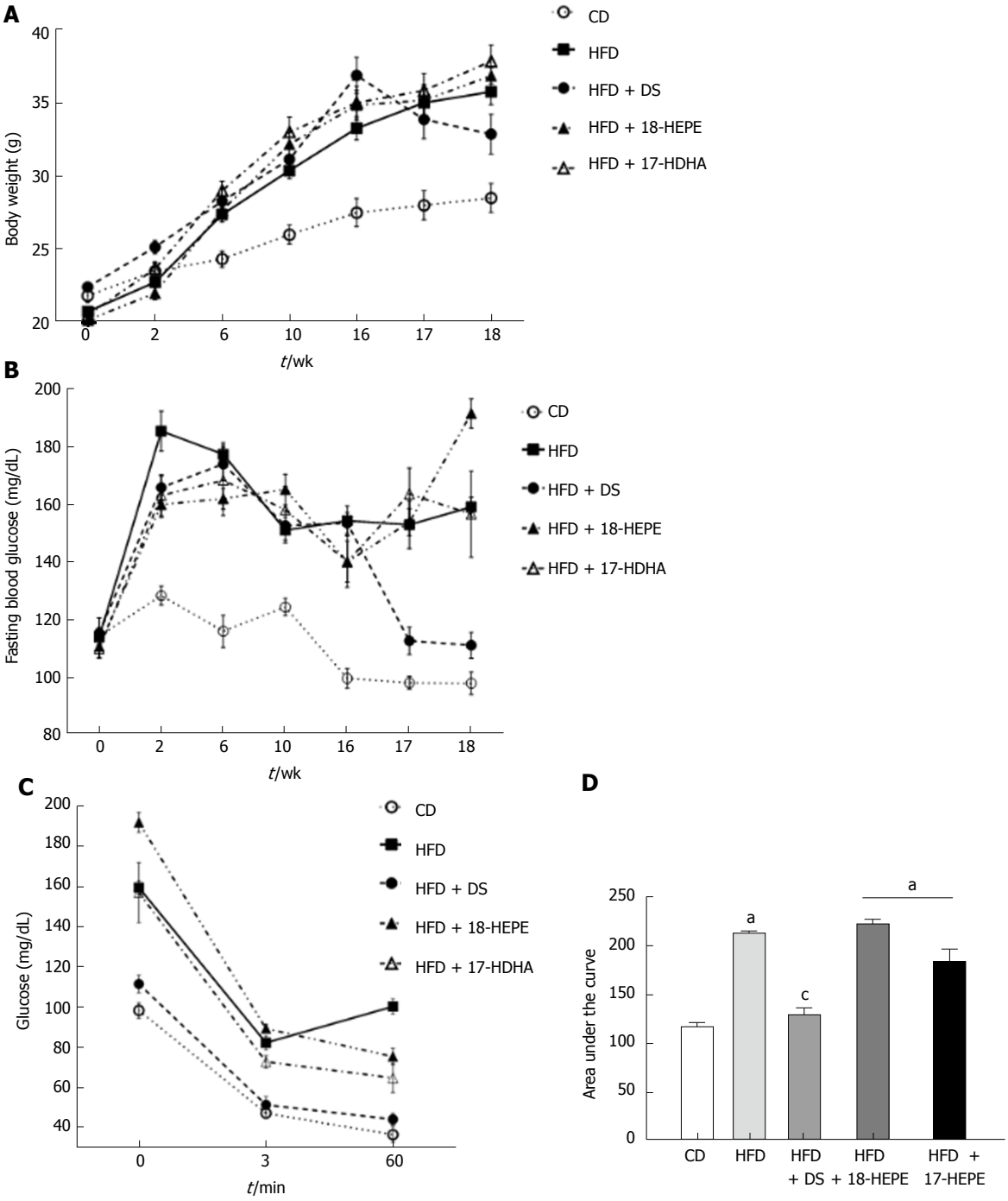

Figure 1 Weight, blood glucose and insulin tolerance test in an obesity/ nonalcoholic fatty liver disease model in C57BL/6 mice. Weight (A), fasting glucose (B), Insulin tolerance test (C) and area under the curve from ITT data (D). All data are mean ± SEM. Groups: CD (n = 6), HFD (n = 6), HFD + DS (n = 6), HFD + 18-HEPE (n = 6), HFD + 17-HDHA (n = 6). [a]P < 0.05 *vs* CD, [c]P < 0.05 *vs* HFD by Mann-Whitney *U* test. HFD: High-fat, fructose-enriched diet.

in both hormones (insulin: 1580 ± 95; glucagon: 239 ± 14 pg/mL). Following with incretins levels, GLP-1 (Figure 2C) remained widely without significant differences among CD, HFD, and HFD+DS groups (42 ± 7, 47

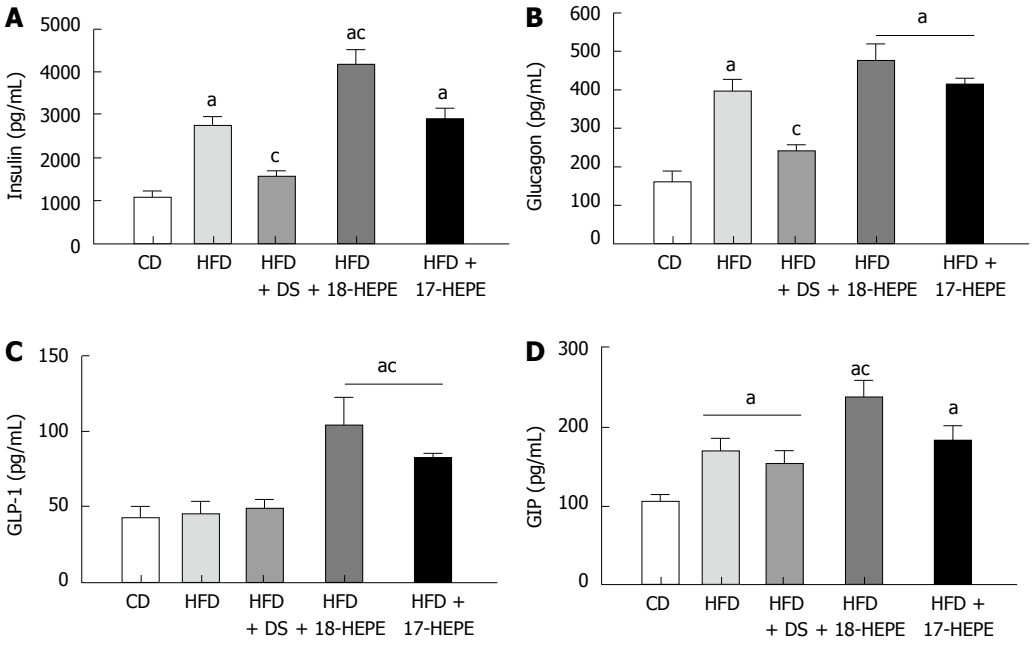

Figure 2 Hormones of glucose homeostasis. Serum insulin (A), glucagon (B) GLP-1 (C), GIP (D). All data are mean ± SEM. Groups: CD (n = 6), HFD (n = 6), HFD + DS (n = 6), HFD + 18-HEPE (n = 6), HFD + 17-HDHA (n = 6). $^{a}P < 0.05$ *vs* CD, $^{c}P < 0.05$ *vs* HFD by Mann-Whitney *U* test. CD: Chow diet; HFD: High-fat, fructose-enriched diet, GIP: Glucose-dependent insulinotropic polypeptide.

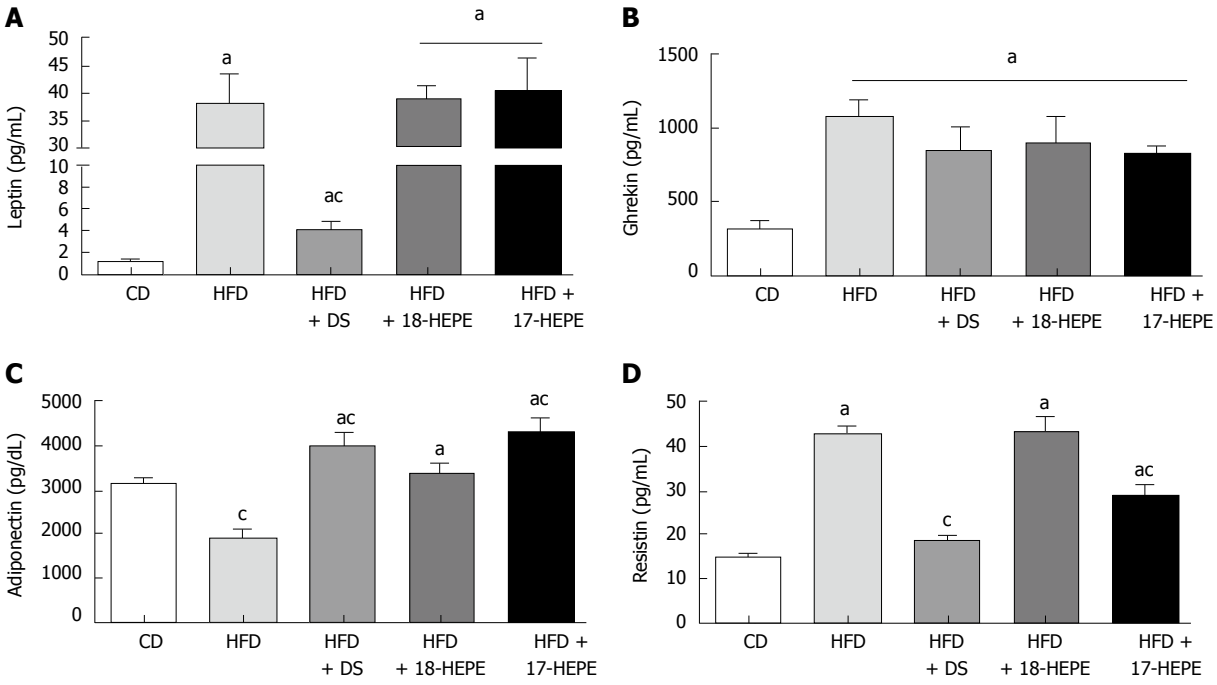

Figure 3 Energy balance hormones and adipokines. Serum leptin (A), ghrelin (B), adiponectin (C) and resistin (D) All data are mean ± SEM. Groups: CD (n = 6), HFD (n = 6), HFD + DS (n = 6), HFD + 18-HEPE (n = 6), HFD + 17-HDHA (n = 6). $^{a}P < 0.05$ *vs* CD, $^{c}P <$ 0.05 *vs* HFD by Mann-Whitney *U* test. HFD: High-fat, fructose-enriched diet.

± 6, and 49 ± 5 pg/mL respectively). Administration of both 18-HEPE and 17-HDHA showed a significant increase in GLP-1 levels (103 ± 18, and 81 ± 4 pg/mL respectively). Finally, GIP levels (Figure 2D) were increased in all HFD-derived groups compared to CD (105.4 ± 8 pg/mL), however HFD + 18-HEPE group showed significantly higher levels compared to HFD group (237 ± 21 *vs* 169 ± 18 pg/mL). No significant differences were found among the rest of the groups or in comparison to HFD group (HFD + DS 151 ± 18 pg/

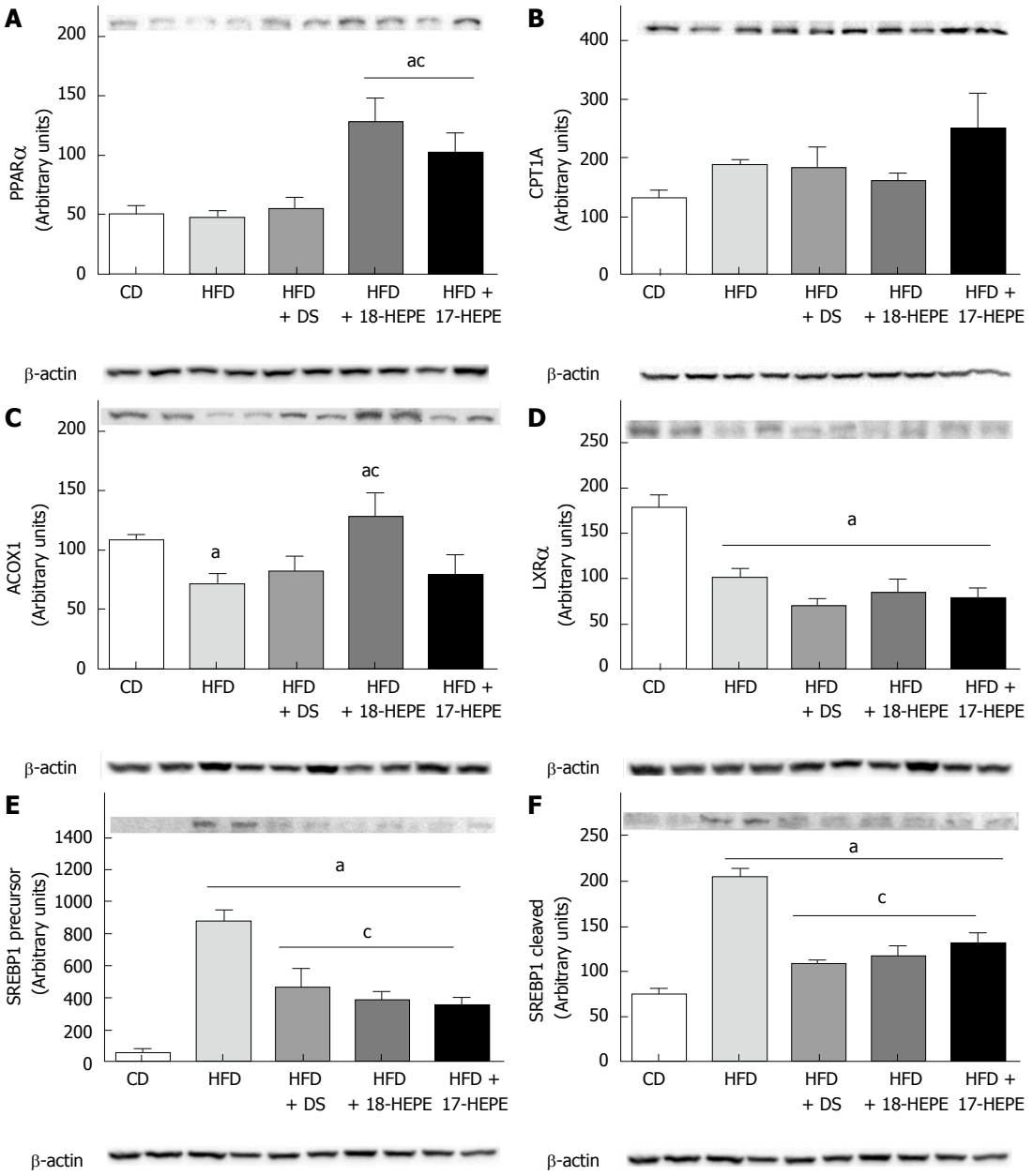

Figure 4 Hepatic proteins involved in lipid oxidation and synthesis. PPARα (A), CPT1A (B), ACOX1 (C), LXRα (D), SREBP1 in both precursor (E) and cleaved form (F). Every two bands from left to right are representative of each group. All data are mean ± SEM. Groups: CD (n = 6), HFD (n = 6), HFD + DS (n = 6), HFD + 18-HEPE (n = 6), HFD + 17-HDHA (n = 6). $^{a}P <$ 0.05 *vs* CD, $^{c}P <$ 0.05 *vs* HFD by Mann-Whitney *U* test. HFD: High-fat, fructose-enriched diet.

mL and HFD + 17-HDHA 181 ± 19 pg/mL).

Energy balance hormones and adipokines

Leptin and ghrelin levels (Figure 3A and B) were significantly increased in HFD, HFD + DS, HFD + 18-HEPE, and HFD + 17-HDHA groups (leptin: 37.8 ± 5.7, 4.1 ± 0.7, 38.4 ± 3.3 and 40.0 ± 6.6 ng/mL; ghrelin: 1070 ± 114, 847 ± 173, 902 ± 176 and 817 ± 68 pg/mL respectively) compared to CD group (leptin: 0.94 ± 0.3 ng/mL; ghrelin: 322 ± 44 pg/mL). However, HFD + DS group showed a significant decrease in leptin levels compared to HFD group. On the other hand, ghrelin remained widely unchanged regardless of treatment. With regards to adipokines, adiponectin (Figure 3C) was significantly reduced in HFD group compared to CD group (1881 ± 213 and 3172 ± 83 pg/mL respectively). Diet switch and administration of both 18-HEPE and 17-HDHA showed a significant increase in adiponectin levels *vs* HFD group (3998 ± 305, 3367 ± 257, and 4297 ± 333 pg/mL respectively). In contrast, resistin levels (Figure 3D) were significantly increased in HFD group compared to CD (42.8 ± 1.9 and 14.8 ± 1.8 ng/mL respectively). Concerning treated groups, only HFD + DS and HFD + 17-HDHA showed decreased levels of resistin compared to HFD group (18.3 ± 1.6 and 28.8 ± 2.5 ng/mL respectively), while administration of HFD + 18-HEPE (43.4 ± 3.6 ng/mL) showed no effect in resistin levels.

Hepatic proteins involved in lipid oxidation and synthesis

Western blot analysis of lipid oxidation-related proteins

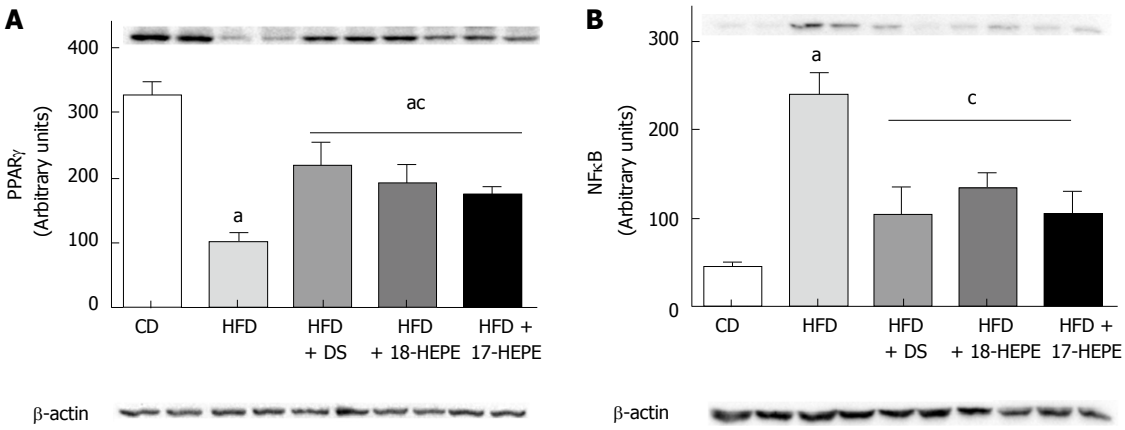

Figure 5 Hepatic proteins involved in inflammatory process. PPARγ (A) and NF-κB (B). Every two bands from left to right are representative of each group. All data are mean ± SEM. Groups: CD (n = 6), HFD (n =6), HFD + CD (n = 6), HFD + 18H (n = 6), HFD + 17-H (n = 6). $^{a}P < 0.05$ *vs* CD, $^{c}P < 0.05$ *vs* HFD by Mann-Whitney *U* test. HFD: High-fat, fructose-enriched diet.

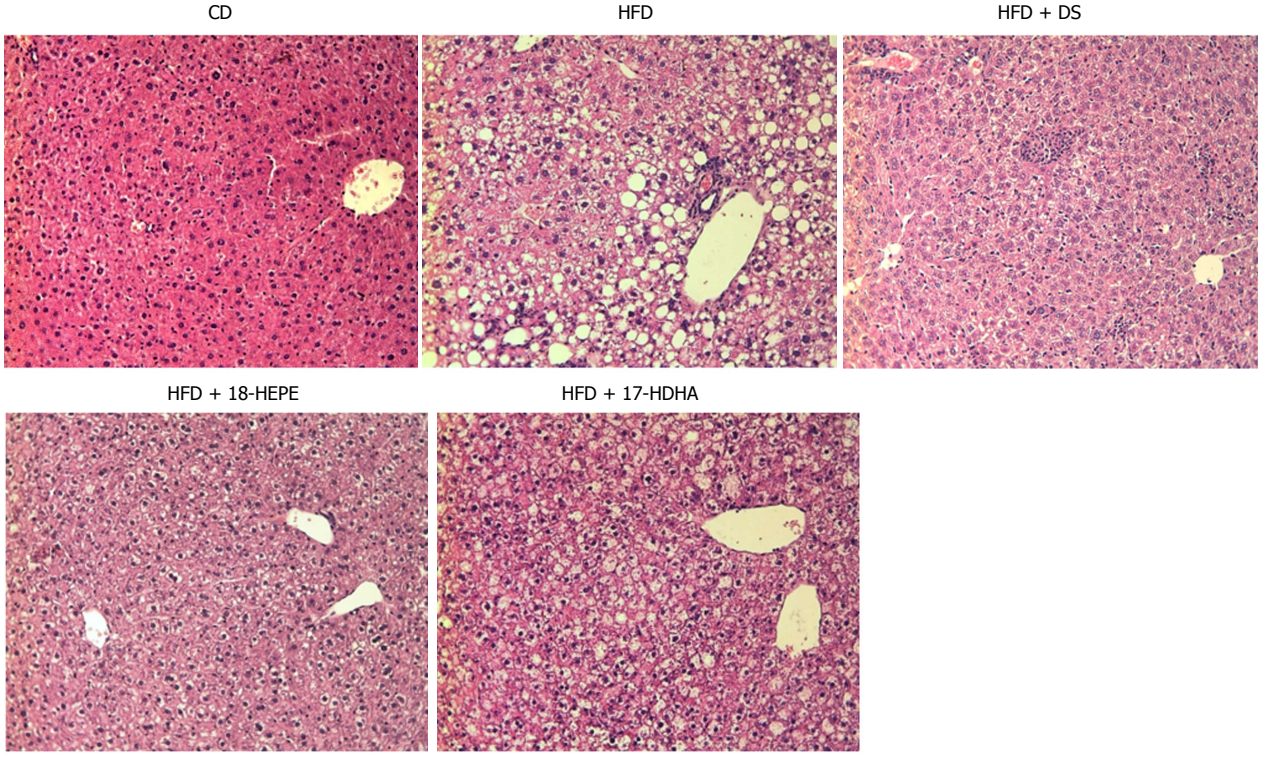

Figure 6 Liver tissue morphology. Representative photomicrographs (Magnification × 20) Hematoxylin-eosin stain. HFD + DS showed a drastic decrease of number in fat vesicles and ballooning degeneration, but minimal change in inflammatory cells. Both groups HFD + 18-HEPE and HFD + 17-HDHA showed noticeable changes in steatosis and hepatocyte ballooning compared to HFD group. Plus, both groups displayed scarce presence of inflammatory cells.

comprised PPARα (Figure 4A) and its target genes CPT1A and ACOX1 (Figure 4B and C). Administration of both 18-HEPE and 17-HDHA produced a significant increase in PPARα compared to both CD and HFD groups. While CPT1A showed no statistical significant differences among groups, ACOX1 revealed to be significantly increased only in HFD + 18-HEPE group. On the other hand, analysis of lipid synthesis-related proteins was conducted by quantifying the relative abundance of LXR (Figure 4D) and its target gene SREBP1 in both, precursor and cleaved forms (Figure 4E and F). In this regard, LXRα was reduced in all HFD-derived groups with or without treatment compared to CD group. Further, SREBP1 in both isoforms was dramatically increased in HFD group *vs* CD, and, noteworthy, diminished in all treated groups.

Hepatic proteins involved in inflammation process

In this regard, two major proteins were analyzed. As shown in Figure 5A, PPARγ showed a dramatic decreased in HFD compared to CD group. Notably, all treated groups produced a significant increase in the

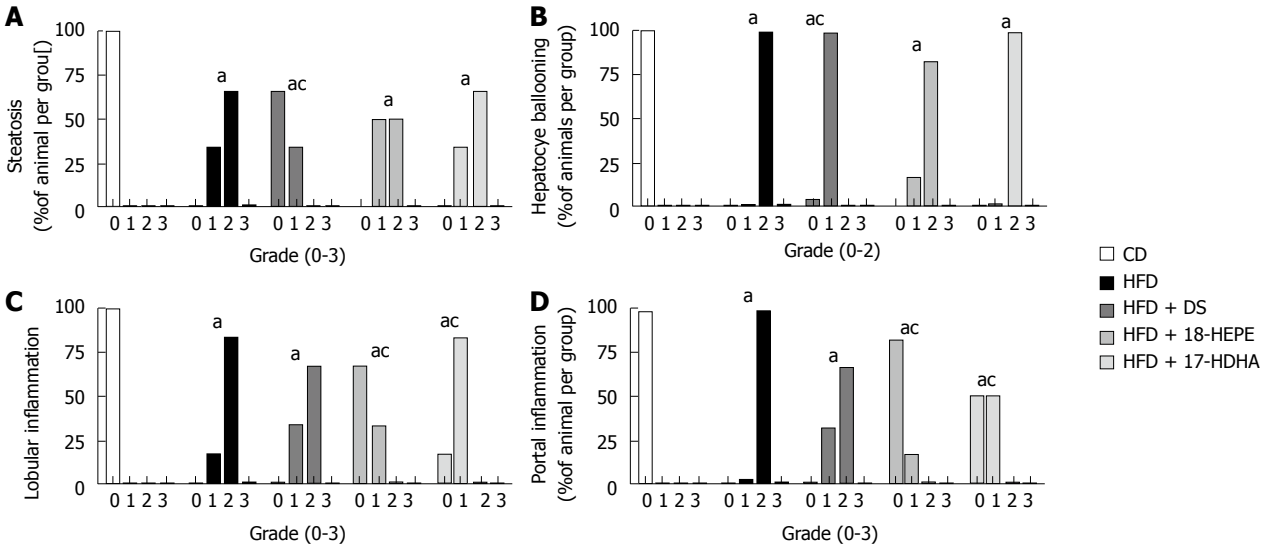

Figure 7 NAS score. (A) Steatosis, (B) hepatocyte ballooning, (C) lobular and (D) portal inflammation. Bars represent the percentage of animals in the group at the given score. According to NAS, steatosis was graded 0-3, hepatocyte ballooning 0-2, lobular inflammation 0-3, and portal inflammation (0-2). Groups: CD (n = 6), HFD (n = 6), HFD + DS (n = 6), HFD + 18-HEPE (n = 6), HFD+17-HDHA (n = 6). $^{a}P < 0.05$ *vs* CD, $^{c}P < 0.05$ *vs* HFD by Fisher's exact test.

relative abundance of PPAR. In contrast, NF-κB showed a significant increase in HFD compared to CD group. It was also observed that all treatments produced a significant decrease in relative abundance of NF-κB (Figure 5B).

Liver histology

Microscopic liver morphology was conducted following HE standard protocol (Figure 6). HFD group was characterized by steatosis, hepatocyte ballooning and inflammatory infiltrate. The main finding in HFD + DS group was the drastic decrease of number in fat vesicles and ballooning degeneration (Figure 7A and B); however, this group presented a moderate inflammatory-cell aggregates on a great proportion of microphotographs showing no statistical differences *vs* HFD group. Further, both groups HFD + 18-HEPE and HFD + 17-HDHA showed noticeable changes in steatosis and hepatocyte ballooning compared to HFD group. Besides, both groups displayed scarce presence of inflammatory cells at lobular and portal zones (Figure 7C and D) in different proportions. Analysis of extracellular matrix was also examined, in which we did not observe differences among all groups. (Figure 8A and B). Furthermore, we conducted an immunohistochemistry analysis to determine early development of fibrosis. Therefore, SMA expression was determined. Interestingly enough, HFD group showed an augmented expression of αSMA along the perisinusoidal space compared to CD group. This fact could be representing the prelude of the fibrogenic process. Noteworthy, HFD + DS and HFD + 17-HDHA groups showed a pronounced reduction in αSMA expression, whereas HFD + 18-HEPE group displayed only a modest decrease (Figure 9).

DISCUSSION

The growing prevalence of obesity and its comorbidities such as NAFLD has urged the need for research on additional alternative therapies. In our study, we demonstrated that administration of 3 hydroxy-fatty acids 18-HEPE and 17-HDHA decreased hepatic inflammation in obese mice. These actions were mainly associated with the up-regulation of PPAR and PPARγ proteins in liver tissue. In addition, evidence for these effects includes the ameliorated production of serum adipokines (*i.e.*, adiponectin and resistin) independently of body weight. Importantly, we compared the effect of these fatty acids to those observed in a group undergoing diet switch (chow) after 16 wk of high-fat, fructose-enriched diet . The importance of approaching obesity and NAFLD with lifestyle modification including diet and exercise is widely accepted and highly recommended[21]. In fact, analysis of on insulin sensitivity in obese mice treated with normocaloric diet for ten wk was examined by Lombardo *et al*[22], reporting weight loss, lower insulin levels, improved insulin tolerance associated with increased expression of Glut4. Remarkably in our study, mice undergoing diet switch displayed weight loss, restored fasting glucose levels, and insulin sensitivity by the end of treatment period. Alongside with these findings, insulin, glucagon, leptin, adiponectin and resistin showed restoration in this mice group which could be largely explained by weight loss. Plus, liver histology showed decreased steatosis and ballooning, but no relevant changes in inflammatory infiltrate, which has been described in humans when rapid weight loss takes place either by bariatric surgery or low fat diets in rodents[23,24]. It is possible that the virtually unaltered liver inflammation in HFD + DS mice is a product of a

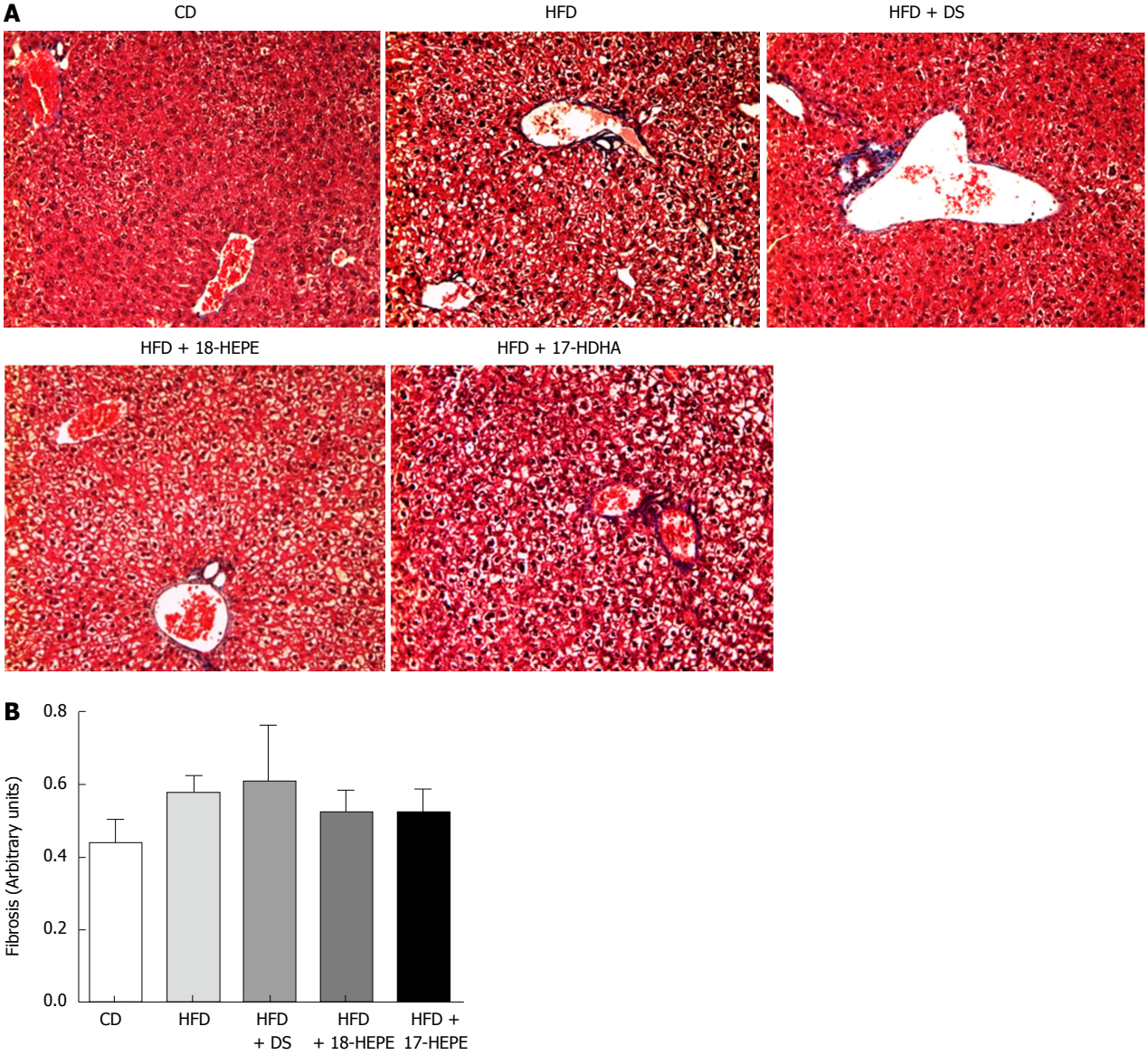

Figure 8 Extracellular matrix in liver tissue. A: Representative photomicrographs (Magnification × 20) Masson's trichrome stain; B: Morphological analysis represented as percentage of extracellular matrix in image. All data are mean ± SEM. Groups: CD (n = 6), HFD (n = 6), HFD + DS (n = 6), HFD + 18-HEPE (n = 6), HFD + 17-HDHA (n = 6). $^{a}P <$ 0.05 *vs* CD, $^{c}P <$ 0.05 *vs* HFD by Mann-Whitney U test.

sustained release of free fatty acids from visceral fat, which in turn may produce a transient activation of inflammatory cells. Therefore, we hypothesized that a longer period of diet switch regimen might produce amelioration in lobular and portal inflammation. Unlike the study carried out by Kohli *et al*[25], we found no fibrosis in liver histology after 16 wk of high-fat, fructose-enriched diet . This might be due to the higher fat percentage in the diet they utilized (60% calories). Nevertheless, similarly to what has been reported to occur in NAFLD in humans, we observed higher serum levels of insulin, glucagon, leptin, ghrelin and resistin in our model[26].

Remarkably, administration of both 18-HEPE and 17-HDHA showed significant higher incretin levels. It has been documented that GLP-1 and GIP secretion can be stimulated by -linolenic acid, EPA, DHA and 5-HEPE through GPR120 in the colon[27,28]. However, in the case of GLP-1, it has become a major target in NAFLD treatment whereas GIP seems to be a controversial piece in glucose homeostasis. It has been reported that suppressing GIP in genetically modified mice is rather beneficial under high fat conditions[29]. Furthermore, GPR120 activation by fatty acids might take place either by oral administration or intracolonic delivery[30], but neither of these techniques were conducted in the animals here studied. Notwithstanding, it has been long reported that intraperitoneal injection may lead to inadvertent administration of some material into the gut, abdominal fat and subcutaneous tissues in a relatively frequent occurrence (14%-24% of cases)[31]. Therefore, our results suggest that the increased GLP-1 and GIP

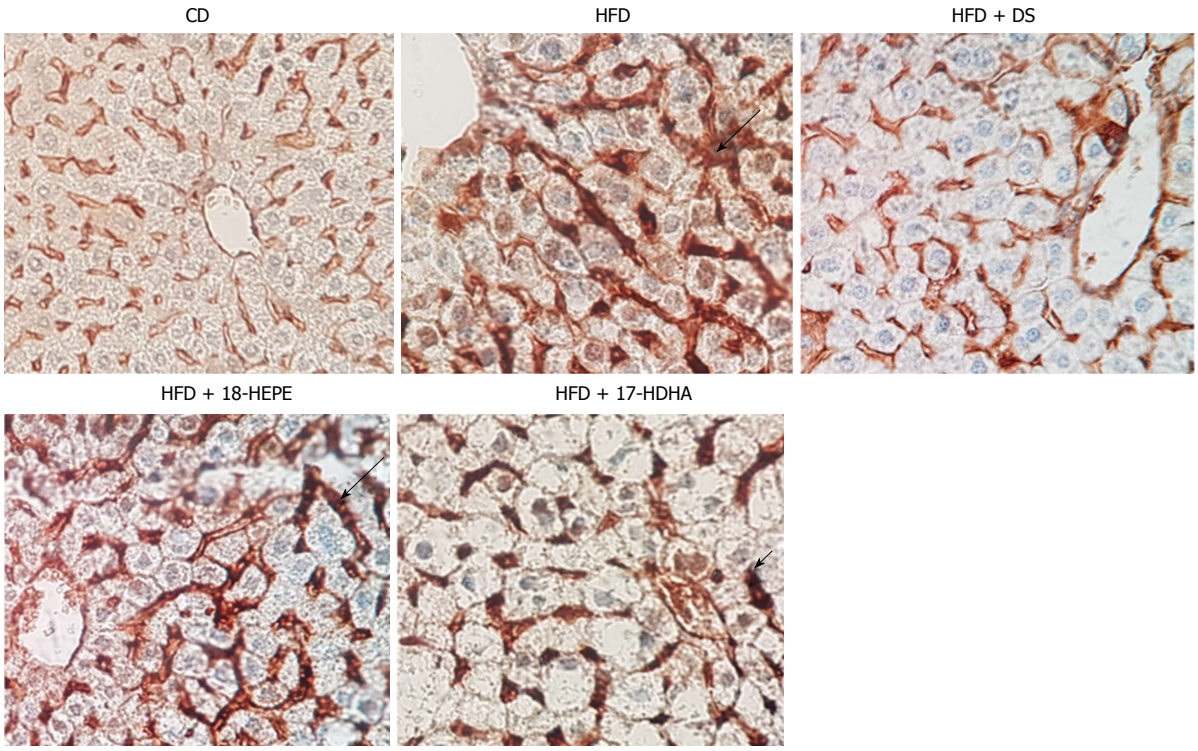

Figure 9 Expression of mouse smooth muscle actin in liver tissue. Representative photomicrographs (Magnification × 40) Immunohistochemistry for α-smooth muscle actin (SMA). Arrows show area in which the expression is marked. SMA expression was determined to analyze early development of fibrosis. HFD group showed an augmented expression of SMA along the perisinusoidal space in compared to CD group. Noteworthy, HFD + DS and HFD + 17-HDHA showed a pronounced reduction, whereas HFD + 18-HEPE groups displayed only a modest decrease.

levels could be in part due to eventual administration of hydroxy-fatty acids into the gut. However, the increase serum levels we observed on GLP-1 along with the amelioration on adiponectin and resistin was not sufficient to produce any improvement on insulin resistance in these groups.

Analysis of liver tissue showed a marked increase in relative abundance of PPARα and PPARγ in mice treated with 18-HEPE and 17-HDHA. These finding supports the previous reports on ligand activities exerted by these fatty acids over both nuclear receptors[15,16]. However, their activities were seemingly distinct. We found a significant increase in ACOX1 by 18-HEPE administrations and a tendency to enhanced production of CPT1A by 17-HDHA (both PPAR target genes). Restoration or enhancement in the abundance of these enzymes is likely to promote fat oxidation and therefore ameliorate steatosis. In fact, we found a modest improvement in fat accumulation in the mice treated with these fatty acids. However, diet switch displayed a remarkable clearance of fat vesicles even with normal ACOX1 and CPT1A protein levels. These differences may be related to the diminished energy intake observed in these mice group during diet switch, leading to a negative energy balance without the need to increment oxidation enzymes production. More to the point, LXRα is a nuclear receptor known for its s capacity to activate lipogenesis mainly through up-regulation of SREBP1. The latter is a protein that in physiological conditions is stimulated by insulin, but it has been described to be paradoxically activated in NAFLD mainly by endoplasmic reticulum stress[32]. We observed that levels of this protein in both precursor and cleaved forms were significantly blunted by all treatments. This effect plays a key role in inhibiting de novo lipogenesis and thus, steatosis exacerbation. Importantly, the effects observed in SREBP1 were independent of the levels we found in LXRα, as this nuclear receptor showed no significant difference among treated *vs* non-treated mice. It is important to mention that the role of LXR in obesity is rather controversial. It has been proposed as a pharmacological target for glucose intolerance[33] through agonists and even it has been reported to be important in inhibiting fibrogenesis[34]. Additionally, both nuclear receptors PPARα and PPARγ display anti-inflammatory actions by inhibiting NF-κB. It has been reported that PPARα can increase NF-κB inhibitor alpha (IκBα) expression and thus, prevent p50/p65 NF-κB translocation into the nucleus for DNA binding[35]. On the other hand, PPARγ has been described to mediate trans repression on inflammatory genes by a SUMOylation dependent pathway also involving p50/p65 NF-κB[36]. These previous data could explain the diminished relative abundance in NF-κB levels observed in liver tissue. Also, lobular and portal inflammation showed marked amelioration in mice groups treated with fatty acids. In our study, hepatoprotective actions elicited by these hydroxy-fatty acids were associated

with increased PPARα and PPARγ proteins in liver tissue. In contrast, a phase 2 trial failed to prove histologic amelioration on individuals with non-alcoholic steatohepatitis using ethyl-eicosapentanoic acid[37]. Explanation for the lack of efficacy seems to lie on the administered dose. The dosing for this trial was selected based on existing data for its efficacy for dyslipidemia in Japan. Thus, it is possible that this dose was not sufficient for an American population. It is important to remark that the dosage used in our study was greatly lower (In average: 950 nanograms per day) compared to previous studies in animals with similar objective using EPA and DHA. Just as previous studies have reported, position of the alcohol group in both EPA and DHA is a relevant fact when it comes down to affinity for nuclear receptors[15,16]. Importantly, these hydroxy-fatty acids originated from ω3 PUFAs exert protective actions noticeable at the nanomolar ranges, many of which have been associated with the resolution of unremitting inflammation[17,38-41]. A major proposed mechanism whereby these novel fatty acids exert anti-inflammatory actions is through enzymatic biotransformation into specialized pro-resolving mediators namely lipoxins, resolvins, protectins and maresins. However, since we did not explore this area, further analyses are required to elucidate these possible mechanisms. Finally, we demonstrated in our work the beneficial properties of 18-HEPE and 17-HDHA in an experimental model under high fat conditions as well as a comparative analysis *vs* dietetic intervention.

In conclusion, We demonstrated that most serum metabolic parameters and histological features in obese mice are reversible by switching diet regimen from high-fat to low-fat for two wk. This finding supports the evidence of diet switch regimen as a valuable reference point for assessing alternative therapies. Finally, administration of 18-HEPE and 17-HDHA exerted hepatoprotective effects in the liver through up-regulation of nuclear receptors PPARα/γ and amelioration of serum adipokines profile.

ARTICLE HIGHLIGHTS

Research background

Nonalcoholic fatty liver disease (NAFLD) is a major chronic liver condition over the last decades. Notably, NAFLD shows a high growth rate worldwide and it is thought to derive mainly from modern lifestyle habits featuring low physical activity and chronic exposure to high-fat, high-fructose diet. Those mentioned factors have dramatically increased the prevalence of obesity and metabolic syndrome along with its comorbidities: dyslipidemia, insulin resistance, and hypertension.

Several drugs have been proposed in the clinical scenario over the last years such as pioglitazone, vitamin E, liraglutide, sitagliptine, elafibranor, obeticholic acid, and pentoxifylline just to name a few. Also, much attention has been paid to the anti-inflammatory and lipid-lowering properties of other types of fats such as 3 polyunsaturated fatty acids (ω3 PUFA), which have long been investigated and showed positive impact on cardiovascular and hepatic alterations as well as in overall health.

Research motivation

Metabolic liver disease is currently a major cause of morbidity worldwide. Research on treatment strategies is in fact an interesting area to explore.

Research objectives

To determine the efficacy of hydroxy-fatty acids in experimental NAFLD/obesity as well as comparing the effects with diet switch regimen.

Research methods

Histological analysis, western bloting analysis and α-mouse smooth muscle actin immunohistochemistry.

Research results

Mice treated with hydroxy-fatty acids 18-hydroxy-eicosapentaenoic acid (18-HEPE) and 17-hydroxy-docosahexaenoic acid (17-HDHA) displayed no weight loss or improved insulin sensitivity. However, these mice groups showed a significant amelioration on serum GLP-1, adiponectin and resistin levels. Also, a significant reduction on inflammatory infiltrate was observed at both portal and lobular zones. Furthermore, up-regulation of PPAR α/γ protein levels was observed in liver tissue and it was associated with decreased levels of NF-κB also determined by western blot analysis. On the other hand, diet switch regimen resulted in a marked improvement in most parameters including: weight loss, increased insulin sensitivity, decreased steatosis, restored levels of insulin, glucagon, leptin, adiponectin and resistin. However, no significant changes were observed regarding inflammatory infiltrate in this last group.

Research conclusions

Most serum metabolic parameters and histological features in obese mice are reversible by switching diet regimen from high-fat to low-fat for two wk. This finding supports the evidence of diet switch regimen as a valuable reference point for assessing alternative therapies. Finally, administration of 18-HEPE and 17-HDHA exerted hepatoprotective effects in the liver through up-regulation of nuclear receptors PPARα/γ and amelioration of serum adipokines profile.

Research perspectives

Just as previous studies have reported, position of the alcohol group in both eicosapentaenoic acid and DHA is a relevant fact when it comes down to affinity for nuclear receptors. Importantly, these hydroxy-fatty acids originated from 3 PUFAs exert protective actions noticeable at the nanomolar ranges, many of which have been associated with the resolution of unremitting inflammation. A major proposed mechanism whereby these novel fatty acids exert anti-inflammatory actions is through enzymatic biotransformation into specialized pro-resolving mediators namely lipoxins, resolvins, protectins and maresins. However, since we did not explore this area, further analyses are required to elucidate these possible mechanisms.

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ORIGINAL ARTICLE

Basic Study

Overexpression of CREPT confers colorectal cancer sensitivity to fluorouracil

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Author contributions: Kuang YS and Wang Y contributed equally to this work; Kuang YS performed the majority of experiments and analyzed the data; Wang Y contributed significantly to staining and analyzing of the immunohistochemistry experiments; Ding LD and Yang L helped perform the analysis with constructive discussions; Wang Y, Zhu BT and Li J helped perform the cell apoptosis detection; Wang XN and Liu HY contributed to obtaining the patients' data; Jia BQ, Chang ZJ and Wang YY contributed to the conception and coordination of the study.

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Institutional review board statement: This study was approved by the Ethics Committee of the PLA General Hospital, Beijing, China. All patients involved in this study gave their informed consent for participation in the study.

Conflict-of-interest statement: We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript titled "Overexpression of CREPT confers colorectal cancer sensitivity to fluorouracil".

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Abstract

AIM

To investigate expression of cell cycle-related and expression-elevated protein in tumor (CREPT) in colorectal cancer (CRC) and determine its prognostic value in response to 5-fluorouracil (5-FU).

METHODS

The relative expression of CREPT in CRC tumor samples was determined using immunohistochemistry. The protein content in cell lines was analyzed by immunoblotting. Cell viability was measured with the CCK-8 assay. Cell cycle and apoptosis analyses were performed with flow cytometry.

RESULTS

CREPT was overexpressed in CRC tissues and correlated with histological grade. Clinicopathological analysis indicated that CREPT was positively related to tumor progression. Exogenous expression of CREPT stimulated cell proliferation and accelerated the cell cycle. More importantly, high expression of CREPT sensitized CRC cells to 5-FU treatment. Furthermore, we demonstrated that 5-FU elicited significant apoptosis in CREPT-positive cells.

CONCLUSION

Aberrant overexpression of CREPT contributes to tumorigenesis of CRC by promoting cell proliferation and accelerating the cell cycle, and confers sensitivity to 5-FU. CREPT is a potential prognostic biomarker for 5-FU in CRC.

Key words: CREPT; Colorectal cancer; 5-Fluorouracil; Apoptosis; Cell proliferation

Core tip: Cell cycle-related and expression-elevated protein in tumor (CREPT) is an oncogene that is preferentially expressed in diverse human tumors. Overexpression of CREPT promotes cell proliferation and tumorigenesis. However, the expression and mechanistic involvement of CREPT in colorectal cancer have not been fully investigated. Despite advances in clinical applications of 5-fluorouracil, drug resistance remains a significant limitation to its clinical use. A prognostic biomarker for administration of this drug is still urgently needed.

Kuang YS, Wang Y, Ding LD, Yang L, Wang Y, Liu SH, Zhu BT, Wang XN, Liu HY, Li J, Chang ZJ, Wang YY, Jia BQ. Overexpression of CREPT confers colorectal cancer sensitivity to fluorouracil. *World J Gastroenterol* 2018; 24(4): 475-483 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i4/475.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i4.475

INTRODUCTION

Colorectal cancer (CRC) is a malignant disease with symptoms such as blood in the stools, aberrant bowel movement, and weight loss[1]. Globally, CRC is the third most common malignancy, accounting for approximately10% of all cases. In China, there were 376 300 newly diagnosed cases of CRC and 191000 deaths in 2015[2]. With advances in early diagnosis and clinical therapeutics, the average five-year survival rate is approaching 70% in the United States. The diagnosis of CRC mainly relies on pathological examination of tissues collected *via* enteroscopy, and evaluation of cancer stage heavily depends on imaging technologies like computed tomography, positron emission tomography and magnetic resonance imaging[3].

Fluorouracil-based chemotherapy is still the mainstay for clinical management of CRC[4]. 5-Fluorouracil (5-FU) is an antimetabolite drug that inhibits the biosynthesis of DNA and thus induces tumor cell apoptosis[5]. The clinical application of 5-FU-based adjuvant chemotherapy in the treatment of late stage CRC improves overall and disease-free survival in 10%-15% of patients[6]. However, the provoked resistance in response to 5-FU seriously compromises its therapeutic efficiency. Therefore, identification and characterization of prognostic biomarkers for screening the potential sensitive population for this drug is crucial.

Cell cycle-related and expression-elevated protein in tumor (CREPT; also named RPR1B) was first identified as an oncoprotein that is highly expressed in most tumors[7]. Principally, CREPT functions as a transcriptional regulator in CCND1 expression in two distinct ways: promoting direct binding of RNA polymerase Ⅱ on the promoter region to activate transcription, or on the termination region before the poly-A site to prevent release from the transcript and allow for recycling[7]. CREPT was later identified to function on the human RNA polymerase Ⅱ C-terminal domain scaffold and participate in phosphorylation of the C-terminal heptapeptide repeat domain[8]. In addition, CREPT induces transcription of several other cell cycle-related genes including CDK2, CDK4, CDK6 and cyclin-E, which eventually accelerates the cell cycle and stimulates cell proliferation[9].

There is accumulating evidence for the crucial role of CREPT in tumor biology in a range of human cancers[10]. However, the expression pattern and mechanistic involvement of CREPT in CRC have not been fully investigated. In this study, we investigated the role of CREPT in tumorigenesis of CRC *via* inducing cell proliferation and stimulating the cell cycle. Overexpression of CREPT rendered cells sensitive to 5-FU, which reinforced the apoptotic response. We propose the prognostic biomarker function of CREPT for clinical application of 5-FU.

MATERIALS AND METHODS

Plasmids and antibodies

The expression plasmid for human CREPT was pCDH/HA-CREPT, which was constructed in our laboratory. The plasmid pBS/U6/CREPT-si was constructed according to a previous protocol. The siRNA target sequence (CREPT-si), GGACCTGAATTCACTAGAGA, was identical for humans and mice. Antibodies against PARP (5625S) were purchased from Cell Signaling Technology (Danvers, MA, United States), anti-actin (AC-15) antibody was obtained from Sigma-Aldrich (St. Louis, MO, United States), and anti-CREPT antibody (3E10) was raised in our laboratory.

Patient specimens and staining

Two hundred and three primary CRC and 13 colorectal adenoma patients who underwent surgical treatment were selected. Formalin-fixed, paraffin-embedded tissue blocks were cut into 4-μm paraffin sections, followed by immunohistochemical analysis. The slides were heated in a tissue-drying oven for 40 min at 65 ℃, followed by deparaffinization in xylene and rehydration in a graded alcohol series. The slides were incubated in sodium citrate solution (pH 6.0) and heated in a boiling water bath for 20 min for antigen retrieval. After endogenous peroxidases were blocked by soaking the slides in 3% H_2O_2, the slides were incubated with anti-CREPT primary antibody (1:20) in a humidity chamber at 4 ℃ overnight. We washed the slides with phosphate-buffered saline (PBS) three times, and applied the EnVision Kit (Dako, Glostrup, Denmark) to the sections on the slides and incubated in a humidified chamber at room temperature for 30 min. Signal detection was performed using diaminobenzidine in the EnVision Kit (Dako). All the slides were examined under a microscope by two blinded pathologists. The proportion of positive cancer cell staining was classified as follows: grade 1 (-) = no positive cells; grade 2 (1+) < 25%; grade 3, 25%-75%; and grade 4, > 75%. All patients gave informed consent for participation in the study. The tissue collection procedure with informed consent was approved by the Ethics Committee of the Chinese PLA General Hospital, Beijing, China.

Construction of lentivirus

Human CREPT gene was subcloned into pCDH-vector with an HA-tag. Short hairpin RNA (shRNA) was designed to downregulate expression of CREPT. Nonoverlapped sequences were designed (shRNA, 5′-GCAAGAACGAAGUGUUAUTT-3′). The shRNA targeting CREPT was selectively subcloned into lentiviral vector pLVX-IRWS-ZsGreen1. pCDH-HA-CREPT was also subsequently cloned into pLVX-IRWS-ZsGreen1. Lentivirus was produced and the titration of purified virus was determined according to our previous study[10]. The virus was stored at -80 ℃ until use.

Cell culture

Human colorectal adenocarcinoma cell lines DLD1 and SW620 were purchased from American Type Culture Collection (Manassas, VA, United States). DLD1 cells were cultured in RPMI 1640 (Life Technologies, Carlsbad, CA, United States) and SW620 cells were cultured in L-15 supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), penicillin (100 U/mL) and streptomycin (100 mg/mL). DLD1 cells were maintained at 37 ℃ in a 5% CO_2-containing atmosphere and SW620 cells were kept at 37 ℃with 100% air.

Western blotting

Cells were harvested and homogenized in RIPA buffer (Cell Signaling Technology), followed by determination of protein concentration using the BCA kit (Life Technologies). Proteins were resolved by 10% SDS-PAGE, and then transferred to 0.45-mm polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% skimmed milk in Tris-buffered saline with Tween 20 (TBST) at 37 ℃ for 1 h and incubated with primary antibody at 4 ℃ overnight. The PVDF membranes were rigorously washed with TBST and subjected to secondary antibody hybridization. The protein bands were visualized using enhanced chemiluminescence (Millipore, Temecula, CA, United States).

CCK proliferation assay

Cells were counted by hemocytometer and then seeded into 96-well plates at 10^4/well, and 5-FU (50 μm/mL) was added 12 h later. CCK-8 (Dojindo, Kumamoto, Japan) buffer was diluted as protocol indicated and added to the wells at indicated time. Absorbance at 450 nm was recorded with a reference filter of 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, United States)

Flow cytometry

Cells were seeded in six-well plates 12 h before 5-FU was added. After incubation for 48 h, cells were harvested and subjected to apoptosis detection by FITC-Annexin V Apoptosis Detection Kit with PI (BioLegend, San Diego, CA, United States). The cells were analyzed by BD FACSCalibur (San Jose, CA, United States).

Statistical analysis

Data were expressed as mean ± standard deviation and were analyzed with unpaired *t* test and analysis of variance followed by a *post hoc t* test. Differences between proportions were assessed by the χ^2 test. Survival analysis was performed by Kaplan-Meier method. All the analyses were conducted using SPSS 17.0 software. Statistical significance was defined as $P < 0.05$.

Table 1 Expression of cycle-related and expression-elevated protein in tumor in colorectal benign tumor and malignant tumor

	CREPT expression			*P* value
	Low	Intermediate	High	
Benign tumor	1	6	6	0.035[a]
Malignant tumor	25	21	157	

[a]$P < 0.05$, CREPT: Cycle-related and expression-elevated protein in tumor.

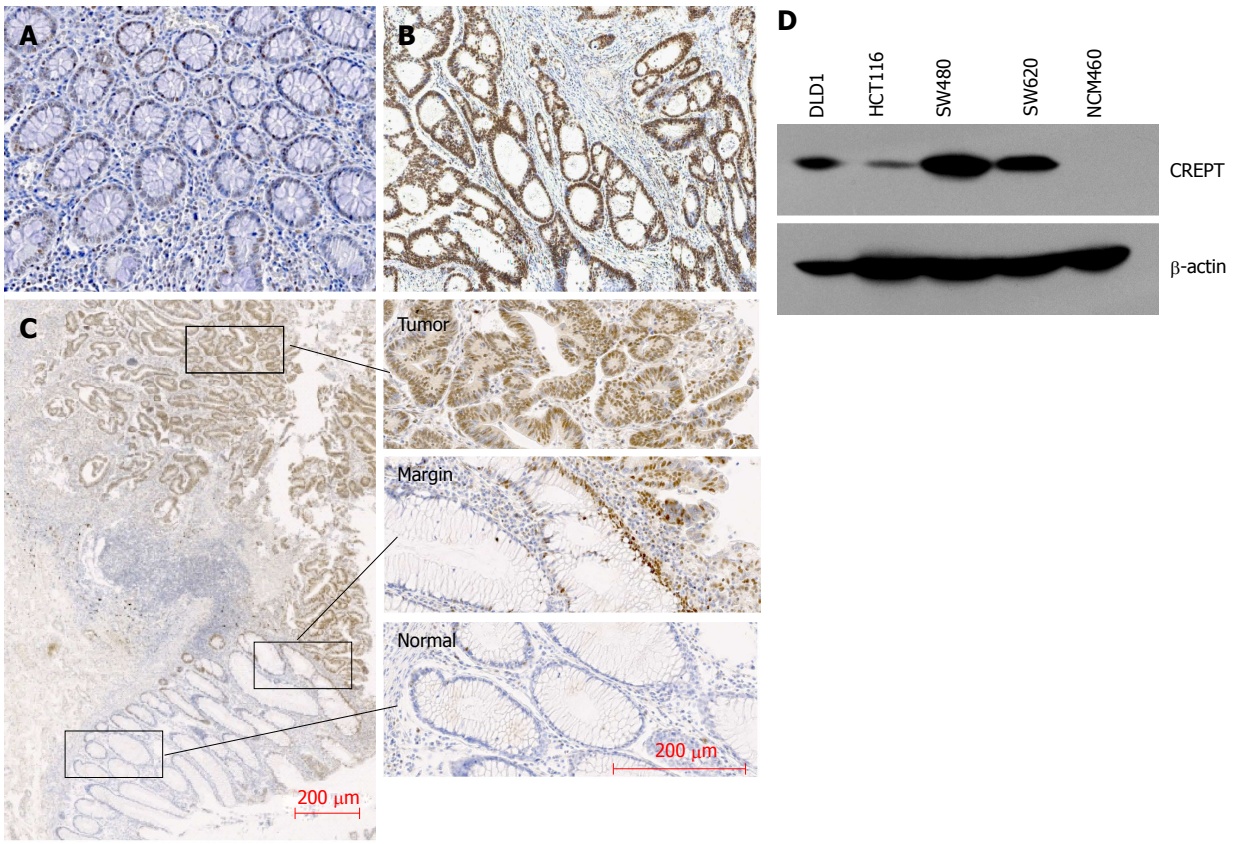


Figure 1 CREPT expression in tumor and adenoma tissues. A: Negative CREPT immunohistochemical staining in colorectal adenoma tissue; B: Positive CREPT immunohistochemical staining in CRC tissue; C: CREPT expression pattern in CRC sample; D: CREPT was determined by immunoblotting in CRC cell lines. CRC: Colorectal carcinoma.

RESULTS

Overexpression of CREPT correlated with clinicopathological features in CRC

To determine the expression pattern of CREPT in CRC clinical samples, 203 CRC tissue slides and 13 benign colorectal adenoma tissues as controls were collected for immunohistochemistry. A significant increase in CREPT was detected in the CRC tissues in comparison with benign tissues (77% *vs* 46%; Figure 1A and B, Table 1). Abundant expression of CREPT was observed in well-differentiated tumors compared to moderately and poorly differentiated tumors (Figure 2A and Table 2). The intensive staining signal was enriched in the malignant region, in contrast to the margin (the benign stromal tissue at the tumor periphery) and normal counterparts in the same slide (Figure 1C).

We analyzed expression of CREPT in CRC cell lines by western blotting (Figure 1D). NCM460, a normal human colon mucosal epithelial cell line, was used for comparative purpose. We did not detect CREPT protein in NCM460 cells. In contrast, CREPT levels were aberrantly upregulated in all four CRC cell lines examined: DLD1, HCT116, SW480 and SW620. Our in vitro expression analysis consolidated the observations from clinical samples.

Next, we attempted to analyze if CREPT had any correlation with clinicopathological features. Our data unambiguously demonstrated the positive association of high CREPT expression with pathological type ($P < 0.05$) and histological grade ($P < 0.005$) (Figure 2A and Tables 1 and 2). Our data suggested that CREPT expression was upregulated with CRC progression, which implicated a crucial role of CREPT in this disease.

CREPT stimulated cell proliferation and cell cycle in CRC cells

Our previous results characterized the aberrant high

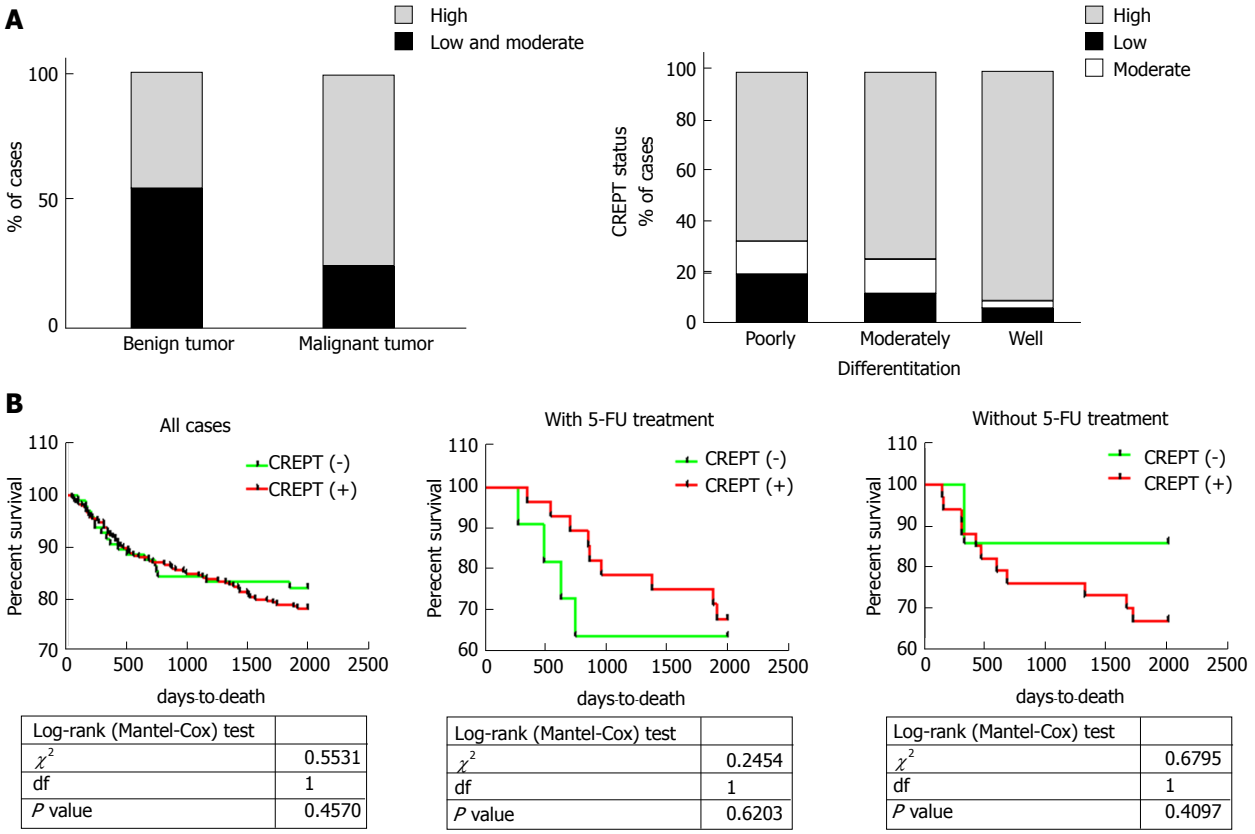


Figure 2 CREPT expression in CRC correlates with clinicopathological characteristics. A: CREPT expression level correlates with pathological type and tumor differentiation; B: Survival curve of CRC patients shows significant difference between patients with or without 5-FU-bases adjuvant chemotherapy. CRC: Colorectal carcinoma.

expression of CREPT in CRC both *in vitro* and *in vivo*. In view of the essential physiological role of CREPT in cell cycle modulation, we investigated whether CREPT was involved in cell proliferation and cell cycle regulation in CRC. To investigate whether CREPT had any influence on the viability of CRC cells, we constructed recombinant lentivirus lenti-HA-CREPT to generate stable overexpression cell lines, and the lentivirus lenti-sh-CREPT to knock down endogenous expression of CREPT. SW620 and DLD1 cells were infected with lentivirus. Both the ectopic expression of CREPT and knockdown efficiency were evaluated by immunoblotting (Figure 3A). Our results confirmed establishment of stable cell lines for further analysis.

Cell viability was determined using CCK-8 assay. Forced expression of CREPT in SW620 cells significantly promoted cell growth, while cell viability was markedly suppressed by CREPT depletion in DLD1 cells (Figure 3B and C). Previous studies have indicated that CREPT affects G1 to S phase transition[10]. In line with this notion, our cell cycle analysis by flow cytometry clearly demonstrated an increase of S and decrease of G phase cells upon exogenous expression of CREPT in DLD1 cells (Figure 3D). All the results suggested that CREPT overexpression played a critical role in stimulation of cell proliferation and the cell cycle in CRC cell lines, which might underlie its oncogenic potential in this disease.

Overexpression of CREPT sensitized CRC cells to 5-FU-induced apoptosis

The aforementioned data demonstrated the aberrant overexpression and oncogenic activity of CREPT *via* promoting cell proliferation and the cell cycle. We investigated whether high expression of CREPT was linked to chemotherapy resistance, especially for 5-FU. We retrieved the relevant data from the Cancer Genome Atlas (TCGA) database, and there was a trend that abundance of CREPT was a favorable indicator for CRC patients who received 5-FU-based chemotherapy (Figure 2B). Therefore, we set out to validate this observation in our *in vitro* system.

We measured the cytotoxic effect of 5-FU in CREPT-silenced DLD1 cells using the CCK-8 method. Knockdown of CREPT markedly suppressed cell proliferation. However, the cell viability of CREPT-silenced DLD1 cells was significantly increased in comparison with control cells in response to 5-FU (50 μg/mL) treatment, which suggested that drug resistance was induced by CREPT deficiency (Figure 4A). We showed that 5-FU elicited dramatic apoptosis in DLD1 cells, while this cytotoxic effect was significantly compromised upon CREPT knockdown (Figure 4B and D).

All these results implied a close link between CREPT expression and 5-FU sensitivity in CRC cells. This observation was consolidated through apoptotic

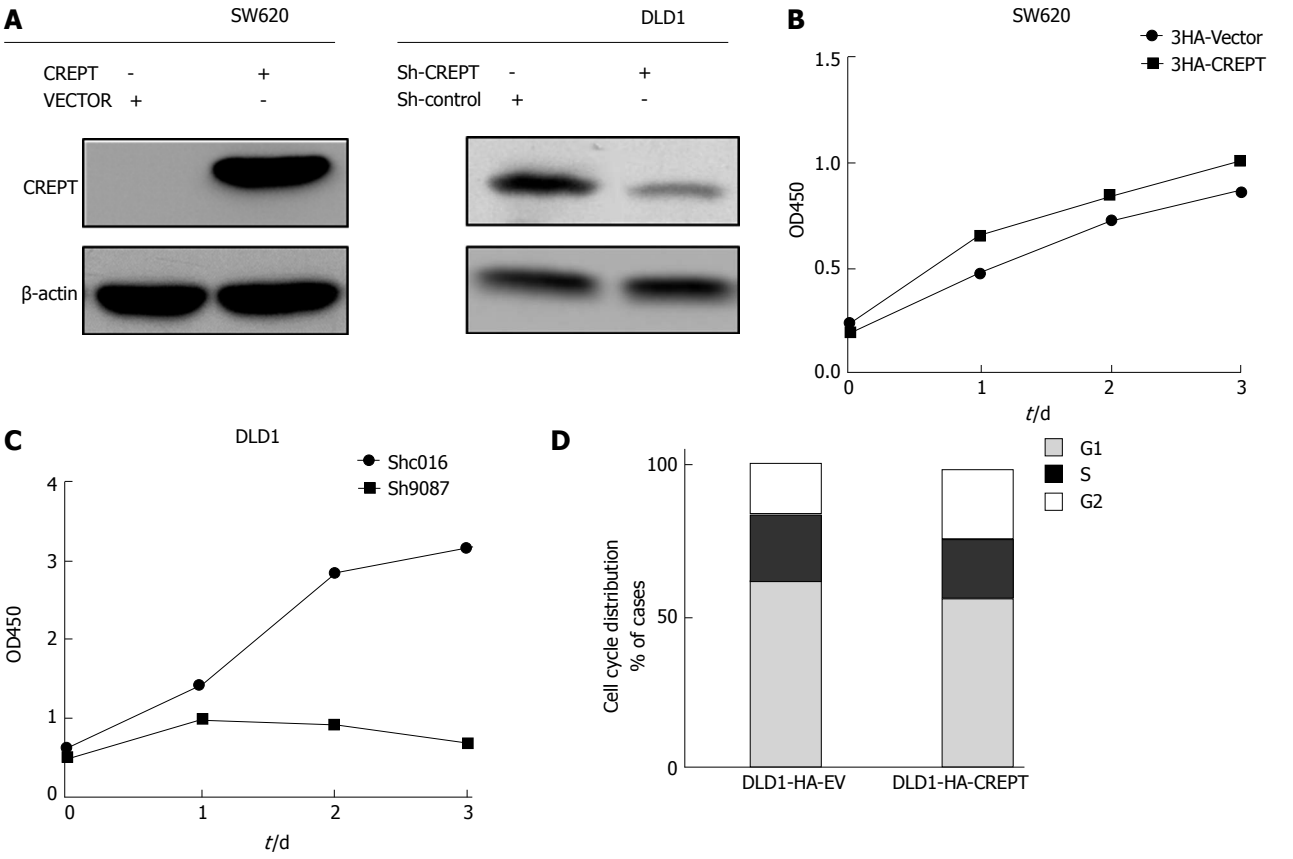


Figure 3 Alteration of CREPT expression in CRC cells affects cell proliferation. A: Western blotting showed that expression of CREPT in SW620 and DLD1 changed after exposed to virus for 48 h; B and C: SW620 and DLD1 cells were incubated in 96-well plates, and CCK-8 assay determined cell viability; D: Cell cycle was detected by flow cytometry and indicated that CREPT accelerated cells through the G1/S check point. CRC: Colorectal carcinoma.

Table 2 Expression of cycle-related and expression-elevated protein in tumor in colorectal cancer according to clinicopathological parameters

	CREPT expression			*P*-value
	Low	Intermediate	High	
Tumor				
T1	2	0	4	0.770
T2	3	2	25	
T3	6	2	20	
T4	14	17	108	
Stage				
Ⅰ	4	2	23	0.700
Ⅱ	11	12	64	
Ⅲ	8	3	56	
Ⅳ	2	4	14	
Histological grade				
Poor	9	6	31	0.004[b]
Moderate	12	13	71	
Well	4	2	55	
Lymph node metastasis				
Negative	15	15	92	0.487
Positive	10	6	65	

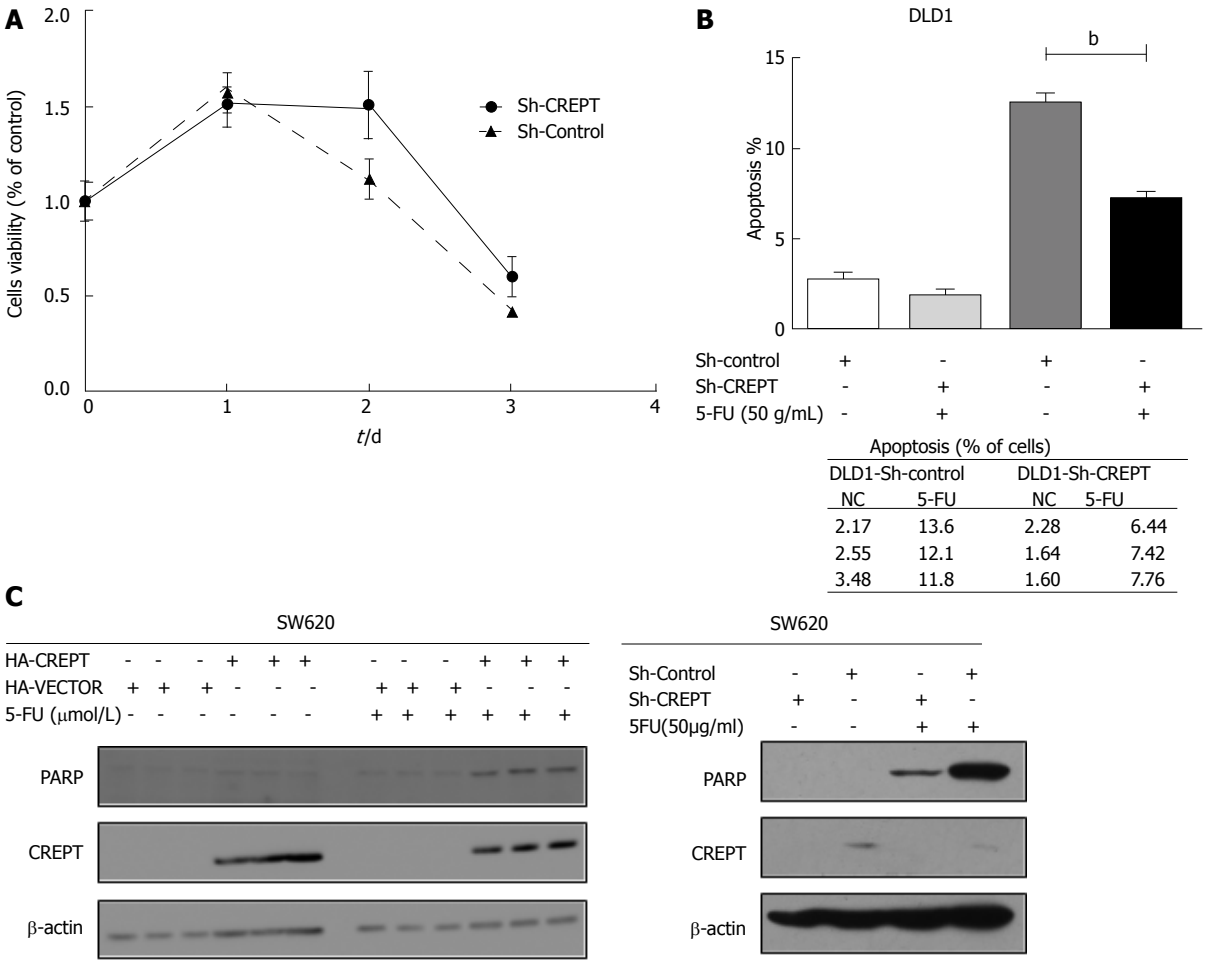
[b]$P < 0.05$, CREPT: Cycle-related and expression-elevated protein in tumor. CRC: Colorectal cancer.

pathway analysis in CREPT-manipulated SW620 and DLD cells. 5-FU treatment stimulated a significantly higher level of poly (ADP-ribose) polymerase (PARP) in CREPT-expressing SW620 cells than in the control ones. Consistently, the PARP level was markedly decreased in CREPT-silenced DLD cells upon 5-FU treatment (Figure 4C). All the results clearly demonstrated that CREPT conferred cells sensitivity to 5-FU *in vitro*.

DISCUSSION

CREPT protein is essentially a transcription regulator that acts *via* modulation of expression of multiple cell cycle-related factors[9]. For example, our previous study indicated that CREPT enhances expression of cyclin D1 by promoting RNA polymerase Ⅱ recycling during transcription of this gene[7]. A later study showed that CREPT promotes transcriptional activity of the β-catenin/TCF4 (transcription factor 4) complex and in turn enhances the Wnt signaling pathway as well[8]. The Wnt pathway consequently regulates diverse biological processes, including cell proliferation, survival, migration and polarity[8].

Accumulating evidence indicates the oncogenic activity associated with aberrant overexpression of CREPT in various human malignances. For instance, Wang *et al*[9] demonstrated that CREPT promotes tumor growth by accelerating the cell cycle in endometrial cancer. Zhang *et al*[8] reported that CREPT is highly expressed in tumors and enhances the β-catenin/TCF4 transcriptional activity in response to Wnt signaling.



Apoptosis (% of cells)

DLD1-Sh-control		DLD1-Sh-CREPT	
NC	5-FU	NC	5-FU
2.17	13.6	2.28	6.44
2.55	12.1	1.64	7.42
3.48	11.8	1.60	7.76

Figure 4 CREPT facilitates CRC cell response to 5-FU chemotherapy. A: Cells were treated with 5-FU (50 μg/mL), and CCK-8 assay estimated their viability; B: Annexin V-PI apoptosis detection show that DLD1 cells were less sensitive to 5-FU chemotherapy when CREPT was knocked down; C: SW620 and DLD1 cells were harvested after exposure to 5-FU (50 μg/mL) for 48 h, and western blotting showed that PARP was highly expressed in cells that expressed more CREPT. CRC: Colorectal carcinoma.

She *et al*[11] suggested that CREPT expression correlates with poor prognosis in patients with retroperitoneal leiomyosarcoma. Similarly, high expression of CREPT in CRC promotes tumor growth and is correlated with poor prognosis[12]. Liu *et al*[13] demonstrated that inhibition of CREPT reduces proliferation and migration of non-small cell lung cancer cells by downregulating cell cycle-related proteins.

Consistent with all these reports, here we demonstrated that CREPT is highly expressed in both CRC tissues and cell lines, and is intimately linked to the pathological stage. Although there was no correlation between overall survival and CREPT expression in any of the CRC cases, stratification into non- and 5-FU treatment groups revealed a significant difference in respect to CREPT status. In line with its well-established role in cell cycle modulation, we further elucidated that CREPT promoted cell growth and accelerated the cell cycle in both CRC cell lines. Consistent with the results from TCGA database analysis, our *in vitro* experiments consolidated that CREPT level was positively associated with sensitivity to 5-FU.

5-FU is the mainstay chemotherapy drug for clinical treatment of CRC. However, only 5%-10% of all CRC patients manifested a favorable response to 5-FU-based regimens, whereas the majority had apparent drug resistance[5]. Several mechanisms underlying the refractory effect have been elucidated. For example, the elevated expression of DNA repair gene ERCC6 confers resistance to 5-FU and is associated with poor patient survival in CRC[14]. Liu *et al*[15] demonstrated that epigenetic silencing of ASPP1 confers 5-FU resistance in clear cell renal carcinoma by preventing p53 activation. In addition, overexpression of long noncoding RNA UCA1 is related to multidrug resistance including 5-FU and cisplatin[16]. The miRNA miR-1290 functions as a biomarker in DNA mismatch repair-deficient colon cancer and promotes resistance to 5-FU by directly targeting hMSH2[17].

Several strategies have been exploited to surmount the resistance developed in response to clinical use of 5-FU. The synthesized peptide of SPARC (secreted

protein acidic and rich in cysteine) interferes with the interaction between caspase 8 and Bcl2 to resensitize chemo-resistant tumors and enhance their regression *in vivo*[18]. With respect to miRNAs, overexpression of miR-122 resensitizes 5-FU-resistant colon cancer cells through inhibition of PKM2 *in vitro* and *in vivo*[19]. Moreover, the chemotherapy response is associated with subsets of tumor-infiltrating lymphocytes in gastric cancer[20]. Together with all these results, here we provide novel evidence that overexpression of CREPT confers sensitivity to 5-FU on CRC cells, which suggests that CREPT has great potential as a prognostic biomarker for clinical application of 5-FU. Therefore, we proposed that relative expression of CREPT in CRC tissues should be determined during biopsy and could serve as prerequisite for the decision to use 5-FU.

Although 5-FU exerts its maximum therapeutic outcome in CRC, it is a broad-spectrum antitumor drug. In the first-line chemotherapy of breast and gastric cancer, 5-FU plays an irreplaceable role. According to our previous study, CREPT has a similar effect on CRC, breast and gastric cancer. This suggests that CREPT is a potential chemotherapy sensitivity indicator in these cancers and further research to verify this is needed.

Despite the well-acknowledged oncogenic role of CREPT in a range of human cancers, here we indicate that high expression of CREPT is favorable in respect to 5-FU administration. However, the molecular events underlying the drug sensitivity induced by CREPT are still elusive. In view of its nature as a transcription regulator of multiple cell cycle-related factors, we hypothesize that CREPT accelerates the cell cycle and exacerbates thymineless death in CRC cells in response to 5-FU challenge. Beyond this study, the issue still to be addressed is how CREPT is upregulated in CRC. Cui *et al*[21] reported that miR-1188 at the imprinted Dlk1-Dio3 domain acts as a tumor suppressor in hepatoma cells and suppresses CREPT expression, which sheds light on the regulatory mechanism underlying the overexpression of CREPT in CRC.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is the third leading cancer and the third most frequent cause of cancer-related death in the United States. Cell cycle-related and expression-elevated protein in tumor (CREPT) is preferentially expressed in many kinds of carcinomas. However, the correlation between CREPT and CRC clinicopathological patterns remains unclear. Study of the impact of CREPT expression on the anticancer drug 5-fluorouracil (5-FU) resistance in CRC has been limited.

Research motivation

We investigated the expression pattern of CREPT in CRC and explored if CREPT rendered CRC cells sensitive to 5-FU.

Research objectives

We investigated the expression pattern of CREPT in CRC. To the best of our knowledge, this is the first study to explore the correlation between CREPT and CRC cell sensitivity to 5-FU. Our results lead us to consider CREPT as a potential chemotherapy predictive biomarker. Moreover, further study on the impact of CREPT on chemotherapy outcome in other cancers and on other antitumor drugs is needed.

Research methods

We analyzed tissue sections from 203 primary CRC patients and 13 benign colorectal adenoma patients using immunohistochemistry with anti-CREPT antibody. CREPT overexpressing/knockdown cell lines were established by lentivirus infection. Expression of CREPT in these cell lines was analyzed by western blotting and the cell viability was measured by CCK-8 assay. The cell lines were subjected to 5-FU treatment. The cytotoxic effect of 5-FU was measured by CCK-8 assay and poly (ADP-ribose) polymerase/flow cytometry analysis.

Research results

CREPT expression correlates with clinicopathological features in CRC. CREPT was abundantly expressed in CRC tissues compared with benign tissues. A significant increase in CREPT was detected in more highly differentiated tumors. The intensive staining signal was enriched in the malignant region in contrast to the margin and normal counterparts in the same slide. CREPT stimulated cell proliferation and the cell cycle in CRC cells. Cell growth was significantly enhanced when CREPT was overexpressed *via* exogenous transfection, while CREPT depletion markedly suppressed cell viability. Overexpression of CREPT sensitized CRC cells to 5-FU-induced apoptosis. Knock down of CREPT markedly suppressed cell proliferation. However, viability of CREPT-silenced DLD1 cells was significantly increased in comparison with control cells in response to 5-FU treatment, indicating that drug resistance was induced by CREPT deficiency. 5-FU elicited dramatic apoptosis in DLD1 cells.

Research conclusions

The impact of CREPT on CRC cell response to 5-FU was identified for the first time. We hypothesize that this phenomenon is attributed to an accelerated cell cycle induced by high expression of CREPT. However, the mechanism of this finding requires further study. Clinically, biomarkers for chemotherapy efficacy prediction are urgently needed and this research provides a candidate.

Research perspectives

There were a few limitations to this study. For example, compared to a public database, first-hand follow-up data of patients are more convincing. For the future, we are working on animal experiments to verify our findings *in vivo*. Then, we will investigate the mechanisms of action of CREPT, and the possibility of clinical application of CREPT as a prognostic indicator.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Early prediction of survival in hepatocellular carcinoma patients treated with transarterial chemoembolization plus sorafenib

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Abstract

AIM

To identify clinical biomarkers that could early predict improved survival in patients with advanced-

stage hepatocellular carcinoma (HCC) treated with transarterial chemoembolization combined with sorafenib (TACE-S).

METHODS

We retrospectively evaluated the medical records of consecutive patients with advanced-stage HCC who underwent TACE-S from January 2012 to December 2015. At the first follow-up 4-6 wk after TACE-S (median, 38 d; range, 33-45 d), patients exhibiting the modified Response Evaluation Criteria in Solid Tumors (mRECIST)-evaluated complete response, partial response, and stable disease were categorized as early disease control. At this time point, multiple variables were analyzed to identify the related factors affecting survival.

RESULTS

Ninety-five patients were included in this study, and 60 of these patients achieved early disease control, with an overall disease control rate (DCR) of 63.2%. Patients who got sorafenib at the first TACE (no previous TACE) and patients without portal vein tumor thrombus (PVTT) had a higher DCR than those who underwent previous TACE before TACE-S (72.4% *vs* 48.6%, $P = 0.019$) and those with PVTT (75.5% *vs* 50.0%, $P = 0.010$). Early disease control after TACE-S, no previous TACE, and no PVTT were the independent prognostic factors for survival in the uni- and multivariate analyses.

CONCLUSION

The first follow-up 4-6 wk after TACE-S can be used as the earliest time point to assess the response to TACE-S, and patients with mRECIST-evaluated early disease control, no previous TACE, and no PVTT had better survival.

Key words: Hepatocellular carcinoma; Transarterial chemoembolization; Sorafenib; Survival; Prognosis

Core tip: There are no clinical data/markers to early predict improved survival in patients with advanced-stage hepatocellular carcinoma treated with transarterial chemoembolization combined with sorafenib (TACE-S). In this study, we found that mRECIST-evaluated disease control (complete response, partial response, and stable disease) at the first follow-up 4-6 wk after TACE-S can be used as an early indicator of better survival from TACE-S. We also found that patients with previous TACE and portal vein tumor thrombus had a poor survival.

Meng XC, Chen BH, Huang JJ, Huang WS, Cai MY, Zhou JW, Guo YJ, Zhu KS. Early prediction of survival in hepatocellular carcinoma patients treated with transarterial chemoembolization plus sorafenib. *World J Gastroenterol* 2018; 24(4): 484-493 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i4/484.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i4.484

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the fourth most prevalent cause of tumor-related deaths[1-4]. Although the surveillance programs for the early detection of HCC have been recommended to high-risk populations, some HCC patients are still diagnosed at an advanced stage, with vascular invasion or distant metastasis. The prognosis of patients with advanced-stage HCC is very poor, with a very short median survival time (less than 6 mo)[5-7]. The Barcelona Clinic Liver Cancer (BCLC) group recommended the tyrosine kinase inhibitor sorafenib as a standard therapy for patients with advanced-stage HCC (BCLC stage C)[8-10]. However, the tumor response rate to sorafenib monotherapy is modest with survival prolonged only for less than three months compared with placebo[9,10]. Recently, a new treatment modality, the combination of delaying intrahepatic tumor progression with transarterial chemoembolization (TACE) and targeting systemic disease (*e.g.*, vascular invasion or extrahepatic metastasis) with sorafenib, is recommended as an alternative for patients with advanced-stage HCC[11-13], and indeed, some studies have demonstrated favorable safety profiles and survival benefits conferred by TACE combined with sorafenib (hereafter, TACE-S)[12-17].

The first follow-up assessment after TACE-S, usually at 4-6 wk after TACE-S, which is considered the earliest assessment time point, may directly guide the decisions about subsequent therapies. However, to date, there has been no specific baseline or clinical biomarker (clinical, radiologic, and/or biochemical) used at the first follow-up assessment to identify those patients who would benefit most from this combination treatment. The modified Response Evaluation Criteria in Solid Tumors (mRECIST) has been proposed for assessing the response to therapy in patients with HCC[18-22]. Indeed, some studies have demonstrated that patients with mRECIST objective responses [complete response (CR) and partial response (PR)] to TACE alone at the first follow-up assessment 4-6 wk after TACE have better survival[18,19]. However, what is the situation after combination therapy with TACE and sorafenib? As sorafenib is part of the combination therapy, the majority of sorafenib adverse events (AEs) appear within the first month of sorafenib treatment. Zhao *et al*[14] demonstrated that ⩾ grade 2 early sorafenib-related dermatologic AEs within the first month of sorafenib initiation could determine the efficacy of TACE-S[23]. This finding implies that sorafenib has had an effect in targeting HCC cells and/or inhibiting tumor angiogenesis within the first month of sorafenib initiation. Thus, we speculated that patients obtaining survival benefits from the combination therapy may

include not only patients with mRECIST-evaluated CR and PR at 4-6 wk after TACE-S but also those patients with mRECIST-evaluated stable disease (SD) because sorafenib might have a tumor stabilizing effect in delaying tumor progression. In fact, in two phase Ⅲ randomized controlled trials of sorafenib in patients with advanced-stage HCC[9,10], the main benefit of sorafenib monotherapy is from the prolonged disease stabilization, which leads to improvement in overall survival (OS). Therefore, in the present study, we designed the first follow-up assessment at 4-6 wk after TACE-S as the earliest observation time point and included mRECIST-evaluated disease control (CR + PR + SD) as one of the early indicators for investigating which patients might benefit the most from TACE-S.

MATERIALS AND METHODS

Study design, patient population, and data collection

This study was a retrospective study in which patients with advanced-stage HCC (BCLC stage C) who had been treated with TACE-S between January 2012 and December 2015 were consecutively enrolled at our institution. HCC was diagnosed according to the non-invasive criteria following the European Association for the Study of Liver/American Association for the Study of Liver Disease guidelines[24]. The inclusion criteria for the study population were: (1) being between 18 and 75 years of age; (2) having an Eastern Cooperative Oncology Group (ECOG) performance status of 0-1; (3) having Child-Pugh class A or B liver disease; (4) having total bilirubin < 51 μmol/ L; and (5) having an abdominal and chest CT or magnetic resonance (MR) scan one week before treatment (at baseline), and by mRECIST criteria[25-27] having at least one target lesion that confirmed the diagnosis of HCC. Patients were excluded from this study if they: (1) had complete main portal vein obstruction without collateral circulation around the portal trunk; (2) had undergone radiofrequency ablation, surgery, or liver transplantation; (3) had undergone other treatments (radiofrequency ablation or ^{125}I seed implantation) besides TACE during this study; (4) had infiltrative lesions not suitable for imaging assessment; (5) had serious medical comorbidities; or (6) had current or a history of malignant tumors in addition to HCC. The study was approved by our institutional review board. Written informed consent was obtained from all patients before treatment.

TACE procedure

TACE was performed with a five-French catheter or microcatheter as selectively as possible through the lobar or segmental arteries, depending on the tumor distribution. Initially, a solution of lobaplatin at a concentration of 0.5 mg/mL was infused into the tumor feeder vessels. The total level of lobaplatin ranged from 20 to 50 mg depending on the patient's body weight. Then, an emulsion of 2-10 mL of lipiodol (Lipiodol Ultrafluido, Guerbet, France) and 20-60 mg of doxorubicin hydrochloride was administered into the feeder vessels. Finally, gelatin sponge particles or polyvinyl alcohol particles (Cook, Bloomington, IN, United States) that were mixed with contrast material were administered into the feeder vessels until stasis of arterial flow was achieved. After embolization, angiography was performed to determine the extent of vascular occlusion.

Sorafenib treatment

Sorafenib 400 mg was orally administered twice daily 3-5 d after TACE, and patients were treated with continuous sorafenib with no breaks before or after repeated TACE. Sorafenib dose reduction was based on the presence of toxicity. If grade 3/4 hematological toxicity, skin toxicity, gastrointestinal toxicity, hypertension, or hepatic dysfunction defined by the National Cancer Institute's Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 4.03 occurred[28], a dose adjustment (400 mg once daily) was required until the AEs were alleviated or eliminated. After dose adjustment, if grade 3/4 AEs continued, sorafenib treatment was halted until the adverse effects were alleviated or disappeared.

Follow-up and repeated TACE

All patients treated in our institution for HCC required follow-up according to our institutional protocol. Each follow-up session included a detailed history and physical examination, laboratory tests, and abdominal contrast material-enhanced three-phase dynamic spiral CT or MR imaging. Laboratory tests included hematological and biochemical analyses, such as complete blood cell count, prothrombin time, α-fetoprotein, aspartate aminotransferase, alanine aminotransferase, total bilirubin, serum albumin, and creatinine. Follow-up of all patients was conducted at a 4-6-wk interval after previous TACE. Patients with intrahepatic residual viable tumor or recurrent tumor on CT/MR imaging underwent repeated TACE, if the Child-Pugh status remained at class A or B and there was no evidence of hepatic decompensation (*e.g.*, uncontrolled ascites or hepatic encephalopathy).

Assessments

The clinical, laboratory, and radiologic records were reviewed. Side effects of sorafenib and TACE were reported according to NCI-CTCAE version 4.03[28]. Follow-up contrast-enhanced CT or MR was performed 4-6 wk after previous TACE to assess the tumor response and to guide timely decision-making for subsequent therapies. Tumor response was assessed according to the overall mRECIST[25-27], which included a combined assessment of target lesions, nontarget lesions, and new lesions. At baseline, measurable lesions with diameters 1 cm or greater, suitability for repeat

Table 1 Baseline patient characteristics and comparison of disease control rate between different baseline characteristics

Characteristic	Overall (n = 95)	CR + PR + SD (n = 60)	DCR (63.2)	P value[2]
Sex				1.000
Male	88	56	63.6	
Female	7	4	57.1	
Age (yr)[1]	48.2 ± 11			0.390
< 60	80	52	65	
⩾ 60	15	8	53.3	
α-fetoprotein level (ng/mL)				0.822
< 400	42	26	61.9	
⩾ 400	53	34	64.2	
ECOG performance				0.752
0	66	41	62.1	
1	29	19	65.5	
Hepatitis B				0.745
No	5	4	80	
Yes	90	56	62.2	
Previous TACE				0.019
No	58	42	72.4	
Yes	37	18	48.6	
Ascites				0.719
Absent	52	32	61.5	
Present	43	28	65.1	
Child-Pugh classification				0.373
A	80	49	61.3	
B	15	11	73.3	
PVTT				0.010
Absent	49	37	75.5	
Present	46	23	50	
Extrahepatic metastasis				0.103
No	69	47	68.1	
Yes	26	13	50	
Number of tumor				0.952
1	16	10	62.5	
⩾ 2	79	50	63.3	
Maximum tumor diameter (cm)[1]	9.5 ± 4.5			1.000
⩽ 3	9	6	66.7	
> 3	86	54	62.8	

[1]Data are mean ± SD; [2]Determined by χ^2 test. CR: Complete response; PR: Partial response; SD: Stable disease; DCR: Disease control rate; ECOG: Eastern Cooperative Oncology Group; TACE: Transarterial chemoembolization; PVTT: Portal vein tumor thrombus; BCLC: Barcelona Clinic Liver Cancer.

measurement, and intratumoral arterial enhancement on contrast-enhanced CT or MR imaging were qualified as target lesions. The longest diameter of the viable tumor (defined as the enhanced area during the arterial phase) was measured on contrast-enhanced CT or MR imaging. Non-enhancing atypical lesions and extrahepatic lesions were assessed using RECIST criteria. The presence or absence of nontarget lesions and the appearance of new lesions were assessed during follow-up. Overall responses were classified into the following four categories: CR, PR, SD, and progressive disease (PD). Patients exhibiting CR, PR, or SD at the first follow-up assessment 4-6 wk after TACE-S were categorized as early disease control, whereas those with PD were classified as non-early disease control. The early disease control rate (DCR) was defined as the percentage of patients who achieved CR, PR, and SD at 4-6 wk after TACE-S. Furthermore, we analyzed the OS. OS was calculated for all patients from the date of their first TACE, with or without sorafenib, until their death or the last follow-up.

Statistical analysis

All statistical analyses were performed using SPSS version 16.0 software. To determine significant differences in DCR between baseline characteristics, chi-square tests were used. OS was compared using Kaplan-Meier curves with the log-rank test. A Cox proportional hazards model was used to examine risk factors associated with survival. A two-tailed P-value less than 0.05 was considered statistically significant. Chen BH and Cai MY, who had learned about biostatistics, performed the statistical analyses together.

RESULTS

Study population

Of the 164 patients initially recruited, 69 were excluded from the study because they met the exclusion criteria (Figure 1). Ultimately, 95 HCC patients were enrolled in this study. The detailed baseline characteristics of these patients are summarized in Table 1. The

Table 2 Univariate Cox proportional hazards regression analysis for overall survival

Factor	HR (95%CI)	*P* value
Sex (Male/Female)	1/0.723 (0.314-1.666)	0.446
Age (< 60/≥ 60 yr)	1/1.233 (0.698-2.179)	0.470
α-fetoprotein (< 400/≥ 400 ng/mL)	1/1.279 (0.821-1.995)	0.277
ECOG performance (0/1)	1/1.058 (0.645-1.735)	0.824
Hepatitis B (No/Yes)	1/2.665 (0.653-10.874)	0.172
Previous TACE (No/Yes)	1/2.997 (1.831-4.903)	< 0.001
Ascites (Absent/ Present)	1/1.440 (0.922-2.250)	0.109
Child-Pugh classification (A/B)	1/1.342 (0.751-2.400)	0.321
PVTT (Absent/ Present)	1/2.678 (1.697-4.227)	< 0.001
Absent	1.000	
Main PVTT	19.206 (8.436-43.727)	< 0.001
Branch PVTT	2.246 (1.386-3.639)	0.001
Extrahepatic metastasis (No/Yes)	1/1.910 (1.182-3.087)	0.008
Number of tumor (1/≥ 2)	1/1.125 (0.620-2.043)	0.698
Maximum tumor diameter (≤ 3/> 3 cm)	1/1.029 (0.472-2.244)	0.944
Early disease control (No/Yes)	1/0.362 (0.227-0.577)	< 0.001

HR: Hazard ratio; CI: Confidence interval; ECOG: Eastern Cooperative Oncology Group; TACE: Transarterial chemoembolization; PVTT: Portal vein tumor thrombus; BCLC: Barcelona Clinic Liver Cancer.

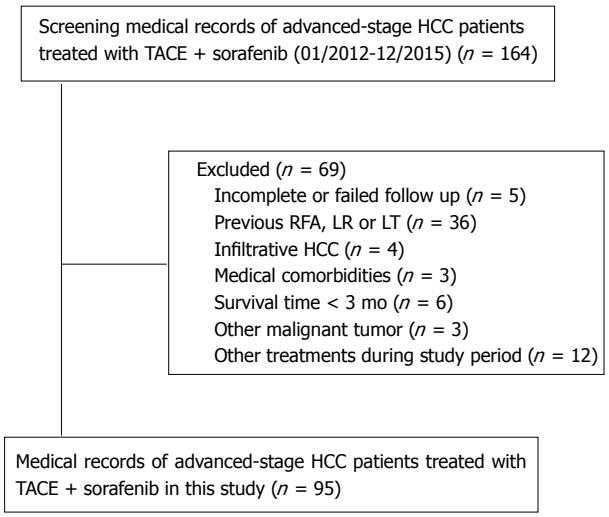

Figure 1 Flow diagram shows the selection of hepatocellular carcinoma patients. HCC: Hepatocellular carcinoma; RFA: Radiofrequency ablation; LR: Liver resection; LT: Liver transplantation; TACE: Transarterial chemoembolization.

patient population consisted of 88 (92.6%) men with an age range of 19-73 years (mean, 48.2 years). Among 95 patients who received TACE-S, 58 (61.1%) got sorafenib therapy 3-5 d after the first TACE (no previous TACE); the remaining 37 (38.9%) patients, who had undergone one or more TACE treatments before TACE-S (previous TACE), received the combination of TACE and sorafenib because of tumor progression after previous TACE. Ninety (94.7%) patients presented with hepatitis B, and 80 (84.2%) patients were classified with Child-Pugh A disease. Forty-six (48.4%) patients had PVTT, including 36 (37.9%) at the portal vein branch and 10 (10.5%) at the main portal vein. Twenty-six (27.4%) patients had extrahepatic metastasis, including 14 patients with extrahepatic metastasis in the lymph nodes, 7 patients in the lung, 3 patients in the bones, and 2 patients in the suprarenal gland. The mean duration of follow-up was 14.6 mo (range, 2-28 mo). The median duration of sorafenib treatment was 13.1 mo (range, 2-26 mo). Eighty-six of the 95 patients (90.5%) underwent repeated TACE after TACE-S, with the mean number of TACE procedures per patient of 3.1 (range, 1-5).

DCR

Among all the 95 patients, 3 (3.2%) achieved CR, 35 (36.8%) achieved PR, and 22 (23.2%) achieved SD at 4-6 wk (median, 38 d; range, 33-45 d) after TACE-S. Thus, a total of 60 patients achieved early disease control (CR + PR + SD), with an overall DCR of 63.2%. The basic characteristics of these 60 patients are shown in Table 1. It was observed that patients who got sorafenib at the first TACE (no previous TACE) had higher DCR than those who underwent one or more TACE treatments before TACE-S (DCR: 72.4% *vs* 48.6%; $P = 0.019$). Similarly, patients without PVTT had higher DCR than those with PVTT (DCR: 75.5% *vs* 50.0%; $P = 0.010$).

OS

Eighty-one (85.3%) of the 95 patients died during the observation period. The 0.5-, 1-, and 2-year cumulative OS rates were 89.5%, 51.3%, and 16.2%, respectively, and the median OS was 12.7 mo (95%CI: 9.4-15.9 mo).

The univariate Cox proportional hazards regression analysis revealed that no previous TACE, the absence of PVTT, the absence of extrahepatic metastasis, and early disease control were significantly associated with a better OS (Table 2). Based on these findings, previous TACE, PVTT, extrahepatic metastasis, and early disease control were included in the multivariate analysis. The multivariate Cox proportional hazards regression analysis found that previous TACE, PVTT,

Table 3 Multivariate Cox proportional hazards regression analysis for overall survival

Factor	HR (95%CI)	*P* value
Previous TACE		
No	1	
Yes	2.552 (1.477-4.412)	0.001
PVTT		
Absent	1	
Present	2.582 (1.608-4.146)	< 0.001
Early disease control		
No	1	
Yes	0.564 (0.339-0.936)	0.027
Extrahepatic metastasis		
No	1	
Yes	1.193 (0.680-2.092)	0. 538

HR: Hazard ratio; CI: Confidence interval; PVTT: Portal vein tumor thrombus; TACE: Transarterial chemoembolization.

Table 4 Adverse events related to sorafenib

Adverse event	All events	Grade 1-2 events	Grade 3 or higher events
Hand-foot skin reactions	78 (82.1)	67 (70.5)	11 (11.6)
Diarrhea	71 (74.7)	62 (65.3)	9 (9.5)
Hypertension	10 (10.5)	8 (8.4)	2 (2.1)
Alopecia	29 (30.5)	29 (30.5)	0
Fatigue	29 (30.5)	29 (30.5)	0
Voice change	1 (1.1)	1 (1.1)	0
Gastrointestinal hemorrhage	7 (7.4)	0	7 (7.4)
Epistaxis	1 (1.1)	1 (1.1)	0

Data shown are number of events. Percentages are in parentheses and were calculated by using number of patients as denominator *n* (%).

and early disease control were identified as independent prognostic factors for OS (Table 3).

Based on the above three factors, Kaplan-Meier survival curves were analyzed (Figure 2A-C). The median OS of patients who got sorafenib at the first TACE (no previous TACE) was 14.9 mo (95%CI: 12.4-17.4 mo), which was significantly longer than the 9.1 mo (95%CI: 7.8-10.3 mo) observed for patients who had received previous TACE (Figure 2A) ($P < 0.001$). The median OS of patients without PVTT was 15.4 mo (95%CI: 11.9-19.1 mo), which was significantly longer than the 8.9 mo (95%CI: 7.9-9.9 mo) observed for patients with PVTT (Figure 2B) ($P < 0.001$). The median OS of patients with early disease control after combined therapy was 15.5 mo (95%CI: 13.7-17.3 mo), which was significantly longer than the 9.1 mo (95%CI: 7.9-10.2 mo) observed for patients without early disease control after combined therapy (Figure 2C) ($P < 0.001$).

OS in patients with different types of PVTT

The median OS of patients without PVTT was 15.4 mo (95%CI: 11.9-19.1 mo), which was longer than the 4.3 mo (95%CI: 3.8-4.9 mo) observed for patients with main PVTT ($P < 0.001$) and the 9.7 mo (95%CI: 9.2-10.2 mo) observed for patients with branch PVTT ($P = 0.001$). There were also significant differences in OS between the patients with main PVTT and patients with branch PVTT ($P < 0.001$) (Figure 2D).

Table 5 Adverse events related to transarterial chemoembolization

Adverse event	All events	Grade 1-2 events	Grade 3 or higher events
New ascites	25 (26.3)	18 (19.0)	7 (7.3)
Liver dysfunction	30 (31.6)	22 (23.2)	8 (8.4)
Pleural effusion	10 (10.5)	8 (8.4)	2 (2.1)
Spontaneous bacterial peritonitis	6 (6.3)	3 (3.2)	3 (3.2)
Gastrointestinal hemorrhage	6 (6.3)		6 (6.3)
Inguinal haematoma	5 (5.3)	5 (5.3)	
Hepatorenal syndrome	1 (1.1)		1 (1.1)
Ischemic cholecystitis	1 (1.1)		1 (1.1)

Data shown are number of events. Percentages are in parentheses and were calculated by using number of patients as denominator *n* (%).

Treatment-related adverse reactions

The most common AEs after sorafenib treatment observed in this study (Table 4) were hand-foot skin reaction (82.1%), diarrhea (74.7%), alopecia (30.5%), and fatigue (30.5%). Most of these adverse reactions were grade 1/2. Grade 3/4 AEs occurred in 29 (30.9%) patients, all of whom required sorafenib dose reductions or interruption. The sorafenib dose was reduced to 400 mg once daily for grade 3 hand-foot skin reactions in 11 (11.6%) patients, grade 3/4 diarrhea in 9 (9.5%) patients, and grade 3/4 hypertension in 2 (2.1%) patients. There were 7 (7.4%) patients with interrupted sorafenib for gastrointestinal hemorrhage. Common AEs associated with TACE in the combination treatment (Table 5) were liver dysfunction (31.6%), new ascites (26.3%), and pleural effusion (10.5%). Most of these AEs were well tolerated because they were grade 1/2 adverse reactions to TACE, suggesting that combination therapy does not increase TACE-related adverse reactions. No treatment-related deaths occurred in this study.

DISCUSSION

In this retrospective study, we found that previous TACE, PVTT, and mRECIST-evaluated disease control (CR, PR, and SD) at the first follow-up assessment 4-6 wk after TACE-S were independent prognostic factors for OS. To the best of our knowledge, this is the first study to use the first follow-up assessment 4-6 wk after TACE-S as the earliest observation time point to predict survival in patients with advanced-stage HCC treated with TACE-S therapy. In prior reports, Prajapati *et al*[19] and Gillmore *et al*[20] demonstrated that patients with mRECIST-evaluated objective responses (CR and PR) at the first follow-up assessment after TACE monotherapy had better survival. In our study,

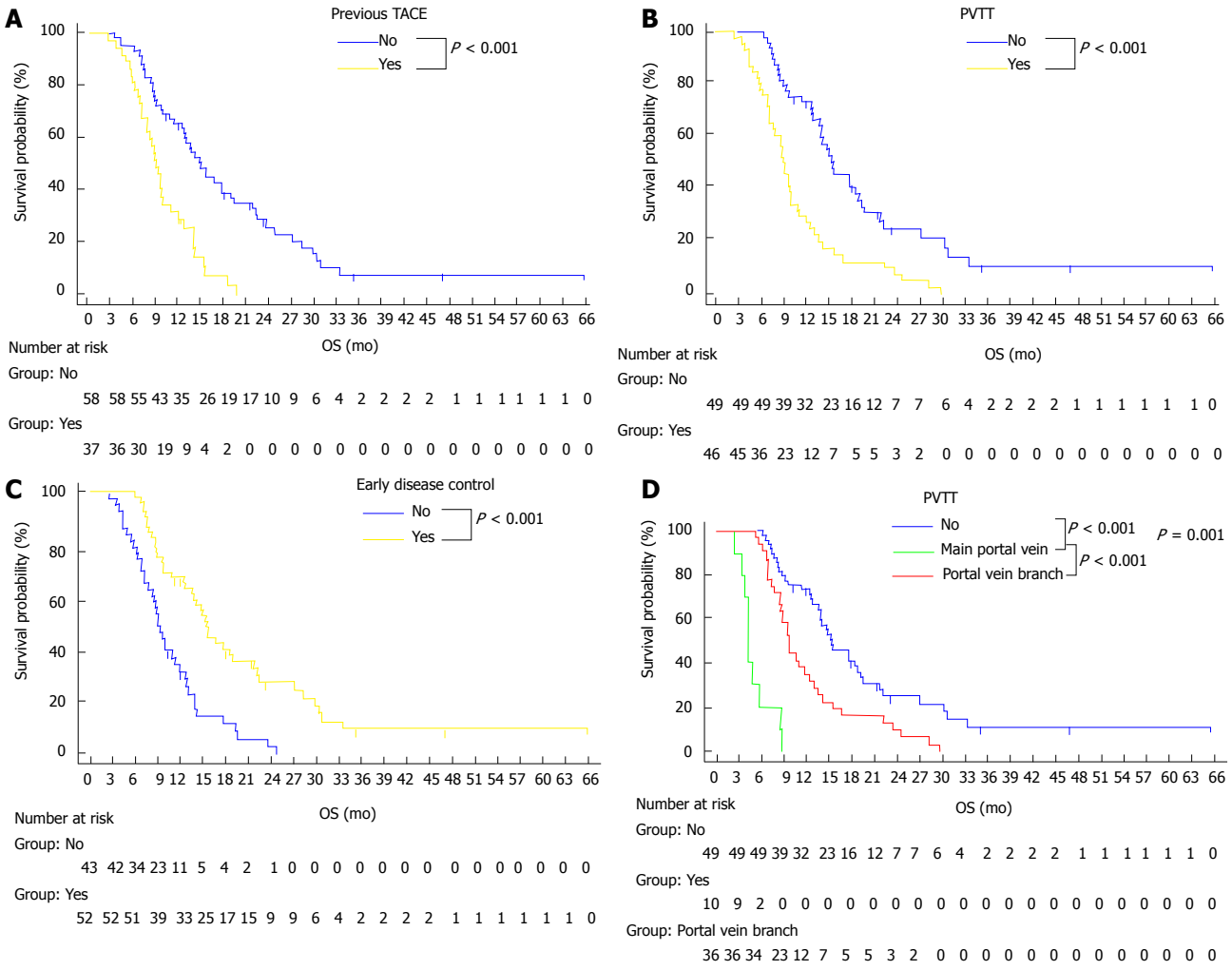

Figure 2 Kaplan-Meier curves of overall survival. A: Comparison of the survival between patients who had previous TACE (Yes group) and patients who had no previous TACE (No group); B: Comparison of the survival between patients who had portal vein tumor thrombus (PVTT) (Yes group) and patients who had no PVTT (No group); C: Comparison of the survival between patients who had early disease control (Yes group) and patients who had no early disease control (No group); D: Comparison of the survival among patients with different types of PVTT: the absence of PVTT (No group), PVTT in the main portal vein (main portal vein group) and PVTT in the portal vein branch (portal vein branch group).

we not only showed that mRECIST-evaluated responses (CR and PR) at the first follow-up assessment were associated with improved survival but also found that patients with mRECIST-evaluated SD had better survival from TACE-S therapy. These results imply that TACE may induce extensive intrahepatic tumor necrosis to reduce the tumor burden, whereas sorafenib may improve local tumor control by blocking HCC cell proliferation and/or inhibiting tumor angiogenesis[29], which may present as a tumor stabilizing agent that delays tumor progression. Our study further proved the results of sorafenib monotherapy in patients with advanced-stage HCC[9,10], and the survival benefit of sorafenib came mainly from the prolonged disease stabilization. Another study[30] supported our results and found a relationship between early tumor growth rate (eTGR) and OS in HCC patients who received sorafenib. eTGR was found to be an independent prognostic factor for OS, and eTGR in patients receiving sorafenib was significantly lower than that in patients receiving the placebo, indicating that sorafenib slowed tumor progression. Tumor shrinkage and tumor stabilization have similar OS outcomes. Our result further supported that SD is an important indicator for improving survival in patients with HCC who were treated with TACE-S.

Wang *et al*[22] combined mRECIST with dermatologic AEs to stratify prognosis in patients with unresectable HCC receiving TACE-S. They found that the earliest time at which mRECIST-evaluated objective responses (CR and PR) and dermatologic responses correlated with survival was 2 mo after TACE-S. Our results advanced the evaluation time point forward to the first follow-up assessment after TACE-S (median, 38 d; range: 33-45 d) and found that mRECIST-evaluated disease control (CR, PR, and SD) could be used as an indicator of better survival with TACE-S. Consequently, we believe that patients with mRECIST-evaluated CR, PR, and SD at the first follow-up assessment 4-6 wk after TACE-S should be considered candidates for continued TACE-S.

Importantly, our study further confirmed that

patients who underwent previous TACE treatment had lower survival than those who received timely sorafenib treatment 3-5 d after the first TACE. This result may be attributed to the low DCR in patients who underwent previous TACE. This low DCR indicated that the residual tumor or tumor progression after TACE may be more difficult to treat with TACE-S, possibly because TACE-induced residual tumor angiogenesis is difficultly controlled by TACE-S or resistant to repeated TACE. Arizumi *et al*[31] also noted that repeated TACE could cause tumor resistance to chemotherapy drugs, thereby increasing the risk of tumor recurrence and metastasis. Therefore, we believe that sorafenib should be orally administered early after the first TACE, which may lead to a greater survival benefit.

PVTT has a profound adverse effect on prognosis, resulting in a very short median survival time (2-4 mo)[5,32]. In our study, the classification of PVTT played an important role in determining disease outcomes, and those patients with main PVTT had worse survival than those with branch PVTT. The results of this study are consistent with our previous findings[14], which showed that PVTT involving the main portal vein was the most important prognostic factor for survival. For HCC patients with main PVTT, the combination of TACE and sorafenib is not recommended because the combined therapy may exacerbate liver function damage in these patients. However, for HCC patients with PVTT confined to portal vein branches, TACE-S had acceptable side effects and may improve OS.

Considering that the cause of death of HCC patients with extrahepatic metastasis is mainly intrahepatic HCC or hepatic failure, rather than extrahepatic metastasis[33,34], a local treatment modality such as TACE is often performed at some centers[14,33]. Our result showed that extrahepatic metastasis was not an independent prognostic factor for worse survival. This implied that the combination of delaying intrahepatic tumor progression with TACE and targeting extrahepatic metastasis with sorafenib might be benefit for survival, although further trials are required to confirm this finding. This study showed that TACE-S in patients with HCC is safe and well tolerated, with the most common drug-related AEs including hand-foot skin reaction, diarrhea, alopecia, fatigue, and hypertension, which were similar to those reported in previous studies with sorafenib as monotherapy[9,10] and with sorafenib in combination with TACE[14]. Furthermore, patients tolerated TACE well, which was similar to that observed in a previous study[15], suggesting that the combination therapy does not increase TACE-related adverse reactions.

Our study had several limitations. First, this study was a single-institution, retrospective study. Therefore, the strength of our conclusions is limited by the retrospective nature of the results. Second, the population used in this study was heterogeneous with regard to the frequency of patients with Child-Pugh B, previous TACE, and different PVTT classifications. However, our population is similar to that of patients who are treated in routine clinical practice. Third, the evaluation of mRECIST may be biased because of investigator-independent factors. However, every evaluation was independently assessed by at least two clinicians, and when there was a discrepancy, a consensus was reached by a panel of clinicians to reduce the error caused by the observers.

In conclusion, our study demonstrated that the first follow-up 4-6 wk after TACE-S can be used as the earliest time point at which the response to TACE-S should be evaluated in patients with advanced-stage HCC. Moreover, mRECIST-evaluated disease control (CR, PR, and SD) was an independent predictor for OS at this early time point and could be considered a valuable early indicator for making subsequent therapeutic decisions and predicting long-term survival. In addition, we found that patients who received previous TACE and patients with main PVTT had worse outcomes. Sorafenib should be orally administered early after the first TACE. We do not, however, recommend the combination of TACE and sorafenib for patients with advanced HCC complicated by main PVTT.

ARTICLE HIGHLIGHTS

Research background

Recently, some studies recommended that the combination of transarterial chemoembolization (TACE) and sorafenib (TACE-S) may be used as an alternative for patients with advanced-stage HCC. However, it is still uncertain which patients can obtain survival benefits from TACE-S treatment.

Research motivation

The aim of this study was to find some clinical biomarkers that can early predict improved survival in patients with advanced-stage HCC treated with TACE-S therapy, which will be beneficial to the choice of the patients who received TACE-S therapy.

Research objectives

The objective of this study was to identify which clinical biomarkers that could early predict improved survival in patients with advanced-stage HCC treated with TACE-S. This may help us make decisions about subsequent therapies and choose the timing of sorafenib treatment.

Research methods

A retrospective study was performed. The mRECIST-evaluated early disease control (including complete response, partial response, and stable disease) and multiple clinical variables at the first follow-up 4-6 wk after TACE-S were analyzed to identify the factors affecting survival.

Research results

No previous TACE, the absence of portal vein tumor thrombus (PVTT), and mRECIST-evaluated disease control at the first follow-up assessment 4-6 wk after TACE-S were independent prognostic factors for better survival. The incidence and severity of adverse events are similar to that observed in previous study.

Research conclusions

The first follow-up 4-6 wk after TACE-S can be used as the earliest time point at which the response to TACE-S should be evaluated in patients with

advanced-stage HCC. At this point, mRECIST-evaluated disease control could be considered a valuable early indicator for making subsequent therapeutic decisions and predicting long-term survival. In addition, patients who received previous TACE or had main PVTT had worse outcomes.

Research perspectives

A further prospective study is needed to confirm mRECIST-evaluated disease control at the first follow-up 4-6 wk after TACE-S as an early indicator for predicting improved survival in patients with advanced-stage HCC treated with TACE-S therapy.

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ORIGINAL ARTICLE

Retrospective Study

Low glucose metabolism in hepatocellular carcinoma with GPC3 expression

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Abstract

AIM

To investigate the relationship between glucose metabolism and glypican-3 (GPC3) expression in hepatocellular carcinoma (HCC).

METHODS

Immunohistochemical staining of pathological samples for GPC3 and glucose transporter 1 (GLUT1), and whole-body ^{18}F-FDG PET/CT for measuring tumour glucose uptake were performed in 55 newly diagnosed HCC patients. The maximum standard uptake value (SUV_{max}) and tumour-to-non-tumourous liver uptake (T/NT) ratio were used to quantify ^{18}F-FDG uptake. *In vitro* ^{18}F-FDG uptake assay of GPC3-expressing HepG2 and non-GPC3-expressing RH7777 cells was used to examine the effect of GPC3 in cellular glucose metabolism. The relationships between GPC3 expression and ^{18}F-FDG uptake, GLUT1 expression, tumour differentiation, and other clinical indicators were analysed using Spearman rank correlation, univariate

and multiple logistic regression analyses.

RESULTS

Positive GPC3 expression was observed in 67.3% of HCC patients, including 75.0% of those with well or moderately differentiated HCC and 36.4% of those with poorly differentiated HCC. There was an inverse relationship between GPC3 expression and SUV_{max} (Spearman correlation coefficient = -0.281, P = 0.038) and a positive relationship between GLUT1 expression and SUV_{max} (Spearman correlation coefficient = 0.681, P < 0.001) in patients with HCC. Univariate analysis showed that two glucose metabolic parameters (SUV_{max} and T/NT ratio), tumour differentiation, lymph node metastasis, and TNM stage were all significantly associated with GPC3 expression (P < 0.05), whereas GLUT1 expression, sex, age, tumour size, intrahepatic lesion number, and distant metastasis showed no statistical association (P > 0.05). Further multivariate analysis revealed that only the T/N ratio was significantly correlated with GPC3 expression in patients with HCC (P < 0.05). *In vitro* assay revealed that the uptake of ^{18}F-FDG in GPC3-expressing HepG2 cells was significantly lower than that of non-GPC3-expressing RH7777 cells (t = -20.352, P < 0.001).

CONCLUSION

The present study demonstrated that GPC3 expression is inversely associated with glucose metabolism, suggesting that GPC3 may play a role in regulating glucose metabolism in HCC.

Key words: Hepatocellular carcinoma; Glypican-3; ^{18}F-FDG; Maximum standard uptake value; T/NT ratio; Glucose metabolism; Glucose transporter 1

Core tip: The present study demonstrated that glypican-3 (GPC3) was positively expressed in 67.3% of hepatocellular carcinoma (HCC) patients. GPC3 expression is found to be inversely associated with the glucose metabolism of HCC tumours in the patient study. Multivariate analysis revealed that only the glucose metabolism was significantly correlated with GPC3 expression (P < 0.05), but not GLUT1 expression, tumour differentiation, or other clinical indicators (P < 0.05). Low glucose metabolism was also observed in positive GPC3-expressing HepG2 cells in cellular uptake assay. Therefore, we suggested that GPC3 may play a role in regulating glucose metabolism in HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and fatal malignancies worldwide and is especially prevalent in China. The outcome of patients with HCC is poor, with a low five-year survival rate of 25%-39%[1]. Surgical resection remains the standard treatment for early stage HCC[2,3]. Unfortunately, most patients present with advanced stage HCC at diagnosis, and there are few treatment options for them since current systemic therapy often cannot effectively control advanced stage HCC[1,2]. Therefore, it is of utmost importance to develop a technique that can accurately diagnose early stage HCC as well as an effective treatment that can control advanced stage HCC.

Glypican-3 (GPC3) is a member of the glypican family of heparin sulfate proteoglycans (HSPGs). This protein has been reported to be highly expressed in HCC, but not in normal liver tissue, cirrhosis tissue, or paracancerous tissue[4-6]. GPC3 plays an important role in regulating malignant transformation and promoting the growth of HCC by stimulating the canonical Wnt signalling pathway[7]. Therefore, GPC3 is suggested to be an important target for diagnosis and therapy. Recently, positron emission tomography (PET) imaging using a ^{89}Zr-conjugated monoclonal antibody or a F(ab')$_2$ fragment directed against GPC3 was shown to successfully enable the non-invasive quantification and visualization of tumour GPC3 expression *in vivo*[8-10], which has potential to be a specific probe for HCC detection. In addition, GPC3-targeted therapies are emerging as novel molecular treatments for HCC patients[11-15].

Malignant cells require accelerated glycolysis to generate ATP, in order to meet their high energy demands for cell proliferation and survival. Accelerated glycolysis has been widely confirmed to be a common biological phenomenon in malignant tumours by positron emission tomography combined with computed tomography (PET/CT) using 2-[fluorine-18]-fluoro-2-deoxy-D-glucose (^{18}F-FDG), a glucose analogue[16-18]. However, glucose metabolism varies greatly in HCC. Low glucose metabolism was often observed in well- and moderately differentiated HCC[19,20]. Previous studies have revealed that low ^{18}F-FDG uptake was correlated with high FDG-6-phosphatase activity, low expression of GLUT1 or GLUT2, and high expression of P-glycoprotein[21,22]. However, Cho *et al*[23] found that GPC3 may also be an important regulator for glucose metabolism in HCC. They reported that GPC3 could bind to GLUT1 and decrease glucose uptake by HCC cells. Nevertheless, this phenomenon has not yet been confirmed in patients. Therefore, we performed the present study to elucidate their relationship in HCC patients. This work may contribute to a better understanding of the biological role of GPC3 in HCC and could be useful to predict the potential utility of GPC3

targeted imaging in the clinic.

MATERIALS AND METHODS

Patients

This study included 55 patients (46 males, 9 females; mean age: 52.9 years [range: 18-78 years]) with newly diagnosed HCC who underwent ^{18}F-FDG PET/CT for staging before local hepatectomy or biopsy at Nanfang Hospital from August 2013 to October 2017. The inclusion criteria were as follows: (1) final diagnosis of HCC established by pathologic examination; (2) no adjuvant therapy administered before the PET/CT scans; and (3) available GPC3 and GLUT1 immunohistochemical staining. A total of 55 patients met the criteria and were enrolled in this study.

^{18}F-FDG PET/CT scans

^{18}F-FDG PET/CT scans were performed using a Biograph mCTx scanner (Siemens, Germany). The patients were instructed to fast for at least 6 h, and their blood glucose levels were monitored with a glucometer prior to ^{18}F-FDG injection. All the patients had blood glucose levels below 7.0 mmol/L. ^{18}F-FDG was manufactured using a tracer synthesis system (TRACERlab FX_{FDG}; GE Healthcare, United States) and had a > 95% radiochemical purity. Approximately 60 min after the intravenous injection of 318-524 MBq (8.6-14.2 mCi, 150 μCi/kg) ^{18}F-FDG, whole-body PET/CT was performed at our centre according to established protocols[24].

Image interpretation

The acquired PET and CT images were registered and analysed using the syngo MI workplace (Siemens, Germany). All the PET/CT images were independently read by two nuclear medicine physicians with over five years of experience. Both physicians were blinded to the findings of other imaging modalities and the GPC3 expression data. For visual analysis, tumours with higher ^{18}F-FDG uptake than that of non-tumour liver tissue were considered PET positive. For the semi-quantitative analysis, a region of interest (ROI) was drawn along the margin of the HCC lesion to measure the SUV_{max}, which was used to quantify glucose metabolism. We also calculated the tumour-to-non-tumourous liver uptake (T/NT) ratio by dividing the tumour SUV_{max} by the SUV_{mean} of the non-tumourous liver tissue, which was measured by automated computation of the average SUV_{mean} of three 1-cm ROIs in non-tumourous liver tissue, two in the right lobe and one in the left lobe, using the syngo MI workplace (Siemens, Germany)[25]. For lesions without obvious ^{18}F-FDG uptake, the ROI was drawn on CT images and copied to the corresponding region on the PET images in order to measure the SUV_{max} and T/NT ratio. Non-contrast-enhanced CT images obtained from PET/CT were reviewed by two experienced radiologists.

Immunohistochemical analysis

HCC tissue samples were acquired *via* biopsy or surgical resection. Paraffin-embedded tissue sections were deparaffinized with xylene and rehydrated in a graded series of ethanol solutions. Antigen retrieval was performed by heating the slides twice in 0.01 mol/L sodium citrate buffer, pH 6.0, in a microwave oven (13 min, 850 W). Endogenous peroxidase was then blocked with 0.3% H_2O_2 in methanol for 15 min at room temperature. Immunohistochemical staining was performed by incubating the slides with a mouse anti-GPC3 antibody (sc-65443 1G12; Santa Cruz Inc., Santa Cruz, CA, United States) or rabbit anti-GLUT1 antibody (ZA-0471; ZSGB-BIO, China) at a dilution of 1:100 at 4 ℃ overnight. Serial sections were stained with a horseradish peroxidase enzyme-labelled polymer conjugated to anti-mouse/rabbit immunoglobulins, according to the instructions of the Chemmate EnVision/Mo&Rb Detection Kit (GK500705, Gene Tech Company Limited, Shanghai, China).

The total GPC3 immunostaining score was calculated based on the percent positivity of stained tumour cells and the staining intensity. The percent positivity was scored as 0 (< 5%), 1 (5%-10%), 2 (11%-50%), or 3 (> 50%). The staining intensity was scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (strong staining). The percent positivity and staining intensity were determined in a double-blinded manner. The GPC3 expression score based on membrane and cytoplasmic immunostaining was calculated as percent positivity score × staining intensity score and ranged from 0 to 9. The GPC3 expression level was defined as -, 0; 1^+, 1-2; 2^+, 3-5; or 3^+, 6-9[26].

Glucose transporter-1 expression in tumour cells was evaluated using a semiquantitative scoring method: score 0 = absence of immunostaining; score 1 = 1%-10% of cells stained; score 2 = 10%-50% of cells stained; and score 3 = > 50% of cells stained. No account was taken for the intensity of staining and necrotic areas were excluded from the evaluation[27]. An immunoreactive score above 2 was defined as high GLUT1 expression, while a score of 0 or 1 was defined as low expression.

In vitro assay of cellular glucose metabolism

In vitro assay was performed to evaluate the effect of GPC3 expression on the cellular glucose metabolism. GPC3-expressing HepG2 and non-GPC3-expressing RH7777 cells[8,10] were seeded into 12-well plates at a density of 5×10^5 cells per well for overnight incubation. Cells were rinsed three times with phosphate buffered saline (PBS), followed by the addition of ^{18}F-FDG (111 kBq/well) to the cultured wells in quadruplicate. After incubation at 37 ℃ for 60 min, cells were rinsed three times with PBS and lysed with NaOH sodium

Table 1 Univariate analysis of the variables related to GPC3 expression in hepatocellular carcinoma *n* (%)

Variable	GPC3 expression		χ^2 or t	*P* value
	Negative	Positive		
Gender			1.458	0.227
Male	13 (28.3)	33 (71.7)		
Female	5 (55.6)	4 (44.4)		
Age (yr)			0.014	0.907
< 50	7 (31.8)	15 (68.2)		
⩾ 50	11 (33.3)	22 (66.7)		
Tumour differentiation			4.341	0.037
Well or moderate	11 (25.0)	33 (75.0)		
Poor	7 (63.6)	4 (36.4)		
Tumour size (cm)			2.542	0.111
< 5	3 (17.6)	14 (82.4)		
⩾ 5	15 (39.5)	23 (60.5)		
^{18}F-FDG			3.135	0.77
Positive	15 (40.5)	22 (59.5)		
Negative	3 (16.7)	15 (83.3)		
Intrahepatic lesion number			0.461	0.497
Solitary	11 (29.7)	26 (70.3)		
Multiple	7 (38.9)	11 (61.1)		
Lymph node metastasis			4.341	0.037
Positive	7 (63.7)	4 (36.4)		
Negative	11 (25.0)	33 (75.0)		
Distant metastasis			0.836	0.361
Positive	5 (50.0)	5(50.0)		
Negative	13 (28.9)	32(71.1)		
TNM stage			4.969	0.026
Ⅰ-Ⅱ	7 (21.2)	26 (78.8)		
Ⅲ-Ⅳ	11 (50.0)	11 (50.0)		
Serum AFP (μg/L)			2.645	0.104
< 20	11 (44.0)	14 (56.0)		
⩾ 20	7 (23.3)	23 (76.7)		
HBV infection			0.836	0.361
Positive	13 (28.9)	32 (71.1)		
Negative	5 (50.0)	5 (50.0)		
Liver cirrhosis			0.445	0.505
Positive	10 (29.4)	24 (70.6)		
Negative	8 (38.1)	13 (61.9)		
GLUT1			1.863	0.172
High	6 (54.5)	5 (45.5)		
Low	12 (27.3)	32 (72.7)		
SUV_{max}	9.56 ± 5.95	6.01 ± 3.55	2.341	0.028
T/N ratio	4.52 ± 2.92	2.62 ± 1.55	2.597	0.017

GPC3: Glypican-3; GLUT1: Glucose transporter 1; SUV: Standard uptake value.

dodecyl sulfate (SDS) (0.2 mol/L NaOH, 1% SDS). The cell lysate was collected and the cell-associated radioactivity was then measured using a gamma counter (GC-1200, USTC Chuangxin Co. Ltd. Zonkia Branch, China). The cell uptake was normalized with inputted radioactivity. Experiments were conducted in quadruplicate[28].

Statistical analysis

All statistical analyses were performed using SPSS version 20.0. Differences in glucose metabolic parameters (SUV_{max}, T/NT ratio) between groups were compared using the *t*-test (unpaired). GPC3 positive rates were compared using the crosstabs χ^2 test. Spearman rank correlation was used to determine the association between GPC3 expression, GLUT1 expression, and glucose metabolism. Univariate and multiple logistic regression analyses were used to analyse the association between GPC3 expression and ^{18}F-FDG uptake, GLUT1, histopathological diagnosis, and other clinical parameters. A *P*-value < 0.05 indicated statistical significance.

RESULTS

Patient characteristics

Of the 55 included patients, 44 (80.0%) were diagnosed with well or moderately differentiated HCC, and 11 (20.0%) were diagnosed with poorly differentiated HCC. Immunohistochemical analysis showed that the expression of GPC3 was positive in 67.3% (37/55) of patients, including 75.0% (33/44) of those with well or moderately differentiated HCC and 36.4% (4/11) of those with poorly differentiated HCC patients. The GPC3 expression score was 3^+ in 34.5% (19/55) of the patients, 2^+ in 14.5% (8/55), 1^+ in 18.2% (10/55), and 0 in 32.7% (18/55). Twenty percent (11/55) of tumours had high GLUT1 expression and 80% (44/55) tumours had low GLUT1 expression. Multiple intrahepatic lesions were found in 18 (32.7%) patients, and solitary lesions were observed in 37 (67.3%) patients. Most intrahepatic lesions (69.1%) were larger than 5 cm in diameter. The disease was categorized into TNM stage I in 29 patients, TNM stage Ⅱ in 4, TNM stage Ⅲ in 6, and TNM stage IV in 16. Other related clinical information is shown in Table 1.

Association of ^{18}F-FDG uptake with GPC3 expression, GLUT1 expression, and tumour differentiation

HCC lesions were noted to be positive for ^{18}F-FDG PET/CT in 37 (67.3%) patients and negative in 18 (32.7%) patients by the visual analysis. The SUV_{max} for primary tumours ranged from 2.07 to 18.60 (7.17 ± 4.73) and the T/NT ratio ranged from 0.86 to 10.0 (3.24 ± 2.26). In the lesions with negative ^{18}F-FDG uptake, GPC3 expression was positive in 15/18 (83.3%) patients. Combining ^{18}F-FDG uptake with GPC3 expression, the total positivity reached 94.5% (52/55).

There was an inverse relationship between GPC3 expression and ^{18}F-FDG uptake (SUV_{max}: Spearman correlation coefficient = -0.281, *P* = 0.038; T/NT ratio: Spearman correlation coefficient = -0.303, *P* = 0.025), and ^{18}F-FDG uptake in HCC lesions with GPC3 positivity was significantly lower than that of lesions with GPC3 negativity (SUV_{max}: 6.01 ± 3.55 *vs* 9.56 ± 5.95, *t* = -2.341, *P* = 0.028; T/NT ratio: 2.62 ± 1.55 *vs* 4.52 ± 2.92, *t* = -2.597, *P* = 0.017) (Figure 1A and B, Figure 2). On the contrary, a positive association was found between GLUT1 expression and ^{18}F-FDG uptake (SUV_{max}: Spearman correlation coefficient = 0.681, *P* < 0.001; T/NT ratio: Spearman correlation coefficient = 0.616, *P* < 0.001). ^{18}F-FDG uptake in the high GLUT1 expression group was significantly higher than that in the low GLUT1 expression group (SUV_{max}: 13.58 ± 3.44 *vs*

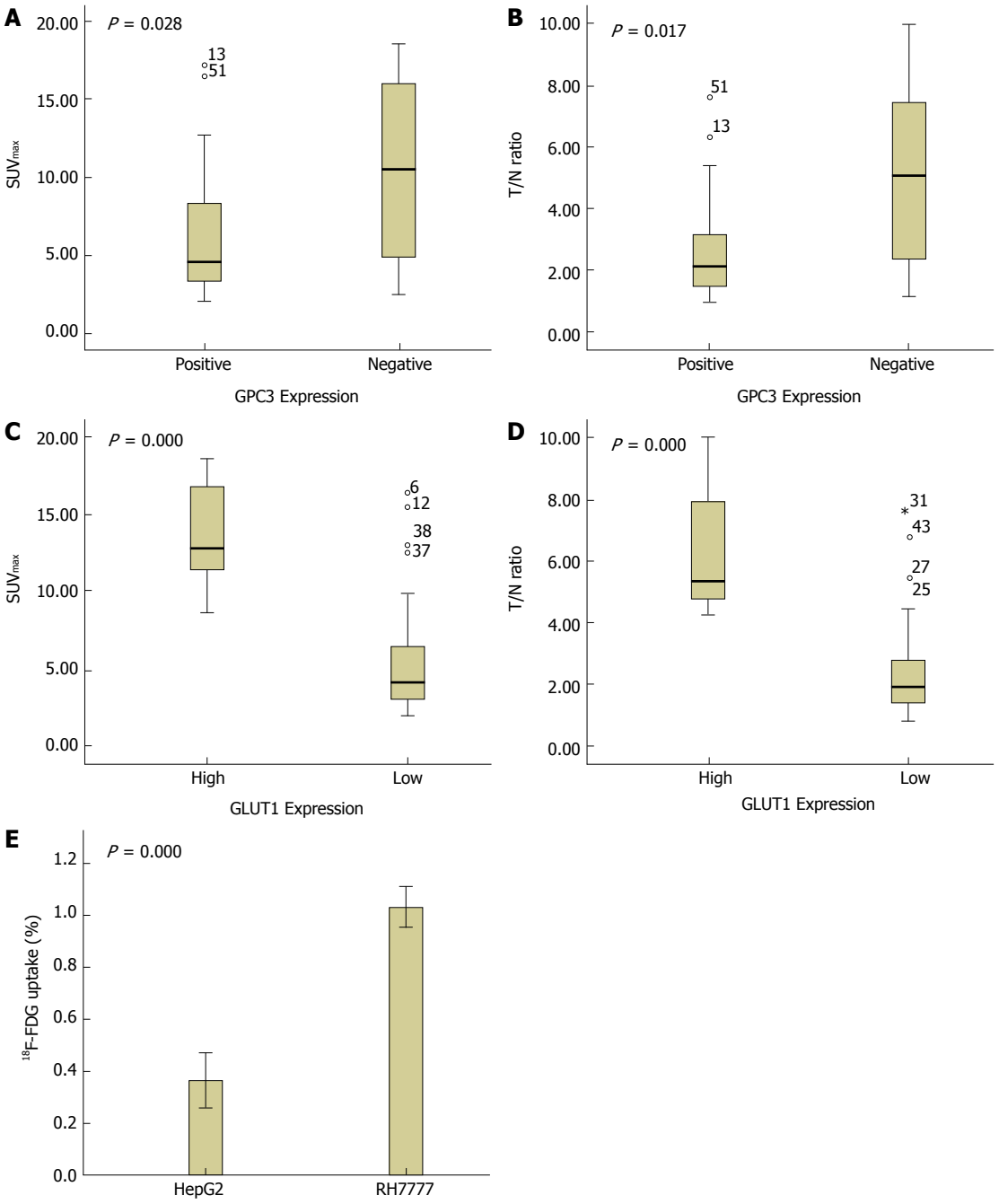

Figure 1 The relationship of ^{18}F-FDG uptake with GPC3 and GLUT1 expression, and the cellular ^{18}F-FDG uptake assay. A and B: ^{18}F-FDG uptake in hepatocellular carcinoma (HCC) lesions with positive and negative GPC3 expression. (A) SUV_{max}: 6.01 ± 3.55 *vs* 9.56 ± 5.95, t = -2.341, P = 0.028; (B) T/NT ratio: 2.62 ± 1.55 *vs* 4.52 ± 2.92, t = -2.597, P = 0.017. C and D: ^{18}F-FDG uptake in HCC lesions with high and low expression of GLUT1. (C) SUV_{max}: 13.58 ± 3.44 *vs* 5.57 ± 3.49, t = 6.898, P < 0.001; (D) T/NT ratio: 6.38 ± 1.91 *vs* 2.46 ± 1.55, t = 6.307, P < 0.001). E: ^{18}F-FDG uptake in GPC3-expressing HepG2 cells and non-GPC3-expressing RH7777 cells (0.37% ± 0.05% *vs* 1.03% ± 0.04% of inputted radioactivity, t = -20.352, P < 0.001).

5.57 ± 3.49, t = 6.898, P < 0.001; T/NT ratio: 6.38 ± 1.91 *vs* 2.46 ± 1.55, t = 6.307, P < 0.001) (Figure 1C and D). We then investigated the relationship between GPC3 and GLUT1 expression. Low GLUT1 expression was found in 86.5% of GPC3-positive tumours and in 66.7% of GPC3-negative tumours, respectively. Although there was an inverse trend of relationship between GPC3 and GLUT1 expression, it did not reach statistical significance (Spearman correlation coefficient = -0.232, P = 0.088).

There were significant differences in SUV_{max} and T/NT ratio between different degrees of tumour differentiation. Poorly differentiated HCC had a significantly higher SUV_{max} and T/NT ratio than well- or moderately differentiated HCC (SUV_{max}: 10.96 ± 6.08 *vs* 6.22 ± 3.86, t = 2.465, P = 0.030; T/NT ratio: 5.16 ± 3.06 *vs* 2.76 ± 1.74, t = 2.499, P = 0.028, respectively).

Univariate and multivariate analyses of the relationship of ^{18}F-FDG uptake, tumour differentiation, and other factors with GPC3 expression

SUV_{max}, T/NT ratio, tumour differentiation, and other

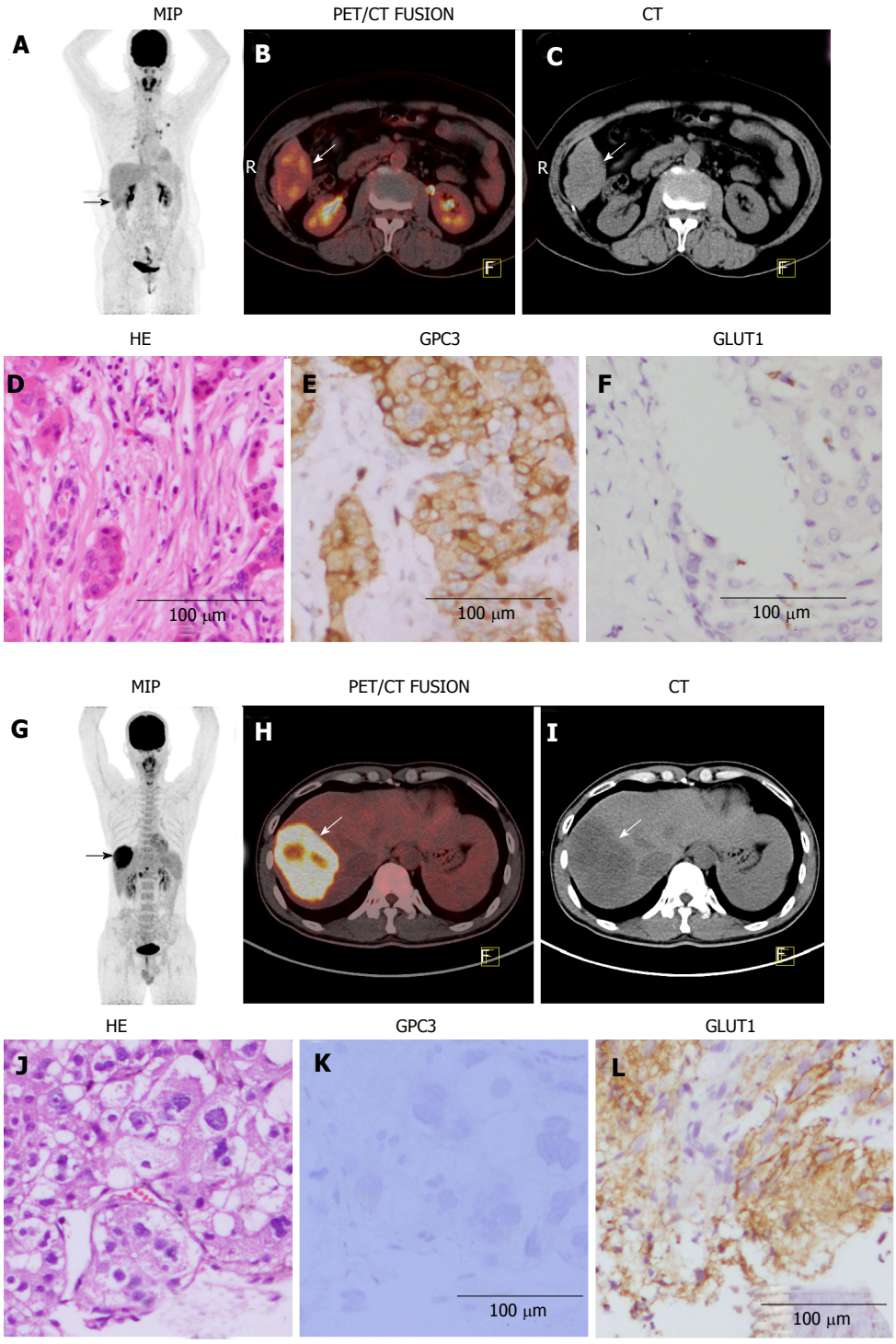

Figure 2 A 60-year-old woman with moderately differentiated hepatocellular carcinoma positive for GPC3 expression (A-E) and a 38-year-old man with poorly differentiated HCC negative for GPC3 (G-J). A-C: ^{18}F-FDG PET/CT showed slight ^{18}F-FDG uptake (SUV_{max} = 3.4, T/NT = 1.54) in the tumour (black arrow in A, white arrows in B and C). D: Moderately differentiated hepatocellular carcinoma (HCC) was diagnosed by pathological examination using HE staining. E: Immunohistochemical analysis revealed positive expression of GPC3. F: Immunohistochemical analysis revealed low expression of GLUT1 in tumour tissue. G-I: ^{18}F-FDG PET/CT scans showed intense accumulation of ^{18}F-FDG (SUV_{max} = 16.5, T/NT = 7.03) in the tumour (black arrow in G, white arrows in H and I). J: Poorly differentiated HCC was confirmed by pathological examination using HE staining. K: Immunohistochemical analysis revealed that the tumour was negative for GPC3 expression. L: Immunohistochemical analysis revealed high GLUT1 expression in tumour tissue.

clinical factors were analysed for their relationship with GPC3 expression. Univariate analysis showed that two glucose metabolic parameters (SUV_{max} and T/NT ratio), tumour differentiation, lymph node metastasis, and TNM stage were all significantly associated with GPC3 positivity in HCC patients ($P < 0.05$) (Table 1). Low ^{18}F-FDG uptake was observed in GPC3-positive HCCs. Well- or moderately differentiated HCCs also showed a significantly higher GPC3 positive rate than poorly differentiated HCC tumours (75.0% *vs*

36.4%, χ^2 = 4.341, *P* = 0.037). Similar trends were observed for lymph node metastasis and TNM stage. Higher GPC3 positivity rates were found in patients with no lymph node metastasis and those with TNM stage Ⅰ-Ⅱ disease, than in patients with lymph node metastasis and TNM stage Ⅲ-Ⅳ disease, respectively (75.0% *vs* 36.4%, χ^2 = 4.341, *P* = 0.037 for lymph node status ; 78.8% *vs* 50.0%, χ^2 = 4.969, *P* = 0.026 for TNM stage). Other clinical factors, such as GLUT1 expression, sex, age, tumour size, ^{18}F-FDG positivity, intrahepatic lesion number, distant metastasis, HBV infection, and liver cirrhosis, were not significantly related to GPC3 expression (*P* > 0.05) (Table 1).

The five factors (two glucose metabolic parameters, tumour differentiation, lymph node metastasis, and TNM stage) that showed a significant relationship with GPC3 expression on univariate analysis were further analysed using multivariate analysis. The multivariate analysis demonstrated that only T/N ratio was significantly correlated with GPC3 expression in patients with HCC (*P* = 0.007, OR = 1.479, 95.0%CI: 1.113-1.964), while SUV_{max}, tumour differentiation, lymph node metastasis, and TNM stage had no significant association (*P* > 0.05).

Effect of GPC3 expression on cellular uptake of ^{18}F-FDG

To evaluate the effect of GPC3 expression on the glucose metabolism, GPC3-expressing HepG2 cells and non-GPC3-expressing RH7777 cells were incubated with ^{18}F-FDG for 60 min and the cellular uptake was measured. The results revealed that HepG2 cells had a significantly lower ^{18}F-FDG uptake than that of RH7777 cells (0.37% ± 0.05 % *vs* 1.03% ± 0.04% of inputted radioactivity, *t* = -20.352, *P* < 0.001) (Figure 1E), which is consistent with the findings in the patient study.

DISCUSSION

^{18}F-FDG PET/CT has often been used to non-invasively evaluate tumour glycolysis *in vivo* by measuring the uptake of ^{18}F-FDG, a glucose analogue[29-33]. This radiotracer is transported into cells *via* glucose transporters (GLUTs) and is then phosphorylated to ^{18}F-FDG-6-phosphate by the rate-limiting glycolytic enzyme hexokinase type 2. ^{18}F-FDG-6-phosphate then becomes trapped within cells[29-33]. High ^{18}F-FDG uptake is indicative of accelerated glycolysis. Although ^{18}F-FDG is consistently taken up intensively by a variety of cancers, ^{18}F-FDG accumulation in HCCs appears to be variable. It is well established that ^{18}F-FDG uptake by well and moderately differentiated HCCs is low, whereas ^{18}F-FDG uptake by poorly differentiated HCC is high[34-36]. On the contrary, ^{11}C-acetate and ^{11}C-choline, which are probes for lipid metabolism, have been reported to be intensively taken up by well- and moderately differentiated HCCs[37,38], indicating that low glucose metabolism and high lipid metabolism are the specific energy metabolism patterns of low grade HCC. In the present study, we found that SUV_{max} was actually lower in well- or moderately differentiated HCC than in poorly differentiated HCC, which consolidated the above views[34-36]. Low ^{18}F-FDG uptake has also been found to correlate with low expression of GLUT1 or GLUT2 and high expression of P-glycoprotein[21,22]. Our study confirmed the above findings that low GLUT1 expressing tumours actually had a significantly low ^{18}F-FDG uptake than that of high GLUT1 expressing tumours (*P* < 0.001).

In the present study, for the first time, we found the phenomenon that low glucose metabolism also occurred in the HCCs with positive GPC3 expression, not only in the patient study, but also in the *in vitro* cellular uptake assay. In the patient study, an inverse association was noted between GPC3 expression and ^{18}F-FDG uptake (*P* < 0.05). ^{18}F-FDG uptake in HCC lesions with GPC3 positivity was significantly lower than that of lesions with GPC3 negativity (SUV_{max}: 6.01 ± 3.55 *vs* 9.56 ± 5.95, *t* = -2.341, *P* = 0.028; T/NT ratio: 2.62 ± 1.55 *vs* 4.52 ± 2.92, *t* = -2.597, *P* = 0.017). Furthermore, multivariate analysis revealed that only the glucose metabolism was significantly correlated with GPC3 expression (*P* < 0.05), but not other clinical factors. *In vitro* cellular uptake assay also revealed that GPC3-expressing HepG2 cells had a low ^{18}F-FDG uptake than non-GPC3-expressing RH7777 cells (0.37 ± 0.05% *vs* 1.03 ± 0.04% of inputted radioactivity, *t* = -20.352, *P* < 0.001). Consistent with these findings, we observed that GPC3 expression was highly expressed in well- or moderately differentiated HCCs, which always have low ^{18}F-FDG uptake [34-36]. Therefore, our study implied that GPC3 may be another underlying factor that contributes to the complex ^{18}F-FDG uptake characteristics in HCCs. Cho *et al*[23] reported that GPC3 could bind to GLUT1 with an equilibrium dissociation constant (K_d) of 1.61 nmol/L and decrease glucose uptake by HCC cells, which might be helpful to explain this phenomenon. However, in the present study, low GLUT1 expression was found in most (86.5%) of GPC3-positive tumours. In addition, although an inverse trend of relationship was observed between GPC3 and GLUT1 expression, their association did not reach statistical significance (Spearman correlation coefficient = -0.232, *P* = 0.088). Therefore, the present study had no enough evidence to identify that GPC3 inversely regulates the glucose *via* GLUT1 and further basic research is warranted to uncover the mechanism.

Both SUV_{max} and T/NT ratio can be used to quantify ^{18}F-FDG uptake in tumours, however, in the present study, multivariate analysis revealed that only the T/NT ratio was significantly correlated with GPC3 expression (*P* < 0.05), but not the SUV_{max} (*P* > 0.05). A rational explanation for this result is that T/NT ratio can be more accurate to define ^{18}F-FDG uptake in HCC since it is not influenced by serum glucose level, the uptake period, or measurement variation, which often make the

measurement of SUV_{max} inaccurate[25].

GPC3 is currently under consideration as a potential molecular therapeutic target for HCC[11-15]. GPC3-targeted treatments that utilize siRNA or anti-GPC3 antibodies have shown potential in altering cell migration, metastasis, and invasion, and in inhibiting xenograft tumour growth[13,15,39,40]. A GPC3-derived peptide vaccine has also been tested in a phase Ⅱ study as an adjuvant therapy for HCC[41]. GPC3-targeted PET imaging might be useful for the non-invasive analysis of GPC3 expression in HCC patients and for selecting those suitable for GPC3-targeted therapy. The present study also indicated that GPC3-targeted PET imaging might be helpful for detection of early stage HCC, which often presents with a low uptake of ^{18}F-FDG and appears as well- and moderately differentiated HCC in pathology tests. In the present study, we demonstrated that GPC3 expression was positive in most (75.0%) of the well- or moderately differentiated HCC tumours. More importantly, in the lesions with negative ^{18}F-FDG uptake, GPC3 expression was positive in 15/18 (83.3%) patients. Combining ^{18}F-FDG uptake with GPC3 expression, the total positivity reached 94.5% (52/55). Therefore, we propose that GPC3-targeted PET imaging may improve diagnostic sensitivity for early stage HCC and can serve as an effective complement to ^{18}F-FDG imaging for diagnosing HCC.

There are some limitations to the present study. First, the sample size of patients was small, especially the number of poorly differentiated HCC patients, which may cause the results of this study to fail to reflect the real correlation between GPC3 and glucose metabolism. Second, this was a retrospective study, and thus, there may have been a certain degree of bias.

ARTICLE HIGHLIGHTS

Research background

Glypican-3 (GPC3) is a cell surface proteoglycan overexpressed in most hepatocellular carcinomas (HCCs), but not in normal liver tissue, cirrhosis tissue, or paracancerous tissue. Therefore, GPC3 is suggested to be an important target for diagnosis and therapy. Elucidating the relationship between GPC3 expression and glucose metabolism may contribute to a better understanding of the biological role of GPC3 in regulating glucose metabolism. In addition, the research also could be useful to predict the potential utility of GPC3-targeted imaging in the clinic.

Research motivation

In this study, we investigated the relationship between GPC3 expression and glucose metabolism in HCC with an aim to uncover how GPC3 regulates the glucose metabolism in HCCs and predict the potential utility of GPC3-targeted imaging in the clinic.

Research objectives

This study aimed to investigate the relationship between glucose metabolism and GPC3 expression in HCC.

Research methods

A retrospective analysis was performed on 55 HCC patients who had undergone ^{18}F-FDG PET/CT before therapy. Tumour SUV_{max} and T/N ratio were used to quantify ^{18}F-FDG uptake. The relationship between ^{18}F-FDG uptake and expression of GPC3 and glucose transporter 1 (GLUT1) was analyzed by immunohistochemical analysis. *In vitro* cellular ^{18}F-FDG uptake was also measured in GPC3-expressing HepG2 and non-GPC3-expressing RH7777 cells to determine the effect of GPC3 on glucose metabolism. The relationships between GPC3 expression and ^{18}F-FDG uptake, GLUT1 expression, tumour differentiation, and other clinical indicators were analysed using spearman rank correlation, and univariate and multiple logistic regression analyses.

Research results

In the present study, we found a phenomenon that the glucose metabolism in the GPC3-expressing HCC tumours is low in the patient study. ^{18}F-FDG uptake in HCC lesions with GPC3 positivity was significantly lower than that of lesions with GPC3 negativity (SUV_{max}: 6.01 ± 3.55 *vs* 9.56 ± 5.95, t = -2.341, P = 0.028; T/NT ratio: 2.62 ± 1.55 *vs* 4.52 ± 2.92, t = -2.597, P = 0.017). Furthermore, multivariate analysis revealed that only the glucose metabolism was significantly correlated with GPC3 expression (P < 0.05), but not other clinical factors. In *in vitro* cellular uptake experiments, GPC3-expressing HepG2 cells were also found to have low ^{18}F-FDG uptake than that of non-GPC3-expressing RH7777 cells (0.37% ± 0.05% *vs* 1.03% ± 0.04% of inputted radioactivity, t = -20.352, P < 0.001). Although an inverse trend of relationship was observed between GPC3 and GLUT1 expression, their association did not reach statistical significance (Spearman correlation coefficient = -0.232, P = 0.088).

Research conclusions

GPC3 was reported to play an important role in regulating malignant transformation and promoting the growth of HCC by stimulating the canonical Wnt signalling pathway. Besides, glucose is very important for malignant cell survival and proliferation. Both of them are very important for tumour growth. Therefore, we suggested that there might be a correlation between GPC3 and tumour glucose metabolism. We used ^{18}F-FDG PET/CT for non-invasively measuring tumour glucose uptake *in vivo* in HCC patients and ^{18}F-FDG uptake assay to measure the cellular glucose metabolism. In conclusion, the expression of GPC3 was observed to be positive in 67.3% (37/55) of HCC patients. The patient study and *in vitro* cellular uptake assay demonstrated that the glucose metabolism is inversely correlated with the expression of GPC3 in HCC. These results implied that GPC3 may be another underlying factor that contributes to the complex ^{18}F-FDG uptake characteristics in HCCs. We believe that it is helpful for clarifying the mechanism of anti-GPC3 treatment by uncovering how GPC3 regulates the glucose metabolism in HCC. In addition, we found that GPC3 expression was positive in 15/18 (83.3%) of the lesions with negative ^{18}F-FDG uptake. Combining ^{18}F-FDG uptake with GPC3 expression, the total positivity reached 94.5% (52/55). Therefore, we propose that GPC3-targeted PET imaging may improve diagnostic sensitivity for early stage HCC and can serve as an effective complement to ^{18}F-FDG imaging for diagnosing HCC.

Research perspectives

For the future research, we want to investigate the mechanism concerning how GPC3 regulates the glucose and lipid metabolism in HCC. In the previous study, we found ^{11}C-choline, as a probe of lipid metabolism, could be highly taken up by well- and moderately differentiated HCC. So, we deduce that GPC3 may have a potential to promote the lipid metabolism in HCC, which may conversely reduce the glucose metabolism. We want to do further basic research confirm this hypothesis.

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ORIGINAL ARTICLE

Clinical Trials Study

Application value of enhanced recovery after surgery for total laparoscopic uncut Roux-en-Y gastrojejunostomy after distal gastrectomy

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Abstract

AIM

To evaluate the safety and feasibility of enhanced recovery after surgery (ERAS) for total laparoscopic uncut Roux-en-Y gastrojejunostomy after distal gastrectomy.

METHODS

The clinical data of 42 patients who were divided into an ERAS group (n = 20) and a control group (n = 22) were collected. The observed indicators included operation conditions, postoperative clinical indexes, and postoperative serum stress indexes. Measurement data following a normal distribution are presented as mean ± SD and were analyzed by t-test. Count data were analyzed by χ^2 test.

RESULTS

The operative time, volume of intraoperative blood loss, and number of patients with conversion to open

surgery were not significantly different between the two groups. Postoperative clinical indexes, including the time to initial anal exhaust, time to initial liquid diet intake, time to out-of-bed activity, and duration of hospital stay of patients without complications, were significantly different between the two groups (t = 2.045, 8.685, 2.580, and 4.650, respectively, $P < 0.05$ for all). However, the time to initial defecation, time to abdominal drainage-tube removal, and the early postoperative complications were not significantly different between the two groups. Regarding postoperative complications, on the first and third days after the operation, the white blood cell count (WBC) and C reactive protein (CRP) and interleukin-6 (IL-6) levels in the ERAS group were significantly lower than those in the control group.

CONCLUSION

The perioperative ERAS program for total laparoscopic uncut Roux-en-Y gastrojejunostomy after distal gastrectomy is safe and effective and should be popularized. Additionally, this program can also reduce the duration of hospital stay and improve the degree of comfort and satisfaction of patients.

Key words: Distal gastrectomy; Enhanced recovery after surgery; Perioperative period; Uncut Roux-en-Y gastrojejunostomy

Core tip: For gastric cancer, uncut Roux-en-Y gastrojejunostomy after distal gastrectomy is still the most important treatment. However, the enhanced recovery after surgery (ERAS) protocol for the safety of gastric surgery is not clear. Therefore, we conducted a long-term follow-up and observation with a large sample. Our study demonstrated that the use of the perioperative ERAS program for total laparoscopic uncut Roux-en-Y gastrojejunostomy after distal gastrectomy is safe and effective and should be popularized.

Zang YF, Li FZ, Ji ZP, Ding YL. Application value of enhanced recovery after surgery for total laparoscopic uncut Roux-en-Y gastrojejunostomy after distal gastrectomy. *World J Gastroenterol* 2018; 24(4): 504-510 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i4/504.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i4.504

INTRODUCTION

Enhanced recovery after surgery (ERAS) is a concept promoted by some countries in Europe and America in recent years[1,2]. It can shorten the hospitalization time and improve the recovery rate after surgery[3]. This concept has been used by surgical centers in Europe and America for herniorrhaphy[4], gastrointestinal surgery[5,6], gynecologic operations[7], and other applications. The ERAS concept emphasizes the reduction of postoperative stress and trauma to the patient due to the surgery during the perioperative period, thereby promoting the rehabilitation of patients[8].

The uncut Roux-en-Y gastrojejunostomy closure of the proximal jejunum without interruption is based on Billroth Ⅱ + Braun anastomosis. It was first reported by Van Stiegmann *et al*[9] in 1988. Then, Uyama *et al*[10], in 2005, and Ahn *et al*[11], in 2014, reported laparoscopic-assisted and single-incision laparoscopic non-interrupted Roux-en-Y anastomoses. The incidence of Roux stasis syndrome (RSS) is 10%-30% after traditional Roux-en-Y anastomosis[12,13]. The advantages of the uncut Roux-en-Y gastrojejunostomy during digestive tract reconstruction include a reduction of the steps involved in freeing the jejunum during the operation, reduced mesangial damage and interference, retention of nerves and normal pacemakers, and significantly reduced occurrence of RSS. Thus, for obese patients or those with short-term mesothelioma, the procedure is particularly applicable[14,15].

This study evaluated the safety and feasibility of ERAS in the uncut operation.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the Second Hospital of Shandong University. Patients or their families signed informed consent forms.

Participants

A total of 42 consecutive patients undergoing total laparoscopic uncut Roux-en-Y gastrojejunostomy after distal gastrectomy (uncut operation) from July 2015 to November 2016 at the Second Hospital of Shandong University in China were included in this study. The data were analyzed in a retrospective cohort study. The patients were randomly divided into an ERAS group and a control group. No significant difference was found between the two groups in sex, age, body mass index, or operation method (Table 1) ($P > 0.05$). Throughout the study period, the same group of surgeons treated all patients and were responsible for all procedures, including the surgical techniques as well as the choice of surgical instruments.

Inclusion and exclusion criteria

The inclusion criteria were: (1) Age ⩾ 18 yr old; (2) Patients who preferred to undergo uncut surgery; (3) Gastrointestinal biopsy and CT examination confirmed gastric malignant tumors before the operation. The clinical stage was assessed by CT examination and found to be $T_{1-3}N_{1-3}M_0$ (7th edition of the UICC-AJCC TNM classification of gastric cancer); and (4) No significant swollen fusion of metastatic lymph nodes

Table 1 Clinical information of the patients

Clinical information	ERAS group (n = 20)	Control group (n = 22)	Statistics	P value
Age (yr, mean ± SD)	71.5 ± 8.1	72.9 ± 6.7	t = 0.613	> 0.05
Sex (male/female)	14/6	17/5	χ^2 = 0.287	> 0.05
Body mass index, (kg/m^2, mean ± SD)	23.6 ± 3.4	22.8 ± 4.6	t = 0.636	> 0.05

ERAS: Enhanced recovery after surgery.

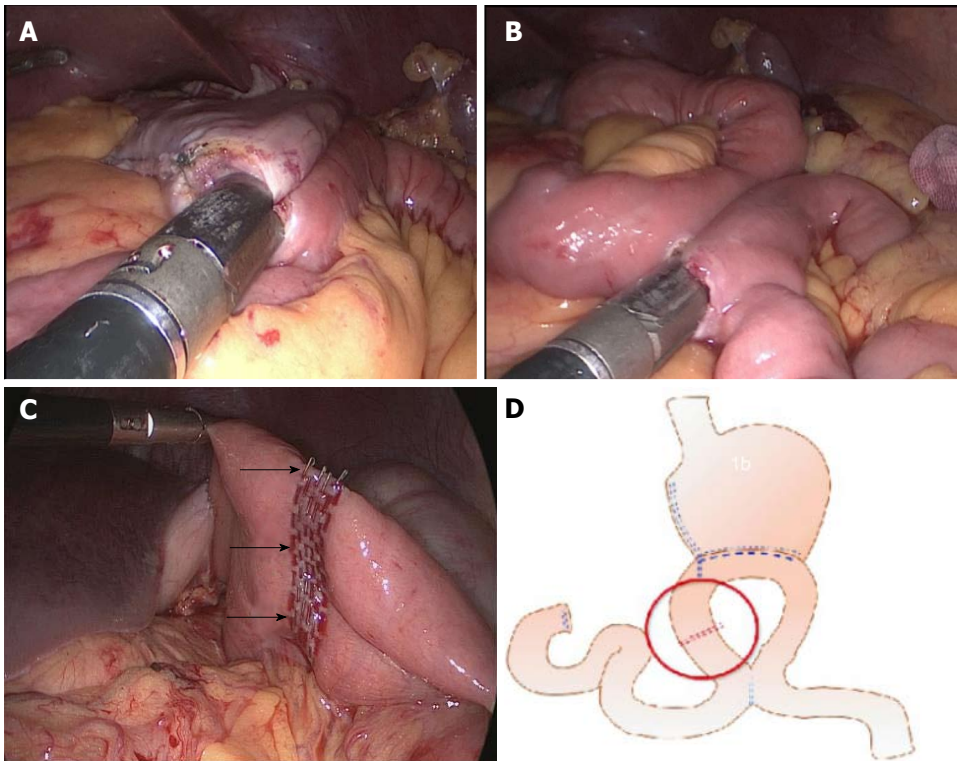

Figure 1 Series of perioperative treatment. A: Gastrojejunostomy; B: Braun anastomosis; C: Uncut reconstruction; D: Schematic diagram of the uncut reconstruction.

was present. The exclusion criteria were: (1) Patients who refused to participate in the study; (2) Patients who had serious underlying diseases; (3) Patients with distant metastases or unresectable tumors; (4) Patients who had serious complications and could not continue to undergo the ERAS procedure; (5) Patients who had a history of other major abdominal surgeries; and (6) Patients who underwent neoadjuvant therapy before surgery.

Perioperative treatment program

Patients in the ERAS group underwent a series of perioperative treatment regimens. The control group underwent a conventional perioperative treatment protocol (Table 2). After routine resection of the D2 group lymph nodes, the duodenum was cut 2 cm from the pylorus, and the stomach was cut 5 cm from the upper edge of the tumor. Then, a side-to-side gastrojejunostomy was performed on the jejunum, which was approximately 20 cm from the ligament of Treitz and the residual stomach (Figure 1A). Approximately 10 cm to 30 cm distal to the ligament of Treitz, a side-to-side jejunojejunostomy *via* a Braun anastomosis was performed to divert the duodenal fluid (Figure 1B). The jejunal occlusion site (uncut reconstruction) was approximately 3 cm from the jejunum (Figure 1C). A schematic diagram of the uncut reconstruction is shown in Figure 1D.

Observation indexes

Observation indicators included: (1) Operation conditions, including operative time, blood loss, and conversion to open surgery; (2) Postoperative clinical indexes, including time to initial anal exhaust, time to initial liquid diet intake, time to out-of-bed activity, time to initial defecation, time to abdominal drainage tube removal, duration of hospital stay of patients without complications, early postoperative complications, and visual analogue scale scores on the first and third days after the operation; and (3) Postoperative serum stress

Table 2 Perioperative treatment program

Perioperative treatment program	ERAS Group	Control group
Preoperative		
Preparation	ERAS-related health education to alleviate tension in patients performed by both surgeons and anesthesiologists. The definition of the visual analog scale was explained to patients.	Regular preoperative education and preoperative conversation only with surgeons, and the definition of the visual analogue scale was explained to patients.
Diet	Patients drank 1000 mL of a 10% glucose solution 10 h before surgery and 500 mL of the 10% glucose solution 2 h before surgery.	Fasting for 12 h before surgery No drinking for 6 h before surgery
Bowel preparation	None	The bowel was cleaned twice the day before surgery and the day of surgery.
Intraoperative		
Nasogastric tube	Not used	Removed after exhaust
Body temperature	Intraoperative warm-air body heating	None
Urinary catheter	Removed after waking from anesthesia	1 d after surgery
Postoperative		
Anesthesia and analgesics	Local anesthesia at surgical incision + PCIA + NSAIDs	PCIA
Diet	Patients chewed gum after waking from anesthesia, drank water 6 h after surgery, and were encouraged to remain on a liquid diet until return to a normal diet.	Patients drank water after anal exhaust and gradually returned to a normal diet.
Activity	Patients were encouraged to get out of bed for more than 6 h a day and walk the length of the ward.	Decided by patients

ERAS: Enhanced recovery after surgery.

Table 3 Operation conditions

Operation parameter	ERAS group (n = 20)	Control group (n = 22)	Statistics	P value
Operative time (min, mean ± SD)	217.9 ± 52.5	225.4 ± 61.7	t = 0.422	> 0.05
Volume of intraoperative blood loss (mL, mean ± SD)	166.1 ± 12.5	150.9 ± 31.7	χ^2 = 2.006	> 0.05
Open surgery	2	1	t = 0.01	> 0.05

ERAS: Enhanced recovery after surgery.

indexes, including detection of the white blood cell count (WBC) and C reactive protein (CRP) and interleukin-6 (IL-6) levels at 1 d and 3 d after the operation.

Statistical analysis

Statistical analyses were performed with SPSS 19.0. Measurement data following a normal distribution are presented as mean ± SD and were analyzed by t-test. A P-value < 0.05 was considered statistically significant.

RESULTS

Operation conditions

The operative time (t = 0.422), volume of intraoperative blood loss (t = 2.006), and number of patients with conversion to open surgery (χ^2 = 0.01) were not significantly different between the two groups (P > 0.05 for all), which indicated that the ERAS program did not affect the surgical results, as illustrated in Table 3.

Postoperative clinical observations

The time to initial anal exhaust, time to initial liquid diet intake, time to out-of-bed activity, duration of hospital stay of patients without complications, and visual analog scale score on the first and third days after the operation were 2.9 ± 1.1 d, 1.6 ± 0.7 d, 2.3 ± 0.8 d, 7.5 ± 1.6 d, 4.0 ± 1.4, and 3.5 ± 1.8, respectively, in the ERAS group and 3.7 ± 1.4 d, 4.1 ± 1.1 d, 4.2 ± 3.2 d, 10.2 ± 2.1 d, 5.4 ± 1.6, and 4.8 ± 1.5 in the control group, respectively, resulting in statistically significant differences between the two groups (t = 2.045, 8.685, 2.580, 4.650, 2.361, and 2.551, respectively; P < 0.05 for all). However, the time to initial defecation, time to abdominal drainage-tube removal, and the early postoperative complications were not significantly different between the two groups.

The rates of anastomotic leakage, postoperative ileus, pneumonia, cardiac disorders, and overall complications did not significantly differ between the two groups, which indicated the safety of the procedure. In addition, the perioperative ERAS program effectively reduced the pain and hospitalization time of patients. Overall compliance with ERAS protocols was good, as illustrated in Table 4.

Postoperative serum stress indexes

Postoperative serum stress indexes are summarized in

Table 4 Postoperative clinical indexes

Index	ERAS group (n = 20)	Control group (n = 22)	Statistics	P value
Time to initial anal exhaust (d, mean ± SD)	2.9 ± 1.1	3.7 ± 1.4	t = 2.045	< 0.05
Time to initial liquid diet intake (d, mean ± SD)	1.6 ± 0.7	4.1 ± 1.1	t = 8.685	< 0.05
Time to out-of-bed activity (d, mean ± SD)	2.3 ± 0.8	4.2 ± 3.2	t = 2.580	< 0.05
Time to initial defecation (d, mean ± SD)	4.2 ± 1.7	4.9 ± 2.0	t = 1.216	> 0.05
Time to abdominal drainage tube removal (d, mean ± SD)	7.3 ± 2.6	8.7 ± 3.2	t = 1.546	> 0.05
Duration of hospital stay of patients without complications (d, mean ± SD)	7.5 ± 1.6	10.2 ± 2.1	t = 4.650	< 0.05
Early postoperative complications	1	1	χ^2 = 0.430	> 0.05
VAS POD1 (points, mean ± SD)	4.0 ± 1.4	5.4 ± 1.6	t = 2.361	< 0.05
VAS POD3 (points, mean ± SD)	3.5 ± 1.8	4.8 ± 1.5	t = 2.551	< 0.05

ERAS: Enhanced recovery after surgery; POD: Postoperative day.

Table 5 Postoperative serum stress indexes

Stress index (mean ± SD)	ERAS group (n = 20)	Control group (n = 22)	Statistics	P value
WBC POD1 (× 10^9/L)	12.7 ± 3.1	15.2 ± 4.2	t = 2.176	< 0.05
WBC POD3 (× 10^9/L)	9.5 ± 2.6	12.4 ± 3.3	t = 3.141	< 0.05
CRP POD1 (mg/dL)	7.5 ± 2.9	11.2 ± 3.4	t = 3.775	< 0.05
CRP POD3 (mg/dL)	5.3 ± 3.1	7.3 ± 2.8	t = 2.197	< 0.05
IL-6 PDO1 (pg/dL)	55.2 ± 21.9	85.7 ± 35.6	t = 3.303	< 0.05
IL-6 POD3 (pg/dL)	20.3 ± 5.7	24.1 ± 6.2	t = 2.061	< 0.05

ERAS: Enhanced recovery after surgery; POD: Postoperative day; WBC: White blood cell count; CRP: C reactive protein; IL-6: Interleukin-6.

Table 5. On the first and third days after the operation, the WBC and CRP and IL-6 levels in the ERAS group were significantly lower than those in the control group, which suggested that the ERAS program significantly reduced the postoperative stress responses.

DISCUSSION

Currently, it is unclear whether the introduction of the ERAS concept benefits the Chinese population. Gastrectomy involving the reconstruction of the digestive tract is extremely specialized, and the application of ERAS after gastrectomy is uncommon. In the present study, the ERAS protocol was novel for gastric surgery[16]. However, the ERAS concept advocates giving patients adequate preoperative preparation (good preoperative communication, nutritional treatment, and comfortable gastrointestinal preparation) to reduce preoperative stress[17,18] The use of warming, planned rehydration, and increased postoperative analgesia after surgery reduces postoperative traumatic stress responses[16]. The ERAS concept emphasizes that patients get out of bed, ingest a liquid diet, and undergo removal of the gastrointestinal decompression tube and catheter earlier to promote postoperative intestinal function recovery and accelerated rehabilitation[19,20]. In the present study, we demonstrated that the ERAS concept can be applied for gastric cancer treatment. This concept can help achieve faster patient recovery without increasing postoperative morbidity.

Surgical treatment of gastric cancer consists of two important parts. The first part is tumor resection. At present, the use of D2 radical surgery has achieved consensus in the field. The second part is the reconstruction of the digestive tract. Due to the development of chemotherapy and targeted drugs, the survival of patients with gastric cancer has significantly improved[21,22]. The uncut Roux-en-Y gastrojejunostomy after distal gastrectomy does not involve cutting the jejunum, which reduces the time needed for dissociation of the jejunum. Therefore, the blood supply of the jejunum remains intact, which reduces intraoperative bleeding and ensures that the function of the intestinal wall nerve is not damaged[12,13]. Simultaneously, this technique can reduce the incidence of RSS[23,24]. Scholars worldwide have applied the coincidence method to endoscopic assisted gastrointestinal reconstruction and total laparoscopic digestive reconstruction and confirmed its safety and effectiveness[10,25]. Uncut surgery in conjunction with the ERAS concept can achieve early recovery in patients.

In this study, the perioperative administration of the ERAS concept showed obvious advantages when intraoperative parameters, postoperative clinical indicators, and postoperative stress responses were compared. The ERAS concept of perioperative treatment significantly shortened the hospital stay after surgery (approximately 2.7 d), significantly improved patient comfort during the perioperative period, and reduced pain without increasing the operating time and blood loss during surgery. The time to initial liquid diet intake, time to out-of-bed activity, and time to postoperative gastrointestinal function recovery were significantly shorter than those in the control group under the premise of the same recovery effect[26,27]. Additionally, the ERAS concept can effectively reduce the level of

postoperative stress in patients. However, some reports have suggested that a delayed return to a normal diet can reduce the incidence of gastrointestinal anastomotic fistula[28]. However, most patients in the study were able to tolerate early postprandial feeding. A large-sample, multicenter, randomized controlled study confirmed the safety for patients with upper gastrointestinal tract surgery to begin a normal diet early. Further research regarding whether the ERAS concept has potential benefits for other patients is necessary. The above results show that health education, the development of a detailed diet, and encouraging patients to eat as soon as possible can effectively promote recovery and shorten hospital stay.

In conclusion, through the optimization and improvement of the traditional perioperative treatment program in this study, our results suggest that the use of the perioperative ERAS program for total laparoscopic uncut Roux-en-Y gastrojejunostomy after distal gastrectomy is safe and effective and should be popularized. This program can also reduce the duration of the hospital stay and improve the degree of comfort and satisfaction of patients.

ARTICLE HIGHLIGHTS

Research background

For gastric cancer, uncut Roux-en-Y gastrojejunostomy after distal gastrectomy is still the most important treatment. However, the safety of enhanced recovery after surgery (ERAS) protocol for gastric surgery is not clear.

Research motivation

Only a few studies have focused on the use of the perioperative ERAS program for total laparoscopic uncut Roux-en-Y gastrojejunostomy after distal gastrectomy. It is unclear whether introduction of the ERAS concept benefits the Chinese population.

Research objectives

This study aimed to evaluate the safety and feasibility of ERAS for total laparoscopic uncut Roux-en-Y gastrojejunostomy after distal gastrectomy. We can use the ERAS protocol for these patients to reduce the duration of the hospital stay and improve the degree of comfort and satisfaction of patients.

Research methods

The clinical data of 42 patients who were divided into a control group of 22 patients and an ERAS group of 20 patients were collected. The observed indicators included operation conditions, postoperative clinical indexes, and postoperative serum stress indexes. Measurement data following a normal distribution are presented as mean ± SD and were analyzed by *t*-test. Count data were analyzed by chi-squared test.

Research results

The operative time, volume of intraoperative blood loss, and number of patients with conversion to open surgery were not significantly different between the two groups. Postoperative clinical indexes, including the time to initial anal exhaust, time to initial liquid diet intake, time to out-of-bed activity, and duration of hospital stay of patients without complications, were significantly different between the two groups. However, the time to initial defecation, time to abdominal drainage-tube removal, and the early postoperative complications were not significantly different between the two groups. Regarding postoperative complications, on the first and the third days after the operation, the white blood cell count and C reactive protein and interleukin-6 levels in the ERAS group were significantly lower than those in the control group.

Research conclusions

We found that the perioperative ERAS program for total laparoscopic uncut Roux-en-Y gastrojejunostomy after distal gastrectomy is safe and effective and should be popularized. In this study, we carried out long-term follow-up and prognosis analysis of patients with gastric cancer who received uncut Roux-en-Y gastrojejunostomy after distal gastrectomy at our center to provide a theoretical basis for prognosis improvement of the patients.

Research perspectives

From this study, we can find that ERAS can be used not only for herniorrhaphy, gastrointestinal surgery, gynecologic operations, and other applications, but also for gastric surgery. The direction of the future research is that an effective perioperative management program specific for gastric cancer is needed to be developed. The best method is to conduct a large-scale clinical trial to verify it.

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ORIGINAL ARTICLE

Observational Study

Influence of *NUDT15* variants on hematological pictures of patients with inflammatory bowel disease treated with thiopurines

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Author contributions: Kojima Y contributed to writing a paper and data acquisition and most of the patients were in his charge; Hirotsu Y, Omata W, Sugimori M and Takaoka S performed genome analysis; Ashizawa H, Nakagomi K, Yoshimura D, Hosoda K and Suzuki Y contributed to the treatment of patients; Mochizuki H contributed to the data analysis; Omata M contributed to the study conception, design, reviewing and final approval of the article.

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Abstract

AIM

The single nucleotide polymorphism (SNP) c.415C>T in exon 3 of *NUDT15* affects thiopurine-induced leukopenia in Asian patients with Crohn's disease. Meanwhile, three additional genetic variants of *NUDT15* were reported in patients with acute lymphoblastic leukemia. We evaluated the effects of these additional genetic variants of *NUDT15* in patients with inflammatory bowel disease (IBD) treated with thiopurines.

METHODS

Ninety-six Japanese patients with IBD were enrolled. Genotyping for the *NUDT15* and *TPMT* genes was performed using Custom TaqMan SNP genotyping assays or Sanger sequencing. The changes in white blood cell (WBC) count, mean corpuscular volume (MCV), platelet count, hemoglobin, CRP, amylase, albumin, AST, ALT, and ESR were evaluated.

RESULTS

Genetic variants of exon 1 and exon 3 of *NUDT15* were identified in 24 of 96 patients (25.0%). C.52G > A and c.36_37insGGAGTC in exon 1 were found in three patients each. All three patients with c.36_37insGGAGTC in exon 1 were heterozygotes of p.Arg139Cys in exon 3. Eighteen patients had p.Arg139Cys in exon 3 alone. The WBC count gradually decreased after initiation of thiopurine treatment in the mutated cases (n = 24), and was significantly lower at 6, 8, 10, and 16 wk (P = 0.0271, 0.0037, 0.0051, and 0.0185, respectively). The WBC counts were also evaluated in patients with and without prednisolone treatment. In the patients with prednisolone treatment, the WBC count tended to show a greater decrease in the mutated cases, with significant differences at 8 and 10 wk (P = 0.012 and 0.029, respectively). In the patients without prednisolone treatment, the WBC count was significantly lower at 2, 4, 8, and 14 wk in mutated cases (P = 0.0196, 0.0182, 0.0237 and 0.0241, respectively). MCV increased after starting thiopurine treatment in the mutated cases, and was significantly higher at 10 wk (P = 0.0085). Platelet count, hemoglobin, CRP, amylase, albumin, AST, ALT and ESR did not differ significantly between the wild-type and mutated cases. *TPMT* mutations were not found in any of the patients.

CONCLUSION

Mutations in exon 1 of *NUDT15* also affect thiopurine-induced leukopenia in patients with IBD. To discuss thiopurine-induced leukopenia in more detail, investigation of SNPs in both exon 1 and exon 3 of *NUDT15* is needed.

Key words: Inflammatory bowel disease; *NUDT15*; Leukopenia; Mean corpuscular volume; Japanese

Core tip: Single nucleotide polymorphism (SNP) in *NUDT15* c.415C>T in exon 3 affects thiopurine-induced leukopenia in Asian Crohn's disease patients. Meanwhile, there is a report of additional three genetic variants of *NUDT15* in patients with acute lymphoblastic leukemia. We evaluated the effect of these additional genetic variants of *NUDT15* on inflammatory bowel disease (IBD) treated with thiopurines. The increase rate of mean corpuscular volume was higher in the variants than the wild, Mutations of *NUDT15* in exon 1 also affects thiopurine-induced leukopenia in patients with IBD. To discuss thiopurine-induced leukopenia, investigating SNPs both exons 1 and exon 3 of *NUDT15* is needed.

Kojima Y, Hirotsu Y, Omata W, Sugimori M, Takaoka S, Ashizawa H, Nakagomi K, Yoshimura D, Hosoda K, Suzuki Y, Mochizuki H, Omata M. Influence of *NUDT15* variants on hematological pictures of patients with inflammatory bowel disease treated with thiopurines. *World J Gastroenterol* 2018; 24(4): 511-518 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i4/511.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i4.511

INTRODUCTION

The number of patients with inflammatory bowel disease (IBD) are increasing worldwide. As the treatment for ulcerative colitis (UC) and Crohn's disease (CD), thiopurine drugs are widely used[1]. For UC, thiopurines are used for both steroid-dependent and steroid-resistant cases. For CD, thiopurines are recommended to be used with infliximab for better efficacy and prevention of events such as infusion reaction[2].

The reported major adverse events associated with thiopurines include leukopenia, pancreatitis, hair loss, and liver dysfunction[3,4]. In European descent, this leukopenia is mainly associated with genetic variations of *TPMT* which encodes thiopurine S-methyltransferase[5]. Meanwhile, in Asian patients, a single nucleotide polymorphism (SNP) in exon 3 of *NUDT15* c.415C>T (encoding p.Arg139Cys), was shown to play an important role in thiopurine-induced leukopenia[6-11]. When p.Arg139Cys occurred, the odds ratio of myelosuppression caused by thiopurines was 35.6 ($P = 4.88 \times 10^{-94}$) in Korean patients with CD[6].

Recently, three additional genetic variants of *NUDT15* were reported to induce leukopenia in patients with acute lymphoblastic leukemia (ALL)[12]. These three genetic variants were c.36_37insGGAGTC (encoding p.Val18_Val19insGlyVal) and c.52G > A (encoding p.Val18Ile) in exon 1 and c.416G > A (encoding p.Arg139His) in exon 3. However, these three SNPs were not examined in Asian patients with IBD.

Table 1 Demographic data and treatment of 96 patients

Patient	*n*
Gender (females/males)	32/64
Median age at presentation	28
Range	10-71
UC; CD	67; 29
Treatment	
6-MP (yes/no)	96/0
5-ASA (yes/no)	90/6
Steroid (yes/no)	55/41
Anti-TNF drugs (yes/no)	36/60

CD: Crohn's disease.

In the present study, we investigated the effects of all four SNPs in exon 1 and exon 3 of *NUDT15* and their correlations with biochemical parameters. We also analyzed three SNPs in the *TPMT* gene that are associated with drug responses and commonly performed in Europe.

MATERIALS AND METHODS

Patients

We enrolled 96 Japanese patients with IBD treated with thiopurines at our hospital between October 2015 and January 2016. These 96 patients comprised 32 females and 64 males with a median age of 28 years at presentation of IBD. Sixty-seven patients had UC and 29 patients had CD (Table 1). The treatment protocols were as follows. All 96 patients were treated with 6-mercaptopurine (6-MP), which was started at a dose of 30 mg daily. Ninety patients received 5-ASA, 55 patients received steroid, and 36 patients received anti-TNF drugs (infliximab, 29; adalimumab, 7) (Table 1). Written informed consent to conduct genetic analysis of *NUDT15* and *TPMT* was obtained from all 96 patients.

Adverse events were examined every week for the first month and then every 1-2 mo thereafter. Blood samples were analyzed for white blood cell (WBC) count, hemoglobin, mean corpuscular volume (MCV), platelet count, amylase, lipase, AST, ALT, albumin, CRP, and ESR.

The protocol was approved by the Institutional Review Board of Yamanashi Prefectural Central Hospital.

DNA extraction

Peripheral blood samples were obtained from the 96 patients. Buffy coats were isolated by centrifugation of the blood samples at 820 × *g* at 25 ℃ for 10 min and stored at -80 ℃ until required for DNA extraction. Buffy-coat DNA was extracted using a QIAamp® DNA Blood Mini QIAcube Kit (Qiagen, Hilden, Germany) with a QIAcube (Qiagen). The total genomic DNA concentration was determined using a Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) as described previously[13,14].

Sanger sequencing

PCR was performed using genomic DNA as a template and primer pairs flanking the SNP sites in exon 1 (rs869320766, p.Val18_Val19insGlyVal; rs186364861, Val18Ile) and exon 3 (rs116855232, Arg139Cys; rs147390019, Arg139His) of the *NUDT15* gene. The PCR products were cleaned up using ExoSAP-IT™ Reagent (Affymetrix, Santa Clara, CA, United States) according to the manufacturer's instructions. Sequencing was performed with a BigDye Terminator v3.1 (Thermo Fisher Scientific) using forward or reverse primers. The PCR products were purified and subsequently analyzed by a 3500 Genetic Analyzer (Thermo Fisher Scientific)[15,16]. The primer sequences are provided in Table 2.

Single nucleotide polymorphism genotyping

Real-time PCR was conducted in a ViiA7 system (Thermo Fisher Scientific) using TaqMan Genotyping Master Mix (1 ×) (Life Technologies Corp.), forward and reverse primers, and specific probes. SNP genotyping was conducted by the allelic discrimination method. *NUDT15* (rs186364861, Val18Ile; rs116855232, Arg139Cys) and *TPMT* (rs1800462, rs1800460, and rs1142345) genotyping primers and probes were purchased from Thermo Fisher Scientific. *NUDT15* SNP typing was validated by the Sanger sequencing results. The GenBank sequences of human *NUDT15* (accession number: NP_060753.1) and *TPMT* (accession number: NP_000358.1) were accessed at the NCBI Reference Sequence Database.

Statistical analysis

All statistical analyses were performed using R version 3.3.3. The statistical significance of differences in mean values between two cohorts was assessed by Student's *t*-test if the variances were equal in an *F* test, or by the nonparametric Mann-Whitney test if the variances were not equal.

RESULTS

Genetic variants of exon 1 and exon 3 of *NUDT15* were identified in 24 of 96 patients (25.0%) (Table 3). All mutated cases were heterozygotes. C.52G > A (p.Val18Ile) in exon 1 was found in three patients (Group A, Table 3). All three patients with c.36_37insGGAGTC (p.Val18_Val19insGlyVal) in exon 1 were heterozygotes for c.415C>T (p.Arg139Cys) in exon 3 (Group B, Table 3).

Eighteen patients had c.415C>T (p.Arg139Cys) in exon 3 alone (Group C, Table 3). The mutations p.Val18Ile and p.Val18_Val19insGlyVal were mutually exclusive. The mutation c.416G > A (p.Arg139His) in exon 3 was not observed in any of the patients.

We investigated the changes in the WBC count. The WBC count gradually decreased after thiopurine

Table 2 Primer sequence for Sanger sequencing analysis of *NUDT15* exon 1 and exon 3

Primer	Primer sequence
NUDT15 exon1 forward	5′-CAAAGCACAACTGTAAGCGACT-3′
NUDT15 exon1 reverse	5′-GAAAGACCCAGCTAGCAAAGAC-3′
NUDT15 exon3 forward	5′-TTGTATAGCCAAGCAAATGCAAAGC-3′
NUDT15 exon3 reverse	5′-TCTGTGTCTGGAATACAATTCAATGAC-3′

Table 3 Genotypes of *NUDT15* and *TPMT*

Patients	Exon1	Exon 3	*TPMT*
Group A (n = 3)			
#1-#3	c.52G > A (p.Val18Ile)	Wild	Wild
Group B (n = 3)			
#4-#6	c.36_37insGGAGTC (p.Val18_Val19insGlyVal)	c.415C>T (p.Arg139Cys)	Wild
Group C (n = 18)			
#7-#24	Wild	c.415C>T (p.Arg139Cys)	Wild
Group D (n = 72)			
#25-#96	Wild	Wild	Wild

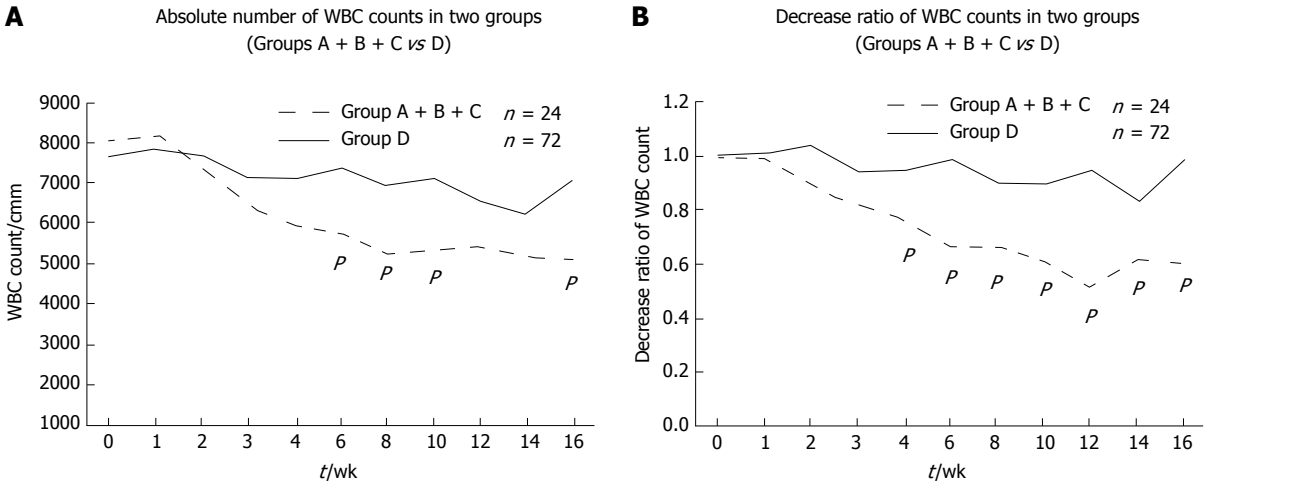

Figure 1 Change of white blood cell counts (Group A, B and C *vs* D). A: Absolute number of WBC counts in two groups (Groups A, B and C *vs* D). WBC gradually decreased after the thiopurine was started in both the mutant (n = 24) and the wild type. The WBC count of the mutant was lower and statistically significant at 6, 8, 10 and 16 wk (P = 0.0271, 0.0037, 0.0051 and 0.0185, respectively); B: Decrease rate of the WBC counts in two groups (Groups A, B and C *vs* D). The decrease rate was higher in the variants (n = 24) than the wild (n = 72) and statistically significant at 4, 6, 8, 10, 12, 14 and 16 wk (P = 0.004, 0.0001, 0.0012, 0.0022, 0.00001, 0.0264 and 0.0031, respectively). We set 1.0 as the WBC count at the beginning of thiopurines. WBC: White blood cell.

treatment was started in both the mutated (n = 24) and wild-type (n = 72) cases (Figure 1A). The WBC count in the mutated cases was significantly lower at 6, 8, 10 and 16 wk (P = 0.0271, 0.0037, 0.0051 and 0.0185, respectively). To examine the decrease rates in the WBC count, we set the WBC count at the beginning of thiopurine treatment at 1.0. The decrease rate was higher in the mutated cases (n = 24) than in the wild-type cases (n = 72), and showed significant differences at 4, 6, 8, 10, 12, 14 and 16 wk (P = 0.004, 0.0001, 0.0012, 0.0022, 0.00001, 0.0264, and 0.0031, respectively, Figure 1B).

We also analyzed the WBC count in the patients with and without prednisolone treatment. In the patients with prednisolone treatment, the WBC count tended to show a greater decrease in the mutated cases (Group A + B + C), with significant differences at 8 and 10 wk (P = 0.012 and 0.029, respectively; Figure 2A). Prednisolone induced dynamic change of WBC counts which varied in each case. Statistical difference was only obtained at 8 and 10 wk. In the patients without prednisolone treatment, the WBC count was significantly lower at 2, 4, 8 and 14 wk in the mutated cases (Group A + B + C) compared with the wild-type cases (Group D; P = 0.0196, 0.0182, 0.0237, and 0.0241, respectively; Figure 2B).

Next, we divided the cases into three categories: Group A + B, Group C and Group D (Figure 3). Group C was already reported cases in IBD with c.415C>T in exon 3 of *NUDT15*. Group A + B did not show any significant differences from Group C, but had a lower WBC count compared with that in Group D.

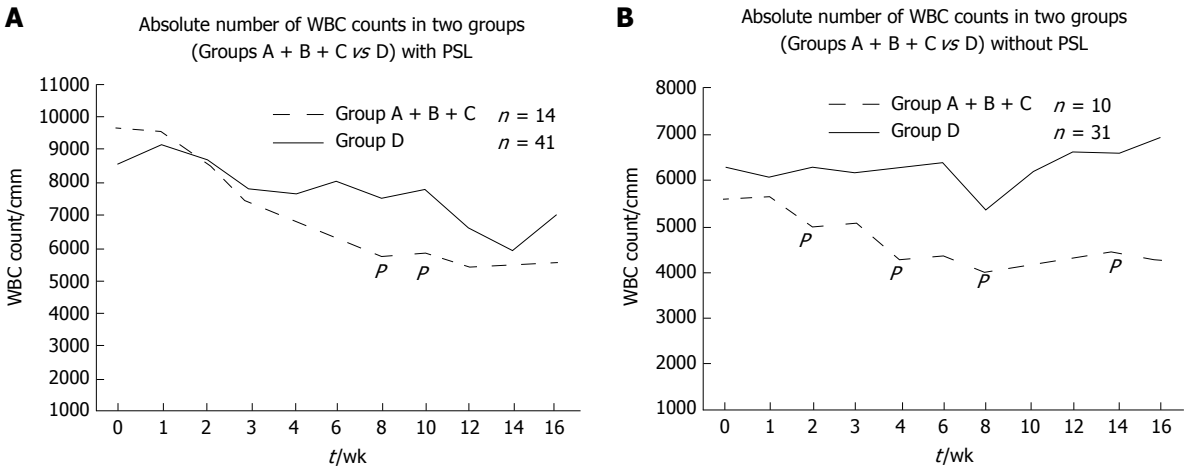

Figure 2 Change of white blood cell counts with or without prednisolone. A: Absolute number of WBC counts in two groups (Groups A + B + C *vs* D) with PSL. In the cases with prednisolone, WBC count tended to decreased more in the mutant cases (Group A + B + C) and was significantly different at 8 and 10 wk (P = 0.012 and 0.029, respectively); B: Absolute number of WBC counts in two groups (Groups A + B + C *vs* D) without PSL. In the cases without prednisolone, WBC count was significantly lower at 2, 4, 8 and 14 wk significantly in the mutant (Group A + B + C) than the wild cases (Group D, P = 0.0196, 0.0182, 0.0237 and 0.0241, respectively). PSL: Prednisolone; WBC: White blood cell.

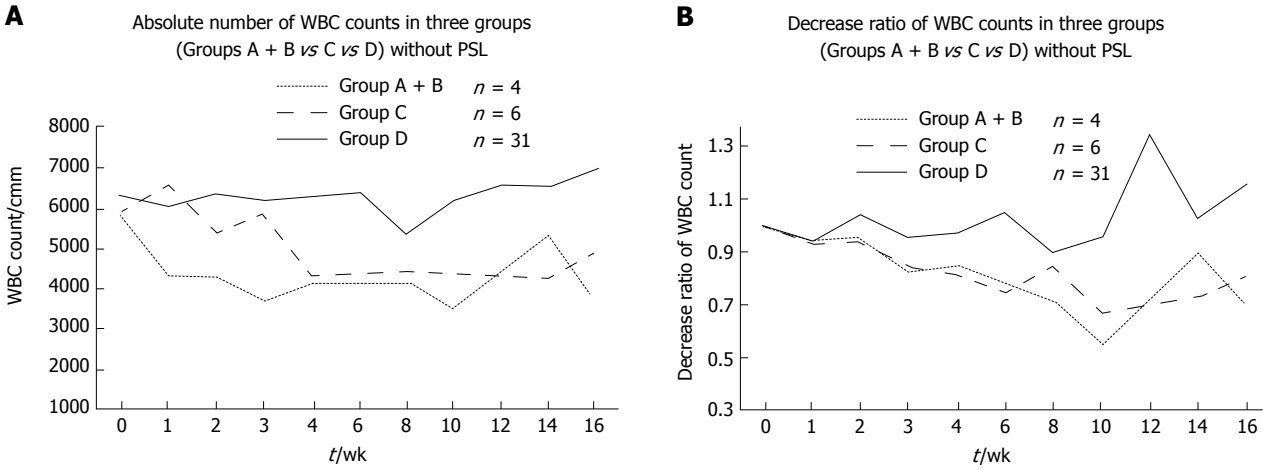

Figure 3 Effect of thiopurine on white blood cell count. A: Absolute number of WBC counts in three groups (Groups A + B *vs* C *vs* D) without PSL. We next divided cases into 3 categories: Group A + B, Group C and Group D. Group C was already reported cases with *NUDT15* c.415C>T in exon 3 in IBD cases. Group A + B included mutations in exon 1 which is not investigated in IBD. Group A + B and Group C was lower in WBC count and decrease rate than Group D; B: Decrease ratio of WBC counts in three groups (Groups A + B *vs* C *vs* D) without PSL; Group A + B and Group C was lower in WBC count and decrease rate than Group D; Group A + B and Group C was lower in decrease rate than Group D. PSL: Prednisolone; WBC: White blood cell; IBD: Inflammatory bowel disease.

As it is well known that thiopurines increase MCV[17], we analyzed the changes in MCV after initiation of thiopurine treatment. MCV increased after starting 6MP in both the mutated (Group A + B + C) and wild-type (Group D) cases (Figure 4). MCV was significantly higher at 10 wk in the mutated cases compared with the wild-type cases (P = 0.0085; Figure 4A). To analyze the increase rate in MCV, we set the MCV at the beginning of thiopurine treatment at 1.0. The increase rate was higher in the mutated cases compared with the wild-type cases, and the difference was significant at 16 wk (P = 0.00198; Figure 4B).

We also investigated the changes in platelet count, hemoglobin, CRP, amylase, albumin, AST, ALT and ESR, but did not observe any significant differences between the mutated and wild-type cases. *TPMT* mutations were not observed in any of the 96 patients.

DISCUSSION

The genotypes of *NUDT15* vary worldwide, according to the 1000 Genomes Project (http://www.1000genomes.org/category/frequently-asked-questions/population), 5000 Exomes Project (NHLBI ESP; https://esp.gs.washington.edu/drupal/), and The Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org). However, detailed information on the different types of *NUDT15* SNPs is not necessarily available for individual diseases.

Recently, new genotypes of *NUDT15* were reported in patients with ALL, including three genetic variants of *NUDT15* that induced leukopenia[12]. Until now, there have been several reports on analyses of *NUDT15* in patients with IBD. However, these studies only evaluated one SNP site (c.415C>T, encoding

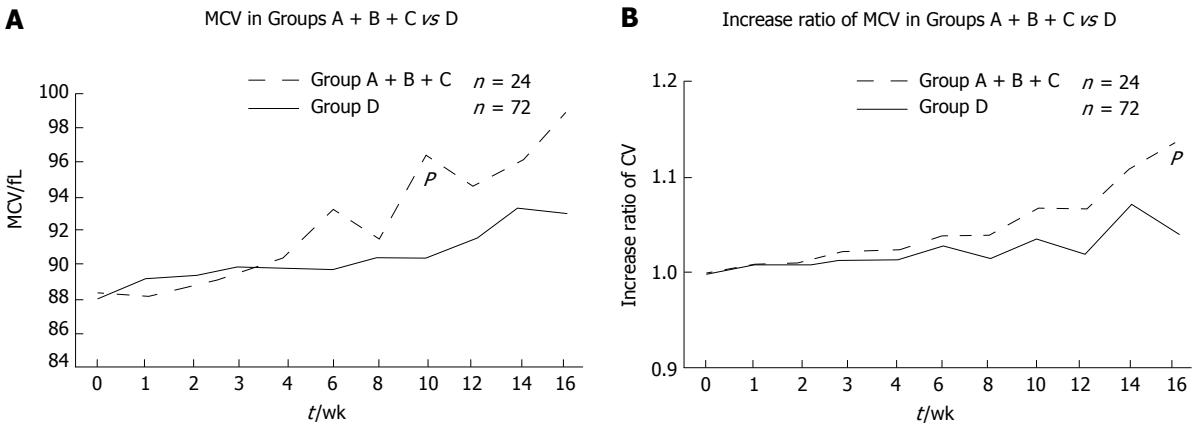

Figure 4 Effect of thipourine on mean corpuscular volume. A: MCV in Groups A + B + C *vs* D. MCV increased after starting 6MP in both the mutant (Group A + B + C) and the wild cases (Group D, Figure 4). MCV was significantly higher at 10 wk in the mutant than the wild cases ($P = 0.0085$); B: Increase ratio of MCV in Groups A + B + C *vs* D. The increase rate was higher in the variants than the wild and statistically significant at 16 wk ($P = 0.00198$). We set 1.0 as the MCV at the beginning of thiopurines. 6MP: 6-mercaptopurine; MCV: Mean corpuscular volume.

p.Arg139Cys) in exon 3[6,7,18]. Therefore, we analyzed the three additional genetic variants of *NUDT15*, namely c.36_37insGGAGTC (encoding p.Val18_Val19insGlyVal) and c.52G > A (encoding p.Val18Ile) in exon 1 and c.416G > A (encoding p.Arg139His) in exon 3, in patients with IBD.

In our 96 patients with IBD, we found six cases with exon 1 mutations. Three exon 1 mutated cases with c.36_37insGGAGTC also had the c.415C>T mutation in exon 3. However, the other three exon 1 mutated cases with c.52G > A had the wild-type in exon 3. Consequently, without analysis of exon 1, 3.1% (3 of 96) of the patients at risk of thiopurine-induced leukopenia would have been missed. Regarding the mutations among the 24 patients with SNPs in either exon 1 or exon 3, 6 (25%) had mutations in exon 1. Thus, to fully evaluate thiopurine-induced leukopenia and other side effects, investigation of both exon 1 and exon 3 of *NUDT15* is necessary.

We also examined MCV and other variables. MCV tended to be higher in the *NUDT15* mutated cases than in the wild-type cases, with a significant difference at 10 wk after the start of thiopurine treatment. We didn' t measure the concentrations of folate and vitamin B12, which affect MCV. Before starting thiopurines, the MCV was in normal range. Previously it is reported that salazosulfapyridine, one of 5ASAs, decreased the absorption of folate, but the only nine patients were taking salazosulphapyridine and the MCV was also in normal range at the initiation of 6MP. MCV was previously shown to be positively correlated with the 6-thioguanine nucleotide (6-TGN) concentration in red blood cells[19]. The role of *NUDT15* SNPs is not totally understood. It was reported that *NUDT15* inactivates thiopurine metabolites and decreases thiopurine cytotoxicity *in vitro*, and that patients with defective *NUDT15* alleles had excessive levels of thiopurine active metabolites and toxicity[12]. Another study that evaluated 6-TGN levels found that thiopurine-induced leukopenia was independent of the 6-TGN concentration[7]. However, that study measured the total amount of 6-TGN and did not differentiate among thiopurine active metabolites (TGTP and DNA-6TG incorporation). Therefore, further investigations are necessary to examine the correlations of *NUDT15* SNPs and thiopurine metabolites, and hence the induction of side effects.

We also tested three SNPs of *TMPT*, and as previously reported, no TPMT variant was found. So it is not necessarily performed in Asian patients. Our results support previous data that *TPMT* variant is low in Asian patients.

Our study has several limitations. Firstly, the number of patients was too small to have definite conclusions. For example, the WBC count in patients with prednisolone treatment was not significant at time points other than 8wk and 10 wk. We have not encountered any patients with agranulocytosis and severe hair loss. If we were able to recruit more patients we may be able to obtain definite conclusions. Secondly, our study was a retrospective in nature, and therefore clinical utility of SNP analysis is not assured to avoid complications related to use of 6MP. Thirdly, we only observed the patients for 16 wk after initiation of thiopurine treatment, the long-term effects of thiopurines remain unclear. We are planning further studies to clarify these limitations.

Recently, three other *NUDT15* variants, c.101G > C (p.R34T), c.103A > G (p.K35E), and c.37_42delGGAGTC (p.G17_V18del), were reported in ALL patients[20]. By using next-generation sequencing, it will become easier to provide information on *NUDT15* SNPs despite changes in SNP numbers. Analysis of *NUDT15* should be routinely performed before starting thiopurine treatment in patients with IBD.

ARTICLE HIGHLIGHTS

Research background

Previous study demonstrated that single nucleotide polymorphism (SNP)

in *NUDT15* c.415C>T (encoding p.Arg139Cys) in exon 3 affects thiopurine-induced leukopenia in Asian patients with inflammatory bowel disease (IBD). In acute lymphoblastic leukemia (ALL), there are other variants of *NUDT15* in exon 1 and exon 3. We demonstrated the variants of c.36_37insGGAGTC (encoding p.Val18_Val19insGlyVal) and c.52G > A (encoding p.Val18Ile) in exon 1 also affect the thiopurine-induced leukopenia. To present thiopurine-induced leukopenia and other side effects, checking both exons 1 and exon 3 of *NUDT15* is definitely needed.

Research motivations

It is well known that leukopenia is one of the most important adverse effects of thiopurines. To distinguish the high risk group of the adverse effects is clinically very important. Thus we investigated other *NUDT15* variants than *NUDT15* c.415 C > T in exon 3.

Research objectives

The main of this paper is to investigate other *NUDT15* variants than c.415 > T have an effect on hematological pictures including WBC count.

Research methods

We enrolled 96 Japanese patients with IBD. Genotyping for *NUDT15* and *TPMT* genes was performed using Custom TaqMan SNP genotyping assays or Sanger sequencing. The changes of white blood cell (WBC) count, mean corpuscular volume (MCV), platelet count, hemoglobin, CRP, amylase, albumin, AST, ALT and ESR were analyzed.

Research results

In 24 out of 96 patients (25.0%), genetic variants of exons 1 and 3 were identified. C.52G > A and c.36_37insGGAGTC in exon 1 was found in 3 cases each. All 3 cases of c.36_37insGGAGTC in exon 1 had heterozygote of p.Arg139Cys in exon 3. Eighteen patients showed p.Arg139Cys in exon 3 alone. WBC count gradually decreased after thiopurine was started in the mutant (n = 24). The WBC count of the mutant was statistically significantly lower at 6, 8, 10 and 16 wk (P = 0.0271, 0.0037, 0.0051 and 0.0185, respectively). We also analyzed WBC count in the cases with and without prednisolone. In the cases with prednisolone, WBC count tended to decrease more in the mutant cases and was significantly lower at 8 and 10 wk (P = 0.012 and 0.029, respectively). In the cases without prednisolone, WBC count was significantly lower at 2, 4, 8 and 14 wk in the mutant than the wild cases (P = 0.0196, 0.0182, 0.0237 and 0.0241, respectively). MCV increased after starting thiopurine in the mutant. MCV was significantly higher at 10 wk in the mutant than the wild cases (P = 0.0085). Platelet count, hemoglobin, CRP, amylase, albumin, AST, ALT, and ESR was not different between the wild and the mutant cases. *TPMT* mutation was not found in any of our Japanese patients.

Research conclusions

We reported *NUDT15* variant in exon 1 also affect thiopurine-induced leukopenia in patients with IBD. Before starting the treatment with thiopurines for patients with IBD, *NUDT15* variant in exon 1 and 3 will be routinely performed for preventing adverse events of thiopurines in the near future.

Research perspectives

There are other *NUDT15* variants which are reported in patients with ALL and near future their role on IBD patients will be investigated.

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META-ANALYSIS

Impact of mechanical bowel preparation in elective colorectal surgery: A meta-analysis

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Author contributions: Rollins KE and Javanmard-Emamghissi H designed this work, collected data, and drafted the manuscript; Lobo DN conceptioned and designed this work, collected and interpreted the data, critically revised the manuscript, and overall supervision; all authors contributed to final approval, accountability for the manuscript.

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Abstract

AIM

To analyse the effect of mechanical bowel preparation *vs* no mechanical bowel preparation on outcome in patients undergoing elective colorectal surgery.

METHODS

Meta-analysis of randomised controlled trials and observational studies comparing adult patients receiving mechanical bowel preparation with those receiving no mechanical bowel preparation, subdivided into those receiving a single rectal enema and those who received no preparation at all prior to elective colorectal surgery.

RESULTS

A total of 36 studies (23 randomised controlled trials and 13 observational studies) including 21568 patients undergoing elective colorectal surgery were included. When all studies were considered, mechanical bowel preparation was not associated with any significant difference in anastomotic leak rates (OR = 0.90, 95%CI: 0.74 to 1.10, P = 0.32), surgical site infection (OR = 0.99, 95%CI: 0.80 to 1.24, P = 0.96), intra-abdominal collection (OR = 0.86, 95%CI: 0.63 to 1.17, P = 0.34), mortality (OR = 0.85, 95%CI: 0.57 to 1.27, P = 0.43), reoperation (OR = 0.91, 95%CI: 0.75 to 1.12, P = 0.38) or hospital length of stay (overall mean difference 0.11 d, 95%CI: -0.51 to 0.73, P = 0.72), when compared with no mechanical bowel preparation, nor when evidence from just randomized controlled

trials was analysed. A sub-analysis of mechanical bowel preparation *vs* absolutely no preparation or a single rectal enema similarly revealed no differences in clinical outcome measures.

CONCLUSION

In the most comprehensive meta-analysis of mechanical bowel preparation in elective colorectal surgery to date, this study has suggested that the use of mechanical bowel preparation does not affect the incidence of postoperative complications when compared with no preparation. Hence, mechanical bowel preparation should not be administered routinely prior to elective colorectal surgery.

Key words: Bowel preparation; Mechanical; Antibiotics; Morbidity; Mortality; Surgery; Outcome complications; Meta-analysis

Core tip: At present there is no evidence that bowel preparation makes a difference to clinical outcomes in either colonic or rectal surgery, in terms of anastomotic leak rates, surgical site infection, intra-abdominal collection, mortality, reoperation or hospital length of stay. Given its potential adverse effects and patient dissatisfaction rates, it should not be administered routinely to patients undergoing elective colorectal surgery.

Rollins KE, Javanmard-Emamghissi H, Lobo DN. Impact of mechanical bowel preparation in elective colorectal surgery: A meta-analysis. *World J Gastroenterol* 2018; 24(4): 519-536 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i4/519.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i4.519

INTRODUCTION

Mechanical bowel preparation (MBP) for colorectal surgery has been surgical dogma for decades, despite increasing evidence from the 1990s refuting its benefits[1,2]. The rationale behind the administration of MBP is that it reduces fecal bulk and, therefore, bacterial colonisation, thereby reducing the risk of postoperative complications such as anastomotic leakage and wound infection[3], as well as to facilitate dissection and allow endoscopic evaluation. Opponents argue that in the 21st century, with rational use of oral and intravenous prophylactic antibiotics there is no longer a place for MBP, that it may cause marked fluid and electrolyte imbalance in the preoperative period, and that evidence has shown that the gut microbial flora load is not reduced grossly by bowel preparation[4]. There is also concern that bowel preparation liquefies feces, thereby increasing the risk of spillage and contamination intra-operatively[5]. Its use remains controversial, particularly within the context of an enhanced recovery after surgery (ERAS) program setting[6,7].

Meta-analyses[8-12] have been published on MBP in elective colorectal surgery showing mixed results, with most studies demonstrating no difference in infective complications between patients receiving MBP or control treatment, although control treatment varied significantly between the use of a rectal enema or absolutely no preparation. Similar results have been found in gynaecological[13,14] and urological[15,16] surgery where studies have shown no benefits in visualisation, bowel handling or complication rates between patients treated with bowel preparation and those given no bowel preparation. As a result of this inconclusive evidence, several studies have established that practice varies significantly between countries, and even surgeons in the same institution[17,18]. Further impediments to the issue are that no consensus has yet been reached regarding the optimal method of bowel cleansing. Various agents such as polyethylene glycol (PEG), sodium phosphate, mannitol, milk of magnesia, liquid paraffin and senna have been used to achieve bowel cleansing.

Infective complications are amongst the leading causes of morbidity and mortality in patients undergoing colorectal surgery[19]. However, MBP is not without its own complications and the process is both time-consuming and unpleasant for patients[20]. It has been shown to cause clinically significant dehydration[21] and electrolyte disturbances, particularly hypocalcaemia and hypokalaemia to which the elderly are especially vulnerable[22-24]. Patient satisfaction is poor for undergoing bowel preparation prior to surgery and colonoscopy, and this may necessitate an additional day preoperatively in hospital, particularly for frail elderly patients.

In the United Kingdom, the National Institution of Health and Clinical Excellence (NICE) does not recommend using MBP routinely to reduce the risk of surgical site infection (SSI)[25] and the ERAS® Society guidelines on perioperative care of patients undergoing colonic resection[6] also recommend against using preoperative bowel preparation. However, for rectal[7] resection the recommendation, albeit weak, is to use MBP for patients undergoing anterior resection with diverting stomas. In recent years further evidence has emerged from large database studies using the National Surgical Quality Improvement (NSQIP) database in America[26-29] showing reduced rates of anastomotic leakage, intra-abdominal abscess formation and wound infection when patients were given MBP with intraluminal antibiotics pre-operatively.

We have assessed this expanding body of evidence in this new comprehensive meta-analysis encompassing both randomised controlled trials and observational studies. We sought to address deficiencies in previous studies by including all levels of evidence, separating those in which patients received a single rectal enema *vs* full or no preparation, and including the recently

published large database studies.

Our aims for this meta-analysis were: (1) To analyse the effect of MBP *vs* no preparation or rectal enema alone on postoperative infective complications in patients undergoing elective colorectal surgery; (2) To examine the differences in results between evidence obtained from randomised controlled trials and observational studies; and (3) To determine what effect, if any, bowel preparation had on postoperative complications in rectal surgery.

MATERIALS AND METHODS

Search Strategy

We performed an electronic search of the PubMed database and the Cochrane Central Register of Controlled Trials to identify studies comparing outcomes in patients undergoing elective colorectal surgery treated with MBP *vs* either no preparation or a single rectal enema (last search on 1st May 2017). We used the search terms "(bowel preparation OR bowel cleansing OR bowel cleaning) AND (surgery OR preoperative)". Further sources were obtained by a manual search of the bibliography of the papers obtained to ensure the search was as comprehensive as possible. We did not apply language restriction or time limitations. Two independent researchers (KER and HJ-E) reviewed the abstracts for inclusion. Where there was a difference of opinion on the inclusion of papers, the opinion of the senior author was sought (DNL). We performed this meta-analysis according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA)[30] and Guidelines for Meta-Analyses and Systematic Review of Observational Studies (MOOSE) statements[31].

Selection of articles

We reviewed full text articles for suitability after excluding studies on the basis of title and abstract. Our inclusion criteria specified that studies must have a minimum of two comparator groups and were either designed as randomised controlled trials or observational studies. Publications comparing preoperative MBP with no preparation or a single rectal enema were included and comparisons with other forms of bowel preparation (*e.g.* intraoperative colonic lavage) were excluded. Only studies on adult patients undergoing elective colorectal surgery were included. We included studies on laparoscopic and open surgical procedures but excluded endoscopic studies. Relevant outcome measures were anastomotic leak, SSI, intra-abdominal abscess, mortality, reoperation and hospital length of stay.

Duplication of results was a particular hazard encountered when selecting which of the studies to include that extracted information from the NSQIP database[26-29,32-36]. The papers were scrutinised for their enrollment dates. There was overlap in these dates and after correspondence with the authors, it was apparent that there was considerable overlap in the data sets used. Hence, we selected the largest study for inclusion with the greatest number of clinically relevant outcome measures[29]. Two further studies[37,38] had duplication of results and in this situation the larger of the two studies was included[38]. One study[39] was a subgroup analysis of patients undergoing anastomosis below the peritoneal reflection taken from a study which was already included[40] in the meta-analysis so this was excluded from the main meta-analysis to prevent dual inclusion of patients. However, this subgroup was included in the separate analysis of rectal surgery. A further study[41] reviewed as a full text article was retracted since its inclusion in the 2011 Cochrane Review[10], so we chose to exclude this. One paper[2] analysed in the Cochrane Review included pediatric patients and so has been excluded from our meta-analysis.

Data extraction

HJ-E extracted the data and they were verified independently by KER. Quantitative data relevant to the endpoints we selected were extracted. Several studies presented hospital length of stay results in formats other than mean and standard deviation. Where this occurred, the authors were contacted for the raw data in order to ascertain the mean and standard deviation necessary for creation of Forest plot. When the raw data were unavailable, mean and standard deviation were calculated using the technique described by Hozo *et al*[42].

Risk of bias and completeness of reporting of individual studies

The risk of bias was assessed using the Cochrane Collaboration tool in RevMan 5.3[43], which focuses upon random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias) and selective reporting (reporting bias).

Statistical analysis

The analysis was performed using RevMan 5.3 software[43]. Continuous variables were calculated as a mean difference and 95% confidence interval using an inverse variance random effects model. Dichotomous variables were analysed using the Mantel-Haenszel random effects model to quote the risk ratio (RR) and 95% confidence interval. These analyses were used to construct forest plots, with statistical significance taken to be a P value of < 0.05 on two tailed testing. A predetermined subgroup analysis was performed for the impact of MBP in rectal surgery specifically using the same methodology. Study inconsistency and heterogeneity were assessed using the I^2 statistic[44].

Protocol registration

The protocol for this meta-analysis was registered

Table 1 Risk of bias of studies included

Ref.	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting
Ji *et al*[76]	NA	NA	NA	NA	NA	NA
Chan *et al*[77]	NA	NA	NA	NA	NA	NA
Hu *et al*[64]	?	?	?	?	?	?
Bhattacharjee *et al*[65]	+	?	-	?	?	?
Allaix *et al*[74]	NA	NA	NA	NA	NA	NA
Kiran *et al*[29]	NA	NA	NA	NA	NA	NA
Yamada *et al*[66]	NA	NA	NA	NA	NA	NA
Otchy *et al*[67]	NA	NA	NA	NA	NA	NA
Kim *et al*[75]	NA	NA	NA	NA	NA	NA
Tahirkheli *et al*[62]	+	?	?	?	-	-
Sasaki *et al*[61]	+	?	?	?	?	?
Bertani *et al*[45]	+	+	?	?	+	+
Roig *et al*[68]	NA	NA	NA	NA	NA	NA
Bretagnol *et al*[46]	+	+	+	+	-	+
Pitot *et al*[69]	NA	NA	NA	NA	NA	NA
Alcantara Moral *et al*[47]	+	+	?	?	?	+
Miron *et al*[70]	NA	NA	NA	NA	NA	NA
Pena-Soria *et al*[48]	+	+	+	+	-	+
Leiro *et al*[59]	+	+	?	?	?	+
Contant *et al*[40]	+	+	- (2)	- (2)	-	+
Bretagnol *et al*[71]	NA	NA	NA	NA	NA	NA
Jung *et al*[49]	+	+	+	+	-	?
Veenhof *et al*[72]	NA	NA	NA	NA	NA	NA
Ali *et al*[63]	?	?	?	?	?	?
Jung *et al*[50]	+	+	+	+	-	?
Platell *et al*[51]	+	+	+	+	-	-
Fa-Si-Oen *et al*[52]	+	+	?	?	+	+
Bucher *et al*[53]	+	+	+	+	+	+
Ram *et al*[54]	+	- (1)	?	?	?	+
Zmora *et al*[37]	+	+	?	?	-	+
Young Tabusso *et al*[55]	?	?	- (2)	- (2)	?	?
Miettinen *et al*[56]	+	+	?	?	+	+
Memon *et al*[73]	NA	NA	NA	NA	NA	NA
Fillmann *et al*[60]	+	+	+	+	+	+
Burke *et al*[57]	?	?	+	+	-	-
Brownson *et al*[58]	?	?	?	?	?	?

NA: Not applicable (observational study); +: Low risk of bias; -: High risk of bias; (1): Allocation concealment utilized identification number of patient (odd or even); (2): Not blinded.

with the PROSPERO database (www.crd.york.ac.uk/prospero) - registration number CRD42015025279.

RESULTS

From 1594 studies identified from the original search, 97 were reviewed as full text articles. Of these, 36 comprising 23[37,40,45-65] randomised controlled trials and 13 observational studies[29,66-77] were eligible for inclusion (Figure 1). The risk of bias of the randomised controlled trials included in this study was moderate (Table 1).

Patient demographics

Overall, 21568 patients were included in the meta-analysis, of whom 6166 had no bowel preparation of any sort, 2739 had a solitary rectal enema and 12663 underwent full MBP as per local policy. Of these, 6277 patients were included in randomised controlled trials and 15291 in observational studies. Demographic details are summarised in Table 2 and of details of interventions (bowel preparation and perioperative antibiotics) in Table 3.

Anastomotic leak

All studies except one[75] included data on the primary outcome measure of this meta-analysis, the incidence of anastomotic leak (Figure 2). When MBP was compared with no MBP (including no preparation at all and those who underwent a single rectal enema), there was no difference in the incidence of anastomotic leak (OR = 0.90, 95%CI: 0.74 to 1.10, P = 0.32). When MBP *vs* absolutely no MBP was analysed[29,40,46,48-50,52,54-65,68,70,71,73], this made no difference to anastomotic leak rates (OR 0.94, 95% CI 0.70 to 1.25, P = 0.67), nor when MBP was compared with a single rectal enema[37,45,47,51,53,66,67,69,72,74,76,77] (OR = 0.92, 95%CI: 0.70 to 1.20, P = 0.52).

When randomised controlled trials alone were included in the analysis[37,40,45-65] (Supplementary Figure 1A), the use of MBP *vs* no MBP did not affect the incidence of anastomotic leak (OR = 1.02, 95%CI: 0.75

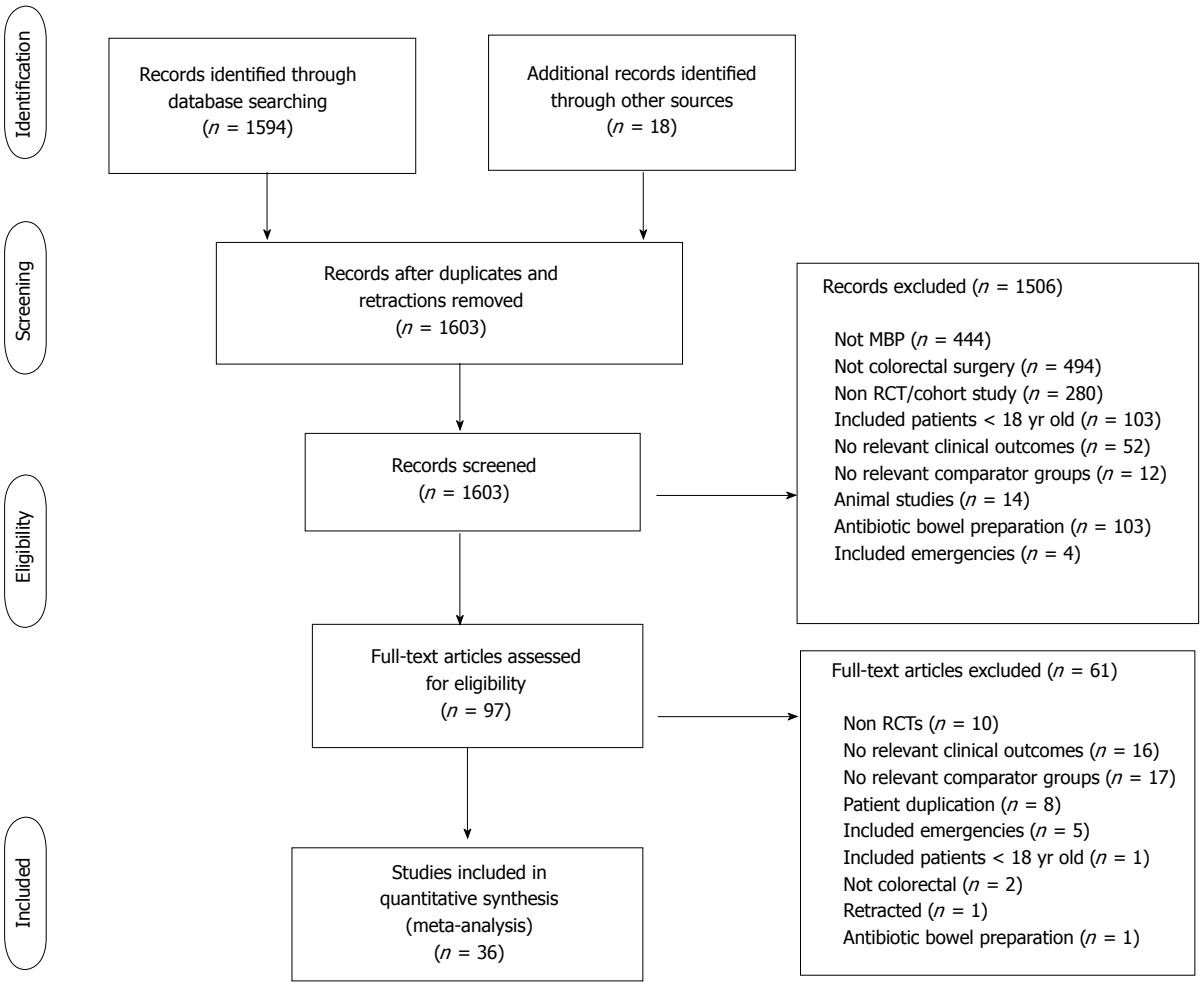

Figure 1 PRISMA diagram showing identification of relevant studies from initial search, PRISMA: Preferred reporting Items for systematic reviews and meta-analyses.

to 1.40, *P* = 0.90), nor when MBP *vs* absolutely no MBP[40,46,48-50,52,54-65] or MBP *vs* single rectal enema[37,45,47,51,53]were considered. When observational studies alone were analysed[66-73,76,77] (Supplementary Figure 1B), the use of MBP *vs* no MBP did significantly affect the incidence of anastomotic leak (OR = 0.76, 95%CI: 0.63 to 0.91, *P* = 0.003), although this was not significant when MBP *vs* single rectal enema[66,67,69,72,74,76,77] and MBP *vs* absolutely no MBP[29,68,70,71,73] were considered separately.

SSI

Data on the incidence of SSI were presented in a total of 19780 patients in 32 studies[29,37,40,45-61,64-70,72-75,77] (Figure 3). There was no difference in the incidence of SSI in those who did *vs* those who did not undergo MBP (OR = 0.99, 95%CI: 0.80 to 1.24, *P* = 0.96), nor in those who had MBP *vs* those receiving a single rectal enema[37,45,47,51,53,66,67,69,72,74,77] (OR = 1.00, 95%CI: 0.57 to 1.76, *P* = 1.00) or those who had MBP *vs* those receiving absolutely no preparation[29,40,46,48-50,52,54-61,64,65,68,70,73,75] (OR = 0.98, 95%CI: 0.78 to 1.24, *P* = 0.87).

When data obtained from 21 randomised controlled trials[37,40,43,45-61,64,65] alone with a total of 5971 patients were included (Supplementary Figure 2A), the use of MBP *vs* no MBP did not impact upon the incidence of SSI (OR = 1.16, 95%CI: 0.96 to 1.39, *P* = 0.12), nor when MBP *vs* single rectal enema[37,45,47,51,53] or MBP *vs* absolutely no preparation[40,43,46,48-50,52,54-61,64,65] were considered. When just observational studies were included[29,66-70,72-75,77] (11 studies, 13809 patients; Supplementary Figure 2B), patients who received MBP had a significantly reduced incidence of SSI than those who did not receive MBP (OR = 0.64, 95%CI: 0.55 to 0.75, *P* < 0.0001), with similar results seen in those who received MBP *vs* absolutely no MBP[29,68,70,73,75], although no difference was seen between those who received full MBP *vs* a single rectal enema[66,67,69,72,74,77].

Intra-abdominal collection

A total of 29 studies[29,37,40,45,46,48,49,51,53-56,58,59,61,62,64-75,77] on 19327 patients included data on postoperative intra-abdominal collections (Figure 4). The administration of

Table 2 Baseline patient demographics for all studies included

Ref.	Year published	Study methodology	Study numbers		Male: Female gender		Indication for surgery	Location	Primary anastomosis	Laparoscopic approach	
			MBP, *n*	No MBP, *n*	MBP	No MBP				MBP, *n*	No MBP, *n*
Ji *et al*[76]	2017	Observational	538	831	Unknown	Unknown	Cancer	Rectum	Y	Unknown	Unknown
Chan *et al*[77]	2016	Observational	159	97	85:74	55:42	Cancer	Colon and rectum	Y	159	97
Hu *et al*[64]	2017	RCT	76	72	Unknown	Unknown	Cancer	Colon and rectum	Y	Unknown	Unknown
Bhattacharjee *et al*[65]	2015	RCT	38	33	21:17	20:13	Cancer, inflammatory bowel disease, volvulus, tuberculosis	Colon and rectum	Y	0	0
Allaix *et al*[74]	2015	Observational	706	829	361:345	432:397	Cancer, adenoma, diverticulitis, reversal of Hartmann's procedure, rectal prolapse	Colon and rectum	Y	829	706
Kiran *et al*[29]	2015	Observational	6146	2296	3030:3116	1111:1185	Unknown	Colon and rectum	N	4443	1389
Yamada *et al*[66]	2014	Observational	152	106	92:60	65:41	Cancer	Colon only	Y	97	64
Otchy *et al*[67]	2014	Observational	86	79	39:47	39:40	Cancer, diverticular disease, IBD, rectal prolapse, ischemic colitis, volvulus, colovaginal fistula	Colon and rectum	Y	37	48
Kim *et al*[75]	2014	Observational	1363	1112	502:694	669:610	Unknown	Colon and rectum	Y	709	472
Tahirkheli *et al*[62]	2013	RCT	48	48	28:20	24:24	Cancer, diverticular disease, IBD, ischemic colitis	Colon and rectum	Y	unknown	unknown
Sasaki *et al*[61]	2012	RCT	38	41	17:21	24:17	Cancer	Colon only	Y	29	19
Bertani *et al*[45]	2011	RCT	114	115	65:49	60:55	Cancer	Colon and rectum	Y	55	51
Roig *et al*[68]	2010	Observational	39	69	Unknown	Unknown	Cancer, diverticular disease, IBD, FAP	Colon and rectum	Y	12	20
Bretagnol *et al*[46]	2010	RCT	89	89	56:33	46:43	Rectal cancer	Rectum only	Y	73	74
Pitot *et al*[69]	2009	Observational	59	127	31:28	53:74	Cancer, diverticular disease, IBD	Colon only	Y	26	30
Alcantara Moral *et al*[47]	2009	RCT	70	69	41:28	48:22	Cancer	Left colon and rectum	Y	12	15
Miron *et al*[70]	2008	Observational	60	39	Unknown	Unknown	Unknown	Colon and rectum	Y	Unknown	Unknown
Pena-Soria *et al*[48]	2008	RCT	65	64	35:29:00	33:22	Cancer, IBD	Colon and rectum	Y	Unknown	Unknown
Leiro *et al*[59]	2008	RCT	64	65	39:25	38:27	Benign and malignant colorectal pathology	Colon and rectum	N	Unknown	Unknown
Contant *et al*[40]	2007	RCT	670	684	337:333	345:339	Cancer, IBD	Colon and rectum	Y	None	None
Bretagnol *et al*[71]	2007	Observational	61	52	42:19	32:20	Rectal cancer	Rectum only	Y	Unknown	27
Jung *et al*[49]	2007	RCT	686	657	306:380	317:340	Cancers, diverticular disease, adenoma	Colon only	Y	None	None
Veenhof *et al*[72]	2007	Observational	78	71	28:43	33:45	Not specified	Colon and rectum	Y	Unknown	Unknown
Ali *et al*[63]	2007	RCT	109	101	Unknown	Unknown	Unknown	Colon and rectum	Y	Unknown	Unknown
Jung *et al*[50]	2006	RCT	27	17	Unknown	Unknown	Cancer, adenoma and diverticular disease	Rectum only	Y	None	None
Platell *et al*[51]	2006	RCT	147	147	Unknown	Unknown	Cancer, IBD, diverticular disease, adenoma	Colon and rectum	N	Unknown	Unknown
Fa-Si-Oen *et al*[52]	2005	RCT	125	125	58:67	56:69	Cancer, diverticular disease	Colon only	Y	None	None
Bucher *et al*[53]	2005	RCT	78	75	47:31	34:41	Cancer, diverticular disease, reversal of Hartmann's procedure, adenoma, endometriosis	Left colon and rectum	Y	20	22
Ram *et al*[54]	2005	RCT	164	165	99:65	102:63	Cancer, diverticular disease, IBD	Colon and rectum	Y	Unknown	Unknown
Zmora *et al*[37]	2003	RCT	187	193	103:84	94:99	Cancer, diverticular disease, IBD	Colon and rectum	Y	Unknown	Unknown
Young Tabusso *et al*[55]	2002	RCT	24	23	12:12	9:14	Unknown	Colon and rectum	Y	Unknown	Unknown
Miettinen *et al*[56]	2000	RCT	138	129	68:70	62:67	Cancer, IBD, diverticular disease	Colon and rectum	91% primary anastomosis in both arms	None	None
Memon *et al*[73]	1997	Observational	61	75	32:29	44:31	Cancer, diverticular disease, IBD, adenoma, lipoma	Left colon and rectum	Y	Unknown	Unknown
Fillmann *et al*[60]	1995	RCT	30	30	Unknown	Unknown	Cancer, diverticular disease, IBD, ischemic colitis	Colon and rectum	N	Unknown	Unknown
Burke *et al*[57]	1994	RCT	82	87	52:30	43:44	Cancer, diverticular disease, IBD	Left colon and rectum	Y	Unknown	Unknown
Brownson *et al*[58]	1992	RCT	86	93	Unknown	Unknown	Cancer and other	Colon and rectum	Y	Unknown	Unknown

FAP: Familial adenomatous polyposis; IBD: Inflammatory bowel disease; MBP: Mechanical bowel preparation; RCT: Randomised controlled trial.

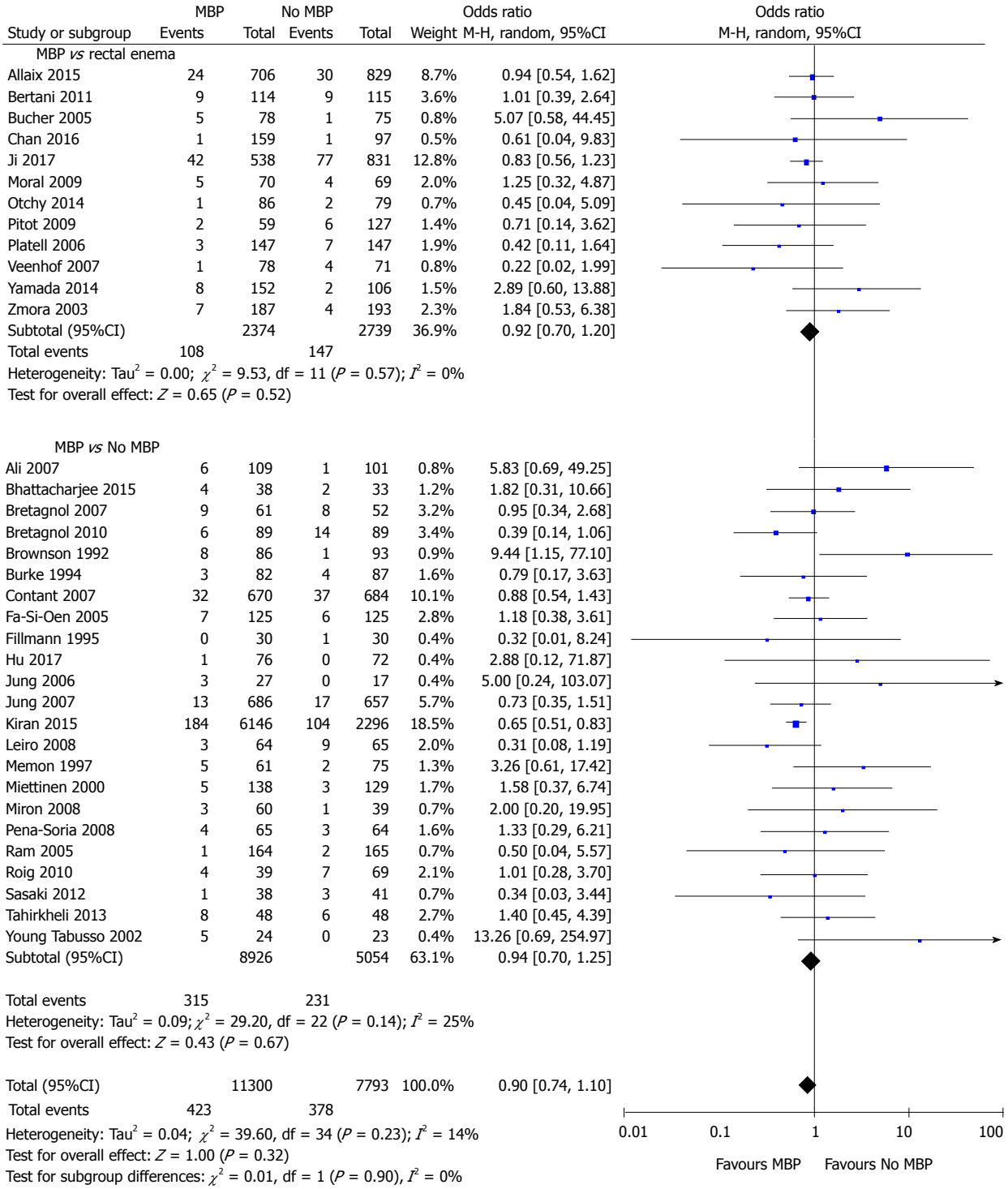

Figure 2 Forest plot comparing overall anastomotic leak rate for patients receiving mechanical bowel preparation *vs* either a single rectal enema (top) or absolutely no preparation (bottom). A Mantel-Haenszel random effects model was used to perform the meta-analysis and odds ratios are quoted including 95% confidence intervals. MBP: Mechanical bowel preparation.

MBP *vs* no MBP did not impact upon the incidence of intra-abdominal collection (OR = 0.86, 95%CI: 0.63 to 1.17, *P* = 0.34), nor when full MBP *vs* single rectal enema[37,45,47,51,53,66,67,69,72,74,77] (OR = 0.83, 95%CI: 0.45 to 1.51, *P* = 0.54) or MBP *vs* absolutely no preparation at all were considered[29,40,46,48-50,52,54-61,64,65,68,70,73,75] (OR = 0.92, 95%CI: 0.62 to 1.34, *P* = 0.65).

When randomised controlled trials alone were considered[37,40,45, 46,48,49,51,53-56, 58,59,61,62,64,65] (Supplementary Figure 3A), no differences were seen in the incidence of intra-abdominal collection between any of the groups (OR = 1.17, 95%CI: 0.66 to 2.10, *P* = 0.59). However, when observational studies were analysed[29,66-75,77] (Supplementary Figure 3B), the incidence of intra-abdominal collection was significantly reduced in those who had MBP *vs* those who did not (OR

Table 3 Nature of the bowel preparation used in studies included in the meta-analysis

Ref.	Details of MBP	Details of no MBP	Antibiotics given
Allaix *et al*[74]	PEG	Enema before left sided operations	As per local policy
Kiran *et al*[29]	As per local policy	Unclear	As per local policy
Yamada *et al*[66]	PEG	Glycerin Enema	Flomoxef at induction and 3 hourly intra op
Otchy *et al*[67]	PEG	Colonic resections- no MBP Rectal resections- single enema	Ertapenem 1 g or levofloxacin/metronidazole 500 mg 1 h post op then continued for 24 h post op
Kim *et al*[75]	As per local policy	Unclear	As per local policy
Tahirkheli *et al*[62]	Saline	No preparation	Oral ciprofloxacin plus unspecified intravenous antibiotics for 24 h post op
Sasaki *et al*[61]	PEG and sodium picosulphate	No preparation	Antibiotic regime not specified
Bertani *et al*[45]	PEG and a single enema	Single enema only	Cefotixin given at induction, 4, 12 and 24 h. Ceftriaxone and metronidazole given for 5 d post op if heavy contamination
Roig *et al*[68]	Mono and di sodium phosphate	No prep	Antibiotic regime not specified
Bretagnol *et al*[46]	Senna plus povidone-iodine enema	No prep	ceftriaxone and metronidazole at induction and every 2 hours intra op
Pitot *et al*[69]	PEG	Rectal resections had single enema	Antibiotic regime not specified
Alcantara Moral *et al*[47]	Sodium phosphate or PEG	Two preoperative enemas	Neomycin and metronidazole 1 d pre op, ceftriaxone and metronidazole at induction
Miron *et al*[70]	PEG and sodium sulphate	No preparation	Antibiotic regime not specified
Pena-Soria *et al*[48]	PEG and standard enema	No preparation	Gentamicin and metronidazole 30 min pre op and 8 hourly post op
Leiro *et al*[59]	Sodium di or monobasic phosphate or PEG	No preparation	Ciprofloxacin and metronidazole 500 mg pre op
Contant *et al*[40]	PEG and bisocodyl/ sodium phosphate	No preparation	Antibiotic regime not specified
Bretagnol *et al*[71]	Senna plus povidone-iodine enema	No preparation	Ceftriaxone and metronidazole at induction and every 2 h intra op
Jung *et al*[49]	As per local policy	No preparation	Trimethoprim + metronidazole or cef and met or dozy and met
Veenhof *et al*[72]	PEG	Single enema	Antibiotic regime not specified
Ali *et al*[63]	Saline	No preparation	Antibiotic regime not specified
Jung *et al*[50]	PEG or sodium phosphate	No preparation	Oral sulphamethoxazole-trimethoprim and metronidazole, cephalsporin and metronidazole, doxycycline and metronidazole
Platell *et al*[51]	PEG	Phosphate enema	Timentin or gentamycin and metronidazole at induction
Fa-Si-Oen *et al*[52]	PEG	No preparation	Ceftriaxone and metronidazole or gentamycin and metronidazole at induction
Bucher *et al*[53]	PEG	Rectal resections had single saline enema	Ceftriaxone and metronidazole at induction and 24 h post op
Ram *et al*[54]	Monobasic and dibasic sodium phosphate	No preparation	Ceftriaxone and metronidazole 1 h pre op and 48 post op
Zmora *et al*[37]	PEG	Rectal resections had a single phosphate enema	Erythromycin and neomycin for 3 doses and then for 24 h
Young Tabusso *et al*[55]	PEG or saline/mannitol	No preparation	Antibiotic regime not specified
Miettinen *et al*[56]	PEG	No preparation	Ceftriaxone and metronidazole at induction
Memon *et al*[73]	Phosphate enema, picolax, PEG, saline lavage	No preparation	Antibiotic regime not specified
Fillmann *et al*[60]	Mannitol	No preparation	Metronidazole and gentamicin 1 h pre op then for 48 h
Burke *et al*[57]	sodium picosulphate	No preparation	Ceftriaxone 1 g, metronidazole at induction and 8 and 16 h
Brownson *et al*[58]	PEG	No preparation	Antibiotic regime not specified

MBP: Mechanical bowel preparation; PEG: Polyethylene glycol.

= 0.67, 95%CI: 0.53 to 0.85, P = 0.0008). A significant reduction in the incidence of intra-abdominal collection was seen in the subgroup of patients who underwent MBP *vs* absolutely no preparation[29,68,70,71,73,75] (OR = 0.65, 95%CI: 0.54 to 0.78, P < 0.0001), however no difference was seen in those undergoing MBP *vs* a single rectal enema[66,67,69, 72,74,77] (OR = 0.80, 95%CI: 0.34 to 1.88, P = 0.60).

Hospital length of stay

Hospital length of stay (LOS) was reported in 20 studies[40,45,46,49,51-56,61,63,67-69,71-74,77] including 7381 patients (Figure 5), with the use of MBP *vs* not (including those who received a single rectal enema) resulting in no significant difference in hospital length of stay (overall mean difference 0.11 d, 95%CI: -0.51 to 0.73, P = 0.72). This was mirrored when just randomised controlled trials were examined[40,45,46,49,51-56,61,63] (Supplementary Figure 4A; overall mean difference 0.22 d, 95%CI: -0.44 to 0.88, P = 0.52) and when just observational studies were included[67-69,71-74,77] (Supplementary Figure 4B; overall mean difference

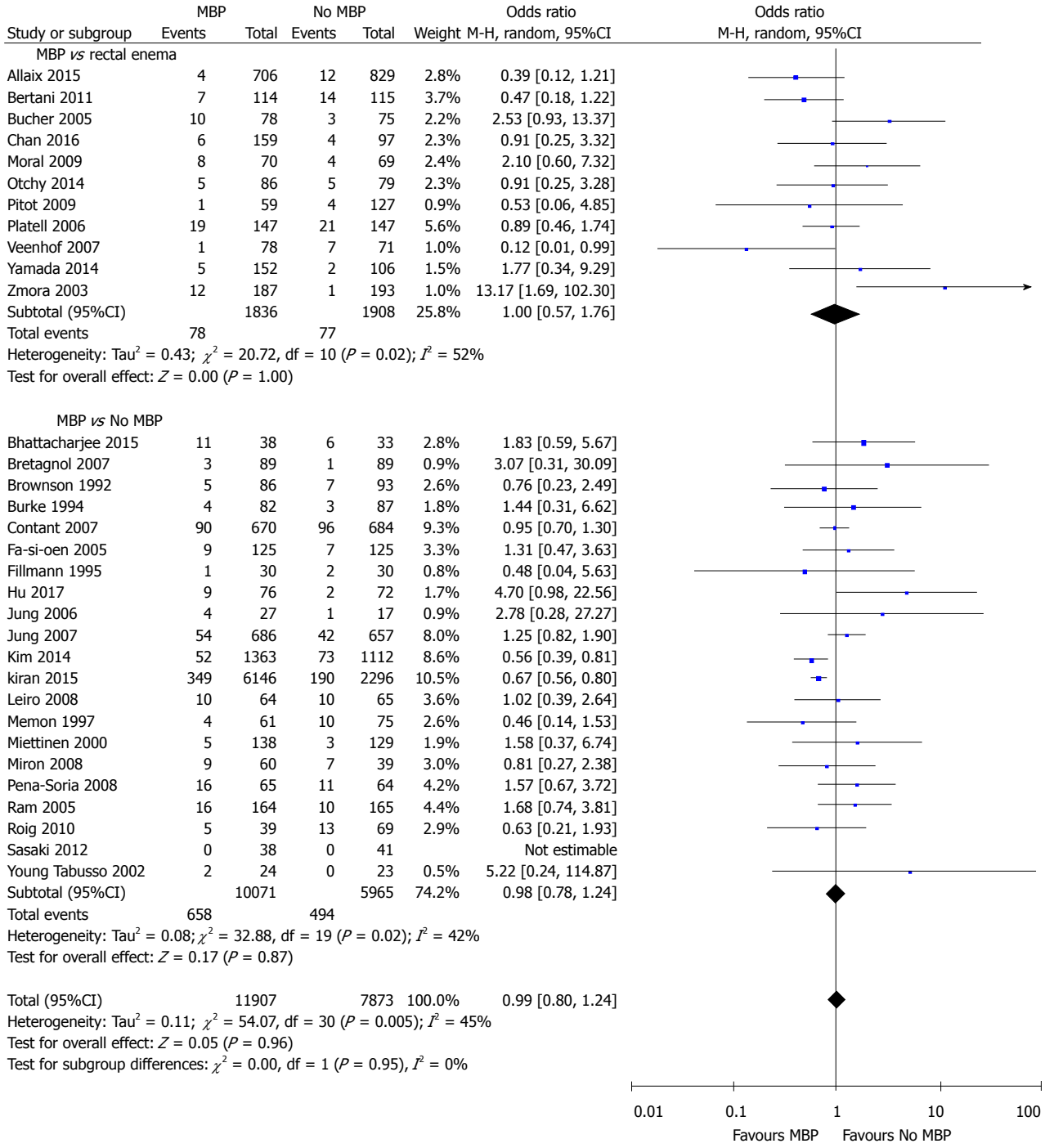

Figure 3 Forest plot comparing overall surgical site infection rates for patients receiving mechanical bowel preparation *vs* either a single rectal enema (top) or absolutely no preparation (bottom). A Mantel-Haenszel random effects model was used to perform the meta-analysis and odds ratios are quoted including 95% confidence intervals. MBP: Mechanical bowel preparation.

-0.12 d, 95%CI: -1.48 to 1.25, *P* = 0.87).

Mortality S

Mortality was reported in 25 studies[29,37,40,45-49,51-54,56,57,59,60,65,66,68,69,71-74,77] that included 16657 patients (Figure 6). The time point this outcome measure was measured was variable between studies, with the majority taken at 30 d[29,37,45-49,51,53,60,65,69,71,73,77], two taken at first outpatient clinic quoted to be approximately two weeks following hospital discharge[40] or four weeks following surgery[66], one at two months[56] and one at three months[52], with six papers not stating when mortality was taken from[54,57,59,68,72,74]. No difference was seen with the use of full MBP, single rectal enema or no preparation at all.

A similar result was seen, with no significant differences, when this comparison was made using only randomised controlled trials[37,40,45-49,51-54,56,57,59,60,65] (Supplementary Figure 5A). However, in observational studies[29,66,68,69,71-74,77], MBP was associated with a

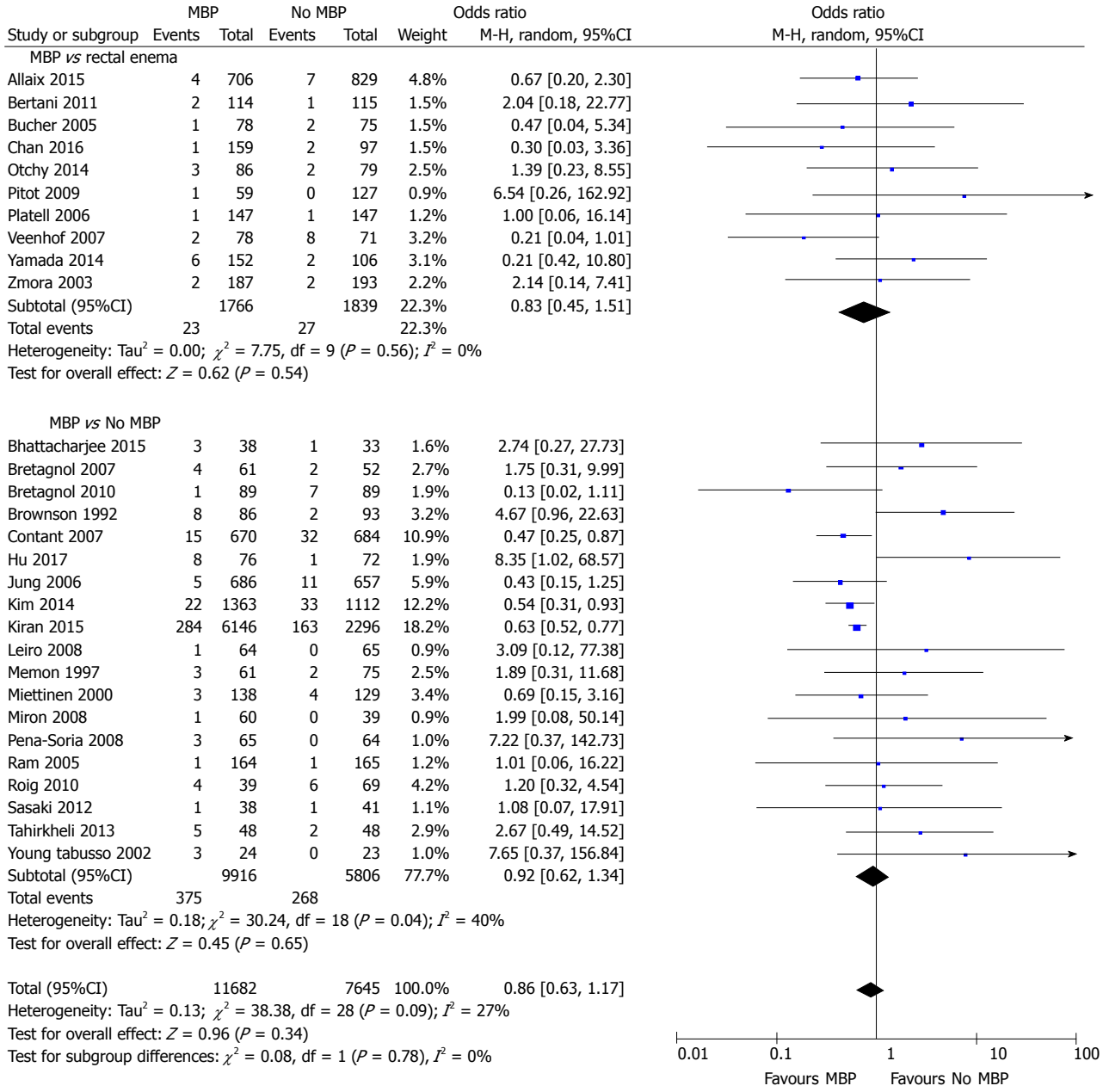

Figure 4 Forest plot comparing overall intra-abdominal collection rates for patients receiving mechanical bowel preparation *vs* either a single rectal enema (top) or absolutely no preparation (bottom). A Mantel-Haenszel random effects model was used to perform the meta-analysis and odds ratios are quoted including 95% confidence intervals. MBP: Mechanical bowel preparation.

significant reduction in mortality (OR = 0.50, 95%CI: 0.34 to 0.74, *P* = 0.0005) (Supplementary Figure 5B). A significant reduction in the incidence of intra-abdominal collection was seen in the subgroup of patients in observational studies who underwent MBP *vs* absolutely no preparation[29,68,71,73] (OR = 0.42, 95%CI: 0.27 to 0.56, *P* < 0.0001). However, no difference was seen in those undergoing MBP *vs* a single rectal enema[66,69,72,74,77] (OR = 0.99, 95%CI: 0.41 to 2.41, *P* = 0.98).

Reoperation

A total of 20 studies on 16742 patients[29,40,46,49,51-57,59,65,68,69,71,72,74,76,77] examined the impact of MBP upon reoperation rates (Figure 7). Overall the use of MBP *vs* no MBP did not impact upon requirement for reoperation[29,40,46,49,51-57, 59,65,68,69,71,72,74,76,77] (OR = 0.91, 95%CI: 0.75 to 1.12, *P* = 0.38), nor when MBP *vs* a single rectal enema[51,53,69,72,74,76,77] (OR = 0.82, 95%CI: 0.42 to 1.60, *P* = 0.56) or MBP *vs* absolutely no preparation[29,40,46,49,52,54-57,59,65,68,71] (OR = 0.85, 95%CI: 0.72 to 1.01, *P* = 0.06) were compared.

When only randomised controlled trials were examined[40,46,49,51-57,59,65] (Supplementary Figure 6A), again no difference was seen by the use of MBP, a single rectal enema or absolutely no preparation. When observational studies were examined[29,68,69,71,72,74,76,77] (Supplementary Figure 6B) overall MPB resulted in no

Table 4 Effect of bowel preparation on outcome in patients undergoing rectal surgery

	Number of participants (MBP *vs* No MBP)	Odds ratio (95%CI), MBP *vs* No MBP	*P* value
Anastomotic leak	2351 (1042 *vs* 1309)	0.86 (0.64 to 1.15)	0.30
Surgical site infection	965 (513 *vs* 452)	1.22 (0.82 to 1.81)	0.33
Intra-abdominal collection	921 (486 *vs* 435)	0.54 (0.21 to 1.38)	0.20
Mortality	813 (419 *vs* 394)	0.73 (0.29 to 1.82)	0.50
Re-operation	1660 (688 *vs* 392)	1.57 (1.02 to 2.43)	0.04

Data from[39,45,46,50,56,57,59,71,75-77]. MBP: Mechanical bowel preparation.

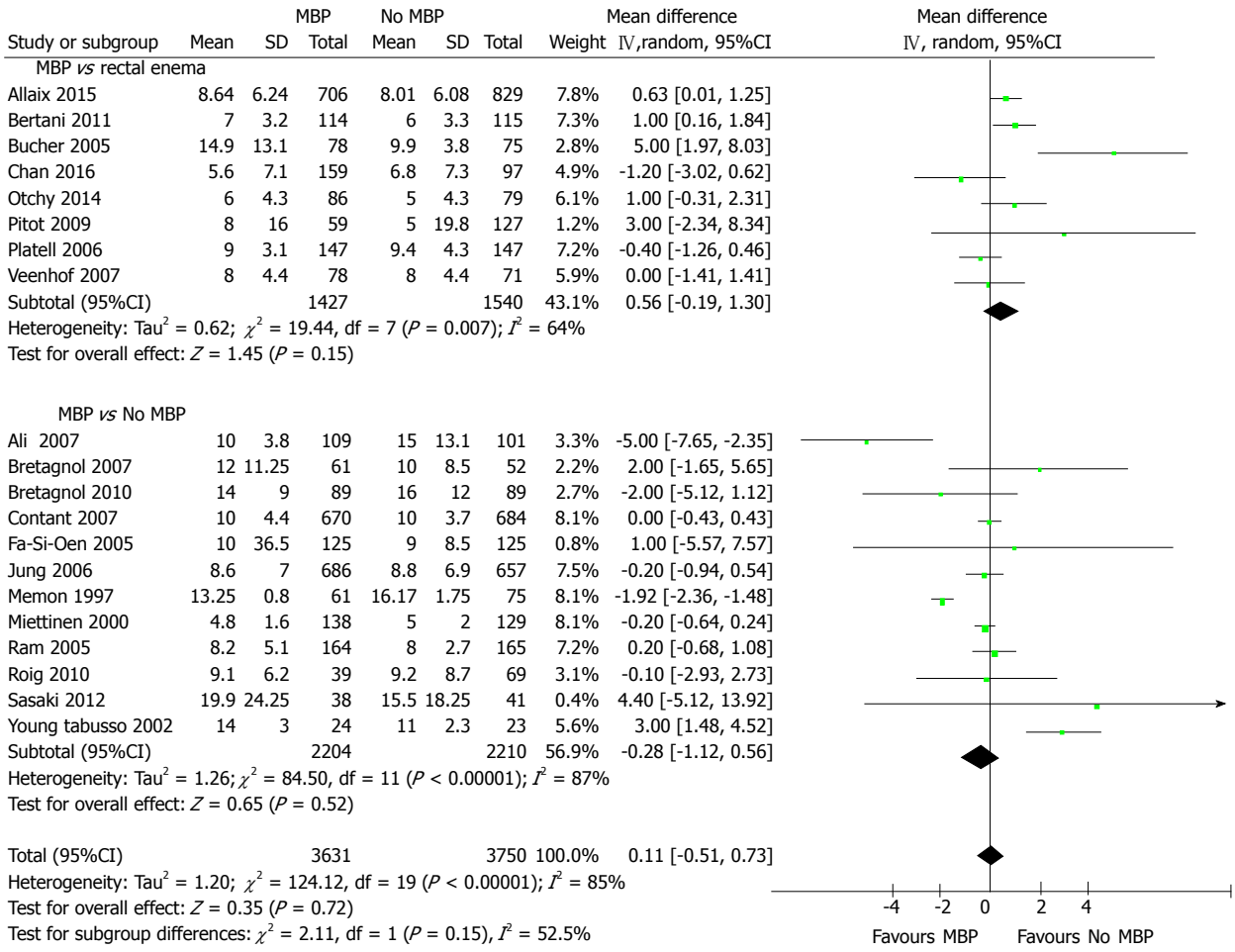

Figure 5 Forest plot comparing overall hospital length of stay for patients receiving mechanical bowel preparation *vs* either a single rectal enema (top) or absolutely no preparation (bottom). An inverse-variance random effects model was used to perform the meta-analysis and mean differences are quoted including 95% confidence intervals. MBP: Mechanical bowel preparation.

significant reduction in the reoperation rate *vs* those who did not have bowel preparation but may have had a rectal enema (OR = 0.86, 95%CI: 0.64 to 1.15, *P* = 0.30), as well as when those who has a single rectal enema (OR = 0.82, 95%CI: 0.44 to 1.52, *P* = 0.52), however a significant difference was seen when MBP was compared with patients who received absolutely no preparation (OR = 0.78, 95%CI: 0.63 to 0.97, *P* = 0.02).

Rectal surgery

A total of 11 studies[39,45,46,50,56,57,59,71,75-77] included either only patients who were undergoing rectal or surgery, or outcome measures for the subgroup of patients who had undergone rectal surgery. Ten studies compared MBP with no MBP, with just one study comparing MBP with a single rectal enema[45]. All studies except one[77] included data on anastomotic leak rates, finding MBP not to be associated with any difference in incidence (OR = 0.86, 95%CI: 0.64 to 1.15, *P* = 0.30). Only seven studies[39,45,46,50,71,75,77] included data on SSI, which also demonstrated no significant difference (OR = 1.22, 95%CI: 0.82 to 1.81, *P* = 0.33). Intra-abdominal collection and mortality data were similarly only available for five[39,45,46,71,77] and four studies[39,45,46,71] respectively, neither of which were associated with the use of MBP (OR = 0.54, 95%CI: 0.21 to 1.38, *P* =

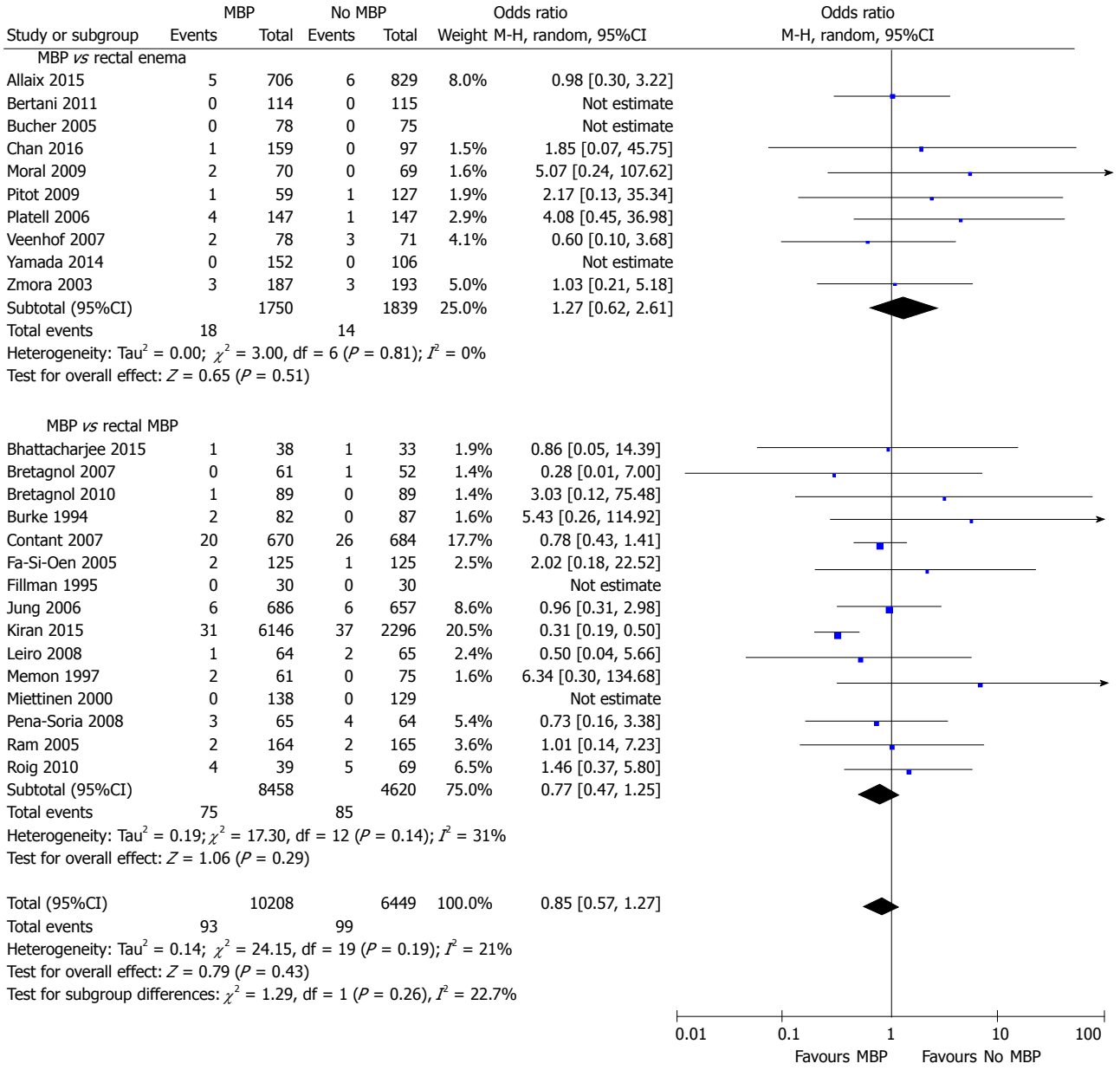

Figure 6 Forest plot comparing overall mortality rates for patients receiving mechanical bowel preparation *vs* either a single rectal enema (top) or absolutely no preparation (bottom). A Mantel-Haenszel random effects model was used to perform the meta-analysis and odds ratios are quoted including 95% confidence intervals. MBP: Mechanical bowel preparation.

0.20; and OR = 0.73, 95%CI: 0.29 to 1.82, *P* = 0.50, respectively). The results in patients undergoing rectal surgery are summarized in Table 4.

DISCUSSION

This meta-analysis of 23 randomised controlled trials and 13 observational studies has demonstrated that, overall, the use of MBP *vs* either absolutely no bowel preparation or a single rectal enema was not associated with a statistically significant difference in the incidence of anastomotic leak, SSI, intra-abdominal collection, mortality, reoperation or total hospital length of stay. When just randomised controlled trial evidence was analysed, there was, again, no significant difference by preparation method in any clinical outcome measure. Finally, when observational studies were analysed, the use of full preparation was associated overall with a reduced incidence of anastomotic leak, SSI, intra-abdominal collection and mortality rates, with these results mirrored in patients receiving MBP *vs* absolutely no preparation, but no significant differences in those receiving MBP *vs* a single rectal enema. When a separate subgroup of just rectal surgery was considered, MBP was not associated with a statistically significant difference in anastomotic leak rates, SSI, intra-abdominal collection or mortality, irrespective of whether patients not receiving MBP were given a single

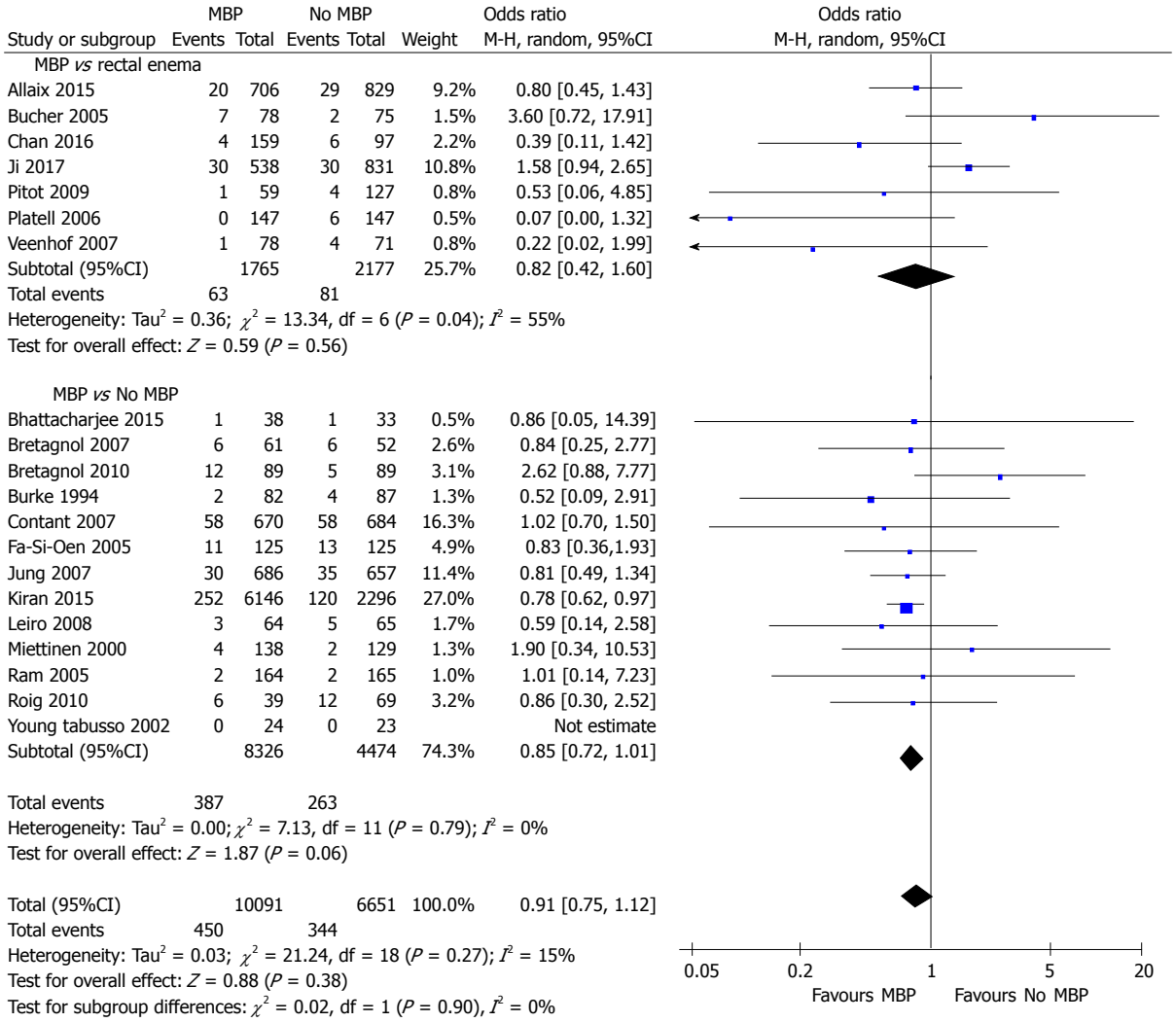

Figure 7 Forest plot comparing overall reoperation rates for patients receiving mechanical bowel preparation *vs* either a single rectal enema (top) or absolutely no preparation (bottom). A Mantel-Haenszel random effects model was used to perform the meta-analysis and odds ratios are quoted including 95% confidence intervals. MBP: Mechanical bowel preparation.

rectal enema.

Strengths of study

This study represents the most comprehensive examination of the role of MBP prior to elective colorectal surgery to date. As part of the study plan, the decision was made to include observational studies as well as randomised controlled trials. However, in order to ensure that inclusion of studies of less rigorous methodology did not exert an undue bias, a predetermined analysis of studies of both methodologies was conducted. This revealed that the overall results and those from analysing just evidence from randomised controlled trials were much the same. However, when analysing evidence from observational studies, this resulted in a significant reduction in anastomotic leak, SSI, intra-abdominal collection and mortality rates. The reasons for this difference in results is not clear from this study, but it is possible that selection bias may exert a confounding effect upon the results, and as such the use of MBP in selected patients as determined by the physician in charge may be appropriate.

With the exception of hospital length of stay (I^2 = 85%), overall study heterogeneity was low to moderate (0%-34%) for all clinical outcome measures, suggesting the studies to be relatively homogeneous. The risk of bias for the randomised controlled trials included in the meta-analysis (Table 1) was relatively low.

Limitations of study

As the raw mean and standard deviation data were not available on the hospital LOS for all studies, despite several attempts at obtaining this directly from the authors, it was necessary to infer this from what was available (either median and range or interquartile range) using statistical techniques previously described[42]. This is a valid technique which has been well described previously, but this may exert some degree of bias upon the results of the meta-analysis.

There was poor documentation within the studies included regarding the side effects of MBP including the incidence of electrolyte disturbance, fluid depletion

and requirement of resuscitation, and renal disturbance or failure, hence this was not included as an outcome within the meta-analysis.

Emerging evidence, much of which has been derived from the studies based upon NSQIP datasets have focused upon the combination between intraluminal antibiotics and MBP and have demonstrated a reduction in SSI rates. However, the data contained within the studies included within this meta-analysis has been scanty regarding the use of intraluminal antibiotics and as such it has not been possible to include this data within the meta-analysis. This may act as a potential confounder when considering the effect of MBP and clinical outcomes.

The studies contained predominantly mixed populations of colonic and rectal procedures, with inadequate documentation to differentiate results between the two, which may be particularly important in addressing the question regarding the use of a single rectal enema as bowel preparation. In addition, there was poor documentation regarding the nature of the anastomoses within the studies included, with a mixture of ileocolic, colon-colon and colorectal. The role of mechanical bowel preparation in various anastomosis types has not been well established. The majority of studies included a predominance of colonic procedures, with some focusing entirely on colonic rather than rectal surgery. Only a small subgroup analysis was available to analyse the impact of MBP in rectal surgery, from which it is very difficult to draw strong conclusions. Further studies are required to discern the importance of a pre-operative enema in this setting. Similarly, the level of documentation in studies regarding laparoscopic *vs* open surgery was not sufficient in terms of correlation with clinical outcome measures to be able to discern the importance of MBP in this setting. Only one recent observational study has focused entirely on laparoscopic procedures[74] which demonstrated no significant difference in the rates of intra-abdominal septic complications by the use of MBP, and prior to this evidence was purely based on several small studies[38,78].

The nature of the MBP used was inconsistent between studies, and this may introduce a further bias[79]. There was also poor documentation regarding antibiotic usage, particularly in the early studies. Much of the recent literature regarding preparation of the bowel has focused upon the use of oral luminal antibiotics in combination with MBP, with these studies suggesting a potential role for this therapy[26,27]. A recent meta-analysis on this topic has demonstrated a significant reduction in the risk of SSI in patients undergoing elective colorectal surgery given oral systemic antibiotics with MBP *vs* systemic antibiotics and MBP[80], thus representing a further weakness in the studies included in this meta-analysis.

Comparison with other studies

A recently published meta-analysis[8] of 18 randomised controlled trials, 7 non-randomised comparative studies, and 6 single-group cohorts compared the use of oral MBP with or without an enema *vs* no oral MBP with or without an enema. This study found that MBP *vs* no MBP was associated with no difference in the rates of all-cause mortality (OR = 1.17, 95%CI: 0.67 to 2.67), anastomotic leakage (OR = 1.08, 95%CI: 0.79 to 1.63), SSI (OR = 1.19, 95%CI: 0.56 to 2.63) as well as wound infections, peritonitis or intra-abdominal abscess or reoperation. This study however found considerable variance in the estimation of treatment effects, possibly due to the large range of study methodology included, which may mask a treatment effect seen.

This topic has been reviewed by the Cochrane Collaboration[81-83], with the most recent review conducted in 2011[10]. This included a total of 18 randomised controlled trials in elective colorectal surgery (5805 patients), and demonstrated no statistically significant evidence to support the use of MBP in either low anterior resection, rectal or colonic surgery in terms of anastomotic leakage or wound infection.

A previous meta-analysis has examined the role of MBP prior to proctectomy[12] from eleven publications (1258 patients), although extractable data were only available in a limited number of studies for outcome measures other than anastomotic leakage rates. This study[12] found no beneficial effect from MBP prior to proctectomy with regards to anastomotic leakage (OR = 1.144, 95%CI: 0.767 to 1.708, P = 0.509), SSI (OR = 0.946, 95%CI: 0.597 to 1.498, P = 0.812), intra-abdominal collection (OR = 1.720, 95%CI: 0.527 to 5.615, P = 0.369) or postoperative mortality.

Health policy implications

Worldwide, elective colorectal surgery is performed frequently. Current opinion regarding the use of MBP prior to this surgery is inconsistent[17,18], despite several previous meta-analyses which have suggested this is not useful in reducing postoperative complications[9,10]. The use of MBP is not without cost implications, including the preparation itself and in elderly and frail patients, MBP may also necessitate an additional stay in hospital prior to surgery due to the risk of dehydration and electrolyte disturbance which is associated with considerable additional healthcare costs. This meta-analysis further reinforces that MBP is not associated with any difference in postoperative complication rates, mortality of hospital length of stay, particularly in elective colonic surgery, and as such should not be administered routinely.

In conclusion, this study represents the most comprehensive meta-analysis to date on MBP in elective colorectal surgery. It has demonstrated that MBP *vs* a single rectal enema or no bowel preparation at all is not associated with a statistically significant difference in any of the clinical outcome measures studied. Given the risks of electrolyte disturbance and patient dissatisfaction, as well as potentially significant levels of dehydration and requirement for pre-admission prior to surgery, MBP should no longer be considered a standard of care prior

to elective colorectal surgery.

ARTICLE HIGHLIGHTS

Research background

Mechanical bowel preparation for colorectal surgery has been surgical dogma for decades, despite increasing evidence from the 1990s refuting its benefits. The rationale behind the administration of mechanical bowel preparation is that it reduces fecal bulk and, therefore, bacterial colonisation, thereby reducing the risk of postoperative complications such as anastomotic leakage and wound infection, as well as facilitate dissection and allow endoscopic evaluation. Opponents argue that in the 21st century, with rational use of oral and intravenous prophylactic antibiotics there is no longer a place for mechanical bowel preparation, that it may cause marked fluid and electrolyte imbalance in the preoperative period. As a result of this inconclusive evidence, practice varies between countries and even surgeons in the same institution. We conducted a comprehensive meta-analysis encompassing both randomised controlled trials and observational studies. We sought to address deficiencies in previous studies by including all levels of evidence, separating those in which patients received a single rectal enema *vs* full or no preparation.

Research motivation

The main topics focused on by this meta-analysis are the role of mechanical bowel preparation *vs* no preparation or rectal enema alone on postoperative infective complications in patients undergoing elective colorectal surgery, as well as in patients undergoing purely rectal resection. This meta-analysis also sought to examine evidence from both randomized controlled trials and observational studies and compare the results of meta-analyses conducted from these evidence sources.

Research objectives

The aims for this meta-analysis were to analyse the effect of mechanical bowel preparation *vs* no preparation or rectal enema alone on postoperative infective complications in patients undergoing elective colorectal surgery, to examine the differences in results between evidence obtained from randomised controlled trials and observational studies, and to determine what effect, if any, bowel preparation had on postoperative complications in rectal surgery. These aims were all achieved by this meta-analysis.

Research methods

We performed an electronic search of the PubMed database and the Cochrane Central Register of Controlled Trials to identify studies comparing outcomes in patients undergoing elective colorectal surgery treated with mechanical bowel preparation *vs* either no preparation or a single rectal enema. We performed this meta-analysis according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement. We reviewed full text articles for suitability after excluding studies on the basis of title and abstract. Our inclusion criteria specified that studies must have a minimum of two comparator groups and were either designed as randomised controlled trials or observational studies. Relevant outcome measures were anastomotic leak, surgical site infection, intra-abdominal abscess, mortality, reoperation and hospital length of stay. The analysis was performed using RevMan 5.3 software. Continuous variables were calculated as a mean difference and 95% confidence interval using an inverse variance random effects model. Dichotomous variables were analysed using the Mantel-Haenszel random effects model to quote the risk ratio (RR) and 95% confidence interval. These analyses were used to construct forest plots, with statistical significance taken to be a P value of < 0.05 on two tailed testing. A predetermined subgroup analysis was performed for the impact of MBP in rectal surgery specifically using the same methodology.

Research results

This meta-analysis of 23 randomised controlled trials and 13 observational studies has demonstrated that, overall, the use of MBP *vs* either absolutely no bowel preparation or a single rectal enema was not associated with a statistically significant difference in the incidence of anastomotic leak, surgical site infection, intra-abdominal collection, mortality, reoperation or total hospital length of stay. When just randomised controlled trial evidence was analysed, there was again no significant difference by preparation method in any clinical outcome measure. Finally, when observational studies were analysed, the use of full preparation was associated overall with a reduced incidence of anastomotic leak, surgical site infection, intra-abdominal collection and mortality rates, with these results mirrored in patients receiving MBP *vs* absolutely no preparation, but no significant differences in those receiving MBP *vs* a single rectal enema.

Research conclusions

This study represents the most comprehensive examination of the role of mechanical bowel preparation prior to elective colorectal surgery to date and has demonstrated that, overall, the use of MBP *vs* either absolutely no bowel preparation or a single rectal enema was not associated with a statistically significant difference in the incidence of anastomotic leak, surgical site infection, intra-abdominal collection, mortality, reoperation or total hospital length of stay. Given the risks of electrolyte disturbance and patient dissatisfaction as well as potentially significant levels of dehydration and requirement for pre-admission prior to surgery, mechanical bowel preparation should no longer be considered a standard of care prior to elective colorectal surgery.

Research perspectives

This study represents the most comprehensive meta-analysis to date on mechanical bowel preparation in elective colorectal surgery. It has demonstrated that mechanical bowel preparation *vs* a single rectal enema or no bowel preparation at all is associated with no difference in any of the clinical outcome measures studied. Mechanical bowel preparation should no longer be considered a standard of care prior to elective colorectal surgery. Emerging evidence, much of which has been derived from the studies based upon NSQIP datasets, has focused upon the combination between intraluminal antibiotics and mechanical bowel preparation and has demonstrated a reduction in SSI rates. However, the data contained within the studies included within this meta-analysis have been scanty regarding the use of intraluminal antibiotics and as such it has not been possible to include these data within the meta-analysis. Further work on this topic should focus upon the role of intraluminal antibiotics in the setting of elective colorectal surgery.

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CASE REPORT

Intraductal papillary bile duct adenocarcinoma and gastrointestinal stromal tumor in a case of neurofibromatosis type 1

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Author contributions: Lee JM and Kim DS performed the procedure and proposed the study; Lee JM wrote the draft; Hyun JJ, Choi HS, Kim JY and Kim ES performed the literature review; Keum B, Jeen YT, Chun HJ, Lee HS and Kim CD reviewed the draft; all authors read and approved the final manuscript.

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Abstract

We report our experience with a synchronous case of gastrointestinal stromal tumor (GIST) and intraductal papillary neoplasm of the bile duct (IPNB) in an

elderly woman with neurofibromatosis type 1 (NF-1). A 72-year-old woman presented with a 2-mo history of right upper abdominal pain unrelated to diet and indigestion. Fourteen years earlier, she had been diagnosed with NF-1, which manifested as café au lait spots and multiple nodules on the skin. Computed tomography (CT) revealed a multilocular low-density mass with septation, and mural nodules in the right hepatic lobe, as well as a 1.7-cm-sized well-demarcated enhancing mass in the third portion of the duodenum. The patient subsequently underwent right hepatectomy and duodenal wedge resection. We present here the first report of a case involving a synchronous IPNB and GIST in a patient with NF-1. Our findings demonstrate the possibility of various tumors in NF-1 patients and the importance of diagnosis at an early stage

Key words: Neurofibromatosis type 1; Intraductal papillary neoplasm of the bile duct; Gastrointestinal stromal tumor; Synchronous

Core tip: We reported the world wide first case of intraductal papillary adenocarcinoma of bile duct in neurofibromatosis type 1 (NF-1) patient. Because patients with NF-1 have a mutation of the *NF-1* gene associated with multiple tumors such as neuroma, and gastrointestinal stromal tumor, consideration for multiple tumors in NF-1 patients would be helpful.

Lee JM, Lee JM, Hyun JJ, Choi HS, Kim ES, Keum B, Jeen YT, Chun HJ, Lee HS, Kim CD, Kim DS, Kim JY. Intraductal papillary bile duct adenocarcinoma and gastrointestinal stromal tumor in a case of neurofibromatosis type 1. *World J Gastroenterol* 2018; 24(4): 537-542 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i4/537.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i4.537

INTRODUCTION

Neurofibromatosis type 1 (NF-1) is an autosomal dominant disorder with an incidence of 1 per 3000 births and a prevalence of 1 per 4-5000 individuals[1]. Typically, NF-1 manifests clinically as café-au-lait spots, cutaneous neurofibromas, cognitive impairment, axillary and/or inguinal freckling, Lisch nodules (*i.e.*, pigmented hamartoma of the iris), and bony dysplasia[2]. Clinical manifestations may vary systemically depending on the type of mutation in NF1[1] ; furthermore, patients with NF-1 have an increased incidence of both benign and malignant neoplasms of the nervous system, skin, muscle, and gastrointestinal tract[2]. Approximately 10%-25% of patients with NF-1 are known to develop neoplasms of the gastrointestinal tract[3]. Gastrointestinal stromal tumor (GIST) is the most common gastrointestinal neoplasm in this population, whereas adenocarcinoma occurs at significantly lower frequency relative to other tumor types although the risk remains high[2,3]. To date, intraductal papillary neoplasm of the bile duct (IPNB) has not been reported in a patient with NF-1 patient. Additionally, few reports have described cases of multiple synchronous gastrointestinal tumors in patients with NF-1. In this report, we present a first case of NF-1 patient with coexisting intra-ductal papillary adenocarcinoma of biliary duct and duodenal GIST.

CASE REPORT

A 72-year-old woman presented with a 2-mo history of right upper abdominal pain unrelated to her diet and indigestion. An examination revealed multiple nodules over her face and body, café au lait spots on the body and limbs, and scoliosis (Figure 1). Fourteen years earlier, she had undergone surgical treatment after receiving a diagnosis of adrenal tumors. Additionally, she was diagnosed with NF-1 after a chromosomal abnormality was detected. Pathology identified the adrenal tumor as a mucinous cystadenoma, but the patient was not subsequently followed up.

At admission, physical examination revealed a negative Murphy's sign. The initial laboratory findings were as follows: aspartate transaminase, 19 IU/L; alanine transaminase, 13 IU/L; total bilirubin, 0.43 mg/dL; alkaline phosphatase, 102 IU/L; γ-glutamyltransferase, 70 IU/L; and carcinoembryonic antigen 3.4 ng/mL. However, her cancer antigen (CA) 19-9 level was elevated to 211.3 IU/mL. Abdominal computed tomography (CT) revealed a low-attenuated multilocular mass with septation and a mural nodule with a soft tissue-enhancing lesion in the right hepatic lobe (Figure 2A). CT also indicated diffuse intrahepatic duct (IHD) dilatation, especially in the right hepatic lobe, where a suspicious soft tissue lesion was connected to the IHD. In addition, a 1.7-cm-sized, well-demarcated enhancing mass was observed in the third portion of the duodenum (Figure 2B) Magnetic resonance (MR) cholangiopancreatography showed a lobulated cystic mass in the right hepatic lobe with multifocal intramural enhancing nodules and suspicious communication with the IHD, indicating a malignant transformation (Figure 3). There were no specific findings on brain MR for central nervous system evaluation. We decided to perform a surgical resection histological diagnostic and treatment purposes. Accordingly, the patient underwent right hepatectomy and duodenal wedge resection.

Pathologically, the resected mass from the third portion of the duodenum had a rounded border, contained spindle cells, and was positive for c-kit immunohistochemistry. This led to a diagnosis of a benign GIST (Figure 4). The resected hepatic mass was a multilocular cystic neoplasm with septation and a mural nodule (Figure 5A). This lesion communicated with the segmental bile duct, but was not connected to the main bile duct (Figure 5B). In the dilated bile duct, a normal epithelial lining

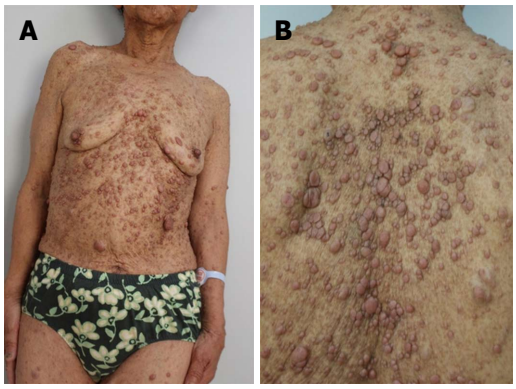

Figure 1 General appearance. A and B: A patient diagnosed with neurofibromatosis type 1 presented with café au lait spots on the skin. 300 mm × 225 mm (300 × 300 DPI).

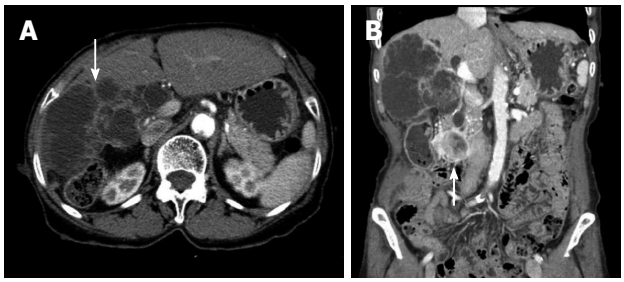

Figure 2 Computed tomography. A: A low-attenuated multilocular mass with septation, and a mural nodule containing a soft tissue-enhancing lesion in the right hepatic lobe (white arrow); B: Diffuse intrahepatic duct (IHD) dilatation, especially in the right hepatic lobe, with a suspicious soft tissue lesion connected to the IHD. A 1.7-cm-sized, well-demarcated enhancing mass is observed in the third portion of the duodenum (white arrow). 300 mm × 225 mm (300 × 300 DPI).

with an abrupt papillary epithelial portion suggested IPNB (Figure 5C). In a microscopy, multifocal stromal invasion and a tubulopapillary mucin component were visible, suggesting an invasive adenocarcinoma with high-grade dysplasia (Figure 5D). This case described very early stage of IPNB having component of invasive carcinoma arising from most high-grade dysplasia without regional organ involvement, and distant metastasis.

The patient was discharged without any post-operative complications and has remained stable for 8 mo after surgery. During every 3 mo follow-up after discharge, there was no recurrence on follow-up CT scan.

DISCUSSION

NF-1, which is also known as von Recklinghausen's disease, is an inherited disorder caused by a mutation of NF1 (chromosome 17q11.2), which acts as a tumor suppressor gene[1]. A previous study reported that intra-abdominal manifestations of NF-1 occurred at frequencies of 5%-25%[4,5]. Intra-abdominal manifestations are known to develop after middle age, particularly following cutaneous manifestations. With age, patients with NF-1 experience increased numbers of both benign and malignant tumors[6]. Neurofibroma is the most common type of benign tumor experienced by NF-1 patients, whereas optic pathway glioma is a common intracranial neoplasm[7].

NF1 encodes a protein called neurofibromin, which regulates RAS activity. RAS activates the stem cell factor/c-KIT signaling pathway and the mitogen-activated protein kinase pathway, leading to cell proliferation[8]. This activation has been linked to a wide spectrum of clinical manifestations of NF-1, including associated tumors[9]. GIST is the most common gastrointestinal manifestation associated with NF-1[9]. Most GISTs in these patients are small and asymptomatic, and follow a benign course. Given the asymptomatic nature of smaller GISTs, early diagnosis is important to reduce the risk of transformation to malignancy. Notably, these GISTs rarely harbor mutations in KIT and PDGFRA (encodes platelet-derived growth factor receptor-alpha)[10,11].

NF-1 is associated with an increased risk of tumors such as neurofibroma and optic pathway ganglioma, but not with gastrointestinal tract adenocarcinoma. As mentioned earlier, IPNB has not previously been reported in patients with NF-1. The term IPNB encompasses both intraductal papillary cholangiocarcinoma and its precursor lesion. IPNB is mainly caused by hepatolithiasis and clonorchiasis, and 40%-80% of these lesions comprise an invasive carcinoma or tubular or mucinous adenocarcinoma[12,13]. The laboratory findings of IPNB mainly involve obstructive jaundice patterns with bilirubin elevation, with elevated CA 19-9 levels in approximately 40% of patients[14].

In the absence of distant metastasis, surgical resection is the first treatment option for IPNB[12,14]. Hepatectomy is performed for IPNB in the IHD, whereas surgical resection is performed for cases involving the common bile duct. In the absence of an increased recurrence risk (*i.e.*, positive lymph node or advanced tumor invasion), subsequent liver transplantation may be an additional treatment option[15]. In the presence of distant metastasis, percutaneous transhepatic biliary drainage, a palliative treatment, helps to improve obstructive jaundice. Patients with IPNB tend to have a better prognosis relative to those with conventional bile duct cholangiocarcinoma[16]. However, prognostic factors include the biology of IPNB and the intraductal growth pattern[12,13,16].

In the present case, a patient with NF-1 presented with various gastrointestinal tumors, including GIST and IPNB, and a benign mucinous cystadenoma of the adrenal gland. As noted earlier, gastrointestinal neoplasms are mostly asymptomatic in patients with NF-1. Kistler *et al*[2] noted that there are currently no screening guidelines for gastrointestinal tumors in patients with NF-1, regardless of tumor type. Although sufficient clinical evidence of the screening of older

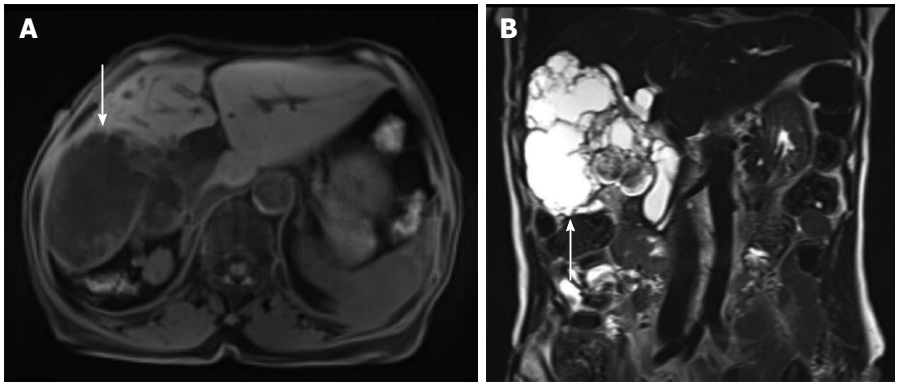

Figure 3 Magnetic resonance cholangiopancreatography. A: A lobulated cystic mass in the right hepatic lobe with multifocal intramural enhancing nodules (white arrow); B: The connection of the cystic mass with the intrahepatic duct indicated a malignant transformation (white arrow). 300 mm × 225 mm (300 × 300 DPI).

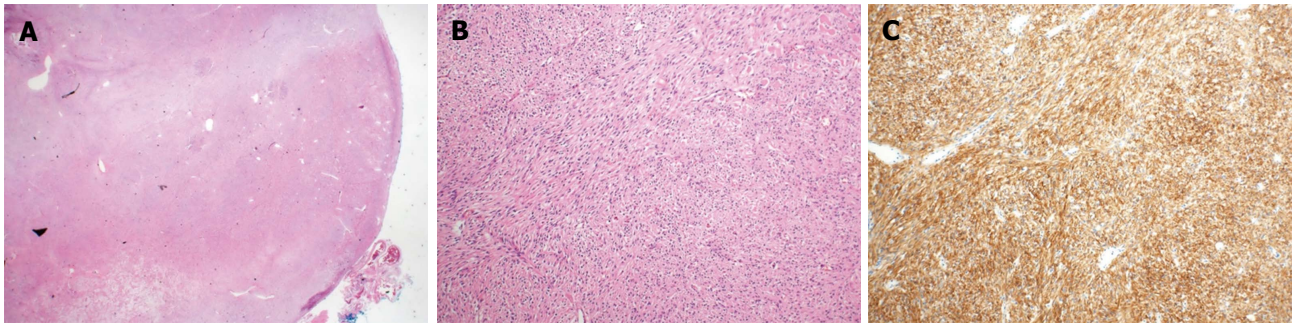

Figure 4 Histologic findings of gastrointestinal stromal tumor. A: A mass with rounded border, resected from the third portion of the duodenum. (HE, 40 × magnification); B: The resected duodenal mass contained many spindle cells. (HE, 100 ×); C: Positive immunohistochemistry for c-kit suggested GIST. (c-kit immunostain, 100 ×). 300 mm × 225 mm (300 × 300 DPI). GIST: Gastrointestinal stromal tumor.

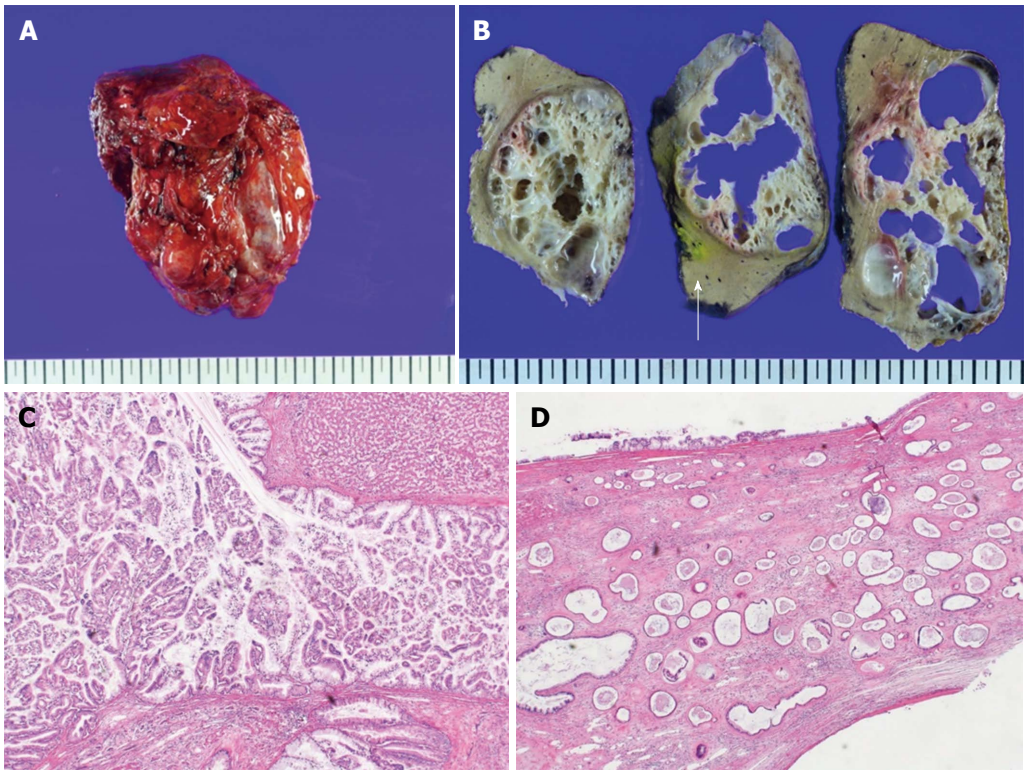

Figure 5 Histologic findings of intraductal papillary neoplasm of bile duct. A: The resected hepatic mass was a multilocular cystic neoplasm with septation and a mural nodule; B: It had communication to segmental bile duct, although it was not connected to main bile duct (white arrow: Main bile duct); C: In the dilated bile duct, normal epithelial lining with abrupt papillary epithelial portions suggests IPNB. (HE, 100 ×); D: Multifocal stromal invasion multifocal stromal invasion with a tubulopapillary mucin component, suggestive of invasive adenocarcinoma with high-grade dyplasia. (HE, 40 ×). 300 mm × 225 mm (300 × 300 DPI).

NF-1 patients is lacking, it would be advisable to routinely perform endoscopic and CT examinations of older (*i.e.*, beyond middle-aged) patients to screen for gastrointestinal neoplasms, including GIST. This case also demonstrates the possibility of an increased CA 19-9 level with IPNB, and suggests that screening procedures should include CA 19-9 and other potential diagnostic tumor biomarkers may be used as diagnostic biomarker for IPNB in NF-1 patients.

Although there were similar cases of periampullary tumors with GIST in NF-1 patients[17,18] or GIST and somatostatinoma in patients with Von Recklinghausen's Disease[19], this is the first case in the global literature to demonstrate synchronous IPNB and GIST in a patient with NF-1. According to the references, the prognosis is determined by depth of invasion, component of invasive carcinoma, and grade[12]. We expected a long recurrence-free survival because this case did not have poor prognostic factor. In previous report, the effect of NF-1 on tumor prognosis has not been reported. However, considering the increased risk of developing tumors due to genetic abnormalities, the possibility that NF-1 may be associated with the prognosis of malignant tumors should be considered. Although these tumors are generally very rare, suspicion and early diagnosis are important for asymptomatic patients with NF-1. Further clinical data is needed to establish a guideline for screening gastrointestinal tumors in this patient population.

ARTICLE HIGHLIGHTS

Case characteristics

A 72-year-old Asian woman who was diagnosed with neurofibromatosis type 1 (NF-1) presented with right upper abdominal pain.

Clinical diagnosis

The patient had a negative Murphy's sign, and no evidence of jaundice. There was no palpable mass in right upper quadrant.

Differential diagnosis

Cholecystitis, choledocholithiasis, cholangiocarcinoma, hepatocellular carcinoma

Laboratory diagnosis

Aspartate transaminase, alanine transaminase, and total bilirubin were within normal range. Alkaline phosphatase, γ-glutamyltransferase was increased slightly.Carcinoembryonic antigen is 3.4 ng/mL, however, cancer antigen 19-9 level was elevated to 211.3 IU/mL.

Imaging diagnosis

Computed tomography showed a low-attenuated multilocular mass with septation, and a mural nodule containing a soft tissue-enhancing lesion in the right hepatic lobe. In addition, a 1.7-cm-sized, well-demarcated enhancing mass was observed in the third portion of the duodenum.

Pathological diagnosis

Histologic results confirmed the diagnosis of benign gastrointestinal stromal tumor (GIST) in the third portion of the duodenum. In addition, the resected hepatic mass was intraductal papillary neoplasm of bile duct (IPNB).

Treatment

Surgical resection was curative treatment. Both GIST and IPNB were successfully resected.

Related reports

NF-1 is associated with an increased risk of tumors. Recently, there were related reports: case of a mixed periampullary adenocarcinoma and a somatostatinoma with a gastrointestinal stromal tumor in NF-1 patients, triad of periampullary carcinoid, duodenal gastrointestinal stromal tumor and plexiform neurofibroma at hepatic hilum in NF-1.

Term explanation

IPNB is a variant of bile duct carcinoma that is characterized by intraductal growth. It have better outcomes compared with common cholangiocarcinoma.

Experiences and lessons

Suspicion and early diagnosis are important for asymptomatic patients with NF-1.

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CASE REPORT

Neuroendocrine carcinoma of the gastric stump: A case report and literature review

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Author contributions: Tian YT and Xue LY designed the report; Xie YB, Zhong YX and Xu Q collected the patient's clinical data; Ma FH and Chen YT analyzed the data and wrote the paper.

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Informed consent statement: Consent was obtained from relatives of the patient for publication of this report and any accompanying images.

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Abstract

We herein report a case of neuroendocrine carcinoma of the gastric stump found 47 years after Billroth II gastric resection for a benign gastric ulcer. A 74-year-old man was referred to another hospital with melena. Endoscopic examination revealed a localized ulcerative lesion at the gastrojejunal anastomosis. The diagnosis by endoscopic biopsy was neuroendocrine carcinoma. A total gastrectomy of the remnant stomach with D2 lymphadenectomy was performed at our hospital. The lesion invaded the subserosa, and metastasis was found in two of nine the lymph nodes retrieved. The lesion was positive for synaptophysin and chromogranin A, and the Ki-67 labeling index was 60%. The diagnosis of neuroendocrine carcinoma of the gastric stump was confirmed using World Health Organization 2010 criteria. Subsequently, the patient underwent one course of adjuvant chemotherapy with the etoposide plus cisplatin (EP) regimen; however, treatment was discontinued due to grade 3 myelosuppression. The patient showed lymph node metastasis in the region around the gastrojejunal anastomosis in the abdominal cavity 7 mo post-surgery. He then underwent radiotherapy and platinum-based combination chemotherapy; however, the disease progressed and liver recurrence was observed on follow-up computed

tomography at 16 mo post-surgery. The patient then received chemotherapy with regimens used for the treatment of small cell lung cancer in first- and second-line settings. The patient died of disease progression 31 months after surgery.

Key words: Gastric stump; Gastric stump cancer; Neuroendocrine carcinoma

Core tip: The most common form of gastric stump cancer is adenocarcinoma. Various types of malignancies have been reported previously, but the development of neuroendocrine carcinoma from the gastric stump is rare. This case might contribute to improving our understanding of the carcinogenesis, biology, and behavior of gastric neuroendocrine carcinoma and gastric stump cancer.

Ma FH, Xue LY, Chen YT, Xie YB, Zhong YX, Xu Q, Tian YT. Neuroendocrine carcinoma of the gastric stump: A case report and literature review. *World J Gastroenterol* 2018; 24(4): 543-548 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i4/543.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i4.543

INTRODUCTION

Gastric stump cancer (GSC) is a well-known long-term complication after distal gastrectomy, and has been reported to account for 1%-8% of all gastric cancers. The most common form of GSC is adenocarcinoma[1], although various types of gastric stump malignancies have been reported[2-6]. Development of neuroendocrine carcinoma (NEC) from the gastric stump is extremely rare. To the best of our knowledge, only a case of NEC in the gastric stump has been reported in the English literature, at the University of Parma, Italy[7]. Herein we report a case of NEC of the gastric stump diagnosed 47 years after distal gastrectomy for a benign gastric ulcer.

CASE REPORT

A 74-year-old man consulted a doctor for melena at another hospital. He had undergone a distal gastrectomy with Billroth Ⅱ reconstruction for a gastric ulcer 47 years ago. He had been having moderate hypertension for 10 years, for which he was taking thiazide daily. A hemorrhage from the upper gastrointestinal tract was suspected. Upper endoscopic examination revealed a localized ulcerative lesion located on the gastrojejunal anastomosis. Contrast-enhanced computed tomography (CT) scans revealed thickening of the stomach wall above the gastrojejunostomy site. There was no evidence of extension of the lesion into the serosa or surrounding soft tissues (Figure 1). An endoscopic biopsy of the tumor was performed. Pathologic examination of the biopsies revealed nests of tumor cells with poor differentiation. The cells showed diffuse positivity for synaptophysin and chromogranin A. Based on biopsy results, the patient was diagnosed with NEC.

A total gastrectomy of the remnant stomach with D2 lymphadenectomy and Billroth Ⅱ reconstruction was performed at our hospital. A low-power histological view revealed that tumor cells had invaded entire layers of the stomach wall and showed infiltrative growth from the muscularis propria to the serosa with angiolymphatic invasion and carcinoma cell embolus (Figure 2). The TNM classification was T3N0M0 (stage IIIA). High-power views revealed monotonous large tumor cells with abundant cytoplasm and large irregular nuclei containing prominent nucleoli; mitotic figures were also observed (60 per 10 high-power fields). Immunohistochemical staining revealed that the tumor cells were positive for chromogranin A, CD56, and synaptophysin. The Ki-67 labeling index was 60%. Thus, the diagnosis of gastric stump large-cell NEC was confirmed.

The patient's postoperative course was favorable, and he was subsequently discharged from the hospital. The patient also commenced a course of adjuvant chemotherapy (EP regimen: 20 mg cisplatin on day 1 and 100 mg etoposide on days 1-4, once a month for one course). However, he experienced grade 3 myelosuppression as a side-effect after this first course of chemotherapy, resulting in treatment suspension due to patient refusal to undergo further treatment. Seven months after the operation, CT scanning revealed lymph node metastasis in the region around the gastrojejunal anastomosis in the abdominal cavity (Figure 4); as a result, the patient received six cycles of chemotherapy (EP regimen: 20 mg cisplatin on days 1-4, and 100 mg etoposide on days 1-3), to which a partial response was achieved. Following this, at 13 mo post-surgery, the patient underwent locoregional radiotherapy, with a total of 60 Gy in 15 fractions. Follow-up CT scanning revealed a recurrence in the liver at 16 mo post-surgery. Two cycles of chemotherapy with the EP regimen were given; however, the patient again experienced grade 3 myelosuppression and disease progression was observed. He then received five cycles of chemotherapy with 240 mg irinotecan on day 1 and 40 mg S-1 on days 1-10, four cycles of chemotherapy with the CAV regimen (0.5 g cyclophosphamide on day 1, 50 mg doxorubicin on days 1-2 and day 21, and 2 mg vincristine on day 1), and two cycles of chemotherapy (200 mg paclitaxel on day 1 and day 14). Despite this treatment, the disease progressed and his performance status deteriorated. He died 31 mo after the operation.

DISCUSSION

GSC was first reported as a disease entity by Balfour in 1922[8]. It was initially defined as a cancer that arose

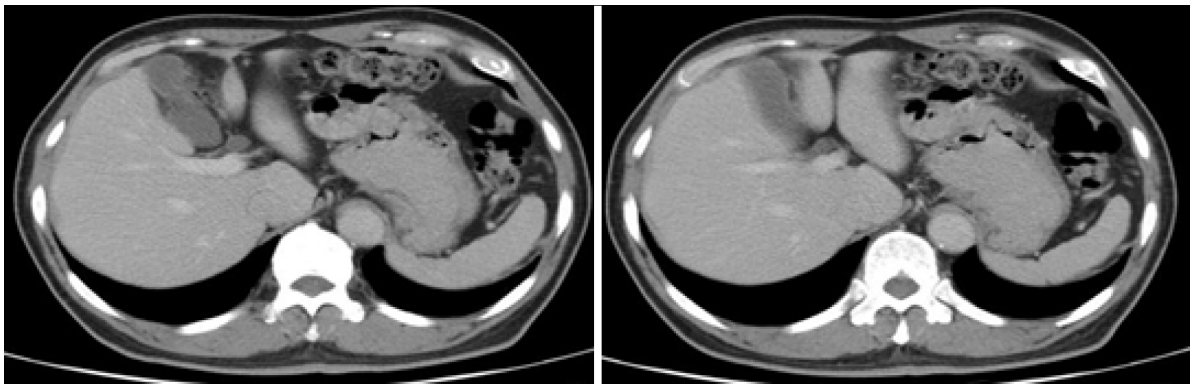

Figure 1 Pre-treatment abdominal contrast-enhanced computed tomography images. These reveal thickening of the stomach wall above the gastrojejunostomy site without enlarged perigastric lymph nodes. There is no evidence of lesion extension into the serosa or surrounding soft tissues.

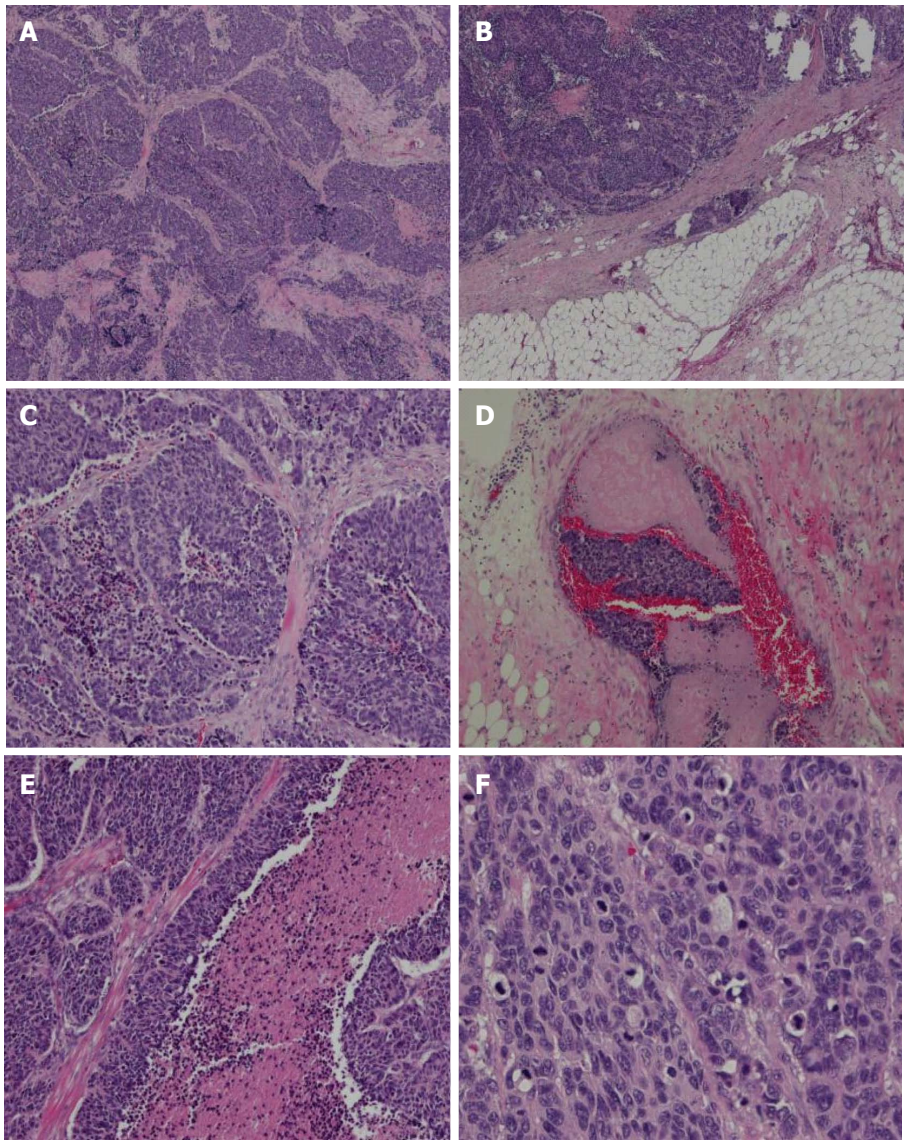

Figure 2 Histological findings. A: A low-power histological view. Tumor cells show infiltrative growth from the muscularis propria to the subserosa (HE, × 40). B: Large-cell carcinoma showing invasion into the subserosa. C: High-power view shows monotonous large tumor cells with abundant cytoplasm and large irregular nuclei with prominent nucleoli (HE, × 100). D and E: Angiolymphatic invasion and carcinoma cell embolus. F: Mitotic figures were also observed (60 per 10 high-power fields). HE: Hematoxylin and eosin.

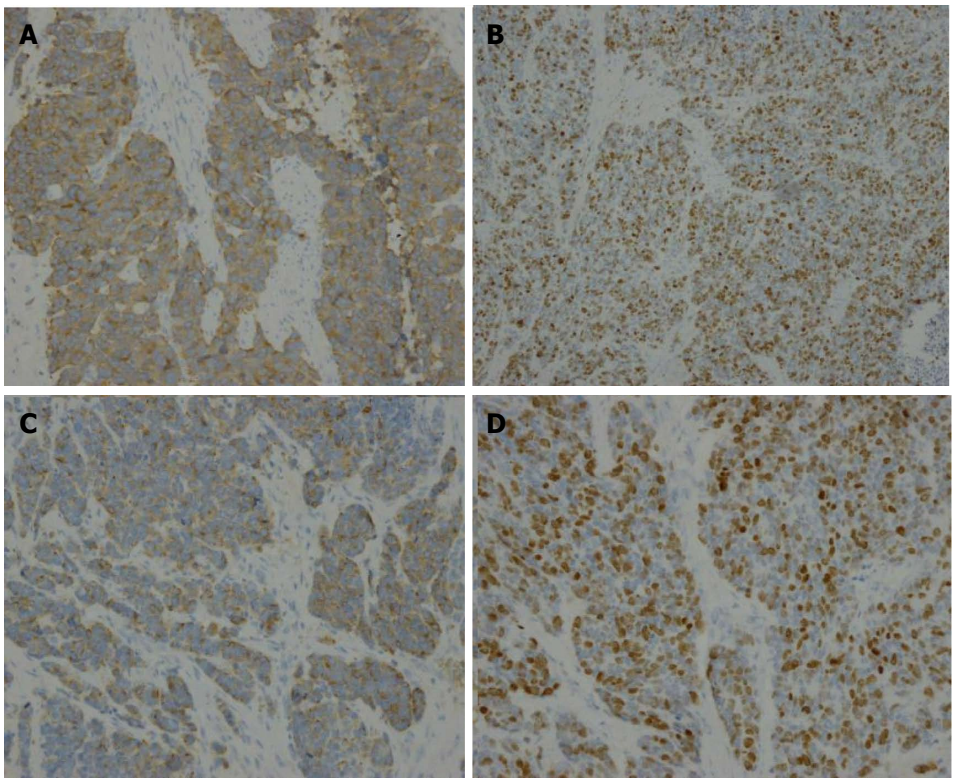

Figure 3 Immunohistochemical staining. Positive immunohistochemical staining for (A) synaptophysin (× 200), (B) CD56 (× 200), and (C) chromogranin A (× 200). D: The Ki-67 index is about 60% (× 200).

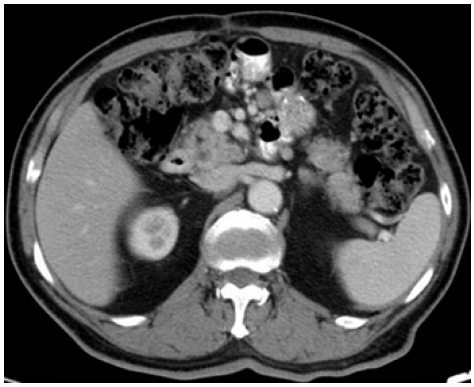

Figure 4 Computed tomography scan 7 mo after the operation. This image reveals lymph node metastasis in the region around the gastrojejunal anastomosis in the abdominal cavity.

in the remnant stomach 5 years after gastrectomy for benign diseases such as peptic ulcers[9]. Currently, the concept of GSC has been expanded to include recurrence after gastric cancer resection, which has been reported to account for 1%-7% of all gastric cancers[10]. Gastric NEC (GNEC) is a rare neoplasm known for its aggressive behavior and poor prognosis, accounting for 0.1%-0.6% of all gastric carcinomas[11]. Primary gastric stump NEC is exceptionally rare. A search of the literature revealed documentation of only one such case, described by D'Adda *et al*[7] who identified a case of metastatic NEC that developed in the gastric stump 25 years after Billroth Ⅱ gastric resection for a duodenal ulcer in 1991.

The carcinogenesis of GSC is strongly associated with chronic duodenogastric reflux of bile and pancreatic juice, and hypochlorhydria secondary to denervation through vagotomy. It has been generally reported that chronic degenerative changes in the gastric mucosa lead to the development of adenocarcinoma with varying degrees of differentiation[12]. The specific carcinogenetic pathways that lead to GNEC are largely unknown. Whether they are related to the classical mechanisms described for GSC development remains to be better understood.

The World Health Organization 2010 classification defined NEC as a subgroup of neuroendocrine neoplasms (NENs). NENs are divided into neuroendocrine tumors (NET) of grade 1 and grade 2 and NEC grade 3 according to the Ki-67 labeling index[13]. The Japanese classification of gastric carcinoma defined NEC as a special type in its histological classification of gastric tumors, and classified it to be either of the small-cell or the large-cell type. In 1993, Rindi *et al*[14] proposed a classification system for gastric NETs (GNETs) wherein tumors were divided into three types by their underlying pathophysiology, etiology, and presentation. According to this classification system, type 1 is associated with chronic atrophic gastritis and hypergastrinemia; type 2 is associated with multiple endocrine neoplasia type 1, Zollinger-Ellison syndrome, and hypergastrinemia; and type 3 is sporadic, gastrin-independent, and is

believed to be the most biologically aggressive GNET. In the present case, based on histological examination and immunohistochemical staining for neuroendocrine markers, the diagnosis was large-cell NEC and the tumor could be classified as a type 3 GNET.

For patients with GNEC, radical gastrectomy plus regional lymph node dissection should be performed for localized disease; adjuvant chemotherapy should also be provided after surgery[15,16]. Given the rarity of these tumors, there is no standardized chemotherapy for GNEC, and therapy is typically done according to the treatment guidelines for small cell lung cancer. A combination of cisplatin and etoposide (EP regimen) is usually proposed as a first-line therapy for extra-pulmonary high-grade NEC[17]. We chose to treat this patient with chemotherapy regimens used for the treatment of SCLC both in first- and second-line settings. Although the addition of radiotherapy has improved the survival of patients with resectable SCLC, its role in the treatment of GNECs is unclear given the extremely limited information on its usage in this type of cancer.

In conclusion, GNEC is rare and this study presents the exceptionally unusual occurrence of NEC in the gastric stump following Billroth Ⅱ gastrectomy. This case will contribute to improvements in our understanding of the carcinogenesis, biology, and behavior of GNEC and GSC. This case may also serve as a reminder to gastroenterologists, surgeons, and pathologists who encounter GSC cases in their clinical practice to consider a diagnosis of NEC and undertake the requisite tests for histological and neuroendocrine markers such as chromogranin A and synaptophysin.

ARTICLE HIGHLIGHTS

Case characteristics
The most common form of gastric stump cancer (GSC) is adenocarcinoma. Various types of gastric stump malignancies have been reported previously, but the development of neuroendocrine carcinoma (NEC) from the gastric stump is rare.

Clinical diagnosis
Gastric ulcer.

Differential diagnosis
Gastric cancer and lymphoma.

Laboratory diagnosis
NEC of the gastric stump.

Imaging diagnosis
Neoplasm of the gastric stump.

Pathological diagnosis
NEC of the gastric stump.

Treatment
Surgery combined with chemotherapy and radiotherapy.

Related reports
A case of neuroendocrine carcinoma in the gastric stump has only been reported once in the English literature from the University of Parma, Italy.

Term explanation
Neuroendocrine carcinoma of the gastric stump.

Experiences and lessons
This case will contribute to improvements in our understanding of the carcinogenesis, biology, and behavior of gastric NEC and GSC. This case may also serve as a reminder to gastroenterologists, surgeons, and pathologists who encounter GSC cases in their clinical practice to consider a diagnosis of NEC and undertake the requisite tests for histological and neuroendocrine markers such as chromogranin A and synaptophysin.

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