PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2001;7(5):642-646
World Journal of Gastroenterology
Copyright © 2001 by The WJG Press ISSN 1007-9327

• ORIGINAL RESEARCH •

# Prokaryotical expression of structural and non-structural proteins of hepatitis G virus

Ning-Shao Xia[1], Hai-Jie Yang[1], Jun Zhang[1], Chang-Qing Lin[1], Ying-Bin Wang[1], Juan Wang[1], Mei-Yun Zhan[2] and MH Ng[3]

[1]Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, Xiamen University, Xiamen 361005, Fujian Province, China
[2]Institute of Virology, Chinese Academy of Preventive Medicine Beijing 100052, China
[3]Department of Microbiology, Hong Kong University, Hongkong, China
**Supported by** National 863 Project, No. 102-07-02-07, and the 9th Five-Year Sci-Tech Plan, No. 96-906A-03-08
**Correspondence to:** Prof. Ning-Shao Xia, Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, Xiamen University, Xiamen 361005, Fujian Province,
Telephone: +86-592-2184110, Fax: +86-592-2184110
**Received** 2001-04-25 **Accepted** 2001-07-30

## Abstract

AIM **To study the epitope distribution of hepatitis G virus (HGV) and to seek for the potential recombinant antigens for the development of HGV diagnositic reagents.**

METHODS **Fourteen clones encompassing HGV gene fragments from core to NS3 and NS5 were constructed using prokaryotic expression vector pRSET and (or) pGEX, and expressed in E.coli. Western blotting and ELISA were used to detect the immunoreactivity of these recombinant proteins.**

RESULTS **One clone with HGV fragment from core to E1 (G1), one from E2 (G31), three from NS3 (G6, G61, G7), one from NS5B (G821) and one chimeric fragment from NS3 and NS5B (G61-821) could be expressed well and showed obvious immunoreactivity by Western blotting. One clone with HGV framment from NS5B (G82) was also well expressed, but could not show immunoreactivity by Western blotting. No obvious expression was found in the other six clones. All the expressed recombinant proteins were in inclusion body form, except the protein G61 which could be expressed in soluble form. Further purified recombinant proteins G1, G31, G61, G821 and G61-821 were detected in indirected ELISA as coating antigen respectively. Only recombinant G1 could still show immunoreactivity, and the other four recombinant proteins failed to react to the HGV antibody positive sera. Western blotting results indicated that the immunoactivity of these four recombinant proteins were lost during purification.**

CONCLUSION **Core to E1, E2, NS3 and NS5 fragment of HGV contain antigenic epitopes, which could be produced in prokaryotically expressed recombinant proteins. A high-yield recombinant protein (G1) located in HGV core to E1 could remain its epitope after purification, which showed the potenti al that G1 could be used as a coating antigen to develop an ELISA kit for HGV specific antibody diagnosis.**

**Subject headings** hepatitis agents; GB/genetics; genes, viral; viral proteins/biosynthesis

Xia NS, Yang HJ, Zhang J, Lin CQ, Wang YB, Wang J, Zhan MY, Ng MH. Prokaryotical expression of structural and non-structural proteins of hepatitis G virus. *World J Gastroenterol*, 2001;7(5): 642-646

## INTRODUCTION

Hepatitis G virus (HGV), also known as GBV-C, is a novel human virus, which can cause acute and persistent infection in humans[1-4]. The clinical significance of HGV infection is still controversial[5-10]. Some studies have reported the seroprevalence of HGV RNA in general population as well as voluntary blood donors between 0.3% and 6%, but the frequency is always significantly higher in high risk groups such as intravenous drug users (IVDU), patients with acute and chronic hepatitis B and C, patients with blood transfusion and hemodialysis, and patients with cryptogenic hepatitis[11-22]. HGV is a member of flaviviridae[3,4,19]. The genome organization of HGV resembles that of hepatitis C virus (HCV). Its positive- stranded RNA genome is about 9.4 kb in length that contains a single open reading frame (ORF), which encodes a polyprotein of about 2900 amino acids. The polyprotein is cleaved by viral and host proteases into structural proteins (Core, E1 and E2) and non-structural proteins (NS2, NS3, NS4, NS5a and NS5b)[1,2,23,24].

Up to now, RT-PCR is the most commonly used method for the diagnosis of HGV infection. It is necessary to develop a convenient antibody detection assay. In this study, we had serial fragments selected from core to NS3 and NS5 region of HGV Chinese strain expressed in *E.coli*, and detected their immunoreactivity by Western blotting and ELISA.

## MATERIALS AND METHODS

### Bacterial strains and plasmids

*E.coli* strain DH5α and BL21(DE3) were stored in our laboratory. Serial expression vectors pRSET and pGEX were purchased respectively from Invitrogen Co. and Pharmacia Co.. Clones include gene fragments of Chinese HGV strain (HGVch, Genbank Accession Number U94695) constructed in our laboratory before[25].

### Enzymes and other biochemical reagents

Restriction endoenzymes, T4 DNA ligase and DNA polymerase Taq were purchased from Promega. Sepharose 4B GST matrix, His Trap™ and isopropyl- β-D-thiogalactopyranoside (IPTG) were purchased from Pharmacia. Goat anti-human IgG-alkaline phosphatase conjugate and substrates BCIP, NBT were purchased from Boehinger Mannheim.

### Serum samples

Three HGV positive serum samples were kindly provided by Dr. Qiu (Beijing Wantai Biological Medicine Co.). Two of them were HGV RNA positive by RT-PCR, the other one was positive in the synthetic peptides based HGV ELISA kits and with high titer anti-HGV antibodies. These three serum samples mixed in equal ratio and were used in Western blotting.

### *Construction of recombinant plasmids*

By the regular molecule biological METHODS, pRSET and pGEX were digested with single endoenzyme or two endoenzymes, gene fragments of HGV in pGEM T-Easy were digested with the same restriction enzyme (s), and they were ligated by T4 DNA ligase, and the expression clones of HGV gene fragments were constructed.

### *Expression of HGV gene fragments in E.coli*

Two mL fresh overnight cultured BL21(DE3) carrying HGV gene fragment expression plasmids were diluted with 200 mL fresh LB medium in the presence of 100 mg·L$^{-1}$ ampicilin and grew to $A_{600}$ = 0.8 at 37 ℃ at a shaki ng speed of 210 r·min$^{-1}$. The culture was induced by adding IPTG to a final concentration of 0.2 mmol·L$^{-1}$ at 37 ℃ for 3 h. One mL culture was harvested by centrifugation, cell pellets were resuspended in 400 μL SDS/PAGE loading buffer, and aliquots were run on 120 g·L$^{-1}$ SDS-PAGE gels. In order to obtain soluble recombinant proteins, induction expression was carried out at 20 ℃ for 3 h.

### *Western blot analysis of recombinant proteins*

Total cell lysates were run on SDS-PAGE gels and transferred electrophoretically to nitrocellulose membrane for 1 h under the voltage of 100 V. The membrane was then incubated in blocking solution (50 g·L$^{-1}$ nonfat milk in Tris-buffered saline, TBS) for 1 hour at room temperature followed by incubation at room temperature for 2 h in the sera that prediluted to 1:200 with blocking solution. The membrane was washed three times with TTBS (0.5 g·L$^{-1}$ Tween-20 in TBS) for 10 min, and alkaline phosphatase-labeled goat anti-human IgG antibodies diluted in TTBS (1:2000) were exposed to the membrane at room temperature for 1h. The membrane was visualized with a substrate solution of BCIP and NBT after another washing for 3 times for 10 minutes with TTBS.

### *Purification of recombinant proteins of HGV*

For soluble recombinant proteins, the harvested bacterial pellets were resuspend ed in PBS, after ultrasonification and high speed centrifugation, supernatants were collected and used for purification. Purification of expressed proteins in pRSET was carried out according to the manual of His Trap™ (Pharmacia Co.), and that in pGEX was done following the manual of GST purification modules (Pharmacia Co.).

For insoluble recombinant proteins, the harvested bacterial pellets were resuspended in PBS, and collected after ultrasonification and high speed centrifugation, and then resuspended in washing buffer (50 mmol·L$^{-1}$ Tris-Cl pH 7.2, 5 mmol·L$^{-1}$ EDTA, 150 mmol·L$^{-1}$ NaCl, 5 g·L$^{-1}$ Triton-x 100). Following washing with 2 mol·L$^{-1}$ and 4 mol·L$^{-1}$ urea, the recombinant proteins in pellets were dissolved in 8 mol·L$^{-1}$ urea, which were run on SDS-PAGE gels and stained with 0.3 mol·L$^{-1}$ $CuCl_2$ solution for 5 minutes. The bands of the recombinant proteins could be seen clearly (background was light blue, and the bands of proteins were blank fielded), they were cut carefully and sealed into dialyzer in elution buffer (250 mmol·L$^{-1}$ EDTA, 250 mmol ·L-1 Tris-Cl pH 9.5) to elute target proteins with electrophoresis.

### *Enzyme linked immunoadsorbent assay (ELISA)*

Purified recombinant antigens were coated to microplate in a amount of 100 ng each well in 0.05 mol·L$^{-1}$ CB (pH 9.6) buffer for 2 h at 37 ℃ and overnight at 4 ℃. Plates were washed with PBS containing 0.5 g·L$^{-1}$ Tween 20 and blocked with blocking buffer (0.5 g·L$^{-1}$ Tween 20 and 10 g·L$^{-1}$ bovine serum albumin in PBS)for 2 hours at 37 ℃. Sera (1:1000) were applied for 30 min at 37 ℃. A peroxidase-conjugated goat anti-human IgG used as secondary antibody was incubated for 30 min at 37 ℃ and visualized with o-phenyl-diamine-2HCl (10 g·L$^{-1}$ in 5 mmol·L$^{-1}$ Tris-HCl, pH 7.0). Wells were washed five times with PBST (0.5 g·L$^{-1}$ Tween 20 in PBS) between each step. The reaction was stopped with 50 μL of 2 mol·L$^{-1}$ $H_2SO_4$. Absorption was measured at $A_{495}$.

## RESULTS

### *Construction and identification of recombinant plasmids*

The recombinant plasmids were digested with proper restriction endoenzymes. Agarose gel electrophoresis showed that all HGV gene fragments were cloned into the vectors with correct orientation and size. The recombinant protein expressed by pGEX vector had a GST fusion protein in N-terminal, while that by pRSET vector had a hexahistidine in N-terminal. The locations of corresponding fragments in HGV genome of these plasmids are listed in Table 1.

**Table 1** Amino acid location of constructed expression vector in HGV CH strain, yields in E.coli and immunoreactivity in Western blotting

| Clone | Vector | Target fragments | Amino acid location in ORF | Molecular mass of recombinant protein (ku) | Yield in *E.coli* | Immuno-reactivity |
|---|---|---|---|---|---|---|
| G1 | pGEX | Core to E1 | 1- 144 | 42 | High | + |
| G2 | pRSET | E1 to E2 | 101- 284 | 23 | No | |
| G3 | pGEX | E2 | 247- 578 | 62 | No | |
| G31 | pGEX | E2 | 491- 578 | 36 | High | + |
| G4 | pRSET | E2 to NS2 | 542- 876 | 40 | No | |
| G5 | pRSET | NS2 to NS3 | 854-1078 | 28 | No | |
| G6 | pGEX | NS3 | 1073-1345 | 56 | High | + |
| G61 | pRSET | NS3 | 1160-1345 | 24 | High | + |
| G7 | pGEX | NS3 | 1267-1427 | 44 | High | + |
| G8 | pRSET | NS5 | 2151-2524 | 45 | No | |
| G81 | pRSET | NS5 | 2151-2412 | 32 | No | |
| G82 | pGEX | NS5 | 2408-2524 | 40 | High | - |
| G821 | pRSET | NS5 | 2357-2524 | 23 | High | + |
| G61-821 | pRSET | NS3+NS5 | 1160-1345+2357-2524 | 44 | High | + |

### *Expression of recombinant proteins in E.coli*

Fourteen clones were constructed, covering the core, E1, E2, NS2, NS3 and NS5 region of HGV (Figure 1). The virus fragment in G1 covered a region from the beginning of the core to the aa144, which was located in the middle of E1. The yield of G1 was about 20% in total bacterial proteins (Figure 2, lane B). No visible expression was found in G2 (covering a region from E1 to E2) and G3 (including almost entire E2). But when the C-terminal 88 residues of G3, named G31, were expressed, a yield of about 30% was obtained (Figure 2, lane C). Both of the clones included NS2 fragment, G4 and G5, and could not produce obvious recombinant proteins. G6 and G7, both located in NS3, were expressed well (Figure 2, lane D and G). To obtain a soluble form of NS3 antigen, a fragment from aa1160 to aa1345 was subcloned from G6 to vector pRSET, the result ed clone G61 was expressed much better than G6 and G7, and the soluble form recombinant protein could be found in the supernatant after centrifugating the ultrasonificated bacteria (Figure 2, lane E and F). The NS5 fragment G8 was not expressed, so did the N-terminal two-three (G81) when G8 was spliced into two parts and subcloned, but the C-terminal one-three (G82) was expressed quite well (Figure 2, lane H). Better expression was found in the yield of G821, resulting from the 78 residues extended from G82 to N-terminal, (Figure 2, lane I), so did the chimerical clone, G61-821 (Figure 2, lane J).

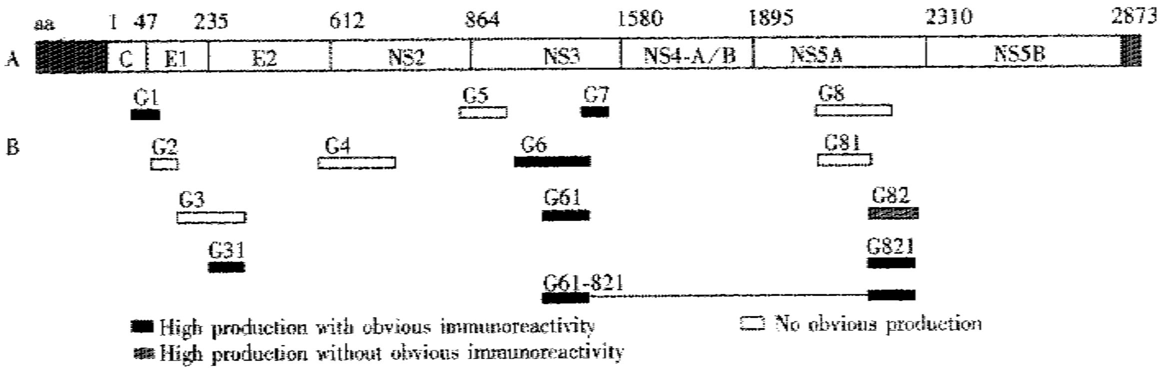


**Figure 1** Schematic representation of HGV genome and ex pressed proteins. A: Putative genomic organization of HGV CH strain. B: Fragments expressed from the HGV CH strain polyprotein.

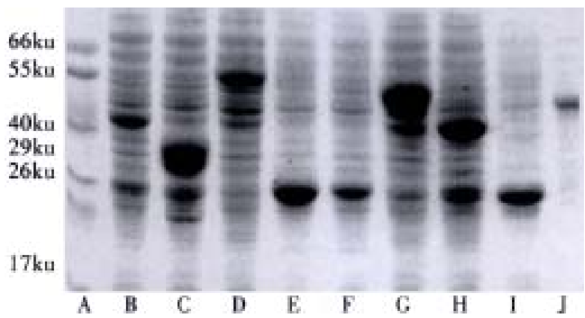


**Figure 2** Expression of the recombinant HGV proteins in *E.coli* analyzed by SDS-PAGE.A. Molecular mass standard; B.G1; C. G31; D.G6; E.G61; F.G61 in soluble form; G.G7; H.G82; I.G821; J. G61-821

### *Western blotting analysis*

Eight well-expressed recombinant proteins were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose for immumoblotting. As shown in Figure 3, except for fragment G82, all the other seven proteins showed immunoreactivity. G1 and G82 had strong reactivity with HGV positive sera, and reactivity of G31 was relatively weaker.

### *Purification and ELISA assay of recombinant proteins of HGV*

Four immunoreactive antigens, G1, G31, G61 and G821, which were located in the core, E2, NS3 and NS5 respectively, and a chimerical antigen G61-821 was selected for further purification. After electrophoretical elution, the purity of these five proteins was higher than 90% (Figure 4). These antigens were coated respectively to microplate in a amount of 100ng each well. Three HGV antibody positive sera and five negative sera were used in ELISA. The results showed that only G1 could detected all positive sera effectively (Figure 5).

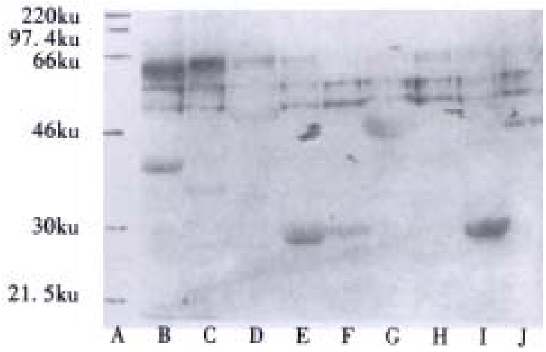


**Figure 3** Western blot analysis of expressed recombinan t HGV proteins. A. Molecular mass standard; B.G1; C.G31; D.G6; E.G61 F. G61 in soluble form; G.G7; H.G82; I.G821; J.G61-821

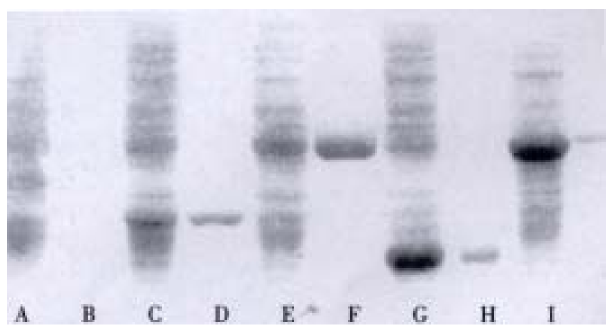


**Figure 4** SDS-PAGE Analysis of purified recombinant HGV proteins. Lane designations refer to purified HGV proteins or corresponding

*E.coli* lysates: A.G1; B.purified G1; C.G31; D.purified G31; E.G61; F.purified G61; G.G821; H.purified G821; I.G61:821; J.purified G61-821

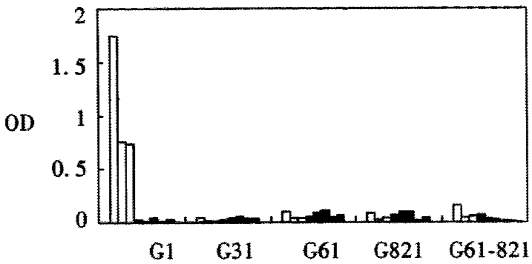


**Figure 5** ELISA results of purified recombinant antigens. □ Positive sera ■ Negative sera

## DISCUSSION

We have obtained full-length sequence of a Chinese HGV strain (HGVch) through overlapping RT-PCR[25]. Seven overlapping clones covering from the beginning of core to the end of NS3, were named G1 to G7, and one clone (G8) was located in NS5. They were subcloned to prokaryotic expression vectors pRSET or pGEX and expressed in E.coli in this study. The SDS-PAGE results showed that only fragments within C-E1 (G1) and NS3 (G6 and G7) could be expressed efficiently, but the clones located in E2 (G2 and G3), NS2 (G4 and G5) and NS5 (G8) could not. To improve the yield, one subclone (G31) for E2 and two subclone s (G81 and G82) for NS5 were constructed. The G31 was located in the C-part of G3, the G81 located in the N-terminal two-three of G8, and the G82 in the C-terminal one-three of G8. The results showed that G31 and G82 expressed well, but not G81.

In order to detect the immunoreactivity of different proteins at the same time, it is important to choose serum containing multiclonal antibodies against different epitopes. Due to the lack of reliable METHODS to detect anti-HGV, in this study, we chose a mixed serum as first antibodies in the immunoblotting assay. The Western blotting showed that G1, G6 and G7 had strong immunoreactivit y, the immunoreactivity of G3 could be identified too, but no reactivity could be found with G82. Then we constructed another clone G821, which resulted from the 78 residues, and extended to N-terminal from G82, its immunoreactivity in Western blotting was quite strong, indicating that this 78 residues played an important role in the epitopes of NS5. Both G6 and G7 showed a strong immunoreactivity, suggesting that the epitopic might cluster in NS3 region. We had a truncated fragment of G6 named G61 expressed as an endeavor to obtain the soluble NS3 antigen, because a soluble antigen usually displays more natural epitopic conformation. But we could not see a stronger reactivity in the soluble form of G61 than the insoluble form of G61 in Western blotting. Chimerical gene G61-821 was expressed as an endeavor for making a better diagnostic antigen, and it showed a strong immunoreactivity in Western blotting.

Among the four immunoreactive antigens, G1, G31, G61 and G821, which were locate d in the core, E2, NS3 and NS5 respectively, the chimerical antigen G61-821 was selected for further purification and coating respectively to microplate to make an ELISA kit. The results showed that only G1 could detect HGV positive sera effectively, the other four recombinant proteins could react with none of the three tested HGV positive sera, which may suggest that the immunodominant epitopes in these four antigens were conformationally depended, and were destructed during our purifying procedure, as sustained by the Western blotting results for these five purified proteins, which showed only the reactivity of G1.

Epidemiological studies of HGV have been hampered by the lack of convenience serologic assays. The virus has not been successfully grown in culture, thus no native antigen is available for serologic detections. Many authors had reported the expression of HGV proteins in prokaryotic systems or eukaryotic systems[26-28]. The recombinant proteins of the core, NS3, NS4 and NS5 gene of HGV expressed in E.coli showed immunoreactivity in Western blot assays, but none of them could be used in ELISA. It may be due to the loss of antigenicity during the purification procedure as shown in this study. Dille *et al*[29] successfully established an ELISA for anti-HGV E2 using CHO expressed E2 antigen, however, the presence of anti-E2 and HGV RNA was almost mutually exclusive: few were positive for both markers at the same time, and the utility of this ELISA in epidemiological studies was very limited[30,31]. Two HGV RNA positive sera were positive in ELISA based on the core-E1 antigen G1 obtained in this study. Although the serum samples in this study are very limited, they suggested the potential utility of this ELISA in epidemiological studies.

Although the prevalence of HGV infection is higher than that of HCV infection in the general population, there is absence of an obvious relationship between elevated level of alanine aminotransferase (ALT) and presence of HGV infection. Besides, whether the liver is the replication site of HGV has remained unclear. Saito *et al*[32] had detected both positive and negative stranded HGV RNA by HGV RT-PCR in the liver tissues of all the six tested HGV infected patients, which indicated that liver might be the primary site of HGV replicatio n, but many other scientists could not repeat these findings in their HGV infected patients by the same METHODS[33-36]. Recently, Reshetnyak et al reported that patients with HGV monoinfection had demonstrated the increase of the DNA single-stranded breaks peripheral blood lymphocytes (PBL) quantity[37]. Whether HGV is pathogenic to the liver or not, we should remain open to the possibility that its major pathological consequences, if any, may lie outside of the liver. A convenient serologic assay is undoubtedly crucial for the clarification of these unclear points.

## REFERENCES


1 Simons JN, Leary TP, Dawson GJ, Pilot-Matias TJ, Muerhoff AS, Schlauder GG, Desai SM, Mushahwar IK. Isolation of novel virus-like sequences associated with human hepatitis. *Nat Med*, 1995;1:564-569

2 Linnen J, Wages JR, Zhang-Keck ZY, Fry KE, Krawezynski KZ, Alter H, Koonin E, Gallagher M, Alter M, Hadziyannis S, Karayiannis P, Fung K, Nakatsuji Y, Shih JW, Young L, Piatak M Jr, Hoover C, Fernandez J, Chen S, Zou JC, Morris T, Hyams K, Ismay S, Lifson JD, Hess G, Foung SK, Thomas H, Bradley D, Margolis H, Kim JP. Molecular cloning and disease association of hepatitis G virus a trans-fusion-transmissible agent. *Science*, 1996;271:505-508

3 Yu JG, Xu CZ, Zhang GS. Progress in research of hepatitis G and HGV. *Huaren Xiaohua Zazhi*, 1998;6:1003-1004

4 Lin WL, Zhou HG, Pan BR. HGV:A new hepatitis virus. *Huaren Xiaohua Zazhi*, 1998;6:1005-1006

5 Liu JP, Li YF. Study on molecular epidemiology of hepatitis G virus infection in Chongqing. *Shijie Huaren Xiaohua Zazhi*, 2000;8:410-412

6 Nie QH, Hu DR, Li MD, Li L, Zhu YH. Detection of hepatitis G virus RNA in liver tissue using digoxigenin labelled probe by *in situ* hybridization. *Shijie Huaren Xiaohua Zazhi*, 2000;8:771-774

7 Ling BH, Zhuang H, Cui YH, An WF, Li ZJ, Wang SP, Zhu WF. A cross-sectional study on HGV infection in a rural population. *World J Gastroenterol*, 1998;4:489-492

8 Zhang F, Kang WZ, Xie YM, Mu XY. Detective significance of mi-

nus-strand RNA of HGV/GBV-C in liver and peripheral blood mononuclear cells of patients with co-infected hepatitis C and G viruses. *Shijie Huaren Xiaohua Zazhi*, 2001;9:383-387
9 Peng XJ, An P, Ren YQ, Chen NL. State of Hepatitis G virus infection in acute hepatitis. *Huaren Xiaohua Zazhi*, 1998;6:546-547
10 Zhao XP, Yang DL, Wang BJ, Yang Y, Shen HX, Peng ZH, Hao LJ. Immunohistochemical study of HGV expression in liver of patients with hepatitis G. *Huaren Xiaohua Zazhi*, 1998;6:585-587ª¤
11 Kar P, Bedi P, Berry N, Chakravorty A, Gupta RK, Saha R, Das BC. Hepat itis G virus (HGV) infection in voluntary and commercial blood donors in India. *Diagn Microbiol Infect Dis*, 2000;38:7-10
12 Chu CW, Hwang SJ, Luo JC, Wang YJ, Lu RH, Lai CR, Tsay SH, Li CP, Wu JC, Chang FY, Lee SD. Clinical, virological, immunological, and pathological significance of GB virus C/hepatitis G infection in patients with chronic hepatitis C. *Hepatol Res*, 2001;19:225-236
13 Tang ZY, Kck J, Moradpour D, Yang DL, Hao LJ, Blum HE. Hepatitis G virus infection in Chinese patients with chronic hepatitis B, C or non-A-E: clinical and molecular aspects. *Hepatol Res*, 1999;13:133-142
14 Yu JG, Hou XR, Pan W, Zhang GS, Zhou XM. PCR detection of hepatitis G virus RNA in sera and liver tissues from patients with chronic hepatitis C. *Huaren Xiaohua Zazhi*, 1998;6:580-581
15 Xu FQ, Tang W, Zhang DL, Wang HM. Detection of HGV-RNA in patients with viral hepatitis and its' clinical significance. *Huaren Xiaohua Zazhi*, 1998;6:642-643
16 Zhu CL, Duan XZ, Li L, Jin B, Du N, Chen JM. Clinical characters and significance for 94 viral hepatitis patients with serum HGVRNA. *Huaren Xiaohua Zazhi*, 1998;6(Suppl 7):189-191
17 Nan YM, Zhang JG, Shang C, Liu JX, Cao ZC, Xie YF. The clinic and significance of hepatitis G virus infection. *Shijie Huaren Xiaohua Zazhi*, 2000;8:597-598
18 Nie QH, Hu DR, Li MD, Xie Q. Expression of virus-related antigens in the livers of patients co-infected with HGV and HCV. *Shijie Huaren Xiaohua Zazhi*, 2000;8:114-115
19 Wang XT, Zhuang H, Song HB, Li HM, Zhang HY, Yu Y. Partial sequencing of 5' non-coding region of 7 HGV strains isolated from different areas of China. *World J Gastroenterol*, 1999;5:432-434
20 Yan J, Dennin RH. A high frequency of GBV-C/HGV coinfection in hepatitis C patients in Germany. *World J Gastroenterol*, 2000;6:833-841
21 Xiong LS, Tang N, Chang MH. Hepatitis B patients coinfected with hepatitis D, E,G virus. *Shijie Huaren Xiaohua Zazhi*, 2000;8:832-833
22 Zhong RX, Luo HT, Zhang RX, Li GR, Lu L. Investigation on infection of hepatitis G virus in 105 cases of drug abusers. *World J Gastroenterol*, 2000;6(Suppl 3):63
23 Leary TP, Muerhoff AS, Simons JN, Pilot-Matias TJ, Erker JC, Chalmers ML, Schlauder GG, Dawson GJ, Desai SM, Mushahwar IK. Sequence and genomic organization of GBV-C: a novel member of the flaviviridae associated with human non-A-E hepatitis. *J Med Virol*, 1996;48:60-67
24 Belyaev AS, Chong S, Novikov A, Kongpachith A, Masiarz FR, Lim M, Kim JP. Hepatitis G virus encodes protease activities which can effect processing of the virus putative nonstructural proteins. *J Virol*, 1998;72:868-872
25 Wang HL, Chen HS, Xia NS, Tan WJ, Chen G, Liu YL, Cong Y, Sun J, Zeng D, Hou YD, Wang Y, Zhan MY. cDNA cloning and sequence analysis of hepatitis G virus genome isolated from a Chinese blood donor. *Chin Med J*, 1999;112:747-749
26 Feucht H, Zollner B, Polywka S, Knodler B, Schroter M, Nolte H, Laufs R. Distribution of hepatitis G viremia and antibody response to recombinant proteins with special regard to risk factors in 709 patients. *Hepatology*, 1997;26:491-494
27 Shi J, Wang YC, Zhang HY, Li HM. Cloning and expression for cDNA of E2 region of hepatitis G virus isolated from Chinese blood donor. *Zhonghua Weishengwuxue He Mianyixue Zazhi*, 1999;19:470-473
28 Ren H, Zhu FL, Zhu SY, Song YB, Qi ZT. Immunogenicity of HGV NS5 protein expressed from Sf9 insect cells. *World J Gastroenterol*, 2001;7:98-101
29 Dille BJ, Surowy TK, Gutierrez RA, Coleman PF, Knigge MF, Carrick RJ, Aach RD, Hollinger FB, Stevens CE, Barbosa LH, Nemo GJ, Mosley JW, Dawson GJ, Mushahwar IK. An ELISA for detection of antibodies to the E2 protein of GB virus C. *J Infect Dis*, 1997;175:458-461
30 Tacke M, Klyosawa K, Stark K, Schlueter V, Ofenloch-Haehnle B, Hess G, Engel AM. Detection of antibodies to a putative hepatitis G virus envelope protein. *Lancet*, 1997;349:318-320
31 Tian DY, Yang DF, Xia NS, Zhang ZG, Lei HB, Huang YC. The serological prevalence and risk factor analysis of hepatitis G virus infection in Hubei Province of China. *World J Gastroenterol*, 2000;6:585-587
32 Saito S, Tanaka K, Kondo M, Morita K, Kitamura T, Kiba T, Numata K, Sekihara H. Plus- and Minus-stranded hepatitis G virus RNA in liver tissue and in peripheral blood mononuclear cells. *Biochem Biophy Res Comm*, 1997;237:288-291
33 Laskus T, Radkowski M, Wang LF, Vargas H, Rakela J. Lack of evidence for hepatitis G virus replication in the livers of patients coinfected with hepatitis C and G virus. *J Virol*, 1998;71:7804-7806
34 Pessoa MG, Terrault NA, Detmer J, Kolberg J, Collins M, Hassoba HM, Wright TL. Quantitation of hepatitis G and C viruses in the liver: evidence that hepatitis G virus is not hepatotropic. *Hepatology*, 1998;27:877-880
35 Abe K, Edamoto Y, Park YN, Nomura AMY, Taltavull TC, Tani M, Thung SN. *In situ* detection of hepatitis B, C, and G virus nucleic acids in human hepatocellular carcinoma tissues from different geographic regions. *Hepatology*, 1998;28:568-572
36 Laskus T, Radkowski M, Wang LF, Vargas H, Rakela J. Detection of hepatitis G virus replication sites by using highly strand-specific Tth-based reverse transcriptase PCR. *J Virol*, 1998;72:3072-3075
37 Reshetnyak VI, Sharafanova TI, Ilchenko LU, Golovanova EV, Poroshenko GG. [JP2]Peripheral blood lymphocytes DNA in patients with chronic liver diseases. *World J Gastroenterol*, 2001;7:235-237

Edited by Ma JY