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A high frequency of GBV - C/HGV coinfection in hepatitis C patients in Germany

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Subject headings GB Virus C; hepatitis G virus; hepatitis C virus; coinfection; polymerase chain reaction; sequencing; dominant viral subtype, Germany

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Abstract

AIM To detect infection rate of GBV-C/HGV in hepatitis C patients, to determine the methods of higher sensitivity and the primers of higher efficiency for GBV-C/HGV RNA detection and to study the dominant subtype and mutation of GBV-C/HGV.

METHODS Quantitative RT-PCR for detection pf HCV RNA concentration in serum samples, RT-nested PCR with two sets of primers for detection of GBV-C RNA, RT-PCR ELISA with two sets of primers for detection of HGV RNA, nucleotide sequence and putative amino acid sequence analysis.

RESULTS The positive rates of GBV-C RNA at the 5′-NCR and NS3 region in 211 serums amples from the patients with HCV infection were 31.8% and 22.8% respectively. The positive rates of HGV RNA at the 5′-NCR and NS5 region in the same samples were 47.9% and 31.8% respectively. The total positive rate of GBV-C/HGV RNA was as high as 55.5%. HCV copy numbers in the patients without GBV-C/HGV coinfection were statistically higher than that in the patients with GBV-C/HGV coinfection ($P<0.01$). Frequent mutation of nucleotide residue was present in the amplification products. Frameshift mutation was found in two samples with GBV-C NS3 region nucleotide sequences. All nucleotide sequences from amplification products showed higher homology to HGV genome than to GBV-C genome even though part of the sequences were amplified with GBV-C primers.

CONCLUSION A high frequency of GBV-C/HGV coinfection existed in the hepatitis C patients. RT-PCR ELISA was more sensitive than RT-nested PCR for detection of GBV-C/HGV RNA. The primers derived from the 5′-NCR was more efficient than those derived from the NS3 and NS5 regions. A reverse relationship was found to exist between HCV RNA concentration and GBV-C/HGV infection frequency. HGV was the dominant subtype of the virus in the local area. The major mutations of GBV-C/HGV genomes were random mutation of nucleotide residue.

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INTRODUCTION

Hepatitis GV virus C (GBV-C) and hepatitis G virus (HGV) were recently identified as novel member of Flaviviridae family associated with human non A-E hepatitis[1-5]. The two viral agents are different isolates of the same virus because of their high homology in nucleotide sequences and putative amino acid sequences[6,7]. GBV-C/HGV can be transmitted parenterally through transfusions of blood, blood products, intravenous drug user, hemodialysis and vertical transmission[8-19]. Although GBV-C/HGV were considered to be the major causative agent of human non A-E hepatitis, many later investigative data revealed that GBV-C/HGV infection rates in non A-E hepatitis patients were lower than 15%[20-25]. GBV-C genome does not have the gene responsible for encoding core protein[26,27]. HGV capsid protein is absent or defective and the capsid may be provided by another virus[2,28]. Therefore, coinfection of GBV-C/HGV with other viruses is an interesting and important subject for investigation. The reported GBV-C/ GV infection rates in hepatitis C patients were approximate 20%[29-32] but a quite lower coinfection rate of HGV and HCV (5.6%) was also reported[33]. Contradictory data about the clinical importance of GBV-C/HGV infection and nucleotide sequence mutation of the viral isolates indicated that the pathogenes is and variability of the virus are not fully understood[34-44].

In this study, GBV-C/HGV RNAs in the 211 serum samples of hepatitis C patients were detected by RT-nested PCR and RT-PCR ELISA with four different sets of primers. The GBV-C/HGV RNA

positive amplification products from part of the serum samples were cloned and then sequenced. The results of this study may contribute to the determination of GBV-C/HGV coinfection frequency in hepatitis C patients, choice of the methods with high sensitivity and the primers with high efficiency for GBV-C/HGV RNA detection and understanding the mutation of GBV-C/HGV genomes.

MATERIALS AND METHODS

Materials

211 serum samples of hepatitis C patients were obtained from the hospitals in Leubeck and in Koln of Germany. The 211 samples were confirmed to be HCV RNA positive by using HCV Monitor-TM Test Kit (Hoffmann LaRoche). This test is a routine work in our laboratory for quantitative detection of HCV RNA. HCV RNA concentration in the 211 samples ranged from 200 to 4 166 000 copies per milliliter of serum. All serum samples were HBV negative by using EIA and PCR. T-A Cloning Kit was purchased from Invitrogen. All other materials used in this study were purchased from Boehringer Mannheim.

Methods

Total serum RNA isolation Highly Pure RNA Isolation Kit was used to prepare total RNA from the samples according to the manufacturer's instruction.

Reverse transcription (RT) reaction Ten μL total RNA preparation was mixed with 10μL RT master mixture containing 0.2mmol/L dNTP, 50nmol/L hexanucleotide, 20U M-MuLV-reverse transcriptase, 20U RNA inhibitor and 4μL 5×RT buffer (pH 8.3), and incubated at 37℃ for 45min.

RT-nested PCR for GBV-C RNA detection Two sets of primers derived from GBV-C 5'NCR and NS3 region were used in the RT-nested PCR [45]. 5'-NCR external primers: 5'-ATGACAGGGTTGGTAG GTCGTAAATC-3' (sense), 5'-CCCCACTGGTC CTTGTCAACTCGCCG-3' (antisense). 5'-NCR internal primers: 5'-TGGTAGCCACTATAGGTGG-GTCTTAA-3' (sense), -5'-ACATTGAAGGGCGACG-TGGACCGTAC-3'(antisense). NS3 region external primers: 5'-GCT CGCCTATGACTCAGCAT-3' (sense), 5'-GTCACCTCAACGACCTCCTC-3' (antisense). NS3 region internal primers: 5'-ATCCATAATTGAGAC AAAGCTGGA-3' (sense), 5'-CCACCAACCCACAGTCGGTG-3' (antisense). For the first PCR round, 10μL- RT product was mixed with PCR master mixture containing 20pmol/L primers, 0.2mmol/L dNTP, 3U *Tag* polymerase and 10μL 10×PCR buffer (pH 9.1) and 25mmol/L $MgCl_2$. For the second PCR round, 5μL product from the first PCR round was used as template and the other reaction reagents were the same as that in the first PCR round except the primers. The volume per reaction in the two rounds was 100μL. The PCR parameters were described as the following : 94℃ 3min(×1); 94℃ 30s, 56℃ 30s, 72℃ 30s, (×10); 94℃ 30s, 56℃ 30s, 72℃ 35s, (×25, 5s in addition for each of the following cycle); and 72℃ 7 min (×1). The products of RT-nested PCR were detected by 2% ethidium bromide stained agarose gel electrophoresis.

RT-PCR ELISA for HGV RNA detection HGV RNA in the samples was detected by Hepatitis G Virus Primer and Capture Probe Set Kit according to the manufacturer's instruction. This kit contains two sets of primers for detection of HGV 5'-NCR and NS5 RNA and two sets of capture probes for detecting PCR products. 5'-NCR primers: 5'-CGGCCAAAAGGTGGTGGATG-3' (sense), 5'-CGACGAGCCTGACGTCGGG-3'(antisense). NS5 region primers: 5'-CTCTTTGTGGTAGTAGC-CGAGAGAT-3' (sense), 5'-CGAATGAGTCAG-AGGACG GGGTAT-3' (antisense). 5'-NCR capture probe: 5'-Biotin-GGTAGCCA CTATAGGTGGG-3'. NS5 region capture probe: 5'-Biotin GTTACTGAGAGCAGCTCAGAT-3'. Taq and two polymerase mix in this kit was used in PCR to allow DIG-11-dUTP incorporating the products during amplification process. The PCR parameters were described as the following: 94℃ 3min (1×); 94℃ 30s, 55℃ 30s, 72℃ 30s (10×); 94℃ 30s, 55℃ 30s, 72℃ 35s (5s in addition for each of the following cycle, 30×); 72℃ 7min (1×).

The RT-PCR products were detected by using PCR ELISA DIG Detection Kit according to the manufacturer's instruction. The PCR ELISA is a liquid phase hybridization/DIG detection system. It was found that DNA fragments amplified from HGV RNA could also be seen in 2% ethidium bromide stained agarose gel if the OD value at 405nm by RT-PCR ELISA was higher than 1.0.

Analysis of nucleotide sequences and putative amino acid sequences The target DNA fragments in GBV-C RT-nested PCR products or HGV RT-PCR products (DIG-11-dUTP replaced by dTTP) were cloned into PCR2.1 plasmid by using T-A Cloning Kit according to the manufacturer's instruction. The plasmid was amplified in E.coli and then recovered by the Sambrook's method[46]. The inserted sequences were analyzed by MWG-BIOTECH. The homology of the nucleotide sequences and putative amino acid sequences was compared with the those thow reported[1,2].

RESULTS

GBV-C and HGV RNA detection rates Seventy-four of the 211 serum samples (35.1%) were GBV-C RNA positive, 41 of the 74 samples (55.4%) were GBV-C RNA positive for both the 5'-NCR and NS3 region, 26 of the 74 samples (35.1%) were only 5'-NCR RNA positive and only 7 of the 74 samples (9.5%) detected NS3 region RNA. A statistically

significant difference (χ^2=4.32, P<0.05) was found to exist between the positive rates of GBV-C 5'-NCR (67/211) and those of NS3 region (48/211).

One hundred and five (49.8%) of the 211 serum samples were HCV RNA positive, 63 of 105 samples (60.0%) were HGV RNA positive for both the 5'-NCR and NS5 regions, 38 of the 105 samples (36.2%) were only 5'-NCR RNA positive and 4 of the 105 samples (3.8%) were only detectable for NS5 region RNA. A statistic ally significant difference (χ^2=11.43, P<0.01)was also found between the positive rates of HGV 5'-NCR (101/211) and NS5 region (67/211). The respective target fragments amplified from GBV-C 5'- NCR, NS3 region and HGV 5'-NCR, NS5 region are shown in Figures 1, 2.

Total positive rate of GBV-C/HGV and distribution of the positive samples One hundred and seventeen (55.5%) of the 211 serum samples were GBV-C and/or HGV RNA positive and 94 of the 211 samples (44.5%) were negative for both viral RNAs, 62, 12 and 43 of the 117 samples were positive for both viral RNAs, GBV-C RNA positive and detectable HGV RNA respectively. The distribution of the 117 samples is shown in Table 1.

Relationship between HCV RNA concentration and GBV-C/HGV infection frequency The range of HCV RNA concentrations of the 211 serum samples was 200-4 166 000 copies/mL. A statistically significant difference (t'=2.559,P<0.01) was present in the HCV RNA copy numbers of the group with GBV-C and/or HGV RNA positive samples (mean =344 000- HCV RNA copies/mL) and the group with both viral RNA negative samples (mean= 556000 HCV RNA copies/mL). In addition, HCV RNA concentration in each of 21 cases of the 211 samples was higher than 1 000 000 copies/mL. Six of the 21 cases distributed in GBV-C and/or HGV RNA positive group and the other 15 cases belonged to the negative group. In comparison with the two percentages (6/211 and 15/211), a statistically significant difference was also found (χ^2=4.06, P<0.05).

Table 1 Distribution of 117 GBV-C and/or HGV positive samples

GBV-C		HGV		Positive	Positive rate
5'-NCR	NS5	5'-NCR	NS5	cases	(%)
+*	+	+	+	26	12.3
+	+	+	-**	7	3.3
+	+	-	+	3	1.4
+	-	+	+	9	4.3
-	+	+	+	3	1.4
+	-	+	-	11	5.2
-	+	+	-	3	1.4
+	+	-	-	5	2.4
-	-	+	+	25	11.9
+	-	-	-	6	2.8
-	-	+	-	17	8.1
-	+	-	-	1	0.5
-	-	-	+	1	0.5
Total				117	55.5

* +: the viral RNA was detectable. ** -: the viral RNA was undetectable.

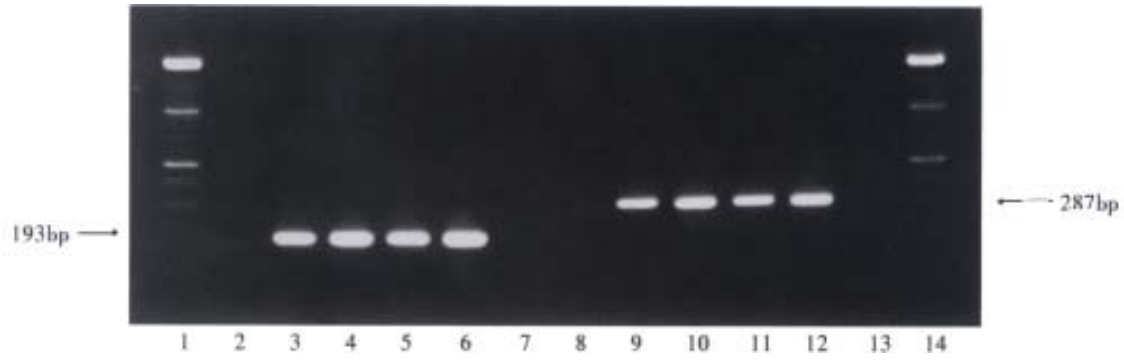

Figure 1 Target amplification fragments from GBV-C 5'-NCR and NS3 region. (1 and 14: markers; 2 and 8: negative serum samples; 7 and 13: blanks; 3-6: four GBV-C 5'-NCR RNA positive serum samples; 9-12: four GBV-C NS3 region RNA positive serum samples)

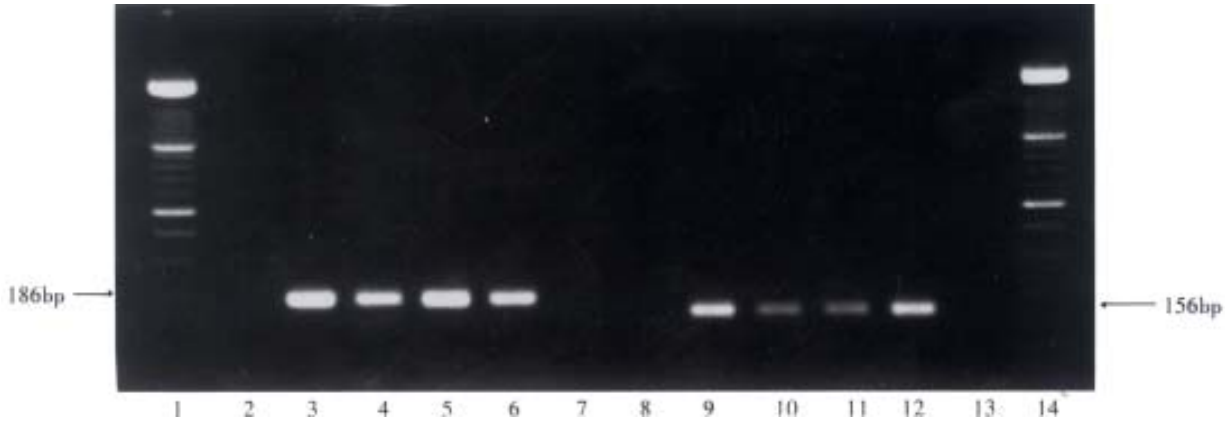

Figure 2 Target amplification fragments from HGV 5'-NCR and NS5 region. (1 and 14: markers; 2 and 8: negative serum samples; 7 and 13: blanks; 3-6: four HGV 5'-NCR RNA positive serum samples; 9-12: four HGV NS5 region RNA positive serum samples)

Nucleotide sequence analysis The homology of the nucleotide sequences of GBV-C 5'-NCR RT-nested PCR products from 3 serum samples compared with the reported GBV-C sequence[1] and the reported HGV sequence[2] was 81.8%, 81.1%, 82.5% and 97.6%, 97.2%, 99.3% respectively. The homology of the two reported sequences at the 5'-NCR was 81.8%. The homology of the nucleotide sequences of GBV-C NS3 region RT-nested PCR products from 6 samples compared with the reported GBV-C sequence[1] and the reported HGV sequence[2] was 80.2%, 82.3%, 81.5%, 84.4%, 85.6%, 85.2% and 86.8%, 88.1%, 88.1%, 88.1%, 86.8%, 87.7% respectively. The homology of the two reported nucleotide sequences at the NS3 region was 84.4%. Although the primers used in the RT-nested PCR derived from GBV-C genome, each of the 9 nucleotide sequences showed a higher homology to HGV than to GBV-C. These nucleotide sequences are shown in Figures 3, 4.

The homology of the nucleotide sequences of HGV 5'-NCR RT-PCR products from 3 samples compared with the reported HGV sequence[2] and the reported GB V-C sequence[1] was 95.2%, 99.3%, 98.6% and 86.4%, 87.1%, 87.8% respectively. The homology of the two reported sequences at the 5'-NCR was 86.4%, whereas the homology of the nucleotide sequences of HGV NS5 region RT-P CR products from 3 serum samples compared with the reported HGV[2] sequence and the reported GBV-C sequence[1] was 95.3%, 96.3%, 96.3% and 94.4%, 94.4%, 93.4% respectively. The homology of the two reported sequences at the NS5 region was 93.4%. These nucleotide sequences are shown in Figures 5, 6.

The homology in comparison of these sequences mentioned above did not contain the primer sequences.

The putative amino acid sequence analysis Putative amino acid sequences translated from the 6 nucleotide sequences from GBV-C NS3 region and the 3 nucleotide sequences from HGV NS5 region were respectively compared with the reported HGV and GBV-C sequences[1,2]. These putative amino acid sequences were shown in Figures 7, 8.

```
(1)   1  TGGTAGCCACTATAGGTGGGTCTTAAGGGGAGGCTACGGTCCCTCTTGCG
(2)   1  ..........................A.A...T..A.A.T.......T.
(3)   1  ..........................A.A...T..A.A.T.......T.
(4)   1  ..........................A.A...T..A.A.T.......T.
(5)   1  ..........................A.A...T..A.A.T.......T.

(1)  51 CATATGGAGGAAAAGCGCACGGT CCACAGGTGTTGGTCCTACCGGTGT
(2)  51 .C.GC..C.AG.CC..... .............C..........GG
(3)  51 .G.GC..G.AG.CC.....C.............C...........G
(4)  51 .C.GC..C.AC.CC..... .............C...........G
(5)  51 .C.GC..C.AG.CC..... .............C...........G

(1)  99 AATAAGGACCCGGCGCTAGGCACGCCGTTAAACCGAGCCCGTTACTCCCC
(2) 100 .......G....A..TC....T..T.....................C.A..
(3) 101 .......G....A..TC....T..T.................A..C.A..
(4) 100 .......G....A..TC....AC.T...........C........C.A..
(5) 100 .......G....A..TC....T..T.....................C.A..

(1) 149 TGGGCAAACGACGCCCACGTACGGTCCACGTCGCCCTTCAATGT
(2) 150 ..........................................
(3) 151 ..................................
(4) 150 .....T.............
(5) 150 ..................
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Figure 3 Homology of the nucleotide sequences from GBV-C 5'-NCR RT-nested PCR products from 3 serum samples as compared with the reported sequences.
(1) the reported GBV-C 5'-NCR sequence, (2) the reported HGV 5'-NCR sequence, (3)-(5) the sequences of GBV-C 5'-NCR RT-nested PCR product from 3 samples. Underlined areas indicated the primers'position.

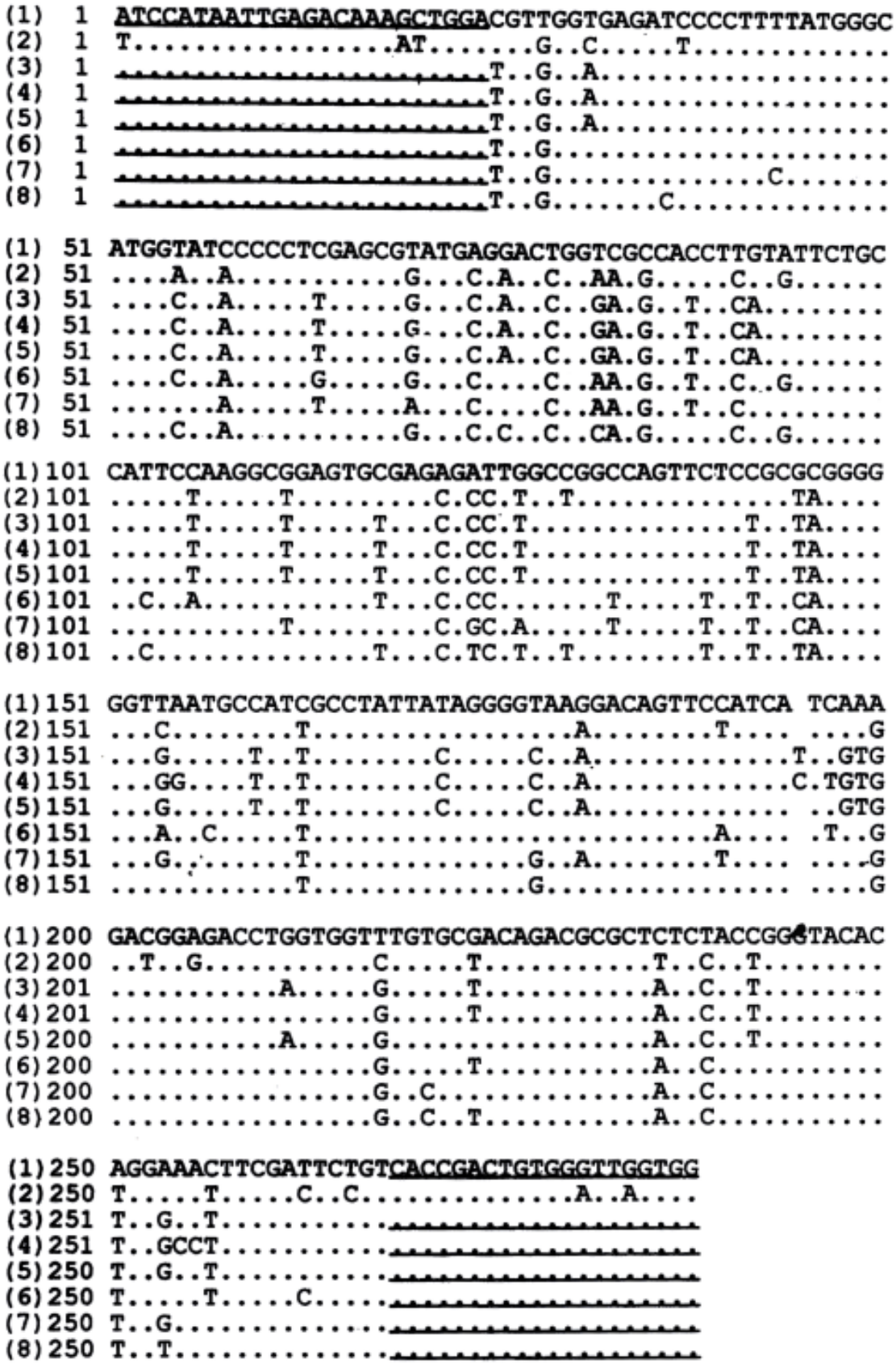

Figure 4 Homology of the sequences from GBV-C NS3 RT-nested PCR products from 6 serum samples compared with the reported sequences.

(1) the reported GBV-C NS3 region sequence, (2) the reported HGV NS3 region sequence, (3)-(8) the sequences from GBV-C NS3 region RT-nested PCR products from 6 serum samples. Underlined areas indicated the primers'position.

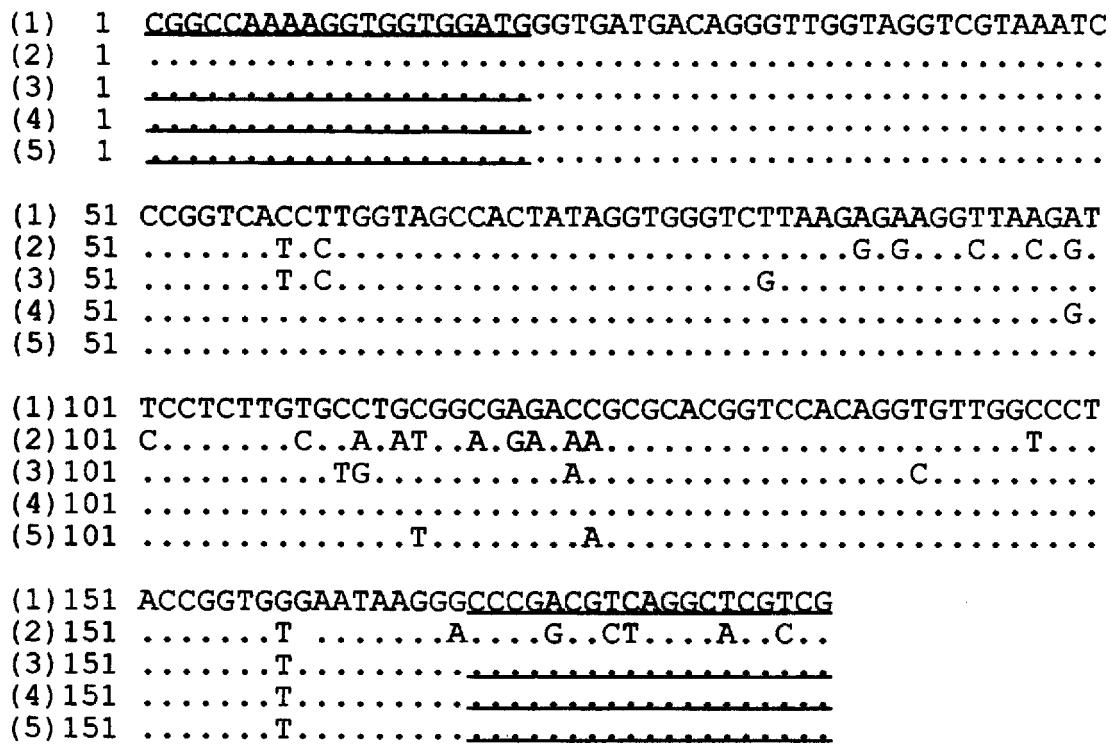

Figure 5 Homology of the nucleotide sequences of HGV 5'- NCR RT-PCR products from 3 serum samples compared to the reported sequences.
(1) the reported HGV 5'-NCR sequence, (2) the reported GBV-C 5'-NCR sequence, (3)-(5) the sequences of HGV 5'-NCR RT-PCR products from 3 samples. Underlined indicated the primers'position.

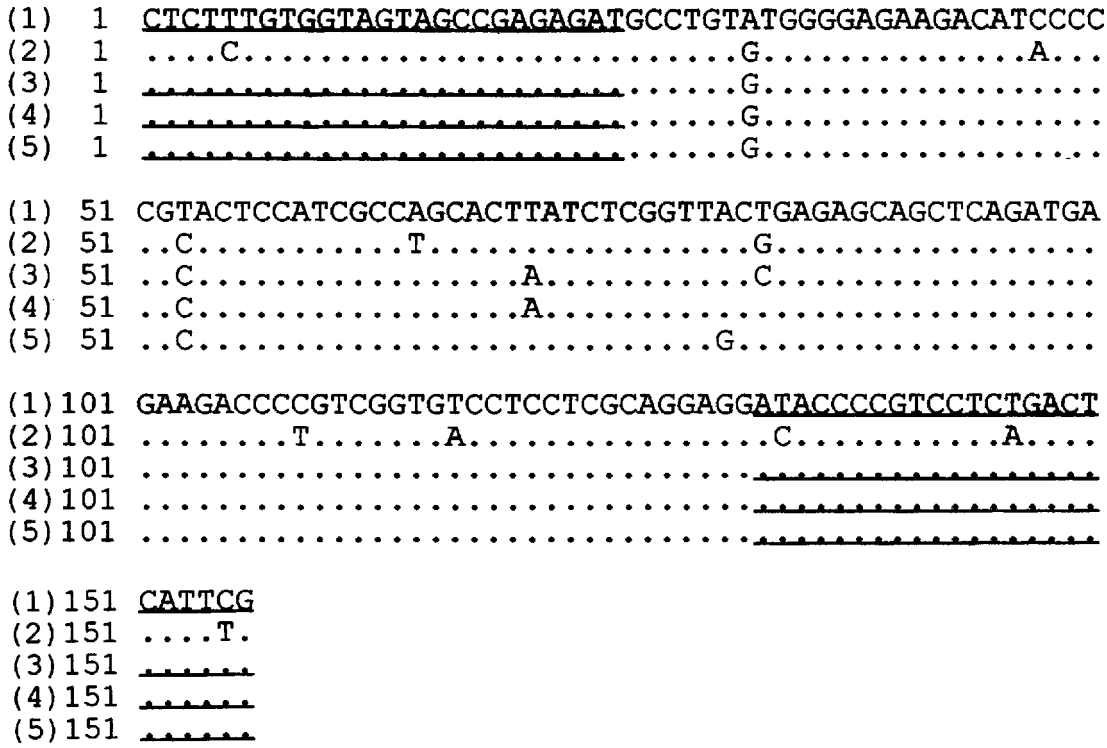

Figure 6 Homology of the nucleotide sequences from HGV NS5 RT-PCR products from 3 serum samples compared to the reported sequences.
(1) the reported HGV NS5 region sequence, (2) the reported GBV-C NS5 region sequence, (3)-(5) the sequences from HGV NS5 region RT-PCR products from 3 serum samples. Underlined areas indicated the primers'position.

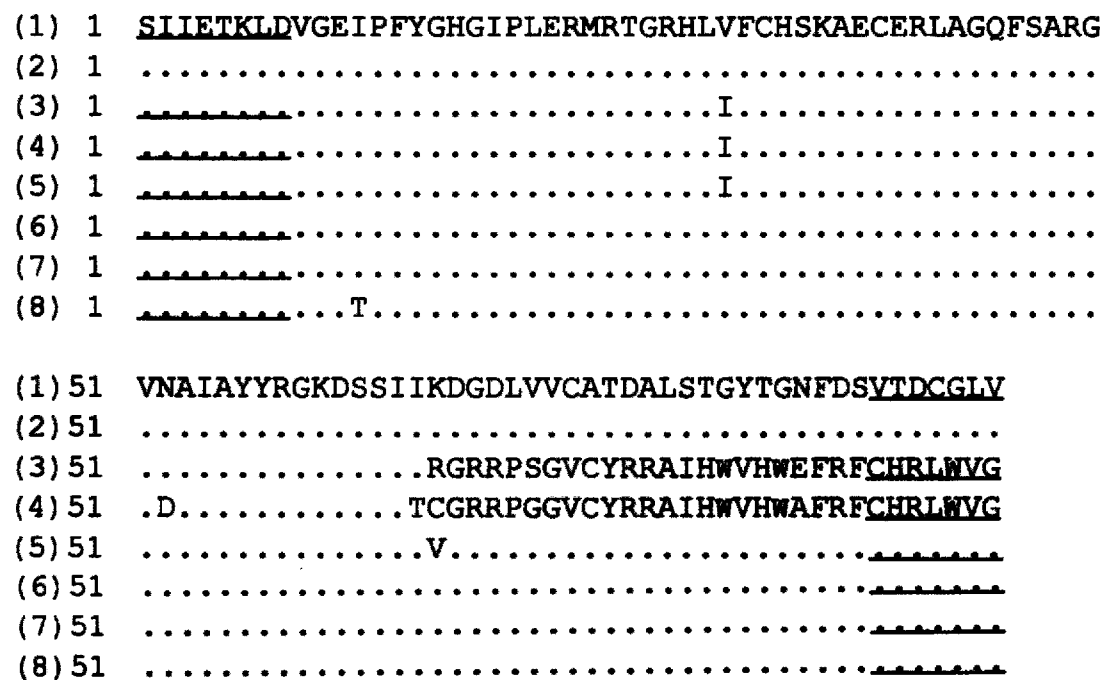

Figure 7 Comparison of the putative amino acid sequences from GBV-C NS3 RT-nested PCR products from 9 serum samples with the reported sequences.
(1) the reported GBV-C sequence, (2) the reported HGV sequence, (3)-(8) putative amino acid sequences translated from the 6 nucleotide sequences from GBV-C NS3 region. Underlined are indicated the primers'position.

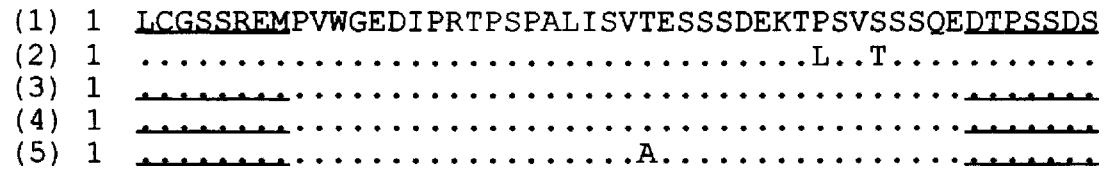

Figure 8 Comparison of the putative amino acid sequences from HGV NS5 RT-PCR products from 3 serum samples with the reported sequences.
(1) the reported HGV sequence, (2) the reported GBV-C sequence, (3)-(5) putative amino acid sequences translated from the 3 nucleotide sequences from HGV NS5 region. Underlined areas indicated the primers'position.

DISCUSSION

As mentioned in the introduction, the reported GBV-C/HGV infection rates in non A-E hepatitis patients were lower than 15% and the reported GBV-C/HGV and HCV coinfection rates were approximate 20%. In this study, a high total positive rate (55.5%) of GBV-C and/or HGV RNA in hepatitis C patients was found. Such a high frequency of GBV-C and/or HGV and HCV coinfection including most of the reported coinfection rates, suggested that GBV-C/HGV coinfected with HCV may be one of the features of its prevalence.

GBV-C RNA positive rate in the 211 samples by using RT-nested PCR was 35.1%. HGV RNA positive rate in the same samples by RT-PCR ELISA was as high as 49.8%. Statistical analysis of the two positive rates indicated that RT-PCR ELISA is more sensitive than RT-nested PCR (χ^2 = 9.32, P<0.01).

In the 211 serum samples, the positive rate (31.8%) of GBV-C 5'-NCR was higher than that (22.8%) of GBV-C NS3 region (χ^2 = 4.32, P<0.05) and the positive rate (47.9%) of HGV 5'-NCR was also higher than that (31.8%) of HGV NS5 region (χ^2=11.43, P<0.01).These data led to the conclusion that the primers derived from the 5'-NCRs were more efficient than those derived from the NS regions[47]. According to the results of nucleotide sequence analysis, this efficiency difference of primers was probably due to the higher conservation of sequences within the 5' -NCRs. In addition, only 26 of the 117 GBV-C and/or HGV RNA positive samples were positive for all the four viral genomic regions. It suggested that application of multiple primers is helpful to increase positive rate of GBV-C/HGV RNA detection.

A reverse relationship of HCV RNA concentration and GBV-C/HGV infection frequency was found in this study. This finding suggested that GBV-C/HGV and HCV might suppress each other *in vivo*. This reverse

relationship seemed to be contradictory to the high GBV-C/HGV and HCV coinfection rate. However, this kind of situation of cocurrence and competition is a normal phenomenon among microorganisms. For example, HBV and HCV coinfection is frequent in Asia but HCV was found to suppress the replication of HBV and vice versa to a lesser extent [48-50].

In this study, the homology of nucleotide sequences in the products amplified with the primers derived from HGV genome was higher in the reported HGV sequence than in the reported GBV-C sequence. Such results could be expected, however, all nucleotide sequences in the products amplified with the primers derived from GBV-C genome also showed higher homology with the reported HGV sequence. These data reveal the fact that: HGV and not the GBV-C, is the dominant subtype in the local area. Besides, these existed random mutations in a large number of nucleotide residue in all amplification products and two showed frameshift mutation which indicate that mutation of GBV-C/HGV genomic sequences occurs easily at the subtype level.

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