

# World Journal of *Hepatology*

*World J Hepatol* 2016 February 18; 8(5): 265-306





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*WJH* covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

We encourage authors to submit their manuscripts to *WJH*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

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ISSN  
ISSN 1948-5182 (online)

LAUNCH DATE  
October 31, 2009

FREQUENCY  
36 Issues/Year (8<sup>th</sup>, 18<sup>th</sup>, and 28<sup>th</sup> of each month)

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PUBLICATION DATE  
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## Controversies in the management of primary sclerosing cholangitis

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**Author contributions:** All authors contributed equally to the paper with concept and design of the review, literature review, drafting, critical revision and editing, and approval of the final version.

**Conflict-of-interest statement:** No potential conflict of interest.

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Received: July 29, 2015

Peer-review started: August 6, 2015

First decision: September 16, 2015

Revised: January 13, 2016

Accepted: January 21, 2016

Article in press: January 22, 2016

Published online: February 18, 2016

### Abstract

Primary sclerosing cholangitis (PSC) remains a rare but significant disease, which affects mainly young males in association with inflammatory bowel disease. There have been few advances in the understanding of the

pathogenesis of the condition and no therapeutics with proven mortality benefit aside from liver transplantation. There remain areas of controversy in the management of PSC which include the differentiation from other cholangiopathies, in particular immunoglobulin G4 related sclerosing cholangitis, the management of dominant biliary strictures, and the role of ursodeoxycholic acid. In addition, the timing of liver transplantation in PSC remains difficult to predict with standard liver severity scores. In this review, we address these controversies and highlight the latest evidence base in the management of PSC.

**Key words:** Immunoglobulin G4 related sclerosing cholangitis; Cholangiocarcinoma; Primary sclerosing cholangitis; Liver transplantation; Dominant strictures

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**Core tip:** There have been few advances in therapeutics for primary sclerosing cholangitis (PSC) and there remain areas of controversy in the management of PSC. In this review, we address these controversies, which include the differentiation of PSC from other cholangiopathies, in particular immunoglobulin G4 related sclerosing cholangitis, the management of dominant biliary strictures, the role of ursodeoxycholic acid, and the timing of liver transplantation.

Nayagam JS, Pereira SP, Devlin J, Harrison PM, Joshi D. Controversies in the management of primary sclerosing cholangitis. *World J Hepatol* 2016; 8(5): 265-272 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v8/i5/265.htm> DOI: <http://dx.doi.org/10.4254/wjh.v8.i5.265>

### INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic cho-



**Table 1 Differential diagnosis for primary sclerosing cholangitis**

Vascular	Hepatic artery thrombosis Portal hypertension bilopathy Portal cavernoma associated cholangiopathy Intra-arterial chemotherapy Sickle cell disease related cholangiopathy
Trauma	Trauma post cholecystectomy Abdominal trauma
Infections	AIDS related cholangiopathy Recurrent pyogenic cholangitis
Benign	Intraductal stone disease
Malignancy	Cholangiocarcinoma
Autoimmune	Autoimmune sclerosing cholangitis IgG4 related sclerosing cholangitis Systemic vasculitis
Other	Recurrent pancreatitis Sclerosing cholangitis in critically ill patient TPN related cholangiopathy Histocytosis X

IgG4: Immunoglobulin G4; TPN: Total parenteral nutrition; AIDS: Acquired immune deficiency syndrome.

lestatic disorder characterised by inflammation and fibrosis of intrahepatic and extrahepatic bile ducts, resulting in multifocal biliary strictures<sup>[1]</sup>. The pathogenesis of PSC remains unclear but hypotheses include genetic factors<sup>[2]</sup>, lymphocyte recruitment and activation<sup>[3]</sup>, portal bacteraemia<sup>[4]</sup> and bile salt toxicity<sup>[5]</sup>.

PSC commonly affects males<sup>[6]</sup> with a median age at diagnosis of 35 years<sup>[6,7]</sup>. In addition, there is a significant association with inflammatory bowel disease (IBD)<sup>[8]</sup>, hepatobiliary malignancies<sup>[9]</sup> and colorectal cancer<sup>[10]</sup>. A key aspect in the management of PSC is surveillance for the development of these conditions. Patients commonly present with cholestatic liver enzymes and a normal bilirubin<sup>[8]</sup>. The demonstration of a cholangiopathy is essential for the diagnosis of PSC<sup>[11]</sup>. Characteristic ERCP findings include diffuse multifocal strictures and irregularities, with normal or minimally dilated segments in between giving rise to the characteristic beaded pattern<sup>[12]</sup>. The use of MRCP has been increasing, partially driven by the complication rate of 11% following ERCP<sup>[13]</sup>, and a comparable sensitivity and specificity to ERCP<sup>[14]</sup>.

PSC is strongly associated with IBD, with prevalence of 63%-81%<sup>[8,15]</sup>, most commonly ulcerative colitis (UC), in 86%-88%<sup>[15,16]</sup>. The temporal relationship of the 2 conditions can be variable, although IBD usually precedes the diagnosis of PSC. Patients with colitis typically have mild symptoms and are sometimes asymptomatic<sup>[17]</sup>. Endoscopic findings are very different between PSC and non-PSC groups, with more pancolitis, backwash ileitis, and rectal sparing in those with PSC<sup>[16]</sup>. Colonoscopy with biopsies is recommended as part of the diagnostic work-up in any new diagnosis of PSC<sup>[18]</sup>. In addition, patients with PSC and IBD have a significantly higher risk of colorectal cancer than those with IBD alone<sup>[10]</sup> (OR = 4.79, 95%CI: 3.58-6.41).

To date, certain aspects remain controversial in the management of PSC. They include the differentiation

of PSC and from other causes of sclerosing cholangitis in particular immunoglobulin G4 related sclerosing cholangitis (IgG4-SC), the optimal management of dominant biliary strictures at endoscopy, the role of ursodeoxycholic acid (UDCA), its optimal dose and likely benefit, and the timing of liver transplantation. In this review article we will address these controversies.

## SEARCH STRATEGY

We searched PubMed using the following terms: "primary sclerosing cholangitis", "secondary sclerosing cholangitis", "cholangiocarcinoma", "IgG4 related disease" and "liver transplantation". We included data from full-text articles, published in English. Further relevant articles were identified from the reference lists of review articles and guidelines from liver societies.

## THE DIFFERENTIAL DIAGNOSIS OF PSC

Secondary sclerosing cholangitis (SSC), includes a heterogeneous group of conditions where different insults (*i.e.*, infections, thrombosis, iatrogenic, trauma) can give rise similar clinical characteristics to PSC<sup>[19]</sup> (Table 1). A single-centre series of 31 patients with SSC, identified a shorter transplant free survival (median 72 mo) when compared to controls with PSC, and with no complicating cases of cholangiocarcinoma (CCA)<sup>[20]</sup>. A more recently described entity is sclerosing cholangitis in critically ill patients. This may be related to hepatic hypoperfusion and biliary cast formation<sup>[21]</sup>, and has a particularly aggressive clinical manifestation with a reported transplant free survival of approximately 1 year<sup>[22]</sup>.

IgG4 disease was first described in 1995 in patients with pancreatitis, raised serum IgG levels and a response to corticosteroids<sup>[23]</sup>, which was termed autoimmune pancreatitis (AIP). Extra-pancreatic biliary changes that were found on ERCP, in addition to lymphoplasmocytic infiltration and fibrosis on liver biopsy, suggested an extra-pancreatic biliary component to the disease<sup>[24]</sup>. This has now been termed IgG4-SC, and is the commonest extra-pancreatic manifestation of AIP<sup>[25]</sup>.

IgG4-SC is an important differential diagnosis for PSC, with a different natural history and treatment profile. There are some subtle differences and similarities between PSC and IgG4-SC (Table 2). Similar to PSC, IgG4-SC has a male preponderance, however it usually presents in older patients<sup>[26]</sup>. The clinical presentation in patients with IgG4-SC is more commonly acute onset of obstructive jaundice, than is seen in classical PSC<sup>[25]</sup>, and was evident in 75% in one series<sup>[27]</sup>. IgG4-SC can also be diagnosed in asymptomatic patients with AIP, either at initial diagnosis or during follow up. The data on rates of co-existent IBD are limited to small case series from Japan<sup>[27]</sup> and United Kingdom<sup>[28]</sup>, which show much lower detection of IBD when compared to patients with PSC. Serum IgG4 is elevated in 76% of patients with AIP, and total IgG is elevated in 42%<sup>[29]</sup>. An elevated

**Table 2 Primary sclerosing cholangitis compared to immunoglobulin G4 related sclerosing cholangitis (adapted from Joshi 2014)**

	PSC	IAC
Gender (M:F)	1.5:1	7:1
Age of onset	Young (< 40 yr)	Older (> 50 yr)
Presentation	Cholestatic liver enzymes	Obstructive jaundice
Cholangiogram	Beading, band-like strictures, peripheral pruning	Long smooth strictures, low CBD strictures
Cholangioscopy	Dilated and tortuous vessels	Scarring, pseudo diverticula
Raised serum IgG4 levels	< 20%	> 70%
Pancreatic involvement	< 5%	> 80%
Cholangiocarcinoma	9%	Rare
Association with IBD	80%	< 10%
Response to steroids	Rare (IgG4 + ve PSC)	97%

M: Male; F: Female; PSC: Primary sclerosing cholangitis; IgG4: Immunoglobulin G4; IBD: Inflammatory bowel disease.

serum IgG4 level however is not sufficient to diagnose IgG4-SC, especially as the optimal cut-off value has not been defined and may differ for subgroups of IgG4-SC<sup>[30]</sup>.

There are 4 cholangiographic patterns of disease which vary in the level of stricture location<sup>[31]</sup>. These are usually long smooth strictures as opposed to beading and band-like strictures in PSC. Depending on the pattern of disease, hepatobiliary malignancy may form part of the differential diagnosis, hence the role for ERCP to obtain samples from dominant strictures<sup>[26]</sup>. Brush cytology during ERCP is beneficial in detecting malignancy, however intra-ductal or ampullary biopsies are required to confirm the diagnosis of IgG4-SC. Histological features of IgG4 related disease are infiltration of plasma cells and IgG4 positive plasma cells, storiform fibrosis, and obliterative phlebitis<sup>[32,33]</sup>. Cholangioscopy in IgG4-SC patients has demonstrated dilated and tortuous vessels in 69%, a feature that was not observed in patients with PSC<sup>[34]</sup>. In addition, less scarring and pseudo diverticula were noted in IgG4-SC patients compared to the PSC patients. When a second procedure was carried out after steroids (32 to 93 d), a significant improvement in stenosis, dilated and tortuous vessels, and mass lesions were identified<sup>[34]</sup>.

The cholangiopathy of IgG4-SC is very responsive to corticosteroids and an improvement in bilirubin can be detected within 8 wk of therapy<sup>[28]</sup>. We recommend 30-40 mg prednisolone o.d. for 4 wk followed by a slow wean, blood test monitoring and imaging at 6 wk<sup>[26]</sup>. Regular clinical assessment in this period is required due to the potential risk of cholangitis and sepsis. A follow up study of IgG4 related disease patients, which included 84 with IgG4-SC, demonstrated a response to steroids in 97% but also a high relapse rate of 50%, which was not predicted by initial or on treatment serum IgG4 levels<sup>[35]</sup>. Five percent were diagnosed with cirrhosis (histological and clinical) and one patient required liver transplantation. It has been hypothesised that unlike PSC, which presents as a more indolent disease with established fibrosclerotic changes, the biliary strictures found in IgG4-SC are at an earlier more inflammatory stage of the disease process which is more responsive

to steroids<sup>[36]</sup>. IgG4-SC patients have not been identified to develop CCA<sup>[37]</sup>, which is in contrast to PSC which confers a significant risk of CCA<sup>[38]</sup>.

## MANAGEMENT OF DOMINANT BILIARY STRICTURE

A dominant stricture is defined as a narrow biliary stricture which impedes normal bile flow, with a diameter < 15 mm in the CBD/CHD or < 10 mm in the hepatic duct<sup>[39]</sup>. In a follow up study of 9.8 years, a new dominant stricture was found in 63% of patients<sup>[40]</sup>. Where liver biopsies were available, those with more advanced liver disease histologically were more likely to have dominant strictures<sup>[39,40]</sup>. The mean survival of patients with dominant strictures was significantly poorer than those without<sup>[40]</sup> (14 years vs 23 years,  $P = 0.01$ ). Data from long-term follow up studies (7.1 and 9.8 years), demonstrates that patients who developed CCA, almost all had pre-existing dominant strictures<sup>[40,41]</sup>.

The most concerning differential diagnosis of a dominant stricture is CCA. When a new dominant stricture is identified, a malignant aetiology needs to be excluded using the combination of axial imaging, biliary cytology and/or histology. Therapy for dominant strictures should be offered to all symptomatic patients, and current guidelines recommend endoscopic dilatation with or without stenting<sup>[40]</sup>. A prospective study of 52 patients with dominant strictures who underwent biliary intervention (stent or dilatation) identified a significantly better survival free of transplantation at 3, 5 and 7 years, when compared to that predicted by the Mayo Risk Score<sup>[39]</sup>.

Despite an improvement in prognosis with biliary intervention, the evidence for the ideal therapeutic strategy is not clear and guidelines suggest endoscopic dilatation, but do not give definitive guidance regarding stenting<sup>[40]</sup>. This decision is a balance between the likelihood of biochemical improvement and the risk of intervention. A large retrospective review of all biliary interventions performed for dominant strictures revealed a similar clinical and biochemical course independent

of modality of intervention, although a lower procedure related complication rate was evident in the balloon dilatation group compared to those who underwent stenting<sup>[42]</sup>. A trial of short term temporary biliary stents (mean 11 d) demonstrated an improvement in symptoms, with only 20% requiring further intervention in 1 year and 40% in 3 years<sup>[43]</sup>. However, within the same study a procedure related complication rate of 15% was reported<sup>[43]</sup>.

A prospective multicentre study of patients with compensated PSC without recent biliary intervention, comparing balloon dilatation to short-term plastic stenting (1-2 wk) is underway (www.clinicaltrials.gov, NCT01398917). The main endpoints of the study include re-intervention free survival time at 2 years, change in cholestatic symptoms and biochemistry at 3 mo, and adverse incidents. Data from this study may guide us towards the optimal management strategy for this group of patients.

Cholangioscopy allows for direct optical visualisation and guided biopsies of the biliary epithelium and biliary lesions. It is another diagnostic tool in the management of dominant strictures. In a multi-centre retrospective study of 52 patients with sclerosing cholangitis (48 PSC, 4 IgG4-SC) who underwent 54 procedures for suspicious biliary strictures, the sensitivity and specificity (50% and 100%, respectively) for diagnosing malignancy was comparable to a control group of patients investigated for a single biliary stricture<sup>[44]</sup>. Failure of cannulation rate was higher in the sclerosing cholangitis group (15%), and was related to difficulty cannulating the narrowed bile duct. The adverse events rate was 17%, with 11% developing cholangitis post procedure despite prophylactic antibiotics.

A further single centre prospective study of patients with PSC referred for cholangioscopy, reported their findings in 41 consecutive patients<sup>[45]</sup>. Cholangioscopy identified ductal stones in 56% of patients (of which 30% were not previously identified on cholangiography), and achieved complete or partial clearance in approximately three quarters of patients. One patient was diagnosed with CCA. Two of the 8 patients who proceeded to transplant were diagnosed with CCA on their explants, both of whom had undergone cholangioscopy directed biopsies which were negative. It appears that the diagnostic accuracy may be related to difficulties in deciding which parts of a stricture to biopsy, especially as it may contain both inflammation and cancer<sup>[44]</sup>. The addition of narrow-band imaging increases the biopsy rate but does not improve the dysplasia detection rate<sup>[46]</sup>.

The use of fluorescence *in situ* hybridisation (FISH) on ERCP brushing samples has been studied in patients with PSC and suspicion of CCA. A recent meta-analysis involving 828 patients from 8 studies, identified a pooled sensitivity and specificity of 68% and 70%, respectively, and 51% and 93% respectively for the 6 studies characterising FISH polysomy<sup>[47]</sup>. In a patient with PSC, a dominant stricture and FISH polysomy, there was a 88% specificity for CCA<sup>[48]</sup>, and where

there was serial FISH polysomy detected with no overt evidence of malignancy, 69% were diagnosed with CCA upto 2.5 years post initial test<sup>[49]</sup>. FISH may play a role in patients with high pre-test probability of CCA, or where CCA is suspected with no clear radiological or histological evidence. However it needs to be used with caution due to the low sensitivity, and importantly the risks of repeated invasive tests and the implications of delaying or excluding patients from liver transplantation.

## THE ROLE OF UDCA

UDCA is a hydrophilic bile acid which was studied in patients with PSC following the discovery of its efficacy in chronic cholestatic conditions, in particular primary biliary cirrhosis<sup>[50]</sup>.

Small prospective studies using UDCA at different doses in patients with PSC showed improvement in symptoms and liver biochemistry (8-16 mg/kg<sup>[51]</sup>, 10 mg/kg<sup>[52]</sup>), as well as histology (13-15 mg/kg<sup>[53]</sup>). Further studies at standard (13-15 mg/kg)<sup>[54]</sup> and higher doses (17-23 mg/kg)<sup>[55]</sup> did not demonstrate any survival advantage or prevention of CCA, although none were powered sufficiently to answer this hypothesis. A more recent randomised placebo controlled study at high dose (28-30 mg/kg) was terminated early due to an interim analysis demonstrating significantly higher rate of serious adverse events in the high dose UDCA group<sup>[56]</sup>. A subsequent meta-analysis on the use of UDCA in PSC concluded that there is no significant difference in mortality, histology or risk of CCA, at both standard and higher dose<sup>[57]</sup>, and guidelines advise against its use in PSC<sup>[18]</sup> (Table 3).

Patients with PSC and IBD have an increased risk of developing colorectal dysplasia compared to patients with IBD alone<sup>[10]</sup>. Although the true mechanisms for this increased risk remains unclear, one proposed hypothesis is the increased exposure of the colon to toxic bile acids<sup>[58]</sup>. Attention has therefore turned to UDCA with some data suggesting a possible chemo-preventative role for UDCA in reducing the incidence of colorectal cancer in PSC and UC patients<sup>[59]</sup>. A meta-analysis also suggested a benefit of using low-dose UDCA (8-13 mL/kg per day) but there was significant heterogeneity of the studies<sup>[60]</sup>. A double-blind, placebo controlled multicentre trial of 56 patients with PSC and UC given high dose UDCA (28-30 mg/kg) identified a significantly increased risk of colorectal cancer and dysplasia on both univariate, HR = 4.44 (1.30-20.1), and multivariate analysis, HR = 5.97 (1.39-41.44), in those receiving UDCA<sup>[61]</sup>. At present based on published guidelines, the use of UDCA for chemo-prevention for colorectal dysplasia is not recommended<sup>[18]</sup>.

## THE ROLE AND TIMING OF LIVER TRANSPLANTATION

Liver transplantation is an effective treatment for PSC,

**Table 3** Important studies involving ursodeoxycholic acid

Ref.	Dose and study design	Number UDCA (number placebo)	Follow up	Parameter	Outcome
Chazouillères <i>et al</i> <sup>[51]</sup>	8-16 mg/kg UDCA alone	15	6 mo	Liver enzymes	Improved
O'Brien <i>et al</i> <sup>[52]</sup>	10 mg/kg UDCA alone	12	37 mo	Liver enzymes	Improved
Beuers <i>et al</i> <sup>[53]</sup>	13-15 mg/kg UDCA <i>vs</i> placebo	6 (8)	12 mo	Liver enzymes Histology	Improved Improved
Lindor <i>et al</i> <sup>[54]</sup>	13-15 mg/kg UDCA <i>v</i> placebo	51 (51)	2.2 yr	Liver enzymes Time to treatment failure Time to liver transplant	Improved No change No change
Olsson <i>et al</i> <sup>[55]</sup>	17-23 mg/kg UDCA <i>vs</i> placebo	110 (109)	5 yr	Liver enzymes Transplant free survival	No change No change
Lindor <i>et al</i> <sup>[56]</sup>	28-30 mg/kg UDCA <i>vs</i> placebo	76 (74)	Terminated	Liver enzymes Primary end-point Serious adverse events	Improved Increased Increased

UDCA: Ursodeoxycholic acid.

**Table 4** Mayo risk score<sup>1</sup>

Parameter	Weighting
Age	+ 0.03 × absolute value
Bilirubin	+ 0.54 × log
Aspartate aminotransferase	+ 0.54 × log
Variceal bleeding	+ 1.24 × yes/no
Albumin	- 0.84 × absolute value

<sup>1</sup>The link is: <http://www.mayoclinic.org/medical-professionals/model-end-stage-liver-disease/revised-natural-history-model-for-primary-sclerosing-cholangitis>.

with survival post-transplant 93.7% at 1 year, 86.4% at 5 years, 69.8% at 10 years<sup>[62]</sup>. The indications for liver transplantation are similar to other aetiologies of chronic liver disease, but also include intractable pruritus and recurrent cholangitis. Organ allocation varies according to national policy. Recurrence of PSC following liver transplantation occurs in up to 20% of patients at 5 years<sup>[63,64]</sup>. The diagnosis of PSC recurrence post-transplant can be challenging due to the variety of causes of biliary strictures and cholangiopathy in the post-transplant setting. After exclusion of these, in combination with a concordant liver biopsy, a diagnosis of PSC recurrence can be made<sup>[65]</sup>. In patients who develop PSC recurrence, re-transplant free survival is 85% at 1 year and 45% at 5 years<sup>[66]</sup>.

Identifying which patients will benefit from liver transplantation, and the optimal timing for this is a challenge in the management of PSC, and many predictive models have been developed to optimise this. The Mayo Risk Score was initially devised following a single centre analysis of 174 patients with PSC<sup>[67]</sup>. Using multivariate analysis, age, log bilirubin, log haemoglobin, presence of IBD and liver biopsy stage, were identified as key parameters in predicting transplant-free survival. These parameters were incorporated into a statistical survival model which stratified patients into "low", "intermediate", and "high" risk<sup>[67]</sup>. The Mayo Risk Score for PSC has had numerous iterations over the last 20 years<sup>[68,69]</sup>. The latest version of the Mayo Risk Score incorporates age,

log bilirubin, log aspartate aminotransferase, variceal bleeding and albumin<sup>[69]</sup> (Table 4). This model predicts survival over 4 years and classifies patients as "low", "medium" and "high" risk.

Cholangiograms of 129 patients with PSC identified that high-grade and diffuse strictures of the intrahepatic ducts were markers of poor prognosis<sup>[70]</sup>. A retrospective review of 181 cholangiograms from 4 centres<sup>[71]</sup>, utilised the Amsterdam Cholangiographic Classification System for PSC<sup>[72]</sup>. The intrahepatic and extrahepatic ducts are scored based on severity of cholangiographic changes, and combined to calculate a prognostic index, where a higher score is associated with a poorer prognosis. If a patient however has a normal cholangiogram, a score of 0 is attributed and a score cannot be calculated, thereby making this model invalid for patients with small-duct PSC. Using this classification, a validation study was able to construct a model which could predict medium and long term prognosis in individual patients with PSC<sup>[73]</sup>.

The enhanced liver fibrosis (ELF) test has recently been assessed to predict clinical outcomes in a cohort of patients with large-duct PSC<sup>[74]</sup>. Median transplant-free survival differed significantly in the tertiles based on ELF score and a cut-off value was calculated to stratify patients into "low-score" and "high-score" groups, with a sensitivity of 67% and specificity of 83%. Patients with higher ELF score had a shorter survival, which was confirmed in a validation group of 138 patients<sup>[74]</sup>. In a multivariate Cox regression analysis, the ELF score was associated with transplant-free survival independent of the Mayo Risk Score.

CCA remains a contraindication to liver transplantation in the majority of liver transplant centres. A protocol was developed at the Mayo Clinic (inclusion criteria: < 3 cm lesion, no metastases, no prior abdominal radiation therapy, no transperitoneal biopsy of the tumour, no prior attempt at resection with violation of the bile ducts), which included pre-transplant neo-adjuvant chemo-irradiation, and a modified post-transplant immunosuppression regimen<sup>[75]</sup>. Using this protocol reported outcomes were comparable to other



indications for liver transplantation<sup>[76]</sup>. Of 215 patients who received neo-adjuvant chemotherapy, 136 patients proceeded to liver transplantation (87 with PSC), with 92% 1 year and 74% 5 year survival. Twenty one percent of those who underwent operative staging were excluded from transplantation due to metastatic disease, and there was a 21% tumour recurrence post transplantation<sup>[77]</sup>. Unfortunately these promising results have not been reproduced at other centres<sup>[78]</sup>. There are currently several studies in progress in order to further understand the role for liver transplantation for CCA, and this data is eagerly awaited<sup>[79]</sup>.

## CONCLUSION

The management of patients with PSC continues to pose a challenge to clinicians worldwide. Although guidelines are available, there are few proven therapeutic options, and there remain clinical scenarios which lack a robust evidence base with which to guide management. Many of the commonly used diagnostic tests, particularly for the detection of hepatobiliary malignancy, lack an appropriate sensitivity and specificity. Until further advances in the field take place, the mainstay of management should involve optimal biliary drainage, timely referral for liver transplantation and a low threshold for investigation for hepatobiliary or colorectal malignancy.

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P- Reviewer: Chetty R, Kaya M, Mudawi HMY, Zhu X  
S- Editor: Kong JX L- Editor: A E- Ed



## Hepatitis B virus and hepatitis C virus infection in healthcare workers

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**Conflict-of-interest statement:** All the authors of the manuscript declare that they have no conflict of interest in connection with this paper.

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Received: July 26, 2015  
 Peer-review started: July 27, 2015  
 First decision: September 30, 2015  
 Revised: October 25, 2015  
 Accepted: January 8, 2016  
 Article in press: January 11, 2016  
 Published online: February 18, 2016

### Abstract

Approximately 3 million healthcare workers per year receive an injury with an occupational instrument, with around 2000000 exposures to hepatitis B virus (HBV) and 1000000 to hepatitis C virus (HCV). Although an effective HBV vaccine has been available since the early eighties, and despite the worldwide application of universal vaccination programs started in the early nineties, HBV still remains a prominent agent of morbidity and mortality. There is no vaccine to limit the diffusion of HCV infection, which progresses to chronicity in the majority of cases and is a major cause of morbidity and mortality worldwide due to a chronic liver disease. Healthcare workers are frequently exposed by a mucosal-cutaneous or percutaneous route to accidental contact with human blood and other potentially infectious biological materials while carrying out their occupational duties. Mucosal-cutaneous exposure occurs when the biological material of a potentially infected patient accidentally comes in contact with the mucous membranes of the eyes or mouth or with the skin of a healthcare worker. Percutaneous exposure occurs when an operator accidentally injures himself with a sharp contaminated object, like a needle, blade or other sharp medical instrument. About 75% of the total occupational exposure is percutaneous and 25% mucosal-cutaneous, the risk of infecting a healthcare worker being higher in percutaneous than in mucosal-cutaneous exposure. All healthcare workers should be considered for HBV vaccination and should meticulously apply the universal prophylactic measures to prevent exposure to HBV and HCV.

**Key words:** Hepatitis B virus infection; Hepatitis C virus infection; Needle-stick injury; Healthcare workers

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**Core tip:** Preventing the transmission of hepatitis B virus

(HBV) or hepatitis C virus infection from source patients to healthcare workers is of vital importance in all healthcare settings worldwide, since these workers are exposed daily to these infections over a period of almost four decades. Needle pricks with contaminated needles, cuts from sharp instruments and blood splashes to the conjunctiva are the most frequent causes of exposure, injuries largely preventable by taking the standard universal precautions. HBV vaccination of anti-HBs-negative healthcare workers is recommended in all countries, but numerous healthcare workers remain exposed to infection because they have eluded HBV vaccination.

Coppola N, De Pascalis S, Onorato L, Calò F, Sagnelli C, Sagnelli E. Hepatitis B virus and hepatitis C virus infection in healthcare workers. *World J Hepatol* 2016; 8(5): 273-281 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v8/i5/273.htm> DOI: <http://dx.doi.org/10.4254/wjh.v8.i5.273>

## INTRODUCTION

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are responsible for the two most widespread forms of chronic hepatitis worldwide<sup>[1-3]</sup>. Healthcare workers are exposed to the risk of acquiring HBV and HCV infection through mucosal-cutaneous exposure (eyes or mouth mucosa or skin) to potentially infectious blood or blood products or through percutaneous exposure to contaminated sharp objects (needles, blades, *etc.*). Twenty-five percent of the total occupational exposure is mucosal-cutaneous and 75% percutaneous<sup>[4]</sup>. The risk of HBV or HCV infecting a healthcare worker is higher in percutaneous than in mucosal-cutaneous exposure. According to the data provided by the World Health Organization (WHO), there are approximately 36 million healthcare workers worldwide, of whom around 3 million per year receive an injury with a sharp instrument, thus resulting in 2000000 subjects contaminated with HBV and 1000000 with HCV<sup>[4]</sup>. Other studies estimated that the incidence of injuries to healthcare workers caused by sharp objects ranges from 1.4 to 9.5 per 100 healthcare workers per year, resulting in 0.42 HBV infections per 100 sharp-object injuries per year<sup>[5]</sup>.

This review article will focus on the risks of healthcare workers acquiring HBV and HCV chronic infections while carrying out their occupational duties.

## HBV INFECTION

HBV infection is a global health problem since 240 to 350 million<sup>[6-9]</sup> people worldwide are estimated to be chronically infected, of whom 312000 die annually of advanced cirrhosis and 341000 of liver cancer<sup>[7]</sup>.

Ten genotypes of HBV have been identified to date, from A to J, based on a genetic diversity of at least 8% in the viral genome<sup>[10,11]</sup>. HBV genotypes show a peculiar

geographical distribution<sup>[12]</sup>. Genotype A predominates in Northern Europe and North America, genotypes B and C in central Asia, genotype D in Mediterranean countries, genotype E in sub-Saharan Africa and in Madagascar, genotype F in South and Central America, genotypes G and H in Mexico and some countries in Central America<sup>[13,14]</sup> and genotypes I and J in Eastern Asia<sup>[15]</sup>.

The age at the time of infection modulates the progression to chronicity of HBV infection, which occurs in around 90% of babies born to hepatitis B e antigen (HBeAg)-positive mothers, a rate that progressively decreases with the increase in age up to 2%-5% in the adult population<sup>[16]</sup>.

The endemicity of HBV infection in a geographical area is classified according to the prevalence of subjects with hepatitis B surface antigen (HBsAg) positivity in the general population as, high (> 8%), intermediate (2.0%-7.9%) and low (< 2%)<sup>[11]</sup>. These categories reflect the predominant patterns of transmission and outcomes of HBV infection. In geographical areas with a high HBV endemicity, such as some countries in Eastern Asia or in sub-Saharan Africa, the majority of HBsAg-positive individuals acquired HBV infection at birth or in early childhood, when the risk of progression to chronicity is very high<sup>[7,15]</sup>. In geographical areas with an intermediate HBV endemicity, such as countries in Northern Africa, the Middle East and Southern Asia, and Eastern Europe, the majority of HBsAg-positive subjects acquired HBV infection in childhood or early adulthood, and family transmission plays an important role in the spread of HBV infection. Finally, in most countries in Western Europe, North, Central and South America and Australia the prevalence of HBsAg-positive individuals is below 2%<sup>[6,7,14]</sup>, the impact of vertical and horizontal transmission in childhood is low, and most HBsAg-positive individuals acquired HBV infection through sexual contact, intravenous drug use (IVDU) or other parenteral exposure to infected blood<sup>[17,18]</sup>.

## HCV INFECTION

HCV infection progresses to chronicity in 70% of cases, a condition that may lead to liver cirrhosis and hepatocellular carcinoma<sup>[19,20]</sup>. According to a WHO report<sup>[21]</sup>, 130-150 million people are chronically infected with HCV. HCV epidemiology shows considerable regional differences. In some geographical areas the endemicity is high, with more than 3.5% of the population having an HCV chronic infection, such as some countries in the Middle East and Central and Eastern Asia and Northern Africa. Several countries in Southern Asia, sub-Saharan Africa, the Andean territories, Central and South America, the Caribbean area, Oceania, Australasia and central and Eastern Europe have a moderate endemicity, with 1.5%-3.5% of subjects carrying HCV infection. The areas considered at low endemicity (< 1.5% of HCV chronic carriers) include countries in the Asian Pacific regions, tropical Latin America, North America and Western Europe<sup>[22]</sup>.



HCV strains have been classified to date into seven genotypes, namely from 1 to 7, on the basis of phylogenetic and sequence analyses of the whole viral genome<sup>[23]</sup>. The global geographical distribution of HCV genotypes is complex. HCV genotype 1 is the most prevalent worldwide, comprising 83.4 million cases (46.2% of all HCV chronic carriers), of whom approximately one-third live in Eastern Asia. HCV genotype 3 is the second most prevalent, and genotypes 2, 4 and 6 are responsible for the majority of the remaining cases worldwide. Eastern Asia has the largest number of carriers with genotype 2 and genotype 6, while Northern Africa and the Middle East have the largest number of carriers with genotype 4. HCV genotype 5 is responsible for around 1.4 million chronic infections (< 1% of all HCV cases worldwide), of which the vast majority in Southern and Eastern sub-Saharan Africa<sup>[22-26]</sup>. Worthy of note, HCV genotypes 1 and 3 generally predominate irrespective of the economic status, while the largest proportions of chronic carriers of HCV genotypes 4 and 5 live in low-income countries.

## RISK FACTORS FOR THE ACQUISITION OF HBV AND HCV INFECTION

HBV circulates in peripheral blood of infected subjects and any parenteral or mucosal exposure to potentially infected blood or blood contaminated material can be considered a risk for HBV transmission to non-immune/non-infected subjects<sup>[18,27]</sup>. In addition, the virus is present at infectious concentrations in semen and cervical secretions, and, consequently, HBV is frequently transmitted also by sexual and vertical routes<sup>[8,16]</sup>. The impact of the various routes of transmission varies significantly from one country to another<sup>[8,16]</sup>. In countries with a high endemicity level, HBV infection is prevalently acquired at birth from an HBeAg-positive mother, in which case it becomes chronic in around 90% of cases, or by horizontal transmission in early childhood through household contact, with a progression to chronicity from 10% to 40% of cases. Promiscuous unprotected sexual activity and IVDU are the major risk factors for acquiring HBV infection in areas with a low-prevalence, such as the United States<sup>[28]</sup>.

Worthy of note, the screening of blood donors for markers of HBV infection has dramatically reduced the risk of HBV transmission through the transfusions of blood and blood products. At present, this risk is estimated as 1-4 cases per million blood components transfused in low-prevalence areas<sup>[29]</sup> and around 1 case per 20000 blood transfusions in high-prevalence regions<sup>[30]</sup>.

The transfusion of infected blood and blood products was the most prominent route of transmission of HCV infection until 1989<sup>[1,31]</sup>. Routine mandatory screening of blood donors for circulating antibodies to HCV that started in the early 1990s has drastically reduced the risk of HCV transmission due to the transfusion of blood and

blood products, currently estimated between 1/500000 and 1/1000000 blood units<sup>[32]</sup>. Some concern for HCV transmission through blood transfusion remains only for some geographical areas with limited resources<sup>[33]</sup>. In developed countries, the sharing of injection equipment among IVDUs is one of the major risk factors for the acquisition of HCV infection, as demonstrated by the high anti-HCV seroprevalence found (70% or more in some studies) in this subset of subjects<sup>[34]</sup>. Conversely, in low-income countries, HCV transmission is frequently due to re-using equipment for injection and other inadequately sterilized therapeutic instruments<sup>[35]</sup>. HCV is rarely acquired through sexual intercourse<sup>[36]</sup>, but some outbreaks of acute HCV infections occurring in men having sex with men in the last decade have attracted the interest of the scientific community on this route of transmission, particularly in human immuno-deficiency virus (HIV)-infected individuals<sup>[37]</sup>. Perinatal transmission of HCV infection from HCV-monoinfected mothers occurs infrequently (around 3% of the cases), whereas it reaches 20% among HIV-coinfected mothers<sup>[38]</sup>. Other risk factors for the acquisition of HCV infection have been described, including acupuncture, tattooing, body piercing, some cosmetic procedures, sharing of shaving razors, nail scissors and other personal effects, and needle-stick injury for healthcare workers<sup>[28]</sup>.

## MODES OF EXPOSURE AND FACTORS ASSOCIATED WITH HBV AND HCV TRANSMISSION IN HEALTHCARE WORKERS

Healthcare workers are exposed to human blood and other potentially infectious biological material more frequently than the general population. Among the 60 or more agents responsible for blood-borne transmissible infectious diseases, HCV and HBV are those most frequently transmitted to healthcare workers. Contact with potentially infectious material occurs in most cases through mucosal-cutaneous or percutaneous exposure. In mucosal-cutaneous exposure, a patient's blood, blood derivative or other potentially infected biological material accidentally enters into contact with the mucous membranes of the eyes or mouth or with the skin, healthy or non-intact, of a healthcare worker<sup>[39]</sup>. Percutaneous exposure occurs when an operator receives an injury with a sharp contaminated object, such as a needle, blade or piece of glass. Around 75% of the total occupational exposure is percutaneous and the remaining 25% mucosal-cutaneous. The risk of HBV or HCV infecting a healthcare worker is higher in percutaneous than in mucosal-cutaneous exposure. The rate of transmission of HCV infection can be five times higher in percutaneous than in mucosal-cutaneous exposure, but the risk of acquiring these infections through conjunctival exposure is also high.

A prominent role in the transmission of an infection



is also played by the degree of infectiveness of the contaminated biological material to which the healthcare worker has been exposed<sup>[40-42]</sup>. The highest rates of transmission of HBV or HCV infection follow exposure to blood or its products, but transmission can also occur, generally at a lower rate, after exposure to ascites, cerebrospinal fluid or solutions from cell cultures. HBV and HCV transmissions have never been observed following exposure to feces, urine, sweat, vomitor tears.

Other main factors significantly affecting the likelihood of transmission of infecting agents are the extent and depth of the cutaneous or mucosal wound and the volume of blood transferred<sup>[40-43]</sup>. All punctures from contaminated needles and sharp objects may be responsible for the transmission of infections. However, the medical devices used to access the blood vessels directly cause most of the conversions to positivity of HCV and HBV serum markers worldwide. Such conversions are less frequent after the intramuscular or subcutaneous use of hollow needles or the use of lancets for capillary blood collection, due to the lesser amount of organic material present on their surface. Nurses generally perform these clinical practices and are the occupational group with the highest risk of needle-stick injuries<sup>[40-43]</sup>.

The risk of exposure is also related to the medical procedure performed. For example, of the 99 percutaneous injuries observed by Tokars *et al.*<sup>[44]</sup> during 1382 surgical operations in five different wards (general, orthopedic, gynecologic, traumatic and cardiac surgery), most (73%) were related with the suturing. Risk factors for percutaneous injuries included the performing an emergency procedure, a patient blood loss greater than 250 mL, and a duration of surgery procedure greater than 1 h<sup>[45]</sup>.

The HBV load in the source patient may influence the risk of transmission of HBV infection to non-immune healthcare workers. In these cases, the risk of HBV transmission is estimated at 19%-30%, if the source patient is HBeAg-positive or shows an HBV load > 10<sup>6</sup> IU/mL and at 5% if the source patient is HBeAg-negative or has a lower HBV load. The anti HBV vaccination of healthcare workers was introduced in the 1980s in most countries, but a substantial number of healthcare workers worldwide have eluded vaccination and, despite the excellent immunogenicity of the vaccine, about 20% of vaccinated subjects show anti-HBs titers lower than 10 IU/mL. It is common opinion, however, that HBV-vaccinated subjects with an anti-HBs titer below 10 IU/mL and those who have become anti-HBs-negative can be considered protected against HBV infection, since the immunological memory for HBsAg may persist even in these cases and ensure a rapid rise in protective antibodies in the case of an HBV assault<sup>[46-48]</sup>. Nevertheless, a highly infectious HBV inoculum might overpower a low antibody titers against HBsAg (anti-HBs) titer during the long professional life of a healthcare worker. In these cases, the administration of a booster dose of HBV vaccine could be considered<sup>[49]</sup>.

The transmission of HCV infection occurs in nearly 10% of the healthcare workers after parenteral exposure to the blood of an HCV-RNA-positive source patient, a rate that may vary according to the HCV load of the source patient<sup>[39,42]</sup>.

## PRE-EXPOSURE MANAGEMENT

The prevention of exposure remains the primary strategy for reducing occupational infections by blood-borne pathogens. The healthcare organizations should make available for their personnel a system that includes written protocols for prompt reporting, evaluation, counseling, treatment, and follow-up of occupational exposures<sup>[39]</sup>. Healthcare workers should be trained to adopt effective measures to prevent infections from occupational exposure to blood, *i.e.*, eliminating unnecessary injections, implementing universal precautions, eliminating needle recapping and disposing of the sharp into a sharps container immediately after use, use of safer devices such as needles that sheath or retract after use<sup>[50]</sup>.

Furthermore, healthcare workers should be aware that any person at risk of contact with blood, blood-contaminated body fluids, other body fluids, or sharps should be vaccinated against HBV<sup>[51]</sup>. The vaccination should happen early after the start of their career to avoid infection and development of carrier status. The healthcare workers should be vaccinated with a standard vaccination schedule and the serum anti-HBs should be assessed 1-2 mo after completion of a 3-dose vaccination series<sup>[52]</sup>. The HBV vaccination is, therefore, an essential part of prevention and control of HBV infection for healthcare workers and its use was one of the causes of drastic reduction of its prevalence in healthcare workers.

## POST-EXPOSURE MANAGEMENT

Although the primary prevention constitutes the first line of defense against the risk of occupational infections by blood-borne viruses, the adequate management of exposures with the post-exposure prophylaxis constitutes a key element in managing and limiting the transmission of these infections to staff exposed.

Regarding HBV infection, the risk of infection and the post-exposure management depends on the HBV status of the source and of the healthcare worker.

The risk of developing clinical hepatitis or serological evidence of HBV infection is high (22%-31% and 37%-62%, respectively) if the source is HBsAg and HBeAg positive, and low (1%-6% and 23%-37%, respectively) if it is HBsAg positive and HBeAg negative<sup>[28]</sup>. Moreover, it needs to evaluate the serological status of the healthcare worker: If HBsAg, anti-HBs (or titer less than 10 IU/mL) and HBV core antigen (anti-HBc) are negative, the healthcare worker is at risk to HBV infection. Precisely, in this case it should be taken in account the post exposure prophylaxis with a first dose of HBV vaccine and anti-HBV-specific immunoglobulin

**Table 1** Prevalence of hepatitis B virus infection in healthcare workers

Ref.	Year of enrollment	Country	No. of subjects	Type of study	HBsAg positive, <i>n</i> (%)	Anti-HBc positive, <i>n</i> (%)
Elzouki <i>et al</i> <sup>[54]</sup>	2008	Libya	601	Cross-sectional	11 (1.8)	51 (8.5)
Alqahtani <i>et al</i> <sup>[55]</sup>	NR	Saudi Arabia	300	Cross-sectional	1 (0.3)	25 (8)
Arguillas <i>et al</i> <sup>[56]</sup>	1990	Philippines	123	Case-control	8 (6.5)	81 (65.8)
Aziz <i>et al</i> <sup>[57]</sup>	NR	Pakistan	250	Cross-sectional	6 (2.4)	
Butsashvili <i>et al</i> <sup>[58]</sup>	2006	Georgia	1386	Cross-sectional	28 (2)	402 (29)
Fisker <i>et al</i> <sup>[59]</sup>	1998	Denmark	960	Cross-sectional	14 (1.5)	
Fritzsche <i>et al</i> <sup>[60]</sup>	2011	Cameroon	237	Cross-sectional	15 (6.3)	174 (73.4)
Kateera <i>et al</i> <sup>[61]</sup>	2013	Rwanda	378	Cross-sectional	11 (2.9)	
Kondili <i>et al</i> <sup>[62]</sup>	2004	Albania	480	Cross-sectional	39 (8.1)	338 (70)
Calleja-Panero <i>et al</i> <sup>[63]</sup>	2007-2010	Spain	4986	Cross sectional	36 (0.77)	
Ozsoy <i>et al</i> <sup>[64]</sup>	1998	Turkey	702	Case-control	21 (3)	
Petrosillo <i>et al</i> <sup>[65]</sup>	1985	Italy	5813	Cross-sectional	108 (1.8)	
Rehman <i>et al</i> <sup>[66]</sup>	1996	Pakistan	95	Case-control	5 (5)	27 (28)
Rybacki <i>et al</i> <sup>[67]</sup>	2009	Poland	520	Cross-sectional	6 (1.2)	99 (19)
Sarwar <i>et al</i> <sup>[68]</sup>	2006	Pakistan	125	Cross-sectional	3 (2.4)	
Slusarczyk <i>et al</i> <sup>[69]</sup>	2008	Poland	961	Cross-sectional	4 (0.4) <sup>1</sup>	151 (15.7)
Thomas <i>et al</i> <sup>[70]</sup>	1991	United States	943	Case-control	1 (0.1)	59 (6.2)
Ciorlia <i>et al</i> <sup>[71]</sup>	1994-1999	Brazil	1433	Cross-sectional	11 (0.8)	

<sup>1</sup>Hepatitis B virus-DNA positivity in anti-HBc-positive subjects. NR: Not reported; HBsAg: Hepatitis B surface antigen; Anti-HBc: Hepatitis B virus core antigen.

(HBIG), that should be initiated as soon as possible, preferably within 24 h of exposure and not more than 7 d. If the healthcare worker is vaccinated with protective antibody response ( $\geq 10$  IU/mL) or is anti-HBc positive, no treatment is needed.

Currently, there is no prophylaxis for HCV infection: In fact, immunoglobulin and antivirals are not recommended and only the observation is indicated. However, recently the available of the second and third wave direct antiviral agents enhanced the efficacy and tolerability of anti-HCV treatment<sup>[53]</sup>; consequently, the traditional management of HCV infection after exposure in healthcare workers probably should be revised.

## STUDIES ON HBV INFECTION IN HEALTHCARE WORKERS

The rates of HBsAg and anti-HBc positivity in healthcare workers reported in several studies published in the last three decades<sup>[54-70]</sup> range from 0.1% to 8.1% and from 6.2% to 73.4%, respectively, depending on the age of the subjects investigated, the spread of HBV infection in their country of origin and on the prevention strategies used by the healthcare workers (Table 1).

Of 5813 healthcare workers tested in Italy in 1985, 21.5% were found to be anti-HBc-positive and 1.8% HBsAg-positive<sup>[65]</sup>. A logistic regression analysis showed that the exposure to HBV infection was associated with male sex, an older age, history of blood transfusion, dental treatment, needle-stick injury and working in a healthcare setting (surgeons and nurses vs others). A similar rate of HBsAg positivity (1.5%) was observed in a study on 960 healthcare workers tested in Denmark in 1998<sup>[59]</sup>. A much higher HBsAg prevalence (8.1%) was detected in 480 healthcare workers investigated by Kondili *et al*<sup>[62]</sup> in 2004 in Albania, in accordance

with the widespread of HBV infection in this country. In this study, the highest rates of HBsAg positivity were found in the youngest age group (11.4% in the aged 20-30) and in the auxiliaries (12.6%), but a high HBsAg prevalence (7.2%-7.5%) was also found in the healthcare workers aged over 30. The anti-HBc seroprevalence was also extremely high (70%) in this study and was associated with an age over 40 (OR = 2.9; 95%CI: 1.9-4.6).

In a study performed in Libya in 2008 on 601 healthcare workers, the rate of HBsAg positivity was 1.8%, higher in nurses (2.3%) and lower in physicians (0.7%) and laboratory staff (0.8%)<sup>[54]</sup>. Of 237 healthcare workers tested in Cameroon, 6.3% were HBsAg-positive and 73.4% anti-HBc-positive, in accordance with the wide spread of HBV infection in this geographical area<sup>[60]</sup>. Seroprevalence studies conducted in Asia showed varying results. Aziz *et al*<sup>[57]</sup>, Rehman *et al*<sup>[66]</sup> and Sarwar *et al*<sup>[68]</sup>, in three different studies conducted in Pakistan, reported that 2.4%, 5% and 2.4% of the healthcare workers, respectively, were HBsAg-positive.

Low rates of HBsAg positivity were found in two seroprevalence studies conducted on healthcare workers in the United States (0.1%) and Brazil (0.8%)<sup>[63,70]</sup>.

Some case-control studies allowing a comparison of the HBsAg prevalence in the healthcare workers with that of the general population provided contrasting results. Rehman *et al*<sup>[66]</sup>, in a small case-control study performed in Pakistan, enrolled 95 healthcare workers and 91 volunteer blood donors as controls and observed higher rates of HBsAg (14% vs 5%) and anti-HBc (36% vs 28%) positivity in the control group. Instead, in a case-control study conducted in Turkey<sup>[64]</sup>, the rate of HBsAg positivity was similar in 702 healthcare workers and 5670 blood donors (3% vs 2.1%), and in the Philippines, Arguillas *et al*<sup>[56]</sup> found 6.5% of 123 healthcare workers and 2.2% of 382 blood donors to be

**Table 2** Prevalence of hepatitis C virus infection in healthcare workers

Ref.	Year of enrollment	Country	No. of patients	Type of study	Anti-HCV positive, <i>n</i> (%)
Elzouki <i>et al</i> <sup>[54]</sup>	2008	Libya	601	Cross-sectional	12 (2)
Alqahtani <i>et al</i> <sup>[55]</sup>	NR	Saudi Arabia	300	Cross-sectional	0
Arguillas <i>et al</i> <sup>[56]</sup>	1990	Philippines	123	Case-control	12 (9.7)
Aziz <i>et al</i> <sup>[57]</sup>	NR	Pakistan	250	Cross-sectional	14 (5.6)
Butsashvili <i>et al</i> <sup>[58]</sup>	2006	Georgia	1386	Cross-sectional	69 (5)
Fisker <i>et al</i> <sup>[59]</sup>	1998	Denmark	960	Cross-sectional	2 (0.14)
Fritzschke <i>et al</i> <sup>[60]</sup>	2011	Cameroon	237	Cross-sectional	4 (1.7)
Kateera <i>et al</i> <sup>[61]</sup>	2013	Rwanda	378	Cross-sectional	5 (1.3)
Kondili <i>et al</i> <sup>[62]</sup>	2004	Albania	480	Cross-sectional	3 (0.6)
Calleja-Panero <i>et al</i> <sup>[63]</sup>	2007-2010	Spain	4981	Cross sectional	31 (0.62)
Ozsoy <i>et al</i> <sup>[64]</sup>	1998	Turkey	702	Case-control	2 (0.3)
Petrosillo <i>et al</i> <sup>[65]</sup>	1985	Italy	5813	Cross-sectional	117 (2)
Rehman <i>et al</i> <sup>[66]</sup>	1996	Pakistan	95	Case-control	4 (4)
Rybacki <i>et al</i> <sup>[67]</sup>	2009	Poland	520	Cross-sectional	4 (0.8)
Sarwar <i>et al</i> <sup>[68]</sup>	2006	Pakistan	125	Cross-sectional	4 (3.2)
Slusarczyk <i>et al</i> <sup>[69]</sup>	2008	Poland	961	Cross-sectional	16 (1.7)
Thomas <i>et al</i> <sup>[70]</sup>	1991	United States	943	Case-control	7 (0.7)
Campello <i>et al</i> <sup>[72]</sup>	1990	Italy	407	Case-control	5 (1.2)
Zaaijer <i>et al</i> <sup>[73]</sup>	2000-2009	Denmark	729	Cross-sectional	1 (0.14)
Okasha <i>et al</i> <sup>[74]</sup>	2008	Egypt	1770	Cross-sectional	141 (8.0)

NR: Not reported; HCV: Hepatitis C virus.

HBsAg-positive. Finally, in the United States, Thomas *et al*<sup>[70]</sup> observed the same HBsAg-positivity rate (0.1%) in 943 healthcare workers and 104239 blood donors, whereas they found a higher rate of anti-HBc positivity in the healthcare workers, 6.2% vs 1.8%, indicating a greater exposure to HBV in these subjects, but exposure was not followed by a chronic infection, most probably because it occurred in adulthood.

## STUDIES ON HCV INFECTION IN HEALTHCARE WORKERS

The prevalence of anti-HCV positivity in healthcare workers<sup>[54-74]</sup> ranges from 0% to 9.7% in different studies worldwide: 9.7% in the Philippines, 8% in Egypt, 3.2%-5.6% in three studies in Pakistan, 5% in Georgia, 0.14% in Denmark, 0.8% in Poland, 0.7% in the United States, 0.6% in Albania and 0.3% in Turkey (Table 2).

The majority of 1386 healthcare workers investigated in Georgia in 2006<sup>[58]</sup> stated an episode of occupational exposure to HCV infection, including accidental needle-stick injuries in 45% of cases, cuts with contaminated instruments in 38% and blood splashes in 46%. For the healthcare workers who received a cut, this unfavorable event occurred during the reassembling of tools or when receiving tools from a colleague in the majority of the cases, and the highest proportion of needle-stick injuries occurred when recapping used needles, more frequently in nurses (39%) than in physicians (22%).

In a cross-sectional study performed on 1770 healthcare workers in Egypt in 2008<sup>[74]</sup>, the anti-HCV seroprevalence was 8.0%, the estimated incidence of HCV infection 7.3 per 1000 persons-year and the risk factors independently associated with HCV seropositivity were an older age, performing a manual job, having a history

of blood transfusion or a history of parenteral treatment for schistosomiasis.

Only five case-control studies compared the prevalence of HCV infection in healthcare workers and in the general population of the same country<sup>[56,64,66,70,72]</sup>. Four of these 5 studies showed similar prevalences of anti-HCV positivity in the two groups of subjects: 0.3% vs 0.4%, respectively, in a study from Turkey on 702 healthcare workers<sup>[64]</sup> and 5670 blood donors, 1.6% in 123 healthcare workers and 2.2% in 382 blood donors investigated in the Philippines<sup>[56]</sup>, 1.2% in 407 healthcare workers and 0.8% in pregnant women studied in Italy<sup>[72]</sup> and 0.7% in 943 healthcare workers and 0.4% in 104239 blood donors in the United States<sup>[70]</sup>. Unexpectedly, but similarly to what was observed for HBsAg by Rehman *et al*<sup>[66]</sup> in a study in Pakistan (see above), they observed that the 95 healthcare workers less frequently than the 91 blood donors were anti-HCV positive (4% vs 14%). The results of this study are of difficult interpretation.

## CONCLUSION

Mucosal-cutaneous and percutaneous exposure to human blood or bloodstained medical instruments occurs more frequently in healthcare workers than the general population. A major role in the transmission of HBV or HCV infection is played by the virus concentration on the infecting materials, high in blood and blood products, much lower in ascites and in cerebrospinal fluid and at non-infectious concentrations in feces, urine, sweat, vomit and tears.

The characteristics of the wound received by a healthcare worker and the volume of blood transferred are other main factors influencing a possible transmission of HBV or HCV infection. In fact, the devices

used to access the blood vessels directly, more frequently than cable needles used for intramuscular or subcutaneous treatments, are responsible for HBV and HCV transmission because of the higher amount of organic material carried on their surface.

The transmission of HBV infection from a source patient to a healthcare worker is also influenced by the natural or vaccine-induced immunological protection against HBV in the healthcare worker. Although HBV vaccination of anti-HBs-negative healthcare workers is highly recommended in all countries, some healthcare workers have eluded vaccination and some were low- or non-responders to the vaccine, indicating that a highly infectious HBV inoculum might overpower low immunological protection.

Since no anti-HCV vaccine is at present available to counteract HCV transmission, healthcare workers should protect themselves by meticulously applying all the universal prophylactic measures whenever potentially exposed.

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**P- Reviewer:** Aghakhani A, D'Amelio R, Honge BL, Panduro A, Wang L

**S- Editor:** Ji FF **L- Editor:** A **E- Editor:** Liu SQ



Basic Study

## Hepatitis C virus inhibitor synergism suggests multistep interactions between heat-shock protein 90 and hepatitis C virus replication

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**Institutional review board statement:** No Ethics Committee approval was required.

**Informed consent statement:** No Ethics Committee approval was required.

**Conflict-of-interest statement:** All of the authors have no conflict of interest to declare in relationship to this article.

**Data sharing statement:** No additional data are available.

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**Received:** September 29, 2015

**Peer-review started:** October 1, 2015

**First decision:** November 4, 2015

**Revised:** December 3, 2015

**Accepted:** January 16, 2016

**Article in press:** January 19, 2016

**Published online:** February 18, 2016

### Abstract

**AIM:** To address the effect of heat-shock protein 90 (HSP90) inhibitors on the release of the hepatitis C virus (HCV), a cell culture-derived HCV (JFH1/HCVcc) from Huh-7 cells was examined.

**METHODS:** We quantified both the intracellular and extracellular (culture medium) levels of the components (RNA and core) of JFH-1/HCVcc. The intracellular HCV RNA and core levels were determined after the JFH1/HCVcc-infected Huh-7 cells were treated with radicicol for 36 h. The extracellular HCV RNA and core protein levels were determined from the medium of the last 24 h of radicicol treatment. To determine the possible role of the HSP90 inhibitor in HCV release, we examined the effect of a combined application of low doses of the HSP90 inhibitor radicicol and the RNA replication inhibitors cyclosporin A (CsA) or interferon. Finally, we statistically examined the combined effect of radicicol

and CsA using the combination index (CI) and graphical representation proposed by Chou and Talalay.

**RESULTS:** We found that the HSP90 inhibitors had greater inhibitory effects on the HCV RNA and core protein levels measured in the medium than inside the cells. This inhibitory effect was observed in the presence of a low level of a known RNA replication inhibitor (CsA or interferon- $\alpha$ ). Treating the cells with a combination of radicicol and cyclosporin A for 24 h resulted in significant synergy (CI < 1) that affected the release of both the viral RNA and the core protein.

**CONCLUSION:** In addition to having an inhibitory effect on RNA replication, HSP90 inhibitors may interfere with an HCV replication step that occurs after the synthesis of viral RNA, such as assembly and release.

**Key words:** Hepatitis C virus; Inhibition of hepatitis C virus release; Cell culture-derived hepatitis C virus; Heat-shock protein 90 inhibitors; Hepatitis C virus RNA replication

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**Core tip:** Hepatitis C virus (HCV) is a major causative agent of hepatocellular carcinoma. Several non-structural proteins of HCV physically and functionally interact with heat-shock protein 90 (HSP90). Although HSP90 inhibitors, which inhibit the chaperone function of HSP90, have been shown to inhibit HCV replication by several groups, a recent report using a reporter system for HCV RNA replication (replicon) suggests that the effect is nonspecific. Thus, the inhibitory mechanism of HSP90 inhibitors remains controversial. Here, we address the effect of HSP90 inhibitors on the release of JFH1/cell culture-derived HCV from Huh-7 cells, and suggested that, HSP90 inhibitors may also interfere with an HCV replication step that occurs after the synthesis of viral RNA, such as assembly and release.

Kubota N, Nomoto M, Hwang GW, Watanabe T, Kohara M, Wakita T, Naganuma A, Kuge S. Hepatitis C virus inhibitor synergism suggests multistep interactions between heat-shock protein 90 and hepatitis C virus replication. *World J Hepatol* 2016; 8(5): 282-290 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v8/i5/282.htm> DOI: <http://dx.doi.org/10.4254/wjh.v8.i5.282>

## INTRODUCTION

Chronic infection with hepatitis C virus (HCV) frequently causes liver cirrhosis and hepatocellular carcinoma<sup>[1]</sup>. Approximately 170 million individuals have been infected by HCV<sup>[2]</sup> and are at risk for developing liver disease<sup>[3]</sup>. HCV has a positive-sense single-stranded RNA genome that encodes a 3000 amino acid polyprotein and also

contains an internal entry site for translation and a non-coding region for genome replication at the 5'- and 3'- flanking region. Translation of the polyprotein is followed by cleavage by the host and viral proteases, which yields three structural proteins (core, E1 and E2) and seven nonstructural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B)<sup>[4]</sup>. Establishment of the cell culture-derived HCV (HCVcc) system (*i.e.*, human hepatoma Huh-7 cells propagating the highly infectious clone of HCV genotype 2a, JFH-1<sup>[5]</sup>) provides a native infection-cycle system, which is suitable for determination of the precise function of HCV proteins and thus the mechanism for replication and secretion of viral particles. Accumulating evidence has indicated that NS3, NS4B and NS5A are required for not only genome RNA replication but also virus assembly<sup>[6]</sup>, whereas P7 and NS2 are dispensable for RNA replication but are required for virus production<sup>[7]</sup>.

Heat-shock protein 90 (HSP90) functions as a molecular chaperone for various client proteins and interacts with a cohort of co-chaperones that modulate the HSP90 ATPase cycle. The ATPase activity of HSP90 is inhibited by HSP90 inhibitors, which compete with ATP for binding and thereby eliminate HSP90 chaperone activity<sup>[8]</sup>. HSP90 clients include not only host proteins but also some virus proteins. Thus, some of the replication steps are required for HSP90 activity<sup>[9]</sup>. In fact, activities of multiple HCV proteins are affected by inhibition of HSP90 by specific inhibitors of HSP90, such as radicicol and geldanamycin and its derivatives. HSP90 inhibitors restrict the activity of NS2/3 protease<sup>[10]</sup>, the stability of NS3<sup>[11]</sup>, and the RNA replication of HCV in cells harboring the HCV replicon by inhibiting a complex consisting of HSP90, NS5A and a human FK506-binding protein (FKBP8)<sup>[12]</sup>. The host factors that are required for HCV replication are also affected by inhibition of HSP90<sup>[13,14]</sup>. Apart from these positive results, Beran *et al.*<sup>[15]</sup> have shown that an HSP90 inhibitor elicits effects similar to those of known cytostatic compounds, abrogating propagation of the mini-genome (subgenomic) replicon of HCV indirectly, through slowing cell growth. HCV subgenomic replicons are generally composed of a selection marker gene, such as G418 resistance, a reporter gene such as luciferase, and a whole HCV genome, except for the regions encoding the structural proteins and NS2 protease, which are deleted<sup>[16]</sup>. The replicons propagating in Huh-7 cells are widely used for studying RNA replication as well as for screening for anti-HCV drugs. However, it is possible that the replicon system does not fully represent the native function of HCV proteins, because mutations that accumulate in NS3 and NS5A in the replicon during enhancement of the RNA replication interfere with virus assembly when the mutation is introduced in the HCVcc construct<sup>[17]</sup>. Hence, the HCVcc system may be more suitable than the subgenomic replicon system to evaluate the anti-viral effect of HSP90 inhibitors. In addition, HSP90 is required for NS3 stability<sup>[11]</sup> and for formation of the complex composing NS4A<sup>[12]</sup>. Because NS3 and NS4A can act on both RNA

replication and HCV assembly, it is possible that HSP90 activity may contribute to post-RNA replication steps, such as virus assembly and release, which is represented by HCVcc but not the replicon system.

In this study, we used JFH1/HCVcc to demonstrate the effect of HSP90 inhibition on HCVcc release from infected Huh-7 cells. Our results showed that the HSP90 inhibitor radicicol preferentially reduced the levels of the core and the HCV RNA released from cells in the medium compared with those in the cells. The HSP90 inhibitor had a more potent effect on viral release in the medium than that of the inhibitor for RNA replication.

## MATERIALS AND METHODS

### Reagents

Radicicol (Sigma-Aldrich, St. Louis, MO, United States) was dissolved in methanol (1 mg/mL). Cyclosporin A [cyclosporin A (CsA); Wako Pure Chemical, Osaka, Japan], 17-AAG [17-(allylamino)-17-demethoxygeldanamycin, Sigma-Aldrich] and 17-DMAG [17-(dimethylaminoethylamino)-17-demethoxygeldanamycin; BIOMOL, Plymouth Meeting, PA, United States] were dissolved in ethanol (1 mg/mL). Interferon- $\alpha$  (IFN- $\alpha$ ; PeproTec EC, London, United Kingdom) was dissolved in water (0.1 mg/mL).

### Culture of human hepatoma Huh-7 cells and preparation of JFH1/HCVcc

Human hepatoma Huh-7 cells (purchased from Human Science Resources Bank, Osaka, Japan) were cultured in DMEM (Nissui Pharmaceuticals, Tokyo, Japan) supplemented with 10% fetal calf serum, 0.06% glutamine, 0.35% glucose and 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA, United States). The cells were maintained at 37 °C in an atmosphere of 5% CO<sub>2</sub>. We synthesized the HCV genomic RNA of genotype 2a (JFH1) *in vitro* using a MEGAscript™ T7 kit (Ambion, Austin, TX, United States) and introduced the RNA into Huh-7 cells by electroporating the cells with the GenePulser II electroporation system (Bio-Rad, Hercules, CA, United States) as previously described<sup>[5]</sup>. The cytotoxic effects of the reagents were examined with Alamar Blue cell viability reagent (Serotec, Raleigh, NC, United States), which allows an estimation of the oxidation levels in the cellular electron-transport pathways with a fluorescent indicator. Alamar Blue was used as described by the manufacturer.

### Quantification of the HCV core protein and genomic RNA

We washed the JFH1/HCVcc cells with PBS and lysed them in lysis buffer (20 mmol/L Tris-Cl, pH 7.5, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 0.1 mmol/L EDTA, 0.1 mmol/L phenylmethanesulfonyl fluoride, 50  $\mu$ mol/L N-*p*-tosyl-L-phenylalanine chloromethyl ketone, 5  $\mu$ mol/L N- $\alpha$ -tosyl-L-lysine chloromethyl ketone hydrochloride, 5  $\mu$ g/mL aprotinin and 5  $\mu$ g/mL leupeptin). We quantified the level of core protein present in

the lysates and spent culture medium using the Ortho HCV antigen ELISA test (Ortho Clinical Diagnostics, Rochester, NY, United States). We used a QIAamp™ Viral RNA Mini kit (Qiagen, Hilden, Germany) to isolate the HCV genomic RNA from the medium. We used an RNeasy™ mini kit (Qiagen) to isolate total RNA from the HCV-infected cells. We quantified the HCV genomic RNA using TaqMan™ EZ RT-PCR Core Reagents (Applied Biosystems, Foster City, CA, United States) and the iCycler™ iQ real-time detection system (Bio-Rad) as previously described<sup>[18]</sup>.

### Effects of radicicol and CsA

The JFH1/HCVcc cells ( $5 \times 10^4$  cells in one well of a 24-well culture dish) were treated with radicicol, CsA and/or IFN- $\alpha$  for 12 h, at which point the medium was replaced by fresh medium that contained the same level(s) of drug(s). After culturing for another 24 h, the core and the HCV RNA in each cell lysate and culture medium were quantified as described above. The synergism between CsA and radicicol was evaluated by the combination index (CI) equation and the Chou and Talalay method<sup>[19,20]</sup> using CalcuSyn software (BIOSOFT, Cambridge, United Kingdom). The CI equation is based on the multiple drug-effect equation of Chou *et al.*<sup>[20]</sup>, which is derived from enzyme-kinetic models. Assuming that CsA and radicicol were mutually non-exclusive drugs that have totally independent modes of action, we used the following equation:

Equation 1 dictates that the combination of drug 1 (D)<sub>1</sub> and drug 2 (D)<sub>2</sub> inhibits a reaction (or phenomenon) by x% in an actual experiment. (D<sub>x</sub>)<sub>1</sub> and (D<sub>x</sub>)<sub>2</sub> are the doses of drug 1 and drug 2 alone that inhibit the same reaction by x%.

$$CI = [(D)_1 / (D_x)_1] + [(D)_2 / (D_x)_2] + \frac{[(D)_1 (D)_2 / (D_x)_2 (D_x)_2]}{[(D)_1 (D)_2 / (D_x)_2 (D_x)_2]} \quad (1)$$

### Statistical analysis

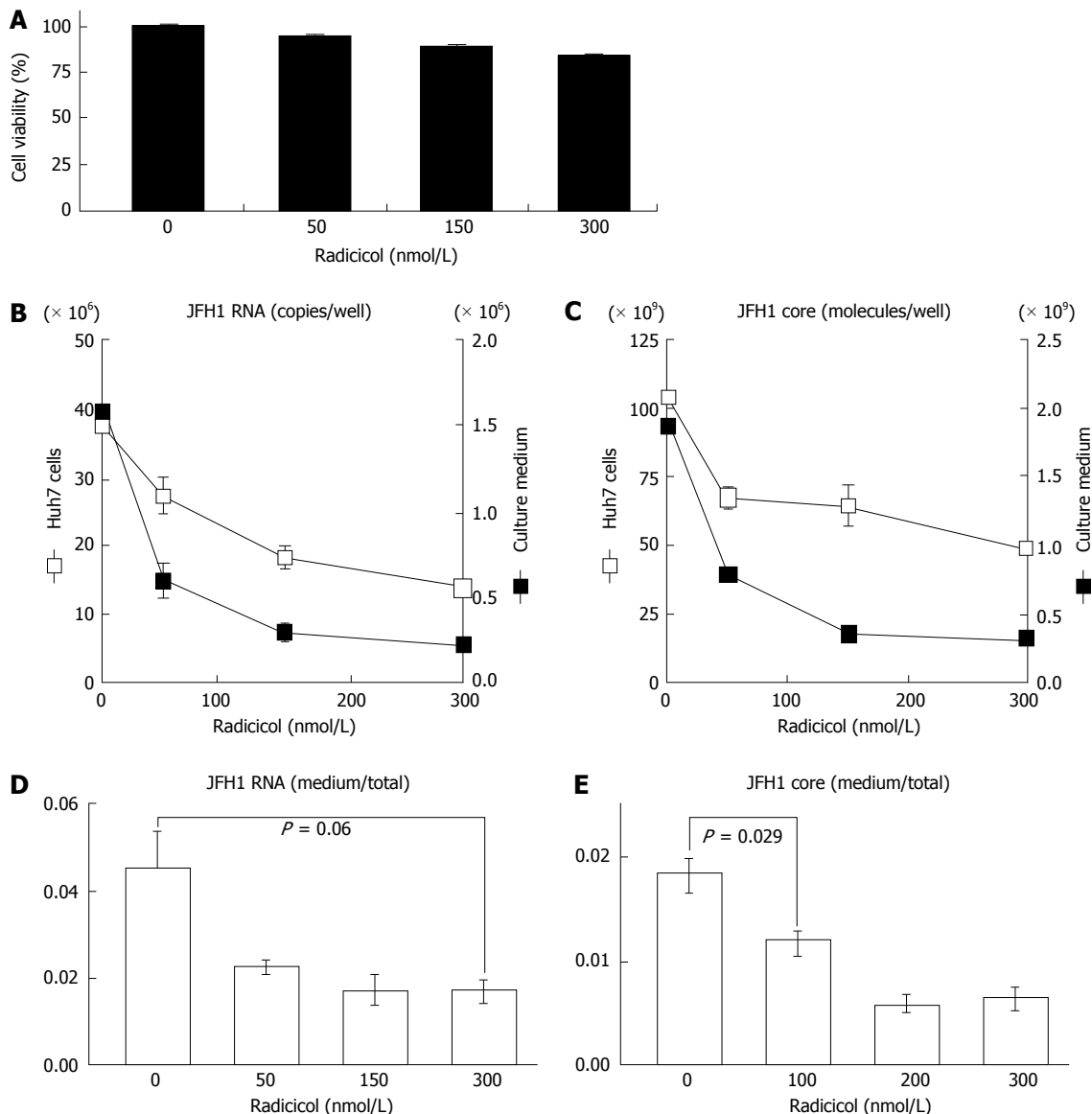
We used Student's *t*-test to examine statistical significance ( $P < 0.05$ ). All of the experiments were performed with multiple independent replicates, and all of the data are presented as the mean results of three independent experiments with the standard error of the mean. The statistical methods of this study were reviewed by professor Kotaro Tanahashi from Mathematics, Tohoku Pharmaceutical University.

## RESULTS

### HCV released into the medium is preferentially reduced by HSP90 inhibitors

To examine the effects of HSP90 inhibitor on the release of HCV, we quantified both the intracellular and extracellular (culture medium) levels of the components (RNA and core) of JFH-1/HCVcc. The intracellular HCV RNA and core levels were determined after the cells were treated with radicicol for 36 h. The extracellular HCV RNA and the core were determined from the medium of the





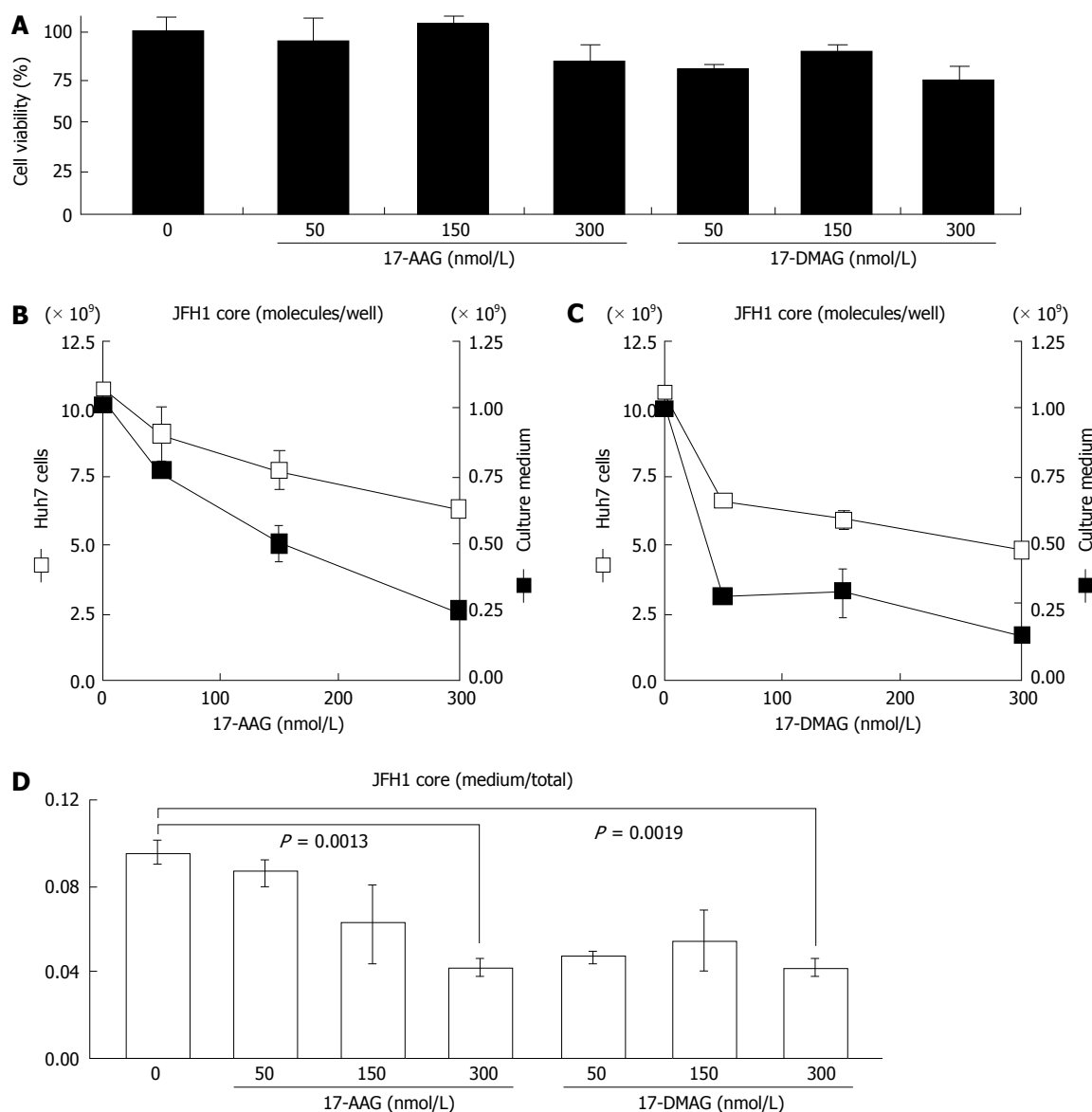
**Figure 1** Radicicol affects the relative level of hepatitis C virus (core and hepatitis C virus RNA) produced from the JFH1/cell culture-derived hepatitis C virus system of Huh-7 cells. A: After the cells were treated with radicicol (at final concentration of 0, 50, 150 and 300 nmol/L) for 12 h, the culture medium was replaced with fresh medium containing the same radicicol levels, and the HCV RNA and core levels released into the medium for the next 24 h and produced within cells were determined as described in the text. The cytotoxic effects of the treatment of radicicol on HCVcc Huh-7 cells were examined as described in the text; B and C: The levels of HCV RNA (B) and core (C) in the HCVcc Huh-7 cells (open squares) and the culture medium (filled squares) were quantified. The scales on the left sides (B and C) indicate the scales for the HCV RNA and the core in the Huh-7 cells. The scales on the right sides (B and C) indicate the scales for the HCV RNA and the core in the culture medium; D: The ratios of HCV RNA in the medium to the total HCV RNA (the sum of HCV RNA in the medium and in the cells) are shown; E: The ratios of the core in the medium to the total core (the sum of the core in the medium and in the cells) are shown. The data represent the mean values ( $\pm$  SEM) of the results from three independent experiments. HCV: Hepatitis C virus; HCVcc: Cell culture-derived HCV.

last 24 h of radicicol treatment. The radicicol treatment (50–300 nmol/L) exhibited no apparent cytotoxic effect (Figure 1A), reduced both the intracellular and extra-cellular (medium) levels of the HCV RNA (Figure 1B) and the core (Figure 1C) in a dose-dependent manner. Interestingly, the RNA level in the culture medium relative to the total RNA level was apparently reduced by radicicol even at a low concentration (50 nmol/L) (Figure 1D). Similarly, the core level in the medium relative to the total core level was also significantly decreased ( $P = 0.029$ ) in the presence of 50 nmol/L radicicol (Figure 1E). Furthermore, two derivatives of the geldanamycin HSP90 inhibitor, 17-AAG and 17-DMAG, also inhibited the

release of the HCV RNA and core more effectively than they decreased the intracellular HCV RNA and core levels (Figure 2).

We next examined whether the integrity of HCV was affected by the radicicol treatment during production of HCV from JFH1/HCVcc. The infectivity of the HCV that had been released into the medium in the presence of radicicol was compared to the infectivity of HCV released in the absence of radicicol. As shown in Figure 3, there was no significant difference ( $P > 0.3$ ) in the infectivity between the HCV produced in the presence and that produced in the absence of radicicol. These results suggested that even though radicicol preferentially reduced





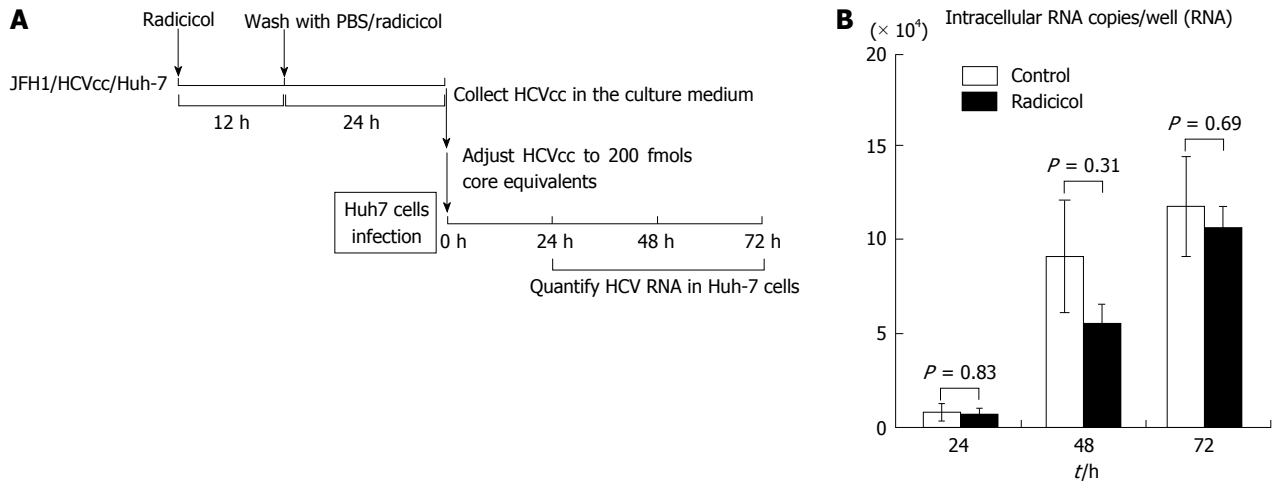
**Figure 2** Effects of the geldanamycin derivatives 17-allylamino-17-demethoxygeldanamycin and 17-dimethylaminoethylamino-17-demethoxygeldanamycin on the release of JFH1. A: The cytotoxic effects of 17-AAG and 17-DMAG on Huh-7 cells carrying JFH1/HCVcc were examined as described in the text; B and C: JFH1-infected Huh-7 cells were treated with 17-AAG (B) or 17-DMAG (C) for 24 h and then the protein samples were isolated and quantified. The levels of the HCV core present in the JFH1-infected Huh-7 cells (open squares) and the culture medium (filled squares) were examined as described in the text. The scales on the left (B and C) and right sides (B and C) indicate the scales for the HCV core in the Huh-7 cells and in the culture medium, respectively; D: The ratios of the core in the medium to the total core are shown. The concentrations of 17-AAG and 17-DMAG (0-300 nmol/L) are shown under the histograms. The data represent the mean values ( $\pm$  SEM) of the results from three independent experiments. HCV: Hepatitis C virus; 17-AGG: 17-allylamino-17-demethoxygeldanamycin; 17-DMAG: 17-dimethylaminoethylamino-17-demethoxygeldanamycin; HCVcc: Cell culture-derived HCV.

HCV release, radicicol did not affect its infectivity.

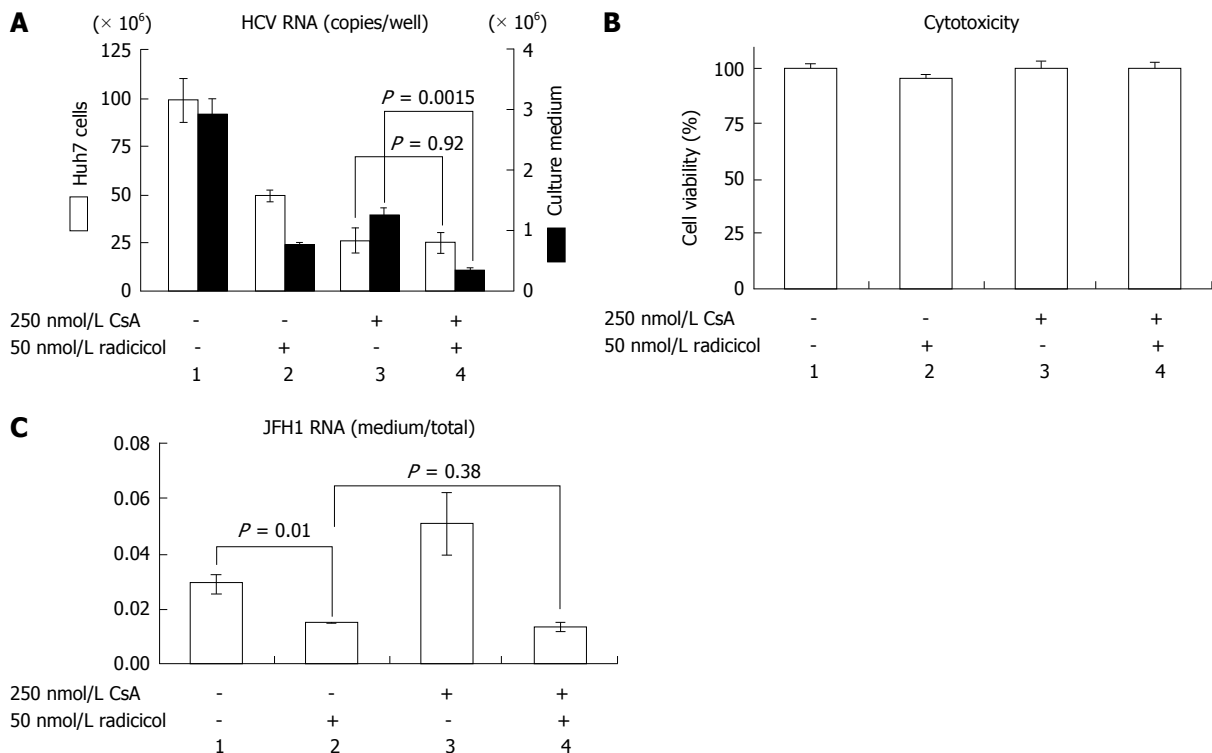
#### **Radicicol preferentially suppressed the HCV RNA release in the presence of CsA or interferon**

NS3 and NS5A, which are known targets of HSP90<sup>[11,12]</sup>, are required for both RNA replication and virus assembly<sup>[6,21]</sup>. Thus, it is difficult to distinguish whether the inhibitory step of the HSP90 inhibitor affects the RNA replication or the assembly. If the HSP90 inhibitor were to preferentially inhibit the post-RNA replication steps, the HSP90 inhibitor might enhance the RNA-replication inhibitor-dependent inhibitory effect on HCV release. CsA an immunosuppressant, inhibits interaction between a

CsA's target cyclophilin A and NS5A and the interaction of the cyclophilin A with the NS5B polymerase/RNA complex, thereby inhibiting RNA replication<sup>[22]</sup>. This inhibitory effect is distinct from CsA's immunosuppressive activity<sup>[23]</sup>. To determine the possible role of the HSP90 inhibitor on HCV release, we examined the effect of a combined application of low doses of radicicol and CsA. By studying the dose-dependent inhibition of HCV RNA production in Huh-7 cells (data not shown), we determined the doses showing the partial effect of CsA (250 nmol/L) and radicicol (50 nmol/L) on the viral RNA production in cells after 36 h of treatment (Figure 4A, column 1, 2 and 3, open bars). CsA alone, radicicol



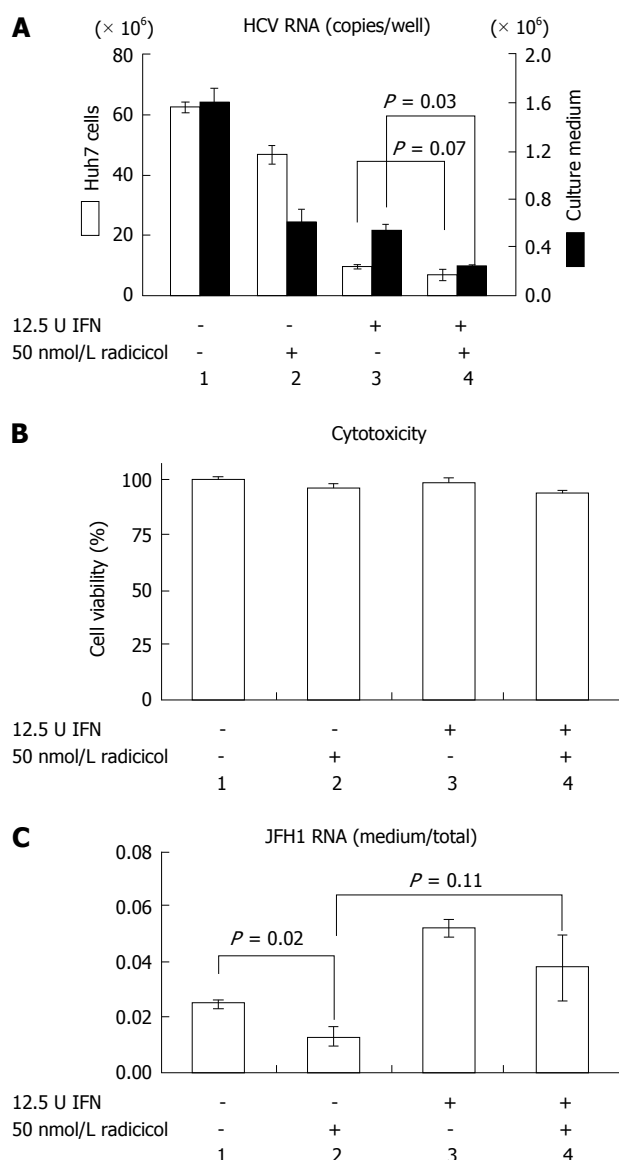
**Figure 3 Infectivity of hepatitis C virus produced from Huh-7 cells that were treated with radicicol.** A: Experimental design. To examine whether the infectivity of the JFH1/HCVcc released from the Huh-7 cells in the presence of radicicol was altered, we infected fresh Huh-7 cells with JFH1/HCVcc prepared in the presence of radicicol. To prepare a viral stock, the JFH1/HCVcc infected Huh-7 cells with 50 nmol/L radicicol were maintained for an additional 36 h. HCVcc released in the medium during the last 24 h was collected and used as the viral stock. We diluted the viral stock 59.7 times (None) and 28.6 times (Radicicol) to reduce the effects of radicicol carryover and to adjust the HCV levels (core protein level: 200 fmols). The final radicicol concentration was 1.75 nmol/L which did not affect HCV propagation. The HCV samples were added to fresh Huh-7 cells and cultured for 24, 48 and 72 h. The HCV RNA in the infected Huh-7 cells was quantified as described in the text. The multiplicities of infection for HCV were 0.2 copies/cell and 0.16 copies/cell for the HCV infection without and with radicicol, respectively; B: The copies of HCV RNA in the Huh-7 cells of each well after infection (24, 48 and 72 h) were determined as described in the text. The value of the RNA copy number in the Huh-7 cells [which were infected with the viral stock from radicicol-treated cells (values of the closed bars)] was adjusted by multiplying by a factor of 1.25. The data represent the mean values ( $\pm$  SE) of the results from three independent experiments. HCV: Hepatitis C virus; HCVcc: Cell culture-derived HCV.



**Figure 4 Radicicol and cyclosporin A have a synergistic effect on hepatitis C virus release into the medium.** A: JFH1/HCVcc-infected Huh-7 cells were treated with 250 nmol/L CsA alone, 50 nmol/L radicicol alone, or both for 36 h. The total RNA was prepared from the Huh-7 cells, and the HCV RNA was quantified by reverse transcription-polymerase chain reaction (open columns, the scale on the left of the panel indicates the copies/well). The medium was replaced by the fresh medium containing the same levels of the drugs after 12 h, and total RNA was prepared from the medium 24 h later. The copy numbers of the HCV RNA in the medium are indicated by the filled columns (scale on the right of the panel); B: The cytotoxic effects of the drugs used in (A) on the Huh-7 cells carrying JFH1/HCVcc; C: The ratios of the RNA in the medium to the total RNA (as described above) are shown. HCV: Hepatitis C virus; HCVcc: Cell culture-derived HCV; CsA: Cyclosporin A.

alone and simultaneous treatment with both drugs did not affect the cytotoxicity in JFH1-infected Huh-7 cells (Figure 4B). When the HCV-infected cells were treated

with the low concentration of both drugs simultaneously, the level of HCV RNA released in the medium after 24 h fell significantly (Figure 4A, comparison of the closed



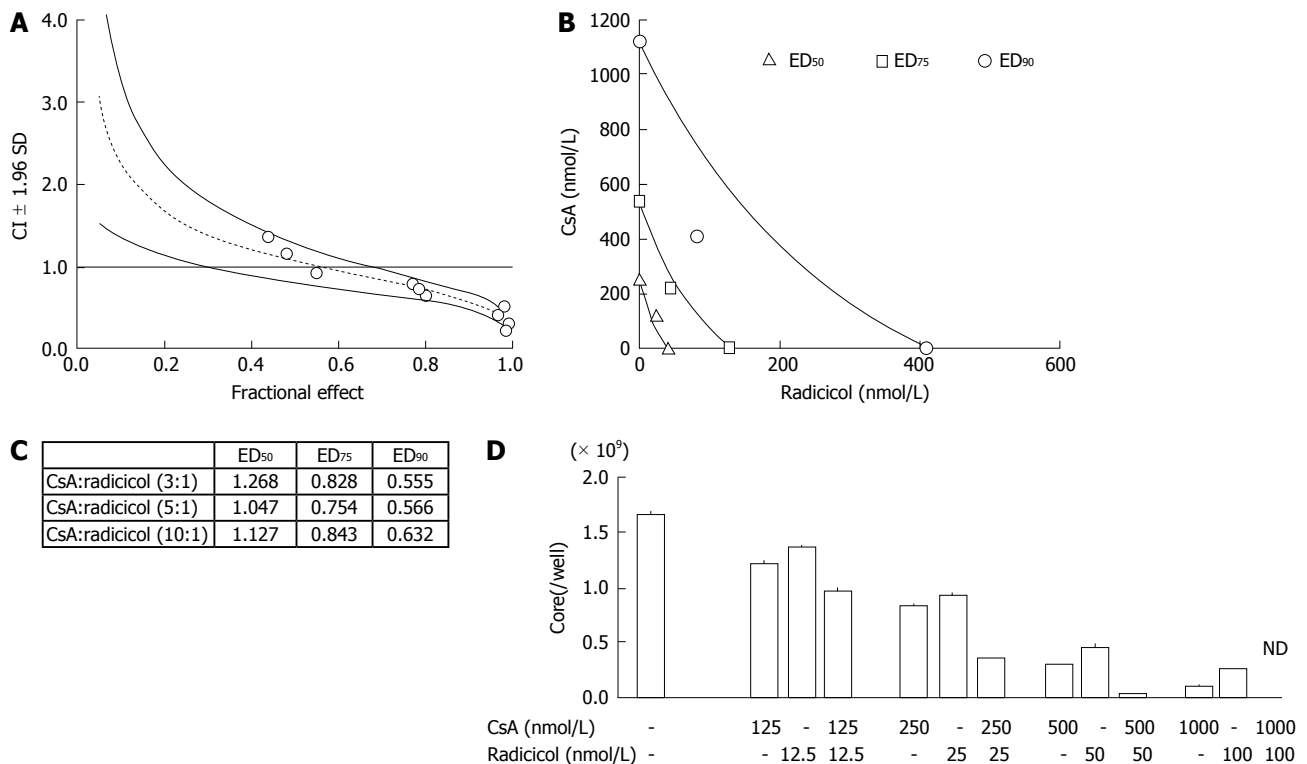
**Figure 5 Radicicol and interferon- $\alpha$  have a synergistic effect on hepatitis C virus release into the medium.** A: JFH1/HCVcc-infected Huh-7 cells were treated with 12.5 U INF- $\alpha$  alone, 50 nmol/L radicicol alone, or both for 36 h. The total RNA was prepared from the Huh-7 cells, and the HCV RNA was quantified by reverse transcription-polymerase chain reaction (open columns, the scale on the left of the panel indicates the copies/well). The medium was replaced by fresh medium after 12 h, and the total RNA was prepared from the medium 24 h later. The copy numbers of the HCV RNA in the medium are indicated by the filled columns (scale on the right of the panel); B: The cytotoxic effects of the drugs used in (A) on the Huh-7 cells carrying JFH1/HCVcc; C: The ratios of RNA in the medium to RNA in the total RNA (as described above) are shown. HCV: Hepatitis C virus; HCVcc: Cell culture-derived HCV; INF- $\alpha$ : Interferon- $\alpha$ .

bars in columns 3 and 4,  $P = 0.0015$ ), whereas the level of HCV RNA in the cells was the same as that in the cells treated with CsA alone (Figure 4A, comparison of the open bars in columns 3 and 4). Intriguingly, the RNA level in the culture medium relative to the RNA levels in the infected cells fell in the presence of radicicol (Figure 4C, comparison of columns 1 and 2). However, this medium-to-total ratio was not affected by simultaneous treatment of CsA with radicicol (Figure 4C, column 2 and 4). Thus, radicicol could have more of an inhibitory effect on viral release from infected cells than CsA.

Previous results have indicated that the combined effect of IFN and CsA on RNA replication is mostly additive<sup>[23]</sup> and have suggested that both CsA and IFN target a similar point in HCV replication. However, Robida *et al.*<sup>[24]</sup> have indicated that CsA resistant mutants maintain their sensitivity to IFN- $\alpha$ . Thus, although both IFN- $\alpha$  and CsA inhibit RNA replication, the inhibitory effects of IFN- $\alpha$  and CsA seems to be different. As shown in Figure 5A, we observed that radicicol efficiently reduced the level of HCV in the medium even in the presence of IFN- $\alpha$ . Although the combined treatment of radicicol and IFN- $\alpha$  and the treatment of IFN- $\alpha$  alone exhibited a similar level of HCV RNA in cells (columns 3 and 4, open bars), HCV RNA in the medium was significantly reduced (columns 3 and 4, closed bars). Again, HCV RNA in the medium was significantly suppressed in the presence of radicicol (Figure 5C). All of these results suggested that HSP90 might be responsible for a post-replication step such as viral release. It should be noted that a similar synergism has been previously reported: A combined administration of an HSP90 inhibitor and polyethylene glycol-conjugated interferon (PEG-IFN) in HCV-infected chimeric mice with humanized livers was more effective at reducing the HCV genomic RNA levels in mouse serum than a single PEG-IFN treatment<sup>[25]</sup>.

#### Analysis of the synergistic effect between radicicol and CsA

Our results suggested that radicicol has a greater inhibitory effect on the release of HCVcc into the medium and that the point of inhibition of HCV production by the HSP90 inhibitor may be different from that of CsA. Thus, we statistically examined the combined effect of radicicol and CsA using the CI and the graphical representation proposed by Chou *et al.*<sup>[19,20]</sup>. We examined the effects of various CsA (125, 250, 750 and 1500 nmol/L) and radicicol (25, 50, 150 and 300 nmol/L) concentrations on the release of the core into the medium after 24 h (data not shown). Next, we examined the effects of a fixed molar ratio of these drugs (5:1 CsA to radicicol). The results (Figure 6A) indicated that CsA and radicicol had a synergistic effect ( $CI < 1$ ) above a fractional effect of 0.5 and close to a strong synergism ( $CI \leq 0.3$ ) at a fractional effect of 1. The combined effect was additive near a fractional effect of 0.5 and antagonistic at a fractional effect less than 0.4. Figure 6B shows the conservation isobologram ( $CI = 1$ ) for the different effective doses of the combination treatment that yielded 50% ( $ED_{50}$ ), 75% ( $ED_{75}$ ) and 90% ( $ED_{90}$ ) inhibition of the core release (constructed using actual experimental data). The combined effect at  $ED_{50}$  ( $CI = 1.05$ ) was additive, whereas the combined effects at  $ED_{75}$  ( $CI = 0.75$ ) and  $ED_{90}$  ( $CI = 0.57$ ) were synergistic (Figure 6). The combined use of 408 nmol/L CsA and 82 nmol/L radicicol yielded  $ED_{90}$  (Figure 6B; if a  $CI = 1$  was expected, an estimated 605 nmol/L CsA and 121 nmol/L radicicol would be required to yield  $ED_{90}$ ). We obtained similar results for the 3:1 and 10:1 molar ratios of CsA



**Figure 6 Analysis of the synergistic effect of cyclosporin A and radicicol.** A: The graphs were constructed using the Chou and Talalay method. The CI and the fractional effect were derived from the release of the HCV core protein into the culture medium of JFH1-infected Huh-7 cells that were treated with a combination of CsA and radicicol (5:1 molar ratio). The open circles indicate actual experimental data. The CI vs fractional effect plots were generated with CalcSyn software. The dotted line and solid lines represent the mean values and standard deviation (1.96), respectively, of three independent experiments. The CI < 1, CI = 1, and CI > 1 indicate synergy, an additive effect and antagonism, respectively; B: The conservation isobologram (CI = 1) depicting different effective doses that yielded 50% (ED<sub>50</sub>), 75% (ED<sub>75</sub>) and 90% (ED<sub>90</sub>) inhibition of viral release by the combination treatment was graphed with actual experimental data (ED<sub>50</sub>, open triangles; ED<sub>75</sub>, open squares; ED<sub>90</sub>, open circles). The data represent the mean values of the results from three independent experiments; C: The CI values of each ED<sub>50</sub>, ED<sub>75</sub> and ED<sub>90</sub> by the combined CsA and radicicol treatment at molar ratios of 3:1, 5:1 and 10:1, respectively; D: The combined effect of CsA and radicicol at a molar ratio of 10:1. The HCV core levels in the medium are shown. The mean values and standard deviation ( $\pm$  SD) of the amounts of the HCV core released into the medium during three independent experiments are shown. The concentrations used in each set of experiments are shown under the histogram. ND: Not detected; CsA: Cyclosporin A; HCV: Hepatitis C virus; CI: Combination index.

to radicicol (Figure 6C). One example, shown in Figure 6D, indicated that treating the cells with both 1000 nmol/L CsA and 100 nmol/L radicicol for 36 h caused the HCV core to be undetectable in the medium for the last 24 h of the combined treatment.

## DISCUSSION

Collectively, our results suggest that HSP90 inhibitors might affect both the RNA replication and the post-RNA replication stage of viral propagation. Previous reports have indicated that HSP90 is required for NS3 stability<sup>[11]</sup> and for the formation of a complex consisting of NS5A and FKBP8<sup>[12]</sup>. Thus, it is possible that HSP90 may affect the post RNA-replication step, such as assembly, through affecting the activity of NS3 and NS5A. Elucidating the precise mechanism for the HSP90 on HCV assembly may provide an alternative drug target for HCV clearance.

## COMMENTS

### Background

Although heat-shock protein 90 (HSP90) inhibitors, which inhibit the chaperone function of HSP90, have been shown to inhibit hepatitis C virus (HCV) repli-

cation by several groups, a recent report using a reporter system for HCV RNA replication (replicon) suggests that the effect is nonspecific. Thus, the inhibitory mechanism of HSP90 inhibitors remains controversial.

### Research frontiers

The authors found that the HSP90 inhibitors had greater inhibitory effects on the HCV RNA and core protein levels measured in the medium than inside the cells. This inhibitory effect was observed in the presence of a low level of a known RNA replication inhibitor [cyclosporin A (CsA) or interferon- $\alpha$ ].

### Innovations and breakthroughs

The authors' results suggested that, HSP90 inhibitors may also interfere with an HCV replication step that occurs after the synthesis of viral RNA, such as assembly and release.

### Applications

This study would benefit the effort to explore new targets for the treatment of HCV infection.

### Terminology

HSP90 inhibitors inhibit the chaperone function of HSP90. HSP90 clients include not only host proteins but also multiple HCV proteins including NS2/3 protease, NS3 and the RNA replication complex consisting of NS5A.

### Peer-review

The authors suggested that HSP90 inhibitors may interfere with an HCV replication step that occurs after the synthesis of viral RNA such as assembly



and release. The results of this study were quite interesting. The data were appropriately presented and interpreted. This manuscript also was well prepared.

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P- Reviewer: Chuang WL, Jin B S- Editor: Qiu S

L- Editor: A E- Editor: Liu SQ



Case Control Study

## High level of serum cholesteryl ester transfer protein in active hepatitis C virus infection

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**Author contributions:** Seki N and Aizawa Y designed research; Satoh K, Nagano T, Seki N, Tomita Y, Aida Y, Sugita T, Itagaki M, Sutoh S, Abe H and Aizawa Y treated patients and collected materials and clinical data; Satoh K, Nagano T, and Aizawa Y analyzed data; Satoh K and Aizawa Y wrote the paper.

**Institutional review board statement:** The study was approved by the ethics committee of Jikei University School of Medicine (Tokyo, Japan).

**Informed consent statement:** All patients gave informed consent.

**Conflict-of-interest statement:** There were no conflict-of-interests which must be declared.

**Data sharing statement:** The technical appendix, statistical code, and dataset are available from the corresponding author at [satoken@jikei.ac.jp](mailto:satoken@jikei.ac.jp).

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Received: August 27, 2015  
 Peer-review started: August 31, 2015  
 First decision: September 28, 2015  
 Revised: December 30, 2015  
 Accepted: January 27, 2016  
 Article in press: January 29, 2016  
 Published online: February 18, 2016

### Abstract

**AIM:** To determine the significance of cholesteryl ester transfer protein (CETP) in lipoprotein abnormalities in chronic hepatitis C virus (HCV) infection.

**METHODS:** We evaluated the significance of the serum concentration of CETP in 110 Japanese patients with chronic HCV infection. Fifty-five patients had active HCV infection, and HCV eradication had been achieved in 55. The role of CETP in serum lipoprotein abnormalities, specifically, in triglyceride (TG) concentrations in the four major classes of lipoproteins, was investigated using Pearson correlations in conjunction with multiple regression analysis and compared them between those with active HCV infection and those in whom eradication had been achieved.

**RESULTS:** The serum CETP levels of patients with active HCV infection were significantly higher than those of patients in whom HCV eradication was achieved (mean  $\pm$  SD,  $2.84 \pm 0.69$   $\mu$ g/mL vs  $2.40 \pm 1.00$   $\mu$ g/mL,  $P = 0.008$ ). In multiple regression analysis, HCV infection status (active or eradicated) was an independent factor significantly associated with the serum CETP level. TG concentrations in low-density lipoprotein (mean  $\pm$  SD,  $36.25 \pm 15.28$   $\mu$ g/mL vs  $28.14 \pm 9.94$   $\mu$ g/mL,  $P = 0.001$ ) and high-density lipoprotein (HDL) (mean  $\pm$  SD,  $25.9 \pm 7.34$   $\mu$ g/mL vs  $17.17 \pm 4.82$   $\mu$ g/mL,  $P < 0.001$ ) were significantly higher in patients

with active HCV infection than in those in whom HCV eradication was achieved. The CETP level was strongly correlated with HDL-TG in patients with active HCV infection ( $R = 0.557$ ,  $P < 0.001$ ), whereas CETP was not correlated with HDL-TG in patients in whom HCV eradication was achieved ( $R = -0.079$ ,  $P = 0.56$ ).

**CONCLUSION:** Our results indicate that CETP plays a role in abnormalities of lipoprotein metabolism in patients with chronic HCV infection.

**Key words:** Hepatitis C virus; Cholesteryl ester transfer protein; High-density lipoprotein triglyceride; Case control study; Lipoprotein metabolism

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**Core tip:** Cholesteryl ester transfer protein (CETP) mediates the transfer of neutral lipids between lipoproteins. Although lipoprotein metabolism abnormalities have been extensively studied, the role of CETP in abnormal lipoprotein profiles in patients with hepatitis C virus (HCV) infection is unknown. Accordingly, we investigated, for the first time, high serum CETP level in patients with active HCV infection. HCV infection was a determinant of the serum CETP level in multiple regression analysis. A high CETP concentration in HCV infection was strongly correlated with excessive triglyceride accumulation in high-density lipoprotein. Thus, CETP may contribute to abnormal lipoprotein metabolism in HCV infection.

Satoh K, Nagano T, Seki N, Tomita Y, Aida Y, Sugita T, Itagaki M, Sutoh S, Abe H, Aizawa Y. High level of serum cholesteryl ester transfer protein in active hepatitis C virus infection. *World J Hepatol* 2016; 8(5): 291-300 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v8/i5/291.htm> DOI: <http://dx.doi.org/10.4254/wjh.v8.i5.291>

## INTRODUCTION

Chronic hepatitis C virus (HCV) infection is one of the most important etiologies of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide<sup>[1-3]</sup>. HCV is a unique virus; its use of host lipid metabolism results in a persistent infection. Therefore, it is very important to understand how HCV uses host lipid metabolism and how host lipid metabolism is affected by HCV infection, because HCV infection represents a unique model in which the virus causes chronic infection while coexisting with the host, simultaneously taking over the host's metabolism<sup>[4]</sup>.

Infectious HCV forms a lipoviral particle that can enter into hepatocytes in the blood<sup>[5]</sup>. The characteristics of HCV lipoviral particles are similar to those of very-low-density lipoproteins (VLDLs)<sup>[6]</sup>. This suggests a close association between HCV infection and VLDL. Both VLDL and HCV lipoviral particles are synthesized,

assembled, and secreted from hepatocytes *via* similar metabolic pathways<sup>[7]</sup>. Consequently, dysregulated lipid metabolism in chronic HCV infection may primarily be caused by VLDL abnormalities. According to some *in vitro* studies, HCV core protein suppressed VLDL production and secretion from the liver by inhibiting microsomal triglyceride (TG) transfer protein<sup>[8,9]</sup>.

In clinical situations, chronic HCV infection alters serum lipid profiles by decreasing the low-density lipoprotein cholesterol (LDL-C) level<sup>[10]</sup> and the VLDL-TG/non-VLDL-TG ratio<sup>[11]</sup>. However, the abnormalities of lipoproteins as a whole in patients with chronic HCV infection have not been clarified. In particular, the abnormal distribution of TGs among lipoprotein subclasses has not been extensively studied, because TG content in each lipoprotein subclass cannot be measured easily by routine laboratory tests.

Cholesterol ester transfer protein (CETP) is a plasma glycoprotein that facilitates the transfer of cholesteryl ester (CE) from high-density lipoprotein (HDL) to other subclasses of lipoprotein [chylomicrons (CM), VLDL, and LDL]<sup>[12]</sup>. The principal effect of CETP on lipoproteins is considered to be the reduction of HDL-C levels and facilitation of reverse cholesterol transport to the liver<sup>[13]</sup>. Accordingly, CETP adjusts the distribution of TG among the different lipoprotein subclasses. Therefore, we speculated that CETP may play an important role in the abnormalities of lipoprotein metabolism in patients with active HCV infection.

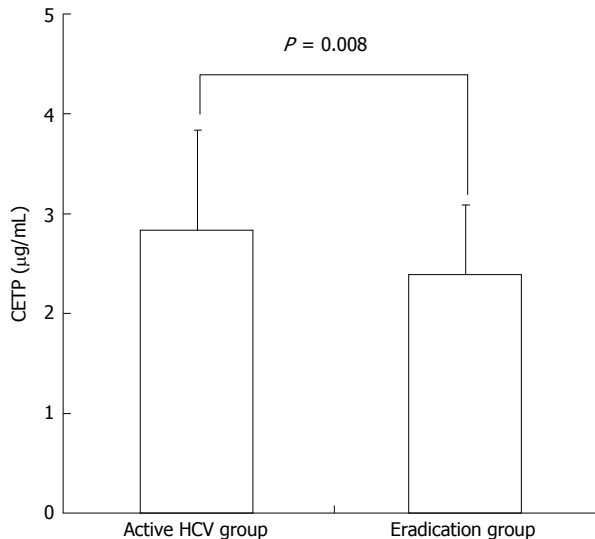
In this study, we determined the serum concentration of CETP in patients with HCV infection and in those in whom HCV was eradicated, to determine the significance of CETP in HCV infection. Furthermore, we investigated the influence of CETP on lipoprotein abnormalities in HCV infection, with particular attention to TG concentrations.

## MATERIALS AND METHODS

The protocol of this case control study was in accordance with the 2004 standards of the Declaration of Helsinki and current ethical guidelines, and was approved by the human ethics review committee of the Jikei University School of Medicine. Written informed consent was obtained from all patients who enrolled in this study.

### Patient population

Japanese patients with active chronic HCV infection (active HCV group) or successfully eradicated chronic HCV infection [negative serum HCV-RNA 6 mo after the end of interferon (IFN)-based therapy] (eradication group) who had been followed up at Jikei University Katsushika Medical Center between September 2013 and October 2014 were randomly considered for enrollment. Patients receiving treatment for diabetes (DM) or hyperlipidemia or hormone replacement therapy and those with hepatitis B virus or human immunodeficiency virus infection were excluded. Additionally, patients who had received IFN within 6 mo or who



**Figure 1** Comparison of the serum cholesteryl ester transfer protein level between the active hepatitis C virus infection group and the hepatitis C virus eradication group. The serum CETP level was significantly higher in patients with active HCV infection than those in whom HCV eradication was achieved ( $2.84 \pm 1.00$  µg/mL vs  $2.40 \pm 0.70$  µg/mL,  $P = 0.008$ ). CETP: Cholesterol ester transfer protein; HCV: Hepatitis C virus.

had been diagnosed with hepatocellular carcinoma or decompensated cirrhosis were excluded.

#### Laboratory tests and demographic data

Demographic data, including age, sex, and body mass index (BMI), and basic laboratory data were obtained from the medical records. The collected basic laboratory data included aspartate 2-oxoglutarate aminotransferase (AST), alanine 2-oxoglutarate aminotransferase (ALT), gamma-glutamyl transpeptidase ( $\gamma$ -GTP), albumin, total bilirubin, fasting blood glucose (FBG), and hemoglobin A1c (HbA1c) levels, hemoglobin (Hb) levels and the platelet count. In addition, basic serum lipid data, including total cholesterol, TG, HDL-cholesterol (HDL-C), and LDL-C were collected. HDL-C had been directly measured by a commercial kit (Kyowa Medex, Tokyo, Japan), and the LDL-C level had been calculated by the Friedewald equation.

#### CETP measurement

The CETP concentration was measured in sera collected after at least a 10-h overnight fast. The CETP mass concentration was measured using a sandwich enzyme-linked immunosorbent assay with two monoclonal antibodies specific to human CETP, JHC1, and JHC2, as previously described<sup>[14]</sup>. The assay was performed in duplicate, and the mean was adopted as the measured value<sup>[15]</sup>.

#### Measurement of cholesterol and TG concentration in the major classes of serum lipoproteins

To examine the distribution of cholesterol and TG in lipoprotein fractions, fresh sera from the collected patient samples were fractionated by high-performance liquid chromatography, and the cholesterol and TG

concentration in the major four lipoprotein classes was measured using the online detection system (Skylight Biotech, Inc., Akita, Japan)<sup>[16,17]</sup>. Serum lipoproteins were classified into four classes according to particle size: CM ( $> 80$  nm), VLDL (30–80 nm), LDL (16–30 nm), and HDL ( $< 16$  nm).

#### Biostatistics

A statistical review of the study was performed by a biomedical statistician.

#### Statistical analysis

Continuous data are expressed as mean  $\pm$  SD. Categorical data are expressed as numbers (%). We used Welch's *t* test or the  $\chi^2$  test for comparisons between the two groups. Correlations between two parameters were evaluated by the Pearson product-moment correlation coefficient. To determine the significance of HCV infection on the serum CETP level, multiple regression analysis was performed, with demographic and basic laboratory data including the HCV infection status (active infection or HCV eradication) as independent variables. The most suitable regression model for explanation of the serum CETP level was constructed by backward elimination of candidate variables.

We performed statistical analyses using STATISTICA software, version 6 (StatSoft Japan Inc. Tokyo, Japan), and two-tailed *P* values of  $\leq 0.05$  were considered significant; *P* values  $> 0.05$  but  $\leq 0.1$  were considered to indicate marginal significance. *P* values less than 0.001 are expressed as  $P < 0.001$ . We determined the multicollinearity of the multiple regression analysis to verify the reliability; the variance inflation factor was  $< 5$ , indicating that our models were reliable.

## RESULTS

#### Study population

In total, 110 patients were included in the study. Fifty-five had active HCV infection, and HCV eradication with IFN-based anti-viral therapy had been achieved in the remaining 55 patients. In the active HCV group, 48 (87%) had HCV genotype (G) 1b infection and 7 (13%) had HCV G2 infection. In the eradication group, 34 (62%) were previously infected with HCV G1b and 21 (38%) were previously infected with HCV G2.

#### Increase in the CETP level in active chronic HCV infection

The serum CETP level was significantly higher in the active HCV group than in the eradication group ( $2.84 \pm 1.00$  µg/mL vs  $2.40 \pm 0.70$  µg/mL,  $P = 0.008$ , Figure 1).

#### Characteristics of the active HCV group and the eradication group

The clinical features of patients in the active HCV group and those in the HCV eradication group are summarized in Table 1. There were significant differences in the proportion of patients with HCV G1b infection. There



**Table 1 Clinical features of patients with active hepatitis C virus infection (active hepatitis C virus group) and of those in whom hepatitis C virus infection was eradicated (eradication group) *n* (%)**

Discrete traits	Active HCV group ( <i>n</i> = 55)	Eradication group ( <i>n</i> = 55)	<i>P</i> value
Sex			0.254
Male	22 (40)	28 (51)	
Female	33 (60)	27 (49)	
HCV genotype			0.003
1b	48 (87)	36 (65)	
2	7 (13)	19 (35)	
Quantitative traits	Mean ± SD	Mean ± SD	
Age (yr)	66.9 ± 11.2	64.3 ± 12.1	0.200
BMI (kg/m <sup>2</sup> )	22.5 ± 3.2	22.8 ± 3.4	0.631
AST (IU/L)	53.1 ± 27.5	24.0 ± 7.2	< 0.001
ALT (IU/L)	49.9 ± 37.1	20.0 ± 11.7	< 0.001
Total bilirubin (mg/dL)	0.8 ± 0.3	0.7 ± 0.3	0.242
γ-GTP (IU/L)	50.4 ± 64	27.1 ± 20	0.011
Albumin (g/dL)	3.9 ± 0.4	4.4 ± 0.3	< 0.001
Hb (g/dL)	13.4 ± 1.7	14.2 ± 1.5	0.010
Platelet (10 <sup>4</sup> /μL)	15.0 ± 6.3	20.0 ± 17.8	0.050
FBG (mg/dL)	108 ± 32	107 ± 16	0.740
HbA1c (%)	5.4 ± 0.6	6.1 ± 0.5	0.030
Lipid profiles			
Total cholesterol (mg/dL)	173.6 ± 31	200.5 ± 37.8	< 0.001
Triglyceride (mg/dL)	107.5 ± 52.0	103 ± 53.1	0.712
LDL cholesterol (mg/dL)	92.1 ± 25.7	117 ± 29.9	< 0.001
HDL cholesterol (mg/dL)	59.9 ± 17.4	64.9 ± 19.0	0.159
CETP level (μg/mL)	2.84 ± 1.00	2.40 ± 0.69	0.008

There were significant differences in the proportion of patients with HCV G1b infection. There were no significant differences in BMI, FBG levels, and HbA1c levels, whereas AST, ALT, and albumin levels differed significantly. TC and LDL-C levels were significantly lower in the active infection group than in the eradication group, whereas TG and HDL-C levels were similar in the two groups. HCV: Hepatitis C virus; BMI: Body mass index; AST: Aspartate-2-oxoglutarate aminotransferase; ALT: Alanine-2-oxoglutarate aminotransferase; γ-GTP: Gamma-glutamyl transpeptidase; Hb: Hemoglobin; FBG: Fasting blood glucose; HbA1c: Glycosylated hemoglobin; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; CETP: Cholesterol ester transfer protein; LDL-C: Low-density lipoprotein cholesterol.

**Table 2 Influence of the hepatitis C virus infection status on the serum cholesterol ester transfer protein level as analyzed by multiple regression analysis**

	B	SE	<i>P</i> value
Constant	0.770	0.541	0.157
HCV infection	0.415	0.158	0.010
Age	0.014	0.006	0.042
Female sex	0.391	0.182	0.034
HDL	0.008	0.004	0.099

HCV infection status was an independent factor that significantly influenced the serum cholesterol ester transfer protein level. HCV: Hepatitis C virus; HDL: High-density lipoprotein.

were no significant differences in BMI, FBG levels, and HbA1c levels, whereas AST, ALT, and albumin levels differed significantly. TC and LDL-C levels were significantly lower in patients with active infection than in patients whose infection was eradicated, whereas TG and HDL-C levels were similar between the two groups.

**HCV infection status (active infection or eradication) as an independent determinant that significantly contributed to the serum CETP level**

To construct a multiple regression model that can suitably

explain the serum CETP concentration, six variables were selected as candidate independent variables. Among categorical data, HCV infection status and sex were selected, because the serum CETP level was significantly higher in female than in male patients ( $2.90 \pm 0.91$  μg/mL vs  $2.28 \pm 0.73$  μg/mL,  $P < 0.001$ ). However, difference in HCV genotype was not selected as a candidate variable because there was no difference in serum CETP level between HCV G1b and G2 patients ( $2.51 \pm 0.68$  vs  $2.66 \pm 0.94$ ,  $P = 0.472$ ). Furthermore, the serum CETP level was similar between HCV G1b patients and G2 patients in the active group ( $2.82 \pm 1.02$  vs  $2.99 \pm 0.76$ ,  $P = 0.685$ ) and the eradication group ( $2.42 \pm 0.75$  vs  $2.36 \pm 0.57$ ,  $P = 0.734$ ).

Among the continuous variables, age ( $R = 0.246$ ,  $P = 0.010$ ), albumin level ( $R = -0.194$ ,  $P = 0.046$ ), Hb level ( $R = 0.249$ ,  $P = 0.009$ ), and HDL-C level ( $R = 0.236$ ,  $P = 0.014$ ) were significantly correlated with the serum CETP level and were thus selected as candidates. Other factors including HbA1c ( $R = 0.084$ ,  $P = 0.538$ ) and FBS ( $R = 0.074$ ,  $P = 0.589$ ) were not selected as candidates, because significant correlation was not verified.

Of these six candidates, HCV infection status, age, sex, and HDL-C level were selected as independent

**Table 3** Comparison of the cholesterol and triglyceride concentrations in the four major lipoprotein fractions according to hepatitis C virus infection status

Major class	Active HCV group	Eradication group	P value	Active HCV group	Eradication group	P value
	Cholesterol (mg/dL)			Triglyceride (mg/dL)		
CM	0.19 ± 0.19	0.19 ± 0.18	0.86	1.21 ± 1.53	1.28 ± 1.31	0.774
VLDL	29.04 ± 12.31	26.12 ± 10.66	0.187	57.66 ± 42.06	65.97 ± 44.18	0.314
LDL	87.94 ± 24.11	117.31 ± 26.91	< 0.001	36.25 ± 15.28	28.14 ± 9.94	0.001
HDL	49.58 ± 15.19	55.19 ± 16.29	0.064	25.9 ± 7.34	17.17 ± 4.82	< 0.001

The cholesterol concentration in LDL-C was significantly lower and HDL-C was marginally lower in the active HCV group, whereas cholesterol concentrations in CM and VLDL were similar between groups. The TG concentrations in LDL-TG and HDL-TG were significantly higher in the active HCV group, whereas TG concentrations in CM and VLDL were similar between groups. HCV: Hepatitis C virus; CM: Chylomicron; VLDL: Very-low-density lipoprotein; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TG: Triglyceride.

variables for the most suitable multiple regression model to explain the serum CETP level. As shown in Table 2, HCV infection status was an independent factor that significantly influenced the serum CETP level. The R<sup>2</sup> value of this model was 0.221, and the adjusted value was 0.191. The R<sup>2</sup> value did not improve significantly by the addition of any other candidate factors. However, elimination of any of these four factors significantly decreased the R<sup>2</sup> values.

The most suitable regression equation was as follows: Serum CETP (μg/mL) = 0.770 + 0.0139 (age) + 0.391 (sex: male: 0, female: 1) + 0.00818 HDL-C (mg/dL) + 0.416 HCV infection status (eradication: 0, active infection: 1).

#### **Differences in the TG concentration in the four major classes of lipoproteins according to HCV infection status**

The cholesterol concentration in LDL-C was significantly lower and the HDL-C concentration was marginally lower in the active HCV group compared to those in the eradication group, whereas cholesterol concentrations in CM and VLDL were similar between the groups.

The TG concentrations in LDL-TG and HDL-TG were significantly higher in the active HCV group compared to the eradication group, whereas TG concentrations in CM and VLDL were similar between groups (Table 3).

#### **Correlation between the serum CETP concentration and TG concentration in the four major lipoprotein classes**

The CETP level had a weak, inverse correlation with CM-C (R = -0.290, P = 0.031) in the eradication group; it had a positive correlation with HDL-C in the active HCV group (R = 0.324, P = 0.015), but this correlation was marginal in the eradication group (R = 0.250, P = 0.064). Significant correlations were not found between groups for the other lipoprotein fractions (Figure 2A).

According to the TG concentration, the CETP level was inversely correlated with chylomicron-TG (R = -0.348, P = 0.009) and VLDL-TG (R = -0.415, P = 0.002) and marginally correlated with LDL-TG (R = -0.247, P = 0.069), but was not correlated with HDL-TG (R = -0.079, P = 0.566) in the eradication group. In contrast, the CETP level was strongly correlated with HDL-TG in

the active HCV group (R = 0.557, P < 0.001). However, significant correlations with TG for other lipoprotein classes were not detected in the active HCV group (Figure 2B).

## **DISCUSSION**

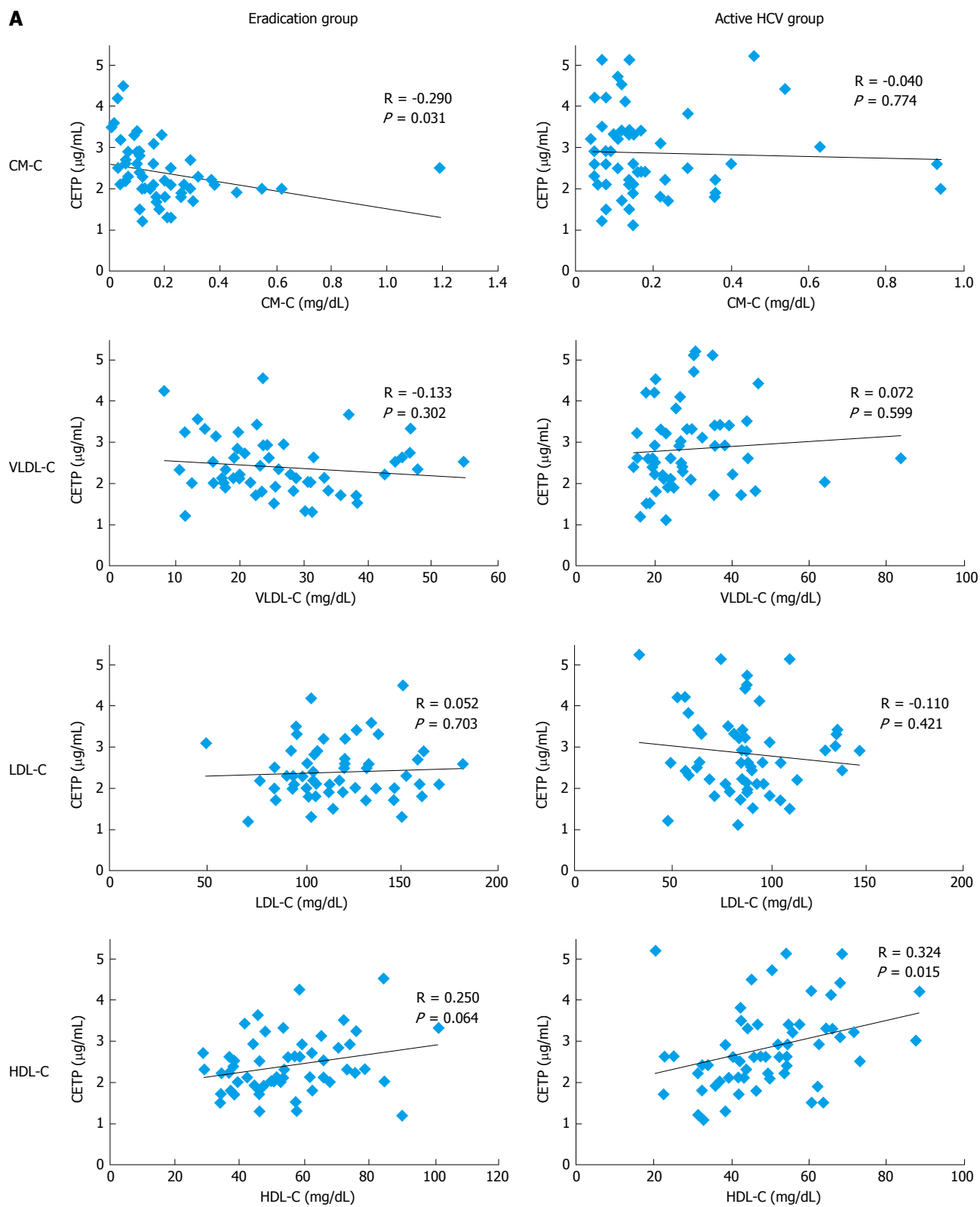
CETP is a glycoprotein that mediates the exchange of CE in HDL for TG in other lipoproteins. In the present study, we investigated the serum concentration of CETP and found that serum CETP levels were significantly higher in the active group than in the eradication group. Moreover, HCV infection was found to be an independent factor in determining the serum CETP level in multiple regression analysis. This suggests that HCV infection promotes the exchange of CE for TG in HDL by increasing the serum CETP concentration.

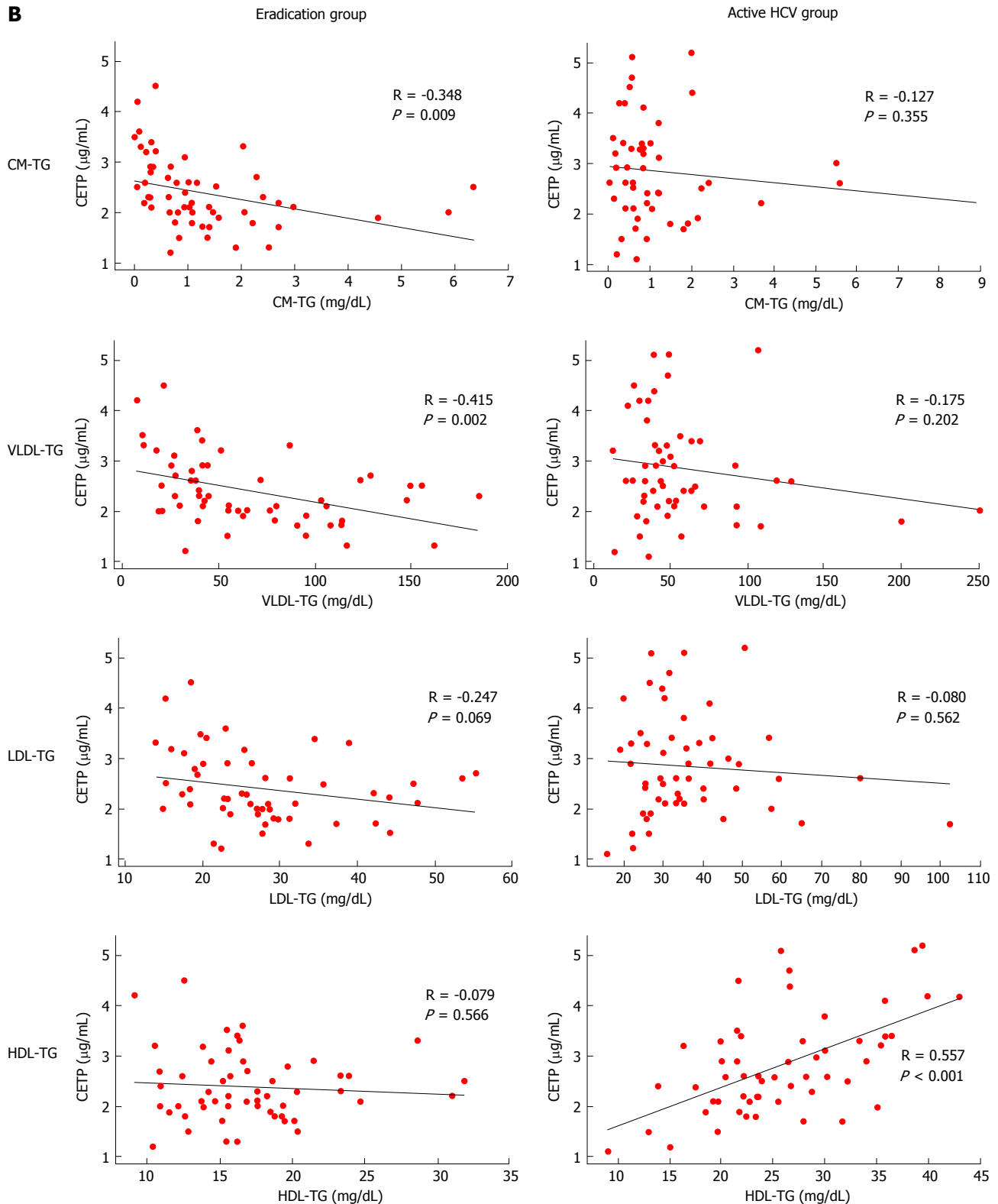
Although we did not evaluate the activity of CETP, it has generally been accepted that the serum CETP concentration reflects CETP activity<sup>[18]</sup>. Thus, an increase in the CETP level in patients with active HCV infection may indicate enhanced CETP activity.

CETP activity is regulated by the amount of dietary cholesterol<sup>[19,20]</sup> and hypertriglyceridemia<sup>[21]</sup>. In addition, CETP activity is dependent on genetic variations in the CETP gene<sup>[22-24]</sup>. However, the effect of HCV infection on the activity of CETP has not been investigated previously. Our results indicate that active HCV infection may promote CETP activity. This enhanced CETP activity may play a role in lipoprotein metabolism abnormalities in patients with active HCV infection<sup>[22-24]</sup>.

An unexpected finding in our study was a positive correlation between the serum CETP level and HDL-C, although the correlation was relatively weak and did not reach the level of significance in the eradication group. One of the major functions of CETP is the removal of CE from HDL. In fact, CETP inhibitors substantially increase HDL-C levels and moderately decrease LDL-C levels in humans<sup>[25]</sup>. There may be a weak negative correlation between CETP and HDL-C in patients with type 2 DM<sup>[26]</sup>. However, the correlation between serum level of HDL-C and CETP in a healthy population has not been observed<sup>[27]</sup>. Therefore, the HDL-C level is not simply determined by the function of CETP because the serum

**A**



**B**

**Figure 2** Correlation between the cholesteryl ester transfer protein level and triglyceride concentration in the four major lipoprotein classes in the hepatitis C virus eradication group and the active hepatitis C virus infection group. A: The CETP level had a weak correlation with HDL cholesterol in the active HCV infection group ( $R = 0.324$ ,  $P = 0.015$ ) and a weak, inverse correlation with chylomicron-cholesterol ( $R = -0.290$ ,  $P = 0.031$ ); B: The CETP level had a strong correlation with HDL-TG in the active HCV infection group ( $R = 0.557$ ,  $P < 0.001$ ). However, significant correlations with TG for other lipoprotein classes were not detected in the active HCV group. HCV: Hepatitis C virus; CM: Chylomicron; VLDL: Very-low-density lipoprotein; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TG: Triglyceride.



level of HDL-C may be dynamically controlled by the balance between HDL synthesis and catabolism, which is not mediated by CETP.

Although there is a consensus that a decrease in serum TC<sup>[28]</sup> and LDL-C<sup>[10]</sup> is a feature of HCV infection, little is known about TG abnormalities. A previous study reported that chronic hepatitis C patients had a lower serum VLDL-TG/non-VLDL-TG ratio<sup>[11]</sup>. Moreover, there was a reported increase in the TG concentration in HDL and LDL in patients with active HCV infection, although the total serum TG level was similar in the active HCV and eradication groups<sup>[29]</sup>.

In a correlation study between CETP and TG concentration in four lipoprotein classes, we found positive and strong correlations between CETP and HDL-TG in the active HCV group. However, this correlation was not found in the eradication group. Conversely, CETP was correlated with CM-TG and VLDL-TG in the eradication group, but was not significantly correlated with CM-TG or VLDL-TG in the active HCV group. These findings indicate that the significance of CETP in the regulation of TG concentration differs according to the HCV infection status. The most striking difference was found in HDL-TG.

The major source of serum TG is a TG-enriched VLDL that is secreted from the liver. TG in VLDL or LDL is transferred to HDL by the action of CETP. In active HCV infection, an increased serum CETP may enhance the transport of TG to HDL. Therefore, the increase in CETP and the strong positive correlation between CETP and HDL-TG in active HCV infection may indicate that HDL has abundant TG, promptly transferred from VLDL, but is not effectively catabolized and eliminated from the serum. Accordingly, as the major metabolic pathway that degrades and eliminates TG in HDL is mediated by hepatic lipase (HL)<sup>[30]</sup>, we speculate that HL activity in active HCV infection is impaired. Our hypothesis of reduced HL activity in active HCV infection is concordant with a previous result that the HL messenger RNA level is lower in the liver of patients with chronic hepatitis C than in the liver of patients with other etiologies and similar disease progression<sup>[31]</sup>. As a consequence of the abnormal retention of TG in HDL, the multifaceted functions of HDL on atherosclerosis<sup>[32]</sup> may be affected, and this could contribute to the progression of atherosclerosis in patients with active HCV infection<sup>[33]</sup>. Furthermore, dyslipidemia, which is caused by high serum CETP activity in active HCV infection, may contribute to intravascular lipoviral particle formation and thus for sustaining HCV infection<sup>[34,35]</sup>.

Our study had some limitations. It included a relatively small sample size, and the degree of atherosclerosis was not determined in the enrolled patients. In addition, we did not examine lipoviral particle and non-lipoviral particle viral load in the active HCV patients. To strengthen our hypothesis that an increase of TG in HDL contributes to atherosclerosis, further large-scale studies including the evaluation of the anti-inflammatory<sup>[36,37]</sup> and proinflammatory<sup>[38]</sup> functions of HDL and measurement

of the degree of atherosclerosis are warranted.

In summary, HCV infection was an independent factor contributing to the increase in serum CETP. The increase in CETP resulted in abnormal retention of TG in HDL. These findings suggest that CETP is one of the factors that contribute to abnormal lipoprotein metabolism in patients with active HCV infection.

## COMMENTS

### Background

Hepatitis C virus (HCV) is a unique virus; its use of host lipid metabolism results in a persistent infection. It is important to understand how HCV uses host lipid metabolism and how host lipid metabolism is affected by HCV infection, because HCV infection represents a unique model in which the virus causes chronic infection while coexisting with the host by taking over the host's metabolism.

### Research frontiers

The effect of HCV infection on the activity of cholesteryl ester transfer protein (CETP) has not been investigated previously. The authors have been the first to clarify that CETP may be increased with HCV.

### Innovations and breakthroughs

The authors confirmed that CETP plays a role in abnormal lipoprotein metabolism in patients with HCV infection.

### Applications

An increase of triglyceride (TG) in high-density lipoprotein (HDL) as a consequence of activated CETP may contribute to progression of atherosclerosis in HCV infection. Furthermore, disturbed lipoprotein metabolism induced by activated CETP may contribute to intravascular formation of HCV lipoviral particles.

### Terminology

CETP is a glycoprotein mediating the exchange of cholesteryl ester in HDL for TG in other lipoproteins.

### Peer-review

In this study, the authors found that HCV infection was an independent factor contributing to the increase in serum CETP, the increase in CETP resulted in abnormal retention of TG in HDL. These findings suggest that CETP is one of the factors that contribute to abnormal lipoprotein metabolism in patients with active HCV infection. This study has scientific basis and is interesting.

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**P- Reviewer:** Felmlee DJ, Wang Y, Zeng Z **S- Editor:** Song XX

**L- Editor:** A **E- Editor:** Liu SQ



Prospective Study

# Blood DNA methylation markers in prospectively identified hepatocellular carcinoma cases and controls from Taiwan

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**Supported by** National Institutes of Health grants, RO1ES005116 (Santella RM) and P30ES009089 (Santella RM).

**Institutional review board statement:** The study was reviewed and approved by the Columbia University Medical Center Institutional Review Board.

**Informed consent statement:** All study participants provided informed written consent prior to study enrollment.

**Conflict-of-interest statement:** There are no conflicts for all authors.

**Data sharing statement:** Detailed data is available from the corresponding author.

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**Received:** March 14, 2015  
**Peer-review started:** March 16, 2015  
**First decision:** April 10, 2015  
**Revised:** January 8, 2016  
**Accepted:** January 21, 2016  
**Article in press:** January 22, 2016  
**Published online:** February 18, 2016

## Abstract

**AIM:** To determine if gene-specific DNA methylation in prospectively collected blood samples is associated with later development of hepatocellular carcinoma (HCC).

**METHODS:** Comparing genome-wide DNA methylation profiles using Illumina Human methylation 450K arrays, we previously identified a list of loci that were differentially methylated between tumor and adjacent nontumor tissues. To examine if dysregulation of DNA



methylation patterns observed in tumor tissues can be detected in white blood cell (WBC) DNA, we conducted a prospective case-control study nested within a community-based cancer screening cohort in Taiwan with 16 years of follow up. We measured methylation levels in ninety-six loci that were aberrant in DNA methylation in HCC tumor tissues compared to adjacent tissues. Baseline WBC DNA from 159 HCC cases and 312 matched controls were bisulfite treated and assayed by Illumina BeadArray. We used the  $\chi^2$  test for categorical variables and student's *t*-test for continuous variables to assess the difference in selected characteristics between cases and controls. To estimate associations with HCC risk, we used conditional logistic regression models stratified on the matching factors to calculate odds ratios (OR) and 95%CI.

**RESULTS:** We found that high methylation level in cg10272601 in *WNK2* was associated with increased risk of HCC, with an OR of 1.91 (95%CI: 1.27-2.86). High methylation levels in both cg12680131 in *TPO* and cg22511877 in *MYT1L*, however, were associated with decreased risk. The ORs (95%CI) were 0.59 (0.39-0.87) and 0.50 (0.33-0.77), respectively, for those with methylation levels of cg12680131 and cg22511877 above the median compared with those with levels below the median. These associations were still statistically significant in multivariable conditional logistic regression models after adjusting for hepatitis B virus infection and alcohol consumption.

**CONCLUSION:** These findings support the measurement of methylation markers in WBC DNA as biomarkers of HCC susceptibility but should be replicated in additional prospective studies.

**Key words:** DNA methylation; Epigenetics; Hepatitis B virus; Hepatocellular carcinoma; White blood cell DNA

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**Core tip:** Hepatocellular carcinoma (HCC) is a highly fatal disease thus, the identification of biomarkers that could predict risk for development could enhance screening/early detection and prognosis. DNA methylation alterations are well established in HCC but whether changes in DNA methylation in white blood cells (WBC) are associated with increased risk of developing HCC is unknown. Taking advantage of a cancer screening program in Taiwan, we measured baseline WBC DNA methylation in prospectively collected blood samples at 96 CpG sites that were identified as differentially methylated in HCC tumors compared to adjacent tissues. Three were significantly associated with later development of HCC suggesting potential utility as a marker of risk.

Wu HC, Shen J, Yang HI, Tsai WY, Chen CJ, Santella RM. Blood DNA methylation markers in prospectively identified hepatocellular carcinoma cases and controls from Taiwan. *World*

*J Hepatol* 2016; 8(5): 301-306 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v8/i5/301.htm> DOI: <http://dx.doi.org/10.4254/wjh.v8.i5.301>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is among the most common cancers around the world<sup>[1]</sup>. Hepatitis B and C virus infection are the most important risk factors of HCC<sup>[2-4]</sup>. More recent studies have also identified the importance of exposure to alcohol, dietary aflatoxins and cigarette smoke<sup>[5-7]</sup>.

The mechanisms of liver cancer induction are now known to include mutations in specific genes and epigenetic alterations such as changes in DNA methylation and microRNA expression. These changes lead to changes in expression of oncogenes and tumor suppressor genes<sup>[8-10]</sup>. DNA hypermethylation can silence tumor suppressor genes while hypomethylation can activate oncogenes<sup>[11,12]</sup>. Using Illumina HumanMethylation 27K and 450K BeadChips, we previously reported a distinct DNA methylation pattern between HCC tumor and paired adjacent nontumor tissues (NCBI's GEO database accession numbers GSE54503 and GSE37988)<sup>[13,14]</sup>. In one of the studies, we found 28017 CpG sites hypermethylated and 102495 hypomethylated in tumor tissues compared with paired adjacent tissues<sup>[14]</sup>, suggesting their role in HCC tumorigenesis.

Using data on baseline white blood cell (WBC) DNA banked up to 16 years before diagnosis, we recently reported that global hypomethylation of Sat2, a repetitive element, was associated with increased HCC risk<sup>[15]</sup> and was also associated with high AFB<sub>1</sub> exposure<sup>[16]</sup>. These results suggest that decreased overall DNA methylation in WBC DNA can be used as a biomarker for HCC risk.

The main aim of this study was to examine whether the dysregulation of DNA methylation markers observed in tumor tissues can be detected in WBC DNA. We measured methylation levels in ninety-six loci in WBC DNA from 159 HCCs who developed cancer after enrollment in a community-based cancer screening program in Taiwan<sup>[5,6,15]</sup> and compared them with 312 controls who remained cancer free in the same cohort.

## MATERIALS AND METHODS

### Study population

This study included individuals who participated in a Cancer Screening Program cohort in Taiwan. This study was approved by both the Institutional Review Board of Columbia University and the Research Ethics Committee of the College of Public Health at National Taiwan University. We obtained written informed consent from all study subjects in this study.

Detail information regarding the cohort description and screening procedure and follow-up was provided in previous publications<sup>[5,6,15,16]</sup>. Between July 1990 and June 1992, 12020 males and 11924 females aged from

**Table 1 Sociodemographic characteristics of hepatocellular carcinoma cases and matched controls**

Variable	Cases <i>n</i> = 159	%	Controls <i>n</i> = 312	%	<i>P</i>
Age (yr, mean, SD)	52.8 (8.0)		53.1 (7.8)		0.72
BMI (mean, SD)	24.3 (3.6)		24.8 (3.7)		0.13
Gender					
Female	77	48	148	47	0.92
Male	82	52	164	53	
HBsAg					
Negative	65	41	238	76	< 0.0001
Positive	93	59	72	23	
Missing	1	< 1	2	< 1	
Anti-HCV					
Negative	109	69	243	78	< 0.0001
Positive	29	18	15	5	
Missing	21	13	54	17	
Smoking					
Never	97	61	184	59	0.67
Ever	62	39	128	41	
Alcohol					
Never	130	82	276	89	0.046
Ever	29	18	36	12	

HBsAg: Hepatitis B virus surface antigen; BMI: Body mass index; HCV: Hepatitis C virus.

30 to 65 years old and who lived in seven towns in Taiwan were enrolled in this study. Each participant filled out a structured questionnaire to collect information including demographic characteristics, history of alcohol intake and cigarette smoking, history of chronic disease and family history of cancers, including HCC. Each participant also donated a fasting blood sample during the time of recruitment.

In this study, we used blood collected from 159 participants who were diagnosed with HCC during the interval between their blood draw and June 2008. We also used blood from 312 controls who remained cancer free in the same cohort. Controls were selected by matching to each case by age (within 5 years), sex, residential area and time of recruitment (within 3 mo). Baseline WBCs were shipped to Columbia University on dry ice for DNA isolation and DNA methylation measurement.

#### DNA bisulfite conversion

We extracted genomic DNA from WBC using a salting out procedure. We bisulfite-treated an aliquot of DNA (500 ng) with EZ DNA methylation kits (Zymo Research, Orange, CA). The bisulfite DNA was resuspended in 20  $\mu$ L of distilled water and stored at -20 °C until use.

#### Loci selection and methylation measurement

We selected 96 CpG sites that previously had shown either hyper- or hypomethylation in HCC tumor compared to paired adjacent nontumor tissues in our 450k array data<sup>[14]</sup>. We selected our target CpG sites from among the top 250 most hyper or hypomethylated sites. Our selection of targets was based on the following criteria: (1) the largest methylation differences

between tumor and adjacent tissues; (2) half of the CpG sites showing hypomethylation and half hypermethylation; and (3) one site per gene. Due to the inability to design primers for some sites, we have 65 CpG sites with hypermethylation and 31 CpG sites with hypomethylation. DNA methylation analysis was measured using an Illumina GoldenGate assay with BeadArray technology. The arrays were customized to measure methylation covering the CpG sites identified in the 450k array. DNA methylation values were scored as  $\beta$ -values which ranges between 0 and 1.

#### Statistical analysis

We used the  $\chi^2$  test and/or student's *t*-test to assess the difference in selected variables between cases and controls. To estimate associations between methylation markers and HCC risk, we used a conditional logistic regression model using PROC PHREG procedure. Subjects were divided into different methylation groups: Those with methylation levels above the median value for all controls sample vs those below the median. In the multivariable model, we modeled the associations of methylation in cg10272601 in *WNK2*, cg12680131 in *TPO* and cg22511877 in *MYT1L* adjusting for, hepatitis B virus surface antigen (HBsAg) (Yes vs No), and history of alcohol intake (Ever vs Never) in the model. All analyses were performed with SAS software 9.2 (SAS Institute, Cary, NC).

## RESULTS

The distributions of subjects' characteristics at baseline for cases and matched controls is given in Table 1. The distributions of matching factors including age, sex were similar between cases and controls. There were 51.7% and 52.5% males in cases and controls, respectively. The distribution of smoking was also similar, while the percentage of ever alcohol consumption was slightly lower in controls (11.5%) than in cases (18.2%). The percents positive for HBsAg and anti-HCV were higher in cases than in matched controls [58.5% vs 23.1% for HBsAg (+) and 18.2% vs 4.8% for anti-HCV (+)].

Table 2 presents the distributions of the 96 methylation markers by HCC status. The mean values of methylation vary by methylation markers. Fifty DNA methylation markers had mean methylation values below 10% in cases and controls. Nineteen DNA methylation markers had mean methylation values above 90%. About 27 DNA methylation markers had mean methylation levels between 10% and 90%. The mean levels of three DNA methylation markers were statistically significantly different between cases and controls, including cg10272601, cg12680131, and cg22511877. The mean methylation beta values for cg1027261 were  $0.30 \pm 0.07$  for cases and  $0.28 \pm 0.08$  for controls ( $P = 0.04$ ). Values for cg12680131 were  $0.80 \pm 0.09$  and  $0.82 \pm 0.11$  for cases and controls, respectively ( $P = 0.02$ ) and for cg22511877,  $0.56 \pm 0.17$  for cases and  $0.60 \pm 0.16$  for controls ( $P = 0.01$ ).

**Table 2** Distribution of DNA methylation by hepatocellular carcinoma status

Locus	Gene	HCC cases		Controls		<i>P</i> <sup>1</sup>
		Mean	SD	Mean	SD	
cg00028598	GABRA5	0.92	0.04	0.92	0.07	0.81
cg00108164	ACP1	0.01	0.02	0.00	0.01	0.55
cg00249511	SCT	0.01	0.04	0.01	0.04	0.80
cg00753478	LDHB	0.09	0.08	0.08	0.06	0.12
cg00817367	GRASP	0.01	0.04	0.01	0.01	0.23
cg00939495	DRD5	0.22	0.10	0.22	0.12	0.95
cg01530024	STK32B	0.97	0.08	0.97	0.07	0.79
cg01566592	RIMS2	0.10	0.09	0.09	0.08	0.32
cg01860297	BASP1	0.96	0.03	0.95	0.08	0.49
cg02527669	OBSL1	0.02	0.02	0.03	0.05	0.53
cg02553663	SECTM1	0.03	0.04	0.03	0.03	0.65
cg02710296	C1orf14	0.33	0.11	0.33	0.11	0.92
cg02736548	FAM109B	0.08	0.09	0.08	0.09	0.46
cg03306486	APC2	0.02	0.02	0.01	0.02	0.44
cg03396005	APCDD1	0.92	0.04	0.92	0.06	0.99
cg03621881	BRUNOL6	0.04	0.05	0.03	0.04	0.70
cg04920951	GSTP1	0.01	0.07	0.00	0.02	0.22
cg05328339	PTPRN2	0.89	0.09	0.88	0.10	0.55
cg05661282	ZNF154	0.03	0.05	0.03	0.08	0.75
cg05699035	KCNK2	0.86	0.07	0.86	0.08	0.99
cg05833351	CUGBP2	0.95	0.07	0.95	0.08	0.70
cg05970721	HS3ST2	0.90	0.10	0.91	0.10	0.49
cg06382344	TBR1	0.02	0.03	0.03	0.05	0.13
cg06445348	ILDR2	0.02	0.06	0.01	0.01	0.24
cg06641285	TIMP2	0.02	0.02	0.02	0.05	0.74
cg07061738	SMOC2	0.94	0.08	0.94	0.11	0.75
cg07689503	MTHFD2	0.00	0.00	0.00	0.01	0.27
cg07759394	GLB1L2	0.01	0.03	0.01	0.02	0.44
cg07765706	KCNQ3	0.95	0.03	0.95	0.08	0.12
cg08328777	DUOX1	0.07	0.05	0.07	0.06	0.30
cg08714590	FZD1	0.86	0.12	0.86	0.12	0.43
cg08738570	C1orf70	0.09	0.10	0.09	0.08	0.69
cg09210956	SNTG2	0.67	0.09	0.67	0.12	0.93
cg09433131	KCNB2	0.94	0.06	0.93	0.10	0.43
cg09489445	ZNF788	0.01	0.03	0.01	0.04	0.92
cg09901035	PLEKHG4B	0.87	0.06	0.87	0.08	0.66
cg10272601	WNK2	0.30	0.07	0.28	0.08	0.04
cg10342963	IGFIR	0.81	0.13	0.79	0.15	0.07
cg11349423	OPCML	0.48	0.15	0.48	0.16	0.93
cg1137136	PKDREJ	0.03	0.03	0.03	0.03	0.69
cg11686528	ABR	0.01	0.07	0.01	0.06	0.60
cg12296772	MTMR7	0.07	0.06	0.07	0.07	0.78
cg12610564	SLC39A12	0.98	0.01	0.97	0.07	0.09
cg12680131	TPO	0.80	0.09	0.82	0.11	0.02
cg12852139	MYO10	0.96	0.02	0.95	0.06	0.70
cg13204512	RNF135	0.01	0.06	0.01	0.02	0.23
cg13517866	SMOC2	0.89	0.11	0.89	0.10	0.58
cg13564825	PPP1R14A	0.01	0.05	0.01	0.02	0.98
cg13604246	ANKMY1	0.11	0.08	0.11	0.09	0.68
cg13611121	COL5A1	0.80	0.08	0.80	0.10	0.73
cg13782274	KCNQ2	0.94	0.08	0.93	0.11	0.39
cg13791254	FOXE1	0.02	0.02	0.01	0.03	0.62
cg13879483	USP44	0.08	0.06	0.07	0.07	0.34
cg13895235	PRKAR1B	0.01	0.01	0.01	0.03	0.39
cg14183206	HLA-L	0.24	0.10	0.23	0.09	0.61
cg14486338	KCN52	0.12	0.07	0.12	0.07	0.53
cg14644001	PRRT1	0.04	0.03	0.04	0.05	0.63
cg14645545	SLC11A1	0.20	0.12	0.19	0.12	0.83
cg14715697	HRNBP3	0.70	0.08	0.71	0.08	0.20
cg14866200	SHISA3	0.02	0.07	0.02	0.06	0.74
cg14988503	CDKL2	0.02	0.03	0.02	0.03	0.85
cg15092343	MSX1	0.07	0.05	0.07	0.04	0.48
cg15167871	TCERG1L	0.92	0.10	0.92	0.11	0.98
cg15549700	AJAP1	0.96	0.05	0.96	0.08	0.53
cg15760257	SARM1	0.01	0.01	0.01	0.05	0.35

cg17264670	RGS17	0.08	0.06	0.08	0.08	0.94
cg17497608	FZD1	0.83	0.11	0.84	0.12	0.43
cg17725364	COL6A3	0.96	0.10	0.96	0.09	0.86
cg18537730	IZUMO1	0.16	0.07	0.16	0.08	0.63
cg19429281	ZNF702P	0.02	0.01	0.02	0.03	0.40
cg19464917	ISL2	0.06	0.04	0.05	0.03	0.17
cg20129213	RIMS2	0.01	0.05	0.01	0.05	0.47
cg20399616	BCAT1	0.05	0.08	0.04	0.08	0.40
cg21385746	LOC150568	0.96	0.10	0.95	0.11	0.80
cg21472506	OTX1	0.01	0.04	0.01	0.04	0.98
cg21790626	ZNF154	0.04	0.04	0.05	0.05	0.32
cg22403469	RIMBP2	0.83	0.05	0.83	0.08	0.63
cg22511877	MYT1L	0.56	0.17	0.60	0.16	0.01
cg22524061	OSR2	0.23	0.09	0.22	0.09	0.48
cg22655988	CRMP1	0.96	0.08	0.96	0.10	0.77
cg22789900	MIXL1	0.00	0.01	0.01	0.04	0.55
cg23004031	MGMT	0.55	0.31	0.58	0.32	0.41
cg23391785	DNM3	0.02	0.06	0.01	0.04	0.28
cg23498518	POM121L12	0.79	0.07	0.80	0.10	0.36
cg23864180	ADARB2	0.90	0.06	0.91	0.07	0.26
cg24274117	C20orf195	0.03	0.07	0.04	0.07	0.52
cg24425838	C2CD4D	0.05	0.08	0.05	0.07	0.98
cg24432073	CDKL2	0.02	0.03	0.02	0.04	0.84
cg24563094	FAM59B	0.10	0.04	0.10	0.05	0.55
cg24602704	ATP10A	0.97	0.02	0.97	0.07	0.46
cg24816460	CDYL	0.03	0.07	0.03	0.07	0.51
cg25480336	ZFP64	0.01	0.02	0.01	0.01	0.16
cg25577023	AMN	0.09	0.09	0.09	0.09	0.82
cg25622366	OTX1	0.02	0.07	0.02	0.05	0.66
cg26010734	EPHX3	0.05	0.05	0.05	0.04	0.43
cg26841013	WNT3A	0.03	0.02	0.03	0.03	0.45

<sup>1</sup>*P* value for student's *t*-test.**Table 3** White blood cell DNA methylation and hepatocellular carcinoma risk

Locus		Cases/ controls	OR (95%CI)
WNK2 cg10272601	Below median (< 0.279)	56/157	1.0
	Above median (≥ 0.279)	103/155	1.91 (1.27-2.86)
TPO cg12680131	Below median (< 0.836)	102/157	1.0
	Above median (≥ 0.836)	57/155	0.59 (0.39-0.87)
MYT1L cg22511877	Below median (< 0.636)	105/159	1.0
	Above median (≥ 0.636)	54/153	0.50 (0.33-0.77)

The association between DNA methylation of cg10272601, cg12680131, and cg22511877 and HCC are given in Table 3. The OR for those with cg10272601 methylation above the median was 1.91 (95%CI: 1.27-2.86). Individuals with a cg12680131 methylation level above the median had lower risk of HCC, with an OR of 0.59 (95%CI: 0.39-0.87). The OR was 0.50 (95%CI: 0.33-0.77) for those with cg22511877 methylation above median.

Table 4 shows the multiple variables conditional logistic regression model. Overall, HBsAg (+) was associated with increased HCC risk (OR = 5.50, 95%CI: 3.34-9.03) compared with HBsAg(-). Ever smokers had a 2.1-fold increased risk of developing HCC (OR = 2.10, 95%CI: 1.08-4.07). The ORs (95%CI) were 2.26 (1.42-3.61), 0.55 (0.34-0.87), and 0.53 (0.32-0.88) for cg10272601, cg12680131, and cg22511877 hypermethylation.



**Table 4 Multiple variables model for DNA methylation and hepatocellular carcinoma risk**

Variable	OR (95%CI)	P
<i>WNK2</i> cg10272601 <sup>1</sup>	2.26 (1.42-3.61)	0.0006
<i>TPO</i> cg12680131 <sup>2</sup>	0.55 (0.34-0.87)	0.01
<i>MYT1L</i> cg22511877 <sup>3</sup>	0.53 (0.32-0.88)	0.01
HBsAg (positive <i>vs</i> negative)	5.50 (3.34-9.03)	< 0.0001
Alcohol (yes <i>vs</i> no)	2.10 (1.08-4.07)	0.03

<sup>1</sup>Above or below the median of 0.279; <sup>2</sup>Above or below the median of 0.836;

<sup>3</sup>Above or below the median of 0.636. HBsAg: Hepatitis B virus surface antigen.

## DISCUSSION

Alterations in methylation of cg10272601, cg12680131, and cg22511877 were associated with risk for later HCC development. Consistent with our tissue data, we found that a high methylation level in cg10272601 was associated with increased risk of HCC, while high methylation levels in both cg12680131 and cg22511877 were associated with decreased risk. In the 450k data, the mean beta values were  $0.52 \pm 0.22$  for cg10272601,  $0.28 \pm 0.21$  for cg12680131, and  $0.34 \pm 0.26$  for cg22511877 in HCC tumors<sup>[14]</sup>. The corresponding beta values were  $0.10 \pm 0.06$ ,  $0.79 \pm 0.08$ ,  $0.87 \pm 0.05$ , respectively, in adjacent nontumor tissues.

cg10272601 is located at transcription start site (TSS) 200 of *WNK2*, a gene encoding a serine-threonine kinase on chromosome 9q22.31<sup>[17]</sup>. *WNK2* acts as a tumor suppressor gene by suppressing the ERK/MAPK-pathway and downstream cell cycle progression<sup>[18]</sup> and *WNK2* expression inhibited colony formation<sup>[19]</sup>, suggesting a role in cell growth suppression. Dense high methylation at the CpG island was associated with decreased *WNK2* expression<sup>[19]</sup>. Hypermethylation of *WNK2* was reported in many cancers, including pancreatic ductal adenocarcinoma<sup>[20]</sup>, HCC<sup>[14,21]</sup>, and gliomas<sup>[22]</sup>.

cg12680131 is located on chromosome 2p25 at TSS 200 of thyroid peroxidase (*TPO*), a key enzyme in thyroid hormone synthesis. Mutations in *TPO* are associated with several disorders of thyroid hormonogenesis<sup>[23]</sup>. The association of methylation and expression of *TPO* has not been studied and the role of *TPO* in carcinogenesis has not been reported. cg22511877 is located at a shore region of myelin transcription factor 1-like (*MYT1L*) also on chromosome 2p25. *MYT1L* is a main member of the MYT/NZF family of transcription factors<sup>[24,25]</sup>. Limited data suggests a polymorphism in *MYT1L* is associated with gastric cancer outcome in a Chinese population<sup>[26]</sup>. Future studies are needed to understand the mechanisms of hypomethylation of both *TPO* and *MYT1L* in hepatocarcinogenesis.

The main limitation of this study is that we did not adjust for multiple comparisons due to the limited sample size. However, in further data analysis, we also observed significant associations of methylation in these 3 CpG sites with HCC risk after adjusting for HBV infection and alcohol consumption, suggesting an independent effect

in HCC risk.

This study, using prospective study design, allowed us to produce causal evidence on DNA methylation in WBC and cancer susceptibility<sup>[27]</sup>. Using information from HCC tumor tissues, our study investigated the associations of HCC-specific differentially methylated loci observed in tumor tissues in WBC DNA with HCC risk.

In summary, we provide new evidence that specific loci methylation in WBC DNA is associated with increased HCC susceptibility. These finding could lead to development of a simple non-invasive blood measure of DNA methylation to identify people at high risk of HCC.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is a highly devastating disease with a poor prognosis. Thus, methods that allow the identification of individuals at elevated risk of HCC should greatly enhance screening for early diagnosis and improve prognosis. While several risk factors are well known such as infection with hepatitis B or C virus, not all viral-infected individuals develop cancer. Additional biomarkers of risk are therefore needed.

### Research frontiers

It is known that tumors release DNA into the blood stream and that this DNA contains the same DNA alterations both mutations and changes in DNA methylation that are found in the tumor. Thus, researchers have been able to develop assays for tumor DNA in plasma/serum for early diagnosis. There is also limited data in some cancers, not HCC, that DNA methylation changes in blood cells differs between cases and controls.

### Innovations and breakthroughs

This study is the first to investigate whether DNA methylation in specific genes in white blood cells is predictive of later HCC development.

### Applications

While the study needs confirmation in another population, it suggests that it may be possible to develop risk prediction models that include white blood cell DNA methylation markers.

### Peer-review

This is a very interesting paper. The authors found the correlation between DNA methylation and HCC occurring. The results provide sufficient experimental evidence or data to draw firm scientific conclusions.

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**P- Reviewer:** Celikbilek M, Dang SS, Luo GH, Morales-Gonzalez J, Romero MR

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