Hepatitis C virus may infect extrahepatic tissues in patients with hepatitis C

Fu Ming Yan[1], An Shen Chen[1], Fei Hao[2], Xi Ping Zhao[3], Chang Hai Gu[2], Li Bin Zhao[1], Dong Liang Yang[3] and Lian Jie Hao[3]

Abstract

AIM   **To explore the status of extrahepatic hepatitis C virus (HCV) infection and replication in hepatitis C patients, and its potential implication in HCV infection and pathogenicity.**
METHODS   **By reverse-transcriptase *polymerase chain reacti on (RT-PCR), *in situ* hybridization (ISH) and immunohistochemistry, HCV RNA, HCV replicative intermediate (minus-strand of HCV RNA), and HCV antigens were detected in 38 autopsy extrahepatic tissue specimens (including 9 kidneys, 9 hearts, 9 pancreas, 5 intestines, 2 adrenal glands, 2 spleens, 1 lymph node, and 1 gallbladder) from 9 hepatitis C patients, respectively; and the status of HCV replication in extrahepatic tissues was studied.**
RESULTS   **By RT-PCR, all 9 patients were positive for HCV RNA in kidney,heart, pancreas, and intestine, but only 6 (66.7%) patients were positive for HCV replicative intermediate. HCV RNA and HCV antigens were detected in kidney, heart, pancreas, intestine, adrenal gland, lymph node, and gallbladder in 5 (55.6%) and 6 (66.7%) patients by ISH and immunohistochemistry, respectively. HCV RNA**
and HCV antigens were not detected in these extrahepatic organs in 3 (33.3%) patients, although their livers were positive for HCV. HCV replicative intermediate detected by RT-PCR was consistent with HCV RNA and HCV antigens detected by ISH and immunohistochemistry (*Kappa* = 0.42-0.75). HCV RNA and HCV antigens were detected in myocardial cells, epithelial cells of intestinal gladular, interstitial cells of kidney, epithelial cells of tubules and glomerulus, pancreas acinar cells and epithelial cells of pancreatic duct, epithelial cells of mucous membrane sinus of gallbladder, cortex and medulla cells in adrenal gland, and mononuclear cells in lymph node. HCV RNA was also detected in bile duct epithelial cells, sinusoidal cells, and mononuclear cells in liver tissues by ISH.
CONCLUSION   **HCV can infect extrahepatic tissues, and many various tissue cells may support HCV replication; extrahepatic HCV infection and replication may be of "concomitant state" in most of patients with hepatitis C. The infected extrahepatic tissues might act as a reservoir for HCV, and play a role in both HCV persistence and reactivation of infection. HCV as an etiologic agent replicating and expressing viral proteins in extrahepatic tissues itself contributes to extrahepatic syndrome associated-HCV infection in a few patients with chronic HCV infection.**

[1]Department of Infectious Diseases, the 161st Central Hospital of Chinese PLA, Wuhan 430010, Hubei Province, China
[2]Department of Infectious Diseases, Southwest Hospital, Third Military Medical University, Chongqing, China
[3]Division of Clinical Immunology, Tongji Hospital, Tongji Medical University, Wuhan, Hubei Province, China
Dr. Fu Ming Yan, graduated from Hubei Medical University in 1983 and got master degree from Third Military Medical University in 1997, now an associate chief physician, specialized research on the pantropism of HCV infection, having more than 10 papers published.
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Correspondence to: Dr. Fu Ming Yan, Department of Infectious Diseases, the 161st Central Hospital of Chinese PLA, Wuhan 430010, Hubei Province, China
Tel. 0086-27-82903100 Ext.2424
Email. ljhao@tjh.tjmu.edu.cn
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INTRODUCTION

Hepatitis C virus (HCV) was discovered in 1989[1], and is in a separate genus of the virus family *Flavividae*. Some aspects of cell tropism of *Flavividae*, such as the yellow fever virus and the dengue fever virus, have been clarified by *in situ* staining technique[2]. Different members of the *Flavividae* family infect a distinct, wide array of cells, resulting in multi-faceted disease expression. It should be noted that even a single amino acid change in the envelope molecule may modulate or even change the cell tropism and virulence of flaviviruses[3,4]. It may only be inferred that HCV may gain access to and replicate in extrahepatic tissues when compared with other flaviviruses. In

1985, the report of Hellings *et al* showed that HCV, which was previously known as the non-A, non-B hepatitis virus, was successfully transmitted via the infusion of PBMC (peripheral blood mononuclear cells) purified from the patients; and then, Nouri Aria *et al* reported that HCV RNA and HCV replicative intermediate (minus-strand of HCV RNA) were detected in the cytoplasm and nuclei of mononuclear and biliary epithelial cells in liver tissue by ISH. Other studies also indicated that HCV can transmit from mother to infant, possibly occurring in utero[5,6]. These results suggested that HCV might replicate in PBMC and nonhepatic cells. In recent years, studies in extrahepatic HCV infection focused on readily obtainable tissue and body fluid samples such as PBMC[7-10], saliva, semen, urine, ascites, and biliary juices[11,12]. Subsequently, HCV replication in cells of the hematopoietic lineage has been demon-strated[8-10,13]. The results showed that it might be of pathological significance in HCV infection[8,10,14,15]. Recently, HCV RNA and HCV replicative intermediate in lymph node, pancreas, adrenal gland, thyroid, spleen, ovary, and uterus were detected by RT-PCR[16,17], but there was lack of *in situ* detection of HCV RNA in these extrahepatic tissues.

During HCV infection, the viral burden and subsequent tissue damage were mainly confined to the liver[18-22]. However, several extrahepatic syndromes associated with HCV infection have been reported[23-26], which include mixed cryoglobulinaemia, glomerulonephritis, lymphoma, and other extrahepatic diseases such as porphyria cutanea tarda[27]. Up to now, little is known about the mechanisms and the role of HCV in the development of these extra hepatic syndromes; and the extrahepatic tissue cells that support viral replication have not been identified. Accordingly, we conducted reverse-transcriptase polymerase chain reaction (RT-PCR), *in situ* hybridization (ISH), and immunohistochemistry for HCV RNA, HCV replicative intermediate, and HCV antigens in several extrahepatic tissues from 9 hepatitis C patients in an attempt to explore the status of extrahepatic HCV infection and replication, and its potential implication in HCV infection and pathogenicity. We believe that the information so derived could lead to a better understanding of the mechanisms of both HCV persistence and reactivation of infection, and the role of HCV in the development of extrahepatic syndromes associated with HCV infection.

MATERIALS AND METHODS
Patients
Nine patients with severe viral hepatitis (1986-1994, diagnosed by autopsy, according to the Diagnostic Criteria of Viral Hepatitis Prevention and Cure Guideline, China'95), 8 male, 1 female, aged 25-48 years, Han nationality, were included in this study. All the patients were positive for HCV antigens and HCV RNA in livers, and their sera were positive for HBV markers. Thirty-eight paraffin-embedded autopsy extrahepatic tissue specimens (including 9 kidneys, 9 hearts, 9 pancreas, 5 intestines, 2 adrenal glands, 2 spleens, 1 lymph node, and 1 gallbladder) were provided by the Department of Pathology and Anatomy, Third Military Medical University, China.

Main reagents
Mouse monoclonal anti-HCV NS3, NS5 and CP10[28]. A digoxigenin-labeled cDNA antisense probe to the 5'-non-coding region of the HCV genome was 145bp in length (provided by the Division of Clinical Immunology, Tongji Medical University). HCV genome primers were derived from the highly conserved 5'-non-coding region of the HCV genome, and were synthesized by Gibco BRL; outer primers: sense 5'-ACTCCACCA TAGATCATCCC 3', antisense 5'-AACACTACTCGGCTAGCAGT-3'; inner primers: sense 5'-TTCACGCAGAAAGCGTCTAG-3', anti-sense 5'-GTTGATCC AAGAAAGGACCC-3'.

RT-PCR
RNA was extracted from 2 to 3 pieces of 5μm thick paraffin-embedded extrahepatic tissue sections (31 specimens, including 9 kidneys, 9 hearts, 9 pancreas, and 4 intestines) cut from the same pathological blocks as used for ISH and immunohistochemistry with TRIzol (Gibco BRL).

RT-PCR was performed as previously described[29,30]. The expected size of the amplified product was 145bp. The PCR products were analyzed by agarose gel electrophoresis. The sera which were negative for HCV RNA and HCV replicative intermediate and the distilled water were used as negative control.

Immunohistochemistry and ISH
Immunohistochemistry and ISH were performed as previously described[28,31]. Liver specimens-positive for HCV RNA and HCV antigens were used as positive control. Ten autopsy extrahepatic tissue specimens from viral hepatitis patients, whose livers were negative for HCV antigens and HCV RNA, and 10 autopsy extrahepatic tissue specimens from patients with rabies were used as negative control. Furthermore, control slides were treated with ribonucleases (RNase) and deoxyribonuclease (DNase) before hybridization; and the digoxigenin-labeled probe was substituted with non-labeled HCV cDNA probe, which was used as control in ISH as well.

RESULTS
Detection rates of HCV RNA and HCV antigen in

the tissues

By RT-PCR, all 9 (positive in 20 specimens, 64.5%) patients were positive for HCV RNA in kidney, heart, pancreas, and intestine, but only 6 (66.7%), (positive in 11 specimens, 35.5%) of 9 patients were positive for HCV replicative intermediate (Figure 1). By ISH and immunohistochemistry, positive staining for HCV RNA and HCV antigens in kidney, heart, pancreas, intestine, adrenal gland, lymph node, and gallbladder were found in 5 (55.6%), (positive in 11 specimens, 28.9%) of 9, and 6 (66.7%), (positive in 23 specimens, 60.5%) of 9 patients, respectively. Positive rates of HCV RNA and HCV antigens in single extrahepatic organ are shown in Table 1. Three (33.3%) of 9 patients were negative for HCV RNA and HCV antigens in these extrahepatic organs, although their livers were positive for HCV.

Table 1 Results of HCV RNA and HCV antigens detection in extrahepatic organs and livers

Tissue	Cases	HCV RNA (+)		HCV antigens (+)		
		RT-PCR	ISH	NS3	NS5	CP10
Kidney	9	7	4*	3	6	5	4
Heart	9	5	3*	2	5	4	4
Pancreas	9	5	3*	2	6	6	5
Intestine	5	3/4	1/4*	1	3	2	0
Adrenal gland	2			1	1	1	0
Spleen	2			0	0	0	0
Lymph node	1			1	1	1	1
Gallbladder	1			1	1	0	1
Liver	9	9	7*	9	8	7	7

Note: *Minus-strand RNA detected.

The positivity of minus-strand RNA detected by RT-PCR was consistent with that of HCV RNA and HCV antigens detected by ISH and immunohistochemistry (*Kappa* = 0.42-0.75).

Expression of HCV RNA and HCV antigens in the tissues

HCV antigen staining was only seen within the cytoplasm with homogenous, inclusive or submembranous distribution. The hybridization signal was observed in both cytoplasm and nuclei, with a greater proportion of cytoplasm signal.

HCV existed in these extrahepatic organs except 2 spleens. There were HCV RNA and HCV antigen positive expressions in myocardial cells, epithelial cells of intestinal gladular, interstitial cells of kidney, epithelial cells of tubules and glomerulus, pancreas acinar cells and epithelial cells of pancreatic duct, epithelial cells of mucous membrane sinus of gallbladder, cortex and medulla cells in adrenal gland, and mononuclear cells in lymph node (Figures 2-10). The amount of HCV positive cells in extrahepatic tissues was obviously less than that in liver tissues. In addition, there were some differences in HCV expression in various cells among different extrahepatic tissues, e.g. the amount of positive cells in heart tissue was less than that in kidney, pancreas, and intestine. In liver tissues, HCV antigens were only detected in hepatocytes, but hybridization signal of HCV RNA was seen in not only hepatocytes, but also bile duct epithelial cells, sinusoidal cells, and mononuclear cells (Figures 10 - 12). The amount of HCV positive cells in liver tissues was lower than 1%[28], but obviously higher than those in extrahepatic tissues.

Negative control

The samples (such as extrahepatic tissues, sera, and the distilled water) from the subjects of control groups were negative for HCV RNA and HCV antigens detected by RT-PCR, ISH, and immunohistochemistry, respectively. Using RNase treatment of the extrahepatic tissues before hybridization, the HCV RNA staining in these tissue cells faded. DNase treatment did not reduce HCV RNA signal in these tissue cells. Using non-labeled cDNA probe, HCV RNA hybridization signal was not observed in these tissues.
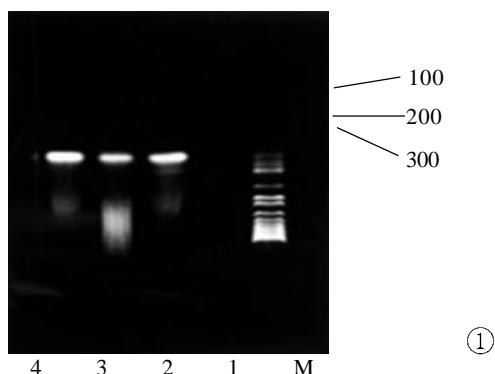

Figure 1 RT-PCR amplification result of minus-strand RNA in extrahepatic tissues.
        M: markers; 1: negative control; 2-4: amplification results of kidney, heart, and pancreas. The expected size of the amplification product is 145bp
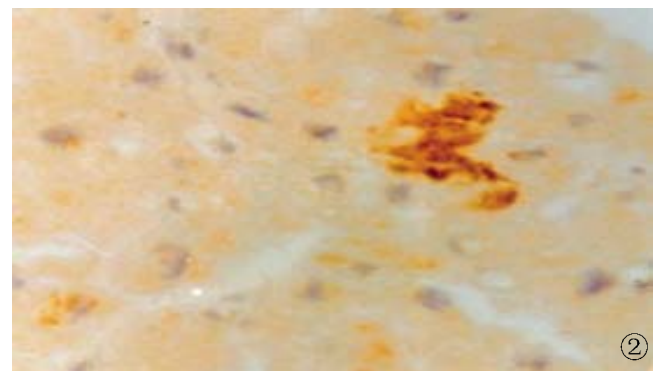**Figure 2** The expression of HCV NS3 in myocardial cells, showing brown yellow. S-P (DAB)×400
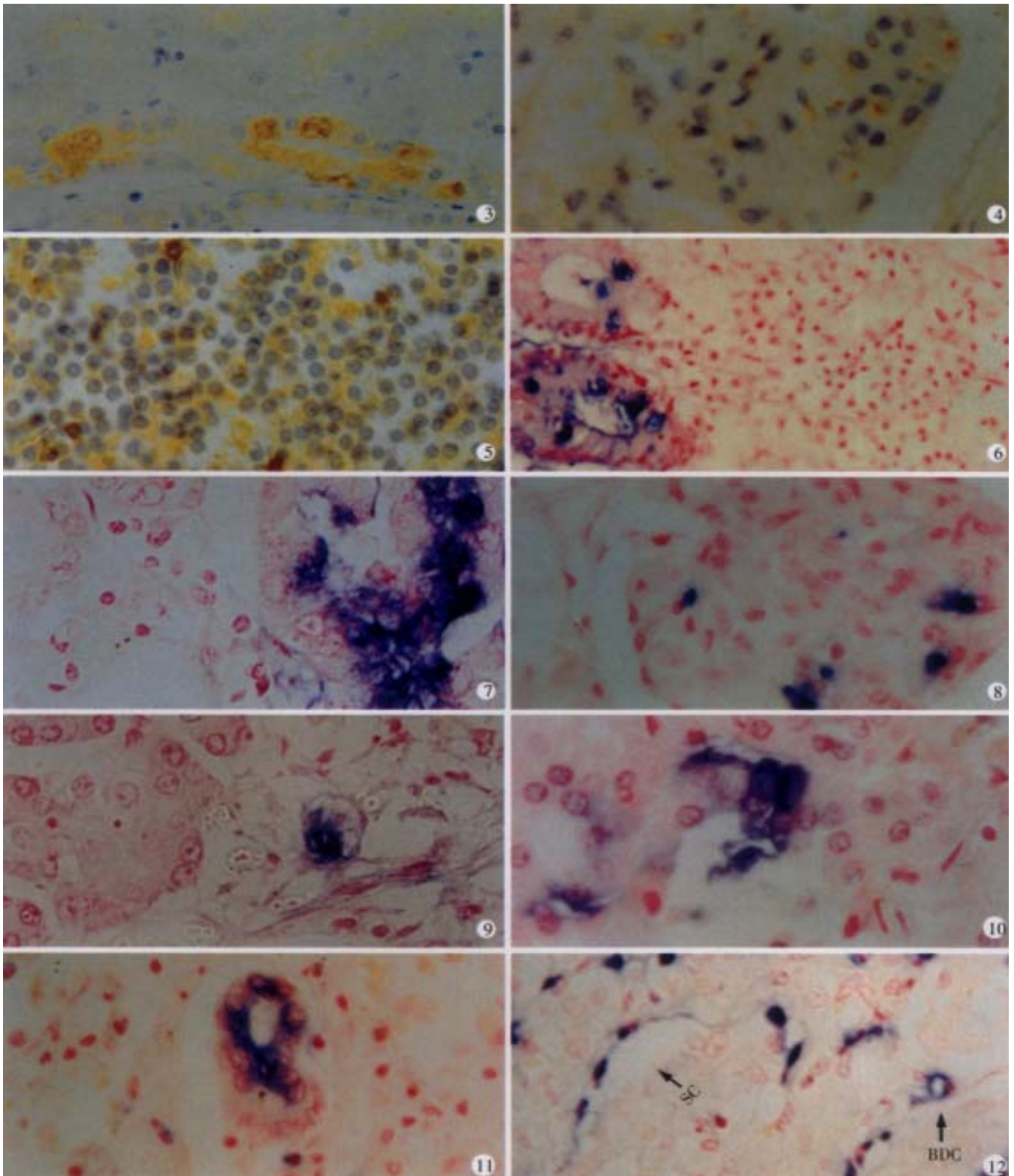
Figure 3   The expression of HCV NS5 in epithelial cells of tubules, showing brown yellow. S-P (DAB)×400
Figure 4   The expression of HCV NS5 in the glomerulus, showing brown yellow. S-P (DAB)×400
Figure 5   The expression of HCV NS3 in mononuclear cells in lymph node, showing brown yellow. S-P (DAB)×400
Figure 6   The expression of HCV RNA in epithelial cells of mucous membrane sinus of gallbladder, showing purple blue. ISH×200
Figure 7   The expression of HCV RNA in epithelial cells of intestinal gladular, showing purple blue. ISH×400
Figure 8   The expression of HCV RNA in the glomerulus, showing purple blue. ISH×400
Figure 9   The expression of HCV RNA in the pancreas acinar cells, showing purple blue. ISH×400
Figure 10   The expression of HCV RNA in cortex cells in adrenal gland, showing purple blue. ISH×400
Figure 11   The expression of HCV RNA in bile duct epithelial cells in liver, showing purple blue. ISH×400
Figure 12   The expression of HCV RNA in bile duct cells (BDC), sinusoidal cells (SC) in liver, showing purple blue. ISH×400

DISCUSSION

Extrahepatic HCV infection was found in hepatitis C patients, recently. But as RT-PCR is fraught with problems[32,33], contradictory data related to extrahepatic HCV infection have been reported[16,17,34-36], and there have been few *in situ* detection of HCV in extrahepatic tissues, the identification of extrahepatic HCV infection and replication has been controversial.

In this study, HCV RNA and HCV replicative intermediate in kidney, heart, pancreas, and intestine were detected by RT-PCR, but more importantly, the localization of HCV RNA and HCV antigens (NS3, NS5 and CP10) in kidney, heart, pancreas, intestine, adrenal gland, lymph node, and gallbladder was demonstrated convincingly by ISH and immunohistochemistry, and various tissue cell types harboring HCV such as pancreas acinar cells were also identified. Moreover, the positivity of HCV replicative intermediate detected by RT-PCR was consistent with that of HCV RNA and HCV antigens in these tissue cells detected by ISH and immunohistoc hemistry (*Kappa*=0.42-0.75). More recently, we used a digoxigenin-labeled HCV oligonucleotide sense probe and detected HCV replicative intermediate in the extrahepatic tissues-positive for HCV RNA and HCV antigens by ISH (data being summarized). These results showed that HCV could infect extrahepatic tissues, and various tissue cells might support viral replication. Recently, Loriot *et al*[37] reported that gallbladder epithelial cells from HCV-negative subjects were successfully infected by HCV *in vitro*, and ISH and immunohistochemical studies identified pancreas and gastric mucosa as the sites of HCV infection[38,39]. These reports support the results of our studies.

So far, there have been few reports on the status of extrahepatic HCV infection and replication in hepatitis C patients. In order to explore this issue, we compared with the status of HCV expression between extrahepatic tissue and liver, and also among different extrahepatic tissues, the results indicated that the detection rates of HCV in extrahepatic tissues were low[40], and the amount of HCV positive staining cells in extrahepatic tissues was also obviously lower than that in livers; and there were differences in HCV expression in various cells among different extrahepatic tissues. These results suggested that the levels of extrahepatic HCV infection and replication were relatively low as compared with those of livers; and there were differences in the status of HCV replication among different extrahepatic tissues. Whether HCV can bring about cell injury of infected extrahepatic tissues is not well understood because of the lack of extrahepatic histopathological observation. Recently, some reports showed that HCV is an causal agent in the pathogenesis of hypertrophic cardiomyopathy and chronic myocarditis[41,42], and that the patients' gastroduodenal mucosal lesions might be associated with HCV infection[43]. However, most of the patients with hepatitis C did not have extrahepatic clinical manifestations[44]. In our previousl studies, we found no relationship between the HCV expression in extrahepatic tissue cells and tissue lesions[45]. We speculate that extrahepatic HCV infection and replication may be of "concomitant state" in most of patients with hepatitis C, while liver was a key organ in HCV infection and pathogenicity[18,21,31,45], i.e. HCV may replicate in various tissue cells, but viral replication may not cause obvious cell injury of these infected tissues.

Zignego *et al*[46] reported that the infected PBMC might act as a reservoir for HCV. In this study, various tissue cells may support viral replication. These extrahepatic tissue and PBMC reservoirs are considered to provide the source of HCV for the patient's liver reinfection and recurrence of infection after liver transplantation for hepatitis C. Once the immunologically privileged sites are infected, HCV is more difficultly eliminated by the host and/or antivirus therapy, which might play a role in both HCV persistence and reactivation of infection after interferon therapy[8,47].

At present, little is known of the mechanisms and the HCV role in the development of HCV-related extrahepatic syndromes. Some reports showed that the vasculitic and kidney damage in mixed cryoglobulinemia and glomerulonephritis associated with HCV infection might be mediated by immune mechanisms[48,49]. However, in this study, HCV may replicate and express viral proteins in many tissue cells such as interstitial cells of kidney, epithelial cells of tubules, and glomerulus. It is noticeable that some of the cell types found to harbor HCV are also associated with HCV-related extrahepatic syndromes such as glomerulonephritis. In addition, HCV antigens were also found in mesangial and paramesangial cells and epithelial cells of tubules in glomerulonephritis kidney tissues[24,48], and in lymphoid cells in hyperplastic reactive lymphad enopathy[50]; HCV RNA and/or HCV replicative intermediate were detected in residual parotid epithelial cells in the parotid non-Hodgkin's lymphoma lesion by ISH[51], and in epineurial cells in mixed cryoglobulinemia-associated neuropathy by *in situ* RT-PCR[52]. HCV as an etiologic agent replicating and expressing viral proteins in extrahepatic tissues itself contributes to extrahepatic syndrome associated-HCV infection in a few patients with chronic HCV infection. For example, a pathogenetic role of HCV as an exogenous trigger might be hypothesized in the parotid non-Hodgkin's lymphoma[51], or in hypertrophic cardiomypathy[41]. In hepatitis B

patients, several extrahepatic syndromes associated-HBV infection have been considered to be mediated by immune complexes or other immunological mechanisms, but various vasculitic and other HBV-related skin lesions are associated with local HBV replication. So Mason et al[53] suggested that once the HBV-related immune complexes are captured by the vascular endothelium, the vasculitic damage might also be mediated by viral replication and immune mechanisms. We speculate that HCV might use a similar mechanism to bring about the vasculitic and kidney damage in the development of mixed cryoglobulinemia and glomerulonephritis associated-HCV infection.

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