

# Mutations in different regions of the genome of hepatitis C virus genotype 1b and association with response to interferon therapy

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**Abstract.** The aim of this study was to investigate the association of mutations in the E2/NS1 [hypervariable regions 1 and 2 (HVR1 and HVR2)] and NS5A regions of the hepatitis C virus (HCV) genome and the effectiveness of interferon (IFN) therapy, and assess whether the degree of heterogeneity of HCV quasispecies predicts response to IFN treatment. Fourteen patients infected with HCV genotype 1b (HCV-1b) who were treated with pegylated IFN- $\alpha$ -2a and ribavirin for 24 weeks, were studied. E2/NS1 and NS5A gene segments were amplified by reverse-transcription polymerase chain reaction. HCV quasispecies heterogeneity in the E2/NS1 region was determined by cloning and sequencing. Mutations in the NS5A region were detected by direct sequencing. The heterogeneity of HCV quasispecies in the HVR1 was significantly greater in the non-responder group than in the responder group, but was not significant for HVR2 or NS5A. The correlation between mutations in IFN sensitivity-determining region (ISDR, NS5A<sub>2209-2248</sub>) and IFN sensitivity could not be supported. The degree of quasispecies heterogeneity in HVR1, but not in HVR2 and NS5A, may be predictive of response to IFN therapy. An ISDR may not apply to patients infected with HCV-1b.

## Introduction

Infection with the hepatitis C virus (HCV) affects approximately 200 million people worldwide. It is a leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). It is estimated that 25% of HCC worldwide is related to HCV. HCV may be implicated in the development of HCC in an indirect way through the induction of chronic inflammation, or directly by means of viral proteins activating several signaling pathways. For patients with chronic hepatitis C, there is a widespread agreement that antiviral therapy is mandatory in order to eliminate the virus. The current standard of care

is a combination of pegylated interferon (IFN) and ribavirin for 24-48 weeks, depending on the genotype. Previous studies have demonstrated that a high HCV load prior to treatment and infection with the HCV genotype 1b (HCV-1b) are independent predictive factors for poor response to IFN treatment (1,2). The majority of Chinese patients are infected with HCV-1b (3). In addition, the response to IFN treatment may vary in patients infected with the same genotype and with similar viral loads, indicating that other factors are involved.

A strong characteristic of HCV infection is its significant genetic diversity, the consequence of the absence of proof-reading activity in RNA-dependent RNA polymerase (4), and the high level of viral replication during its life cycle (5). As a result, the infecting HCV clones in each patient invariably demonstrate population diversity with a high degree of genetic heterogeneity. The collection of viruses in a population of closely related but non-identical genomes is referred to as a quasispecies (6,7), and the dominant viral population may evolve as a result of its viral replicative fitness and concurrent immune selection pressures that drive clonal selection.

It is reasonable to assume that the viral pathogenesis and sensitivity to treatment are affected by the generation of escape mutants through immune evasion and the modification of virulence characteristics by anti-viral treatment (8). Thus, certain viral mutations have important implications for the pathogenesis of viral disease and the sensitivity to antiviral therapy.

Previous reports have suggested that the degree of quasispecies heterogeneity of the hypervariable region (HVR)1 and NS5A regions is predictive of the effectiveness of IFN therapy (9,10); however, but there have been conflicting opinions (11,12). In this study, mutations in different regions of the HCV genome, including HVR1, HVR2 and NS5A, were identified in 14 Chinese patients with chronic HCV-1b infection, with the objective of assessing the correlation between mutations and the therapeutic efficacy of IFN.

## Patients and methods

**Patients.** Fourteen patients infected with HCV-1b were enrolled in the study. Patients included were 8 men and 6 women, with ages ranging from 23 to 45 years. This study was conducted in accordance with the Declaration of Helsinki and approval from the Ethics Committee of Sheng Jing Hospital of China Medical University. Written informed consent was obtained

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Table I. Primer sequences for E2/NS1 and NS5A.

Region	Primer sequences	Nucleotide position
E2/NS1	Outer primer 1 5'-CTACTCCGGATCCCACAAGC-3'	1009-1028
	Outer primer 2 5'-GGGCTCGGAGTGAAGCAATA-3'	1519-1538
	Inner primer 1 5'-GGATTCATTCCTTGGTGGGGAACTGG-3'	1085-1104
	Inner primer 2 5'-GAATCCGCAATACACTGGACCACACA-3'	1504-1523
NS5A	Outer primer 1 5'-TTGGCCAGCTCTTCAGCTAGC-3'	6921-6936
	Outer primer 2 5'-CCAGGACTCTAGCAGTGGAC-3'	7211-7232
	Inner primer 1 5'-TCAGCTAGCCAATTGTCTGC-3'	6933-6952
	Inner primer 2 5'-CAGTGGAGGGTTGTAGTCTG-3'	7200-72200

from all participants. The patients received pegylated IFN- $\alpha$ -2a (180  $\mu$ g/week) and ribavirin (1,000 mg/day) for 24 weeks. This was the first treatment for each patient. After the completion of treatment, patients with normal levels of serum alanine aminotransferase (ALT) and who were negative for HCV RNA for longer than 6 months were classified as sustained virological responders, and the others were classified as non-responders. Prior to treatment, sera were obtained from the patients and stored at -70°C until analysis.

**Amplification of E2/NS1 and NS5A gene segments.** HCV RNA was extracted from serum samples, and the E2/NS1 and NS5A gene segments were amplified using reverse-transcription polymerase chain reaction (RT-PCR), following previously published methods (13). The primer sequences are listed in Table I; the sizes of the E2/NS1 and NS5A products were 388 and 573 bp, respectively (Fig. 1).

**Quasispecies determination for HCV E2/NS1 regions.** We used primer 5.0 software to design the primers for E2/NS1 PCR. The forward primer contained a *Bam*HI (Takara D1010A) digestion site, and the reverse primer contained an *Eco*RI (Takara D1040A) digestion site. The sequences of both primers were the following for RT-PCR (Takara DR027A): P1, 5'-GGA TTCATTCCTTGGTGGGGAACTGG-3'; and P2, 5'-GAAT CCGCAATACACTGGACCACACA-3'. The PCR product was identified by 1.5% agarose gel electrophoresis, and a corresponding DNA band of interest was purified using the DNA gel purification kit (Takara DV805A). The concentration of purified DNA was measured.

The purified DNA of interest (0.3 pmol) was ligated with the pMD18-T simple vector (Takara D103A) at 16°C overnight. The ligated product was transformed into *E. coli* EZ competent cells. Single positive clone colonies were selected to extract the plasmids of interest using the plasmid miniprep kit (Takara DV801A). *Bam*HI and *Eco*RI were used for double digestion. Agarose gel electrophoresis was used for identification before and after digestion.

The DNA of interest was sent to be sequenced by the Shanghai United Gene Technology Group Company, and the sequencing results were analyzed using computer software.

**Evaluation of NS5A genetic variation.** PCR products of NS5A were purified, and the nucleotide sequences were determined

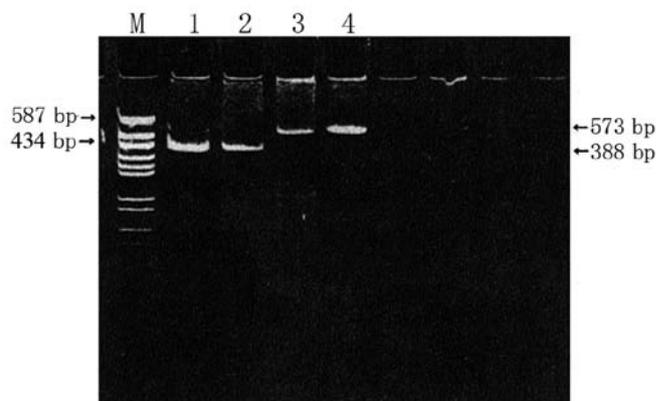


Figure 1. Products of RT-PCR amplification of E2/NS1 and NS5A regions from selected samples were separated by electrophoresis. E2/NS1 and NS5A products were 388 and 573 bp, respectively.

by direct sequencing. The evaluation of NS5A genetic variation was finished by the Shanghai United Gene Technology Group Company.

**Statistical analysis.** Statistical analysis was performed with the aid of SPSS 15.0 software. Differences between 2 groups were performed using the Chi-square test and t-test. P-values <0.05 were considered to indicate a statistically significant difference.

## Results

**E2/NS1 quasispecies heterogeneity and IFN plus ribavirin therapy.** PCR amplification of the E2/NS1 region was positive in 10 of 14 patients. Out of the 10 patients, 4 were responders and 6 were non-responders. A total of 93 sequences were obtained among 100 clones. The amino acid sequences of HVR1 and HVR2 are shown in Figs. 2 and 3. The degree of complexity (number of quasispecies) and nucleotide diversity (mean nucleotide variation rate/site) of HVR1 quasispecies was greater in the non-responder group than in the responder group. There were no significant differences observed for HVR2 quasispecies between the responders and non-responders (Tables II and III).

P1-1	HTVGGAGGRSFKFTNIPSSGFSQK	P2-1	ATTVVVGGMAGSSSLHTFTSFFNFGPSQK
P1-2	-----	P2-2	-----
P1-3	-----	P2-3	T-H-A--V--Q-AWGL--LISR-SA--
P1-4	-----	P2-4	-----Q-AWGL--LISR-SA--
P1-5	-----	P2-5	T-H-A--V--Q-AWGL--LISR-SA--
P1-6	-----	P2-6	-----
P1-7	-----	P2-7	-----
P1-8	-----	P2-8	-----
P1-9	-----	P2-9	-----
P3-1	NTRVTGGTQTHSLWTLTSLFRPGPSQR	P4-1	S-RLTGGAAAGHNAYGLTSLASGPSQR
P3-2	-----K	P4-2	-----K
P3-3	-----K	P4-3	GPSVI---SYT-H-----
P3-4	-----K	P4-4	TRSVV---SYT-H-----
P3-5	-----K	P4-5	-HSM---SYT-H-----
P3-6	-----K	P4-6	-SSVI---**RQ-IP***M--R--LK
P3-7	-----K		
P3-8	-----K		
P3-9	-----K		
P3-10	-----K		
P5-1	NRRVVGGVKSHFAQLTSLFTLGP AQN	P6-1	DTHVS GGTA GRTHGLASLFTPGASQN
P5-2	-----	P6-2	-----
P5-3	SHS--I-AA-YT-HG--FLAS--S-R	P6-3	-----Y-F-----P-----
P5-4	SHS--I-AA-ATYG--FLAS--S-R	P6-4	-----I--F-----P-----
P5-5	S-T-M-AA-G-TVHFF--LVK--S-R	P6-5	-----N-L-FT--I-RL-----
P5-6	S+ LT-AA-G-AA-YG--FLAS--S-R	P6-6	-----N-L-FT--I-RL-----
P5-7	S+ LT-AA-G-AA-YG--FLAS--S-R	P6-7	G-----Y-TG--A-----
P5-8	S+ LT-AA-G-ATYG--FLAS--S-R	P6-8	-----Y--G--A-----
P5-9	S+ LT-A-G--ATYG--FLAS--S-R	P6-9	-----F--I--P-----
		P6-10	-----T--F--P-----
P7-1	ETHVTGGSASHTTSMFASLLSPGPSQK	P8-1	NRRVTGGTQTHSLWTLTSLFRPGPSQK
P7-2	-----	P8-2	-----
P7-3	-----	P8-3	-----
P7-4	-----A--	P8-4	-----
P7-5	-----	P8-5	-----
P7-6	-----	P8-6	-----
P7-7	-----A-T--L-----	P8-7	-----
P7-8	-----A-T--L-A-----	P8-8	-----
P7-9	-RA--ELGR--FR-TA--FNF-N-	P8-9	-----
P7-10	-----	P8-10	-----
P9-1	NRRVVGGVKSHFAQLTSLFTLGP AQN	P10-1	DTHVS GGTA GRTHGLASLFTPGASQN
P9-2	-----	P10-2	-----
P9-3	SHS--I-AA--ATYG--FLAS--S-R	P10-3	-----I--F-----P-----
P9-4	SHS--I-AA--ATYG--FLAS--S-R	P10-4	-----I--F-----P-----
P9-5	-----	P10-5	-----N-L-FT--I-RL-----
P9-6	-----	P10-6	-----
P9-7	-----	P10-7	-----
P9-8	-----	P10-8	-----
P9-9	-----	P10-9	-----
P9-10	-----	P10-10	-----

Figure 2. Amino acid sequences of HVR1 from 10 patients infected with HCV-1b. Patients nos. 1, 2, 3 and 8 were responders. Patient nos. 4, 5, 6, 7, 9 and 10 were non-responders. \*Represents absent mutation.

P1-1	TKPDSFD	P2-1	TEPDSSD	P3-1	AEPDSSD	P4-1	VEPDSSD	P5-1	VEPDSSD
P1-2	-----	P2-2	-----	P3-2	-----P	P4-2	-----	P5-2	-----
P1-3	-----	P2-3	AG-----	P3-3	-----	P4-3	-----	P5-3	-----
P1-4	-----	P2-4	-----	P3-4	-----	P4-4	4A-----	P5-4	-G-----
P1-5	-----	P2-5	AG-----	P3-5	-----	P4-5	-----	P5-5	-----
P1-6	-----	P2-6	-----	P3-6	-----	P4-6	WMS-LR-	P5-6	-----
P1-7	-----	P2-7	-----	P3-7	-----			P5-7	-K-----
P1-8	-----	P2-8	-----	P3-8	-----			P5-8	-----
P1-9	-----	P2-9	-----	P3-9	-----			P5-9	-----
				P3-10	-----				
P6-1	AEPDSSD	P7-1	TEPNSSD	P8-1	AKPDSPD	P9-1	TEPDSSD	P10-1	AGPDSSD
P6-2	-----	P7-2	-----	P8-2	-----	P9-2	-----	P10-2	-----
P6-3	-----	P7-3	-----	P8-3	-----	P9-3	-----	P10-3	TE-----
P6-4	-----	P7-4	-----	P8-4	-----	P9-4	-----	P10-4	TE-----
P6-5	-----	P7-5	-----	P8-5	-----	P9-5	-----	P10-5	-----
P6-6	-----	P7-6	-----	P8-6	-----	P9-6	-----	P10-6	-----
P6-7	-----	P7-7	-----	P8-7	-----	P9-7	-----	P10-7	-----
P6-8	-----	P7-8	-----	P8-8	-----	P9-8	-----	P10-8	-----
P6-9	-----	P7-9	---D-P-	P8-9	-----	P9-9	-----	P10-9	-----
P6-10	-----	P7-10	-----	P8-10	-----	P9-10	-----	P10-10	-----

Figure 3. Amino acid sequences of HVR2 from 10 patients infected with HCV-1b. Patient nos. 1, 2, 3 and 8 were responders. Patient nos. 4, 5, 6, 7, 9 and 10 were non-responders. \*Represents absent mutation.

NS5A gene variation and IFN therapy. Out of the 14 patients, 11 were positive for NS5A RT-PCR amplification. The number of mutant nucleotides before IFN treatment is shown in Table IV. Patients 3 and 4 were responders to the treatment and the other patients were non-responders. There was no significant difference in the number of nucleotide mutations between the responder and non-responder groups ( $t=0.86$ ,  $P>0.05$ ). Compared with the HCV-J strain, the number of nucleotide mutations was not  $>10\%$ , and most were synonymous mutations. These results indicate that the NS5A region is highly conserved. There was no association between mutations leading to amino acid changes in NS5A<sub>2209-2248</sub> and response to IFN therapy (Fig. 4).

### Discussion

To date, there is no effective vaccine for the prevention of HCV infection. IFN plus ribavirin remains the primary treatment.

Table II. HVR1 and HVR2 quasispecies in HCV 1b patients undergoing IFN therapy.

Group	No. of patients	Clone no.	HVR1 quasispecies (n)	HVR2 quasispecies (n)
Responder	4	38	7	6
Non-responder	6	55	32 <sup>a</sup>	11 <sup>b</sup>

Comparison of 2 groups <sup>a</sup>P<0.01, <sup>b</sup>P>0.05.

Table III. Genomic diversity of responders and non-responders.

Group	HVR1		HVR2	
	Mean nucleotide variation rate/site	Mean amino acid variation rate/site	Mean nucleotide variation rate/site	Mean amino acid variation rate/site
Responder	1.146x10 <sup>-2</sup>	4.156x10 <sup>-2</sup>	0.89x10 <sup>-2</sup>	2.55x10 <sup>-2</sup>
Non-responder	3.563x10 <sup>-2a</sup>	7.625x10 <sup>-2a</sup>	1.19x10 <sup>-2</sup>	3.26x10 <sup>-2</sup>

Comparison of 2 groups <sup>a</sup>P<0.01.

Table IV. Nucleotide sequences of NS5A of patients compared with HCV-J.

No. of patients	No. of nucleotide mutations	Nucleotide mutation rate (%)
1	9	5.2
2	10	5.8
3	10	5.8
4	9	5.2
5	13	7.6
6	8	4.7
7	8	4.7
8	15	8.8
9	11	6.4
10	9	5.2
11	9	5.2

The rates of sustained virological response to IFN therapy is approximately 70%. Certain refractory patients do not respond to the treatment or relapse after treatment. To improve the response rate, the prediction of treatment efficacy to obtain satisfactory treatment options is critical. Among the HCV genotypes, HCV-1b, which is the predominant cause of infection in China, has a lower response rate to IFN treatment compared to other genotypes. Therefore, predicting the response to IFN treatment in patients with HCV-1b infection is clinically important.

In this study, the association between HCV gene variation in different regions of the genome and response to IFN therapy was assessed in 14 patients infected with genotype 1b. We found that the degree of HVR1 quasispecies heterogeneity in the non-responders was higher than in the responders,

	2209	2248 complete response
HCV-J	PSLKATCTTHHVSPDADLIEANLLWRQEMGGNITRVESEN	
1	-----	NO
2	-----	NO
3	-----	YES
4	-----	YES
5	-----	NO
6	-----	NO
7	-----A-----	NO
8	-----	NO
9	-----	NO
10	-----	NO
11	-----	NO

Figure 4. Changes in amino acid sequences in NS5A<sub>2209-2248</sub> from HCV-1b patients compared with strain HCV-J, and response to IFN therapy.

and there was no significant difference observed for HVR2 quasispecies heterogeneity. These results indicate that the greater the degree of HVR1 quasispecies heterogeneity in an infected patient, the more likely the patient will not respond to IFN treatment. However, Abbate *et al* (11) reported that the complexity of HVR1 quasispecies can only be used to predict earlier response rates to IFN therapy and is not associated with sustained virological response rates.

Patients with a higher degree of HVR1 quasispecies heterogeneity before treatment had lower IFN response rates, suggesting that there may be strains resistant to IFN in these infected patients. The development of resistant strains may be associated with immune selective pressure, and HVR1 is located on the virus where it may be subjected to immune pressure (14,15). IFN therapy may enhance the antiviral immune response, but also increases immune selective pressure against HVR1, leading to the development of greater variation. When the replication of IFN-sensitive strains is inhibited, mutant strains that adapt to the new environment become dominant.

In the E2/NS1 region, the degree of variation in HVR2 is second to variation in HVR1. HVR2 may be associated with immune recognition. In this study, the genomic complexity and diversity of HVR2 quasispecies were less than those observed for HVR1, and had no association with response to IFN. These findings should be confirmed by additional studies with larger numbers of patients.

Previously, Enomoto *et al* (16) reported that a small region in NS5A (NS5A<sub>2209-2248</sub>) of HCV-1b was associated with sensitivity to IFN. Compared with the HCV-J strain, mutant-type NS5A<sub>2209-2248</sub> regions (number of amino acid mutations  $\geq 3$ ) were sensitive to IFN therapy; intermediate-types (amino acid mutations =1-3) and wild-types were resistant to IFN. They designated NS5A<sub>2209-2248</sub> the IFN sensitivity-determining region (ISDR). However, these results have not been confirmed in European countries (17-19). In our study, we found that NS5A<sub>2209-2248</sub> was highly conserved; among 11 patients with HCV-1b infection, 10 patients were infected with wild-type HCV and 1 was infected with the intermediate type. The 2 responders were in the wild-type group. There were no significant differences in the nucleotide and amino acid sequences between the responders and non-responders. Therefore, the amino acid sequence of NS5A<sub>2209-2248</sub> may not be predictive of response to IFN therapy in Chinese patients. Chayama *et al* (20) also found that substitutions in NS5A<sub>2209-2248</sub> were rare and that therefore the concept of ISDR was not applicable.

Since the number of patients in our study was very small, and no mutant-type infections were observed, whether or not mutant-type HCV is sensitive to IFN treatment could not be ascertained. By contrast, the study reported by Enomoto *et al* (16) had many patients infected with mutant-type HCV-1b. These differences may be a result of the geographical variation in HCV-1b infections. These differences need to be further investigated.

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