Original Article

Sequence variation of the HVR1 region of Hepatitis C virus in response to interferon-α and ribavirin treatment

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Abstract

Introduction: Hepatitis C virus (HCV), which is a major cause of liver diseases worldwide, undergoes genetic variation during the course of infection. The aim of this study was to examine sequence variations within the HVR1 region of HCV genotype 4 in infected Saudi patients treated with a combination therapy of interferon-α and ribavirin.
Methodology: cDNA of the HVR1 region of HVC-4 from one responder and one non-responder patients was generated, cloned and sequenced. Ten clones were randomly selected and analyzed for changes in nucleotide and amino acid sequences before the start of treatment, and subsequently three and six months after the start of the therapy course.
Results: Based on nucleotide and amino acid sequence variations, the HVR1 region is highly sequence variable. In both the responder and the non-responder patients, amino acid sequence variations were observed and a clear distinction between patients was evident. The amino acid changes after the treatment course were different in the responder compared to the non-responder subject. Five amino acids (residues 364 to 367, 381 and 409) were unique in the non-responder patient.
Conclusion: Considerable amino acid variations were observed in the HVR1 region in both responder and non-responder patients. These findings could have implications for the development of an HCV vaccine as well as treatment protocols for HCV infections.

Introduction

Hepatitis C virus (HCV) belongs to the *Flaviviridae* family and it possesses a positive, single-stranded RNA genome. The genetic material encodes a single, large open reading frame that produces one large protein which is subsequently processed to produce all proteins of the virus [1]. Clinically, HCV is a major cause of liver complications such as cirrhosis and hepatocellular carcinoma (HCC) worldwide [2,3]. HCV exhibits great genetic variations during its course of infection, a biological phenomenon attributed to several reasons including the lack of a proof-reading mechanism of its RNA polymerase and the host immune pressure that could force the virus to undergo genetic changes allowing it to escape the killing power of host defenses [4]. Such genetic variability across the whole genome could reach 30% between all genotypes worldwide and 8-10% among subtypes of the same genotype [1]. Other changes also include synonymous changes in the RNA that do not result in amino acid alterations. Although this type of mutation is silent, it is believed that it makes changes on the secondary structure of the RNA which may affect its interaction with some cellular proteins [5]. Non-synonymous changes result in amino acid alterations of some HCV proteins [5,6]. Both synonymous and non-synonmous genetic changes could influence antiviral treatment [7,8]. Therefore, it is hypothesized that virus particles with certain genetic sequences are selected during the course of treatment to continue to proliferate, giving rise to a population that is resistant to antiviral therapy [9]. However, the molecular mechanisms through which this resistant population is selected is not understood [10].

The hypervariable region 1 (HVR1) is part of E2 glycoprotein and consists of 27 amino acids and is said to be an immunodominant epitope for activation of specific T and B cells [11]. HVR1 undergoes variations during HCV infection in a rate much higher than that of other regions of the genome. The exact mechanisms that control the variations in this region are largely unknown. It has been suggested that variations in this region might contribute to HCV persistence for long periods of time within the host [12-14]. However, other reports did not find any correlation between HVR1 variation and resistance to anti-HCV treatment [6,15]. Therefore, the aim of this study was to use sequence analysis to examine the variation within the HVR1 region in Saudi patients infected with HCV genotype 4 (HCV-4). We compared the sequence variation in two patients; one underwent successful elimination of the virus after treatment with alpha interferon (IFN-α) and ribavirin and the other exhibited resistance to this well-established anti-HCV treatment. Due to the extensive sequencing and laborious investigations of various clones from the amplified HCV-4 RNA, the comparison was initiated for two patients with the purpose of demonstrating the variation, if any, in Saudi patients.

Methodology

Patients

Two HCV-4 patients were selected randomly from the naive group in a previous study. At that time, patients were treated with IFN-α and ribavirin. Characteristics of these patients were previously described [16]. Virus titer (viral load) for the one responding patient was 15.19, 0.219, and < 0.2 genome copies/mL X 10^6 at 0, 3, and 6 months of treatment, respectively, while the virus titer for the one non-responding patient was 40.76, 4.22, and 14.38 genome copies/mL X 10^6 at 0, 3, and 6 months of treatment, respectively.

Amplification, cloning and sequencing

HCV RNA was extracted from 100 μl of serum using Q1Aamp Viral RNA Kit (QIAGEN Inc. Santa Clarita, CA, USA). The RNA preparation, cDNA synthesis and PCR amplification were described previously [17]. PCR was performed as described before [18] and numbering of amino acid residues of HVR1 was used as described by Timm *et al.* [19]. The amplification products were cloned into PCR 2.1 TA vector (InVitrogen, San Diego, CA, USA) according to the manufacturer's instructions and were

used to transform *E. coli* INVα F' competent cells (InVitrogen). The bacteria cells were grown on Luria-Bertani (LB) plates containing ampicillin (50μg/ml) at 37°C overnight and ten colonies were randomly selected. The plasmids were isolated using QIAprep spin columns (QIAGEN, Santa Clarita, CA, USA). The recombinant plasmids were sequenced in both directions using the dideoxynucleotide chain termination method (Taq Dye Deoxy Terminator Cycle Sequencing Kit, Applied Biosystems GmbH, Weiterstadt, Germany). PCR primers QP3 (5'-ACTGGGGTGTCCTCGTGGG-3') and QP4 (5'-AACAGCAATGGGAGCTGGCA-3') were used as sequencing primers. The sequences were obtained using ABI 373A Autosequencer (Applied Biosystems, Foster City, CA, USA).
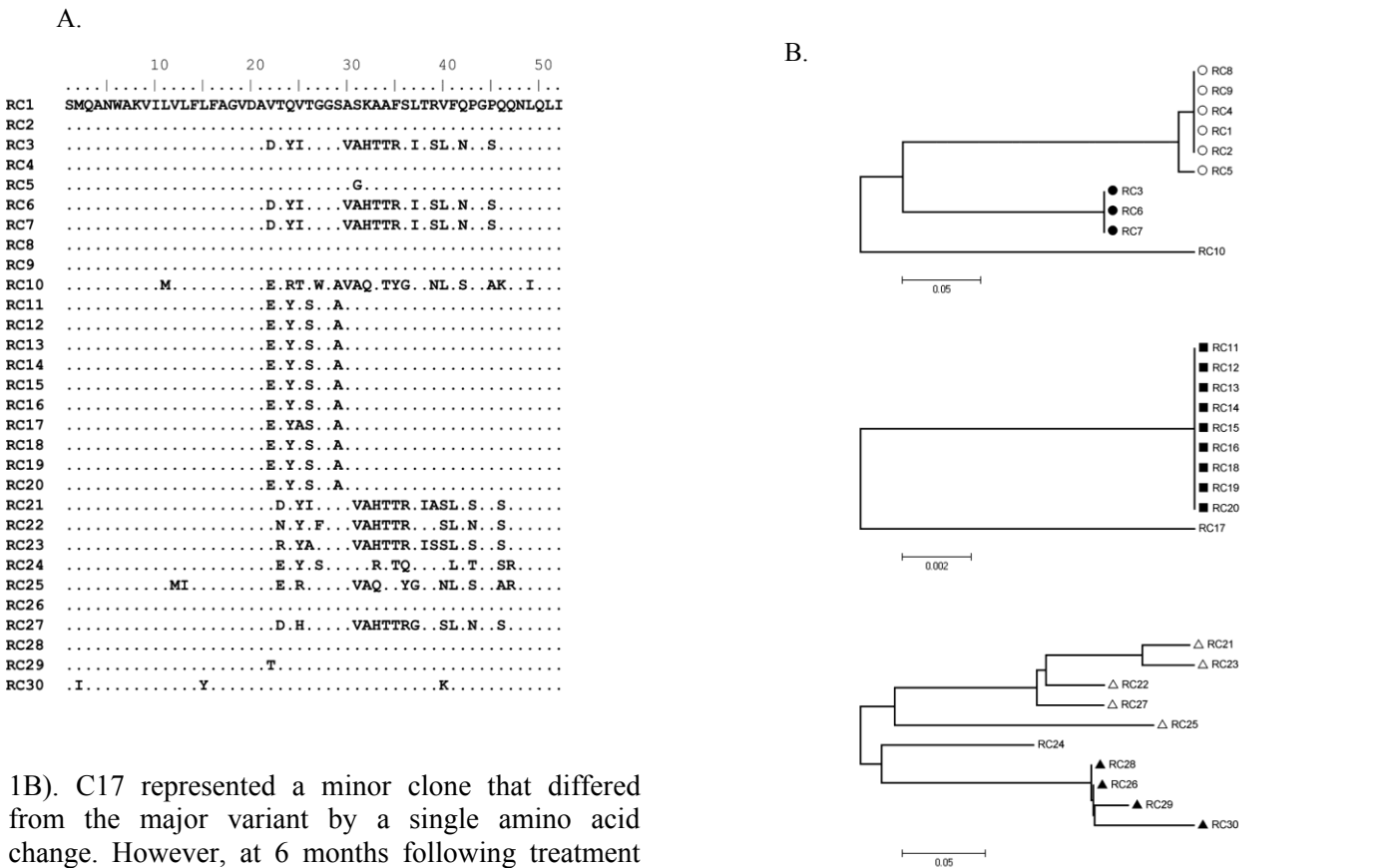
Alignment and phylogenetic analysis

Nucleotide sequences of positive isolates before initiation of therapy and at three and six months after initiation of therapy were compared by multiple sequence alignment using the Lasergene Navigator computer program (DNA Star Sequence Analysis Software, Madison, WI). Sequence divergence and phylogenetic analysis of HVR1 cDNA clones were performed according to the neighbor-joining method [20] using the software Clustal V [21].

Results

Sequence analysis of amplified products from the responder

To examine the sequence variation of HCV genome during the course of treatment, HVR1 PCR product from a representative sample of patient who responded to the combination therapy were cloned before and three and six months after initiation of therapy. Ten clones were chosen randomly from the resultant HVR1 cDNA library and sequenced. The deduced amino acid sequences are shown in Figure 1A. Before initiation of therapy and based on amino acid sequence variation, three HCV variants were present in the serum. One comprised of 6 of the 10 clones (C1, C2, C4, C5, C8, and C9), the second consisted of three clones (C3, C6, and C7), and the third is a single clone represented by C10 (Figure 1B). The amino acid sequence of the C10 differed by as many as 18 amino acid residues in the putative antigenic domain spanning codons 384-410 (Figure 1A). Three months following initiation of treatment, a predominant major variant representing nine of ten clones (C11-C20, 90%) was evident (Figures 1A and

Figure 1. (A) Alignment of deduced amino acid sequence of HVR1 cDNA clones detectable in serum before and after treatment from a responder patient (A) Amino acid sequence of 10 HVR1 clones in the serum before (C1-C10), 3 months (C11-C20) and 6 months (C21-C30) after combination therapy. Dashes indicate amino acid identity with predominant sequence in serum before initiation of therapy. **(B)** Phylogenetic analysis of deduced amino acid sequences of the HVR1 cDNA clones.

A.

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                10        20        30        40        50
        ....|....|....|....|....|....|....|....|....|....|..
RC1     SMQANWAKVILVLFLFAGVDAVTQVTGGSASKAAFSLTRVFQPGPQQNLQLI
RC2     ...................................................
RC3     ....................D.YI....VAHTTR.I.SL.N..S........
RC4     ...................................................
RC5     ..............................G....................
RC6     ....................D.YI....VAHTTR.I.SL.N..S........
RC7     ....................D.YI....VAHTTR.I.SL.N..S........
RC8     ...................................................
RC9     ...................................................
RC10    ..........M.........E.RT.W.AVAQ.TYG..NL.S..AK..I...
RC11    ....................E.Y.S..A.......................
RC12    ....................E.Y.S..A.......................
RC13    ....................E.Y.S..A.......................
RC14    ....................E.Y.S..A.......................
RC15    ....................E.Y.S..A.......................
RC16    ....................E.Y.S..A.......................
RC17    ....................E.YAS..A.......................
RC18    ....................E.Y.S..A.......................
RC19    ....................E.Y.S..A.......................
RC20    ....................E.Y.S..A.......................
RC21    ....................D.YI....VAHTTR.IASL.S..S.......
RC22    ....................N.Y.F...VAHTTR...SL.N..S.......
RC23    ....................R.YA...VAHTTR.ISSL.S..S........
RC24    ....................E.Y.S....R.TQ....L.T..SR.......
RC25    ..........MI........E.R.....VAQ..YG..NL.S..AR......
RC26    ...................................................
RC27    ....................D.H.....VAHTTRG..SL.N..S.......
RC28    ...................................................
RC29    ..................T................................
RC30    .I..........Y......................K...............
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B.

1B). C17 represented a minor clone that differed from the major variant by a single amino acid change. However, at 6 months following treatment (C21-C30), three variants were present and consisted of C21, C22, C23 and C27 in one group, C24, C26, C28, C29 and C30 in another, and C25 which represented a distinct variant that differed by as many as 17 amino acids from the major variant (Fig. 1A and 1B).

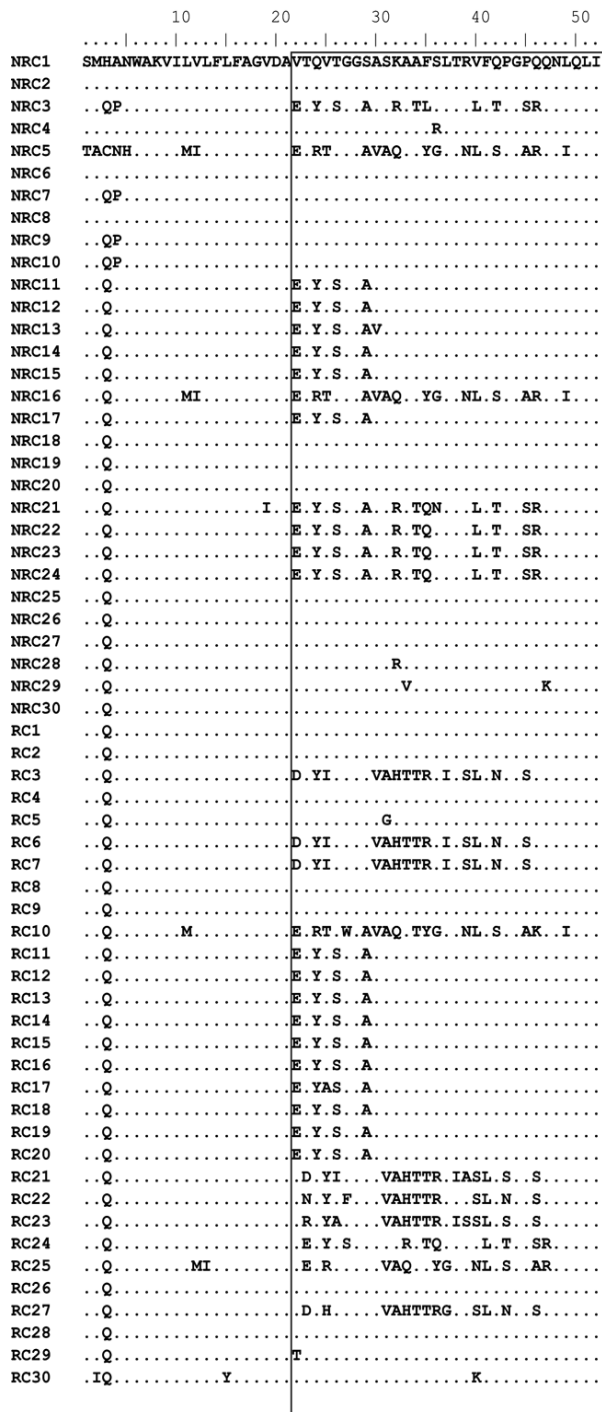*Sequence analysis of amplified products from the non-responder*

In a representative sample from patients who did not respond to the treatment, sequence analysis of 10 clones from the HVR1 cDNA library also showed nucleotide sequence variations. The deduced amino acid sequences are shown in Figure 2A**.** Before initiation of therapy, four HCV variants were present in the serum. The first variant included C1, C2, C4, C6 and C8; the second consisted of C7, C9 and C10; the third was a C3 clone that differed from the other variants by 13 amino acids; and the fourth variant was C5 clone that differed by as many as 22 amino acids (Figures 2A and 2B). Three months after initiation of therapy, the nucleotide sequence and subsequently the amino acid composition differed significantly from the initial

composition. The original variants were replaced with a novel major variant representing five of ten clones (C11, C12, C13, C14, C15 and C17), a second variant consisted of three clones (C18, C19 and C20), the third (C16, presented as a single clone) differed from the original variant by 18 amino acids. At six6 months of treatment, two variants were evident; one was comprised of four clones (C21 and C22, C23, and C24) and the other was represented by six clones (C25, C26, C27, C28, C29 and C30). Of importance, the amino acid changes occurred more significantly in the region from 384 to 410 within HVR1, while other regions were more conserved with minor changes observed (Figure 3).

HVR1 amino acid changes

Analysis of the frequency of variations at each codon position of HVR1 in all sixty clones has shown that variations at amino acids 364 to 467, 381 and 409 are unique to the non-responder patient (Figure 4).

Figure 2. (A) Alignment of nucleotide sequences of HVR1 cDNA clones detectable in serum before and after treatment from a non-responder patient (A) Amino acid sequence of 10 HVR1 clones in the serum before (C1-C10), 3 months (C11-C20) and 6 months (C21-C30) after combination therapy. Dashes indicate amino acid identity with predominant sequence in serum before initiation of therapy. **(B)** Phylogenetic analysis of deduced amino acid sequences of the HVR1 cDNA clones.

A.

B.
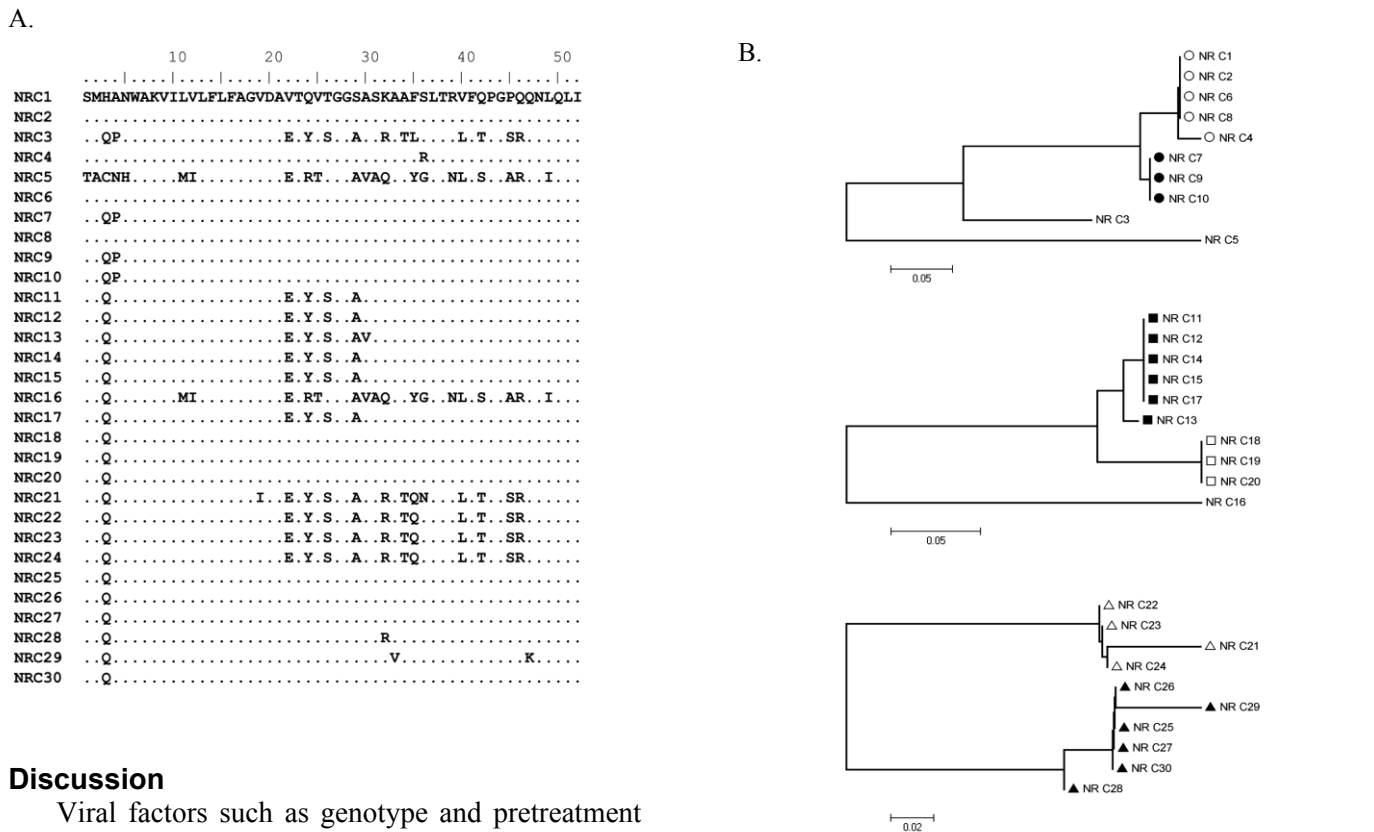
Discussion

Viral factors such as genotype and pretreatment titer are known to influence the outcome of therapy of HCV infected patients with IFN–α [22,23]. Genetic heterogeneity of the virus was also shown in recent studies to be a possible independent predictor of response to IFN-α therapy [24]. It is hypothesized that responsive patients could possess quasispeceis with less nucleotide sequence variations in pretreatment sera than do non-responsive patients [25-28]. Interferon-α had been the treatment of choice for HCV infection until recently when a combination regimen of IFN-α with ribavirin was demonstrated in clinical trials to produce higher rates of sustained virologic, biochemical, and histologic response, particularly in relapsed patients, than the conventional IFN-α monotherapy [29,30]. The role of HCV genetic heterogeneity, however, on the outcome of IFN-α-ribavirin combination therapy of HCV infected patients has not been thoroughly studied. Data from a our previous study, which examined the effect of combination therapy of IFN-α-ribavirin on chronic HCV genotype 4 patients (the predominant genotype in Saudi Arabia), showed a poor response among the patients with only 23% of the patients showing a sustained biochemical and 12% showing a

sustained virologic response at the end of a follow-up period of 24 weeks [16]. We subsequently showed that quasispeceis evolution during the treatment course does not significantly correlate with response to treatment [18].

In the present study, we utilized DNA sequencing analysis to evaluate HCV genetic complexity in sera of two patients, a responder and a non-responder, before initiation of combination therapy to assess changes in amino acid sequence of HVR1 after three and six months following therapy. The HVR1 region is known to be the most heterogeneous in the virus genome and is therefore considered to reflect most accurately the genetic heterogeneity nature of the virus in the serum of the individual patient. We have shown in our previous study that single-stranded conformational polymorphism techniques provides an overall view of quasispecies; however, detailed analysis of nucleotide variation necessitates sequencing of PCR products. Several studies [31,32] have shown that direct sequencing could detect the nature of the heterogeneous population of the virus.

Figure 3. All clones sequenced for HVR1 region from both patients. Region extending from position 384 to 410 is indicated by a rectangle box.

Figure 4. Graph reporting the frequency of changes in each amino acid in all clones isolated from responder and non-responder patients. Amino acids that were found at each position are shown below.
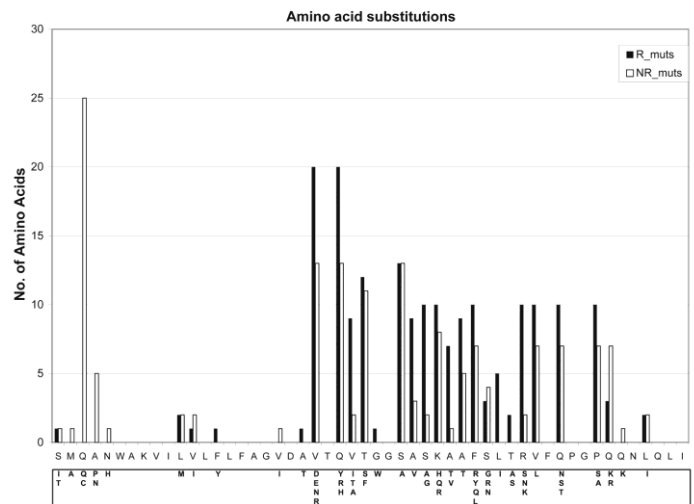
months post therapy. In the responder patient, a major variant present before treatment was replaced entirely by a different predominant variant three months following treatment with a distinct nucleotide and amino acid sequence in the *hvr1* region. Only minor variants remained at six months following treatment, out of which three had the identical amino acid sequence as the pretreatment dominant variant in the putative antigenic region. It was postulated that the antiviral and immunodulatory effects of IFN-α are effective in eliminating predominant HCV species [3,29,33]. Our sequence data corroborate the previously reported studies whereby dominant strains with certain amino acid composition could disappear and minor or new resistant variants may become predominant [14,28,34]. In the non-responder patient, composition of the HCV species differed from that of the responder patient. Three major variants were present initially and were replaced subsequently with a novel major variant following three months of treatment. At six months following therapy, two novel variants emerged, one of which was homologous to one of the major variants in the pretreatment serum in the putative antigenic region. We recognize that, due to the limited number of clones (n = 10) sequenced, not all variants present at each time point were identified. However, the new variants also could have arisen from mutations during replication. Alternatively, drug and immune pressure have eliminated most of the variants except those

In both patients, we found that virus variants present before initiation of treatment bore little resemblance to the variants present at three and six

with certain sequences that are resistant to antiviral activities. The differences in the virus sequence variants present in the representative patient samples prior to therapy and the uniqueness of the changes at the time points following treatment suggest that factors determining the variants present and progress of the infection are not limited to the virus [24]. It is more likely that a combination of host and viral factors determine which variant is most fit to persist within a host. Antibodies to peptide epitopes of HVR1 have been shown, however, to be neutralizing in cell culture systems [35-37]. It is likely that cytotoxic lymphocyte epitopes also play a role in the host immunity. Therefore, changes in amino acid sequences observed in this study could contribute to evasion of the virus from the immune system. Although the underlying mechanisms for viral fitness selection remain to be elucidated, it is likely to include both virus and host interactions.

Acknowledgments

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