Identification of antigenic escape variants in an immunodominant epitope of hepatitis C virus

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Abstract
Numerous investigators have postulated that one mechanism by which hepatitis C virus (HCV) may evade the immune system is through the formation of escape mutants. This hypothesis is based largely on the observed mutability of the viral genome resulting in evolution of diverse quasispecies over the course of infection. That such diversification is a product of viral RNA polymerase infidelity, immune-driven selection or a combination of the two processes has not been addressed. We have examined sequence variability in a specific segment of HCV RNA encoding a known immunodominant region of the viral helicase, amino acids 358–375 of the non-structural 3 protein. Using sequence-specific oligonucleotide probe hybridization and automated DNA sequencing, we report a high frequency of mutations, essentially all of which result in amino acid replacements. To assess the biological impact of such mutations, corresponding chemically synthesized peptides were compared to wild-type peptide in T cell proliferation assays. We observed that a sizeable fraction of such peptides stimulated attenuated or negligible levels of proliferation by peripheral T cells from a chronically infected patient. This observation is consistent with expectations for immune-mediated selection of escape variants at the epitope level. We postulate that such a mechanism may be important in the immunopathogenesis of HCV infections.

Introduction
Hepatitis C virus (HCV) is the leading cause of chronic liver disease due to a persistent infection that lasts over decades despite evidence of humoral and cellular immunity (1). Little is known about the structural basis by which HCV may evade immune responses, although mechanisms involving cytokine shifts and antigenic escape have both been postulated (2–6). We have argued that a key to understanding chronic persistence of HCV lies in an appreciation for the location and diversity of immunodominant epitopes recognized by CD4 T cells (1,7). Not that antibody and CD8 T cell-mediated immune responses are unimportant, but because CD4 T cells drive both humoral and killer cell responses through production of specific cytokines, T<sub>H</sub> cells are likely to play a major role in the outcome of HCV infection.

The non-structural (NS) 3 protein of HCV is a bi-functional molecule with protease and helicase activities critical to viral infection and replication (8,9). We have identified an immunodominant epitope that is comprised of amino acids 358–375 and that is not located in any known functional region of the NS3 molecule (7). We also know that NS3<sub>358–375</sub> represents a cryptic IL-2-producing epitope because, in synthetic peptide form, it stimulates IFN-γ and IL-2 production by peripheral T cells even though intact NS3 fails to stimulate IL-2 secretion (7,10). Both intact NS3 and peptide NS3<sub>358–375</sub> stimulate strong proliferation by peripheral blood CD4 T cells and intact NS3, unlike NS3<sub>358–375</sub>, stimulates high levels of IL-10. Thus, NS3<sub>358–375</sub> represents a cryptic T<sub>H</sub>1 epitope within an antigen to which the overall immune response is productive of several type 2 cytokines. How the T<sub>H</sub>1 response to NS3<sub>358–375</sub> is suppressed when presented in the context of the intact NS3 antigen is unknown. The response to NS3<sub>358–375</sub> is restricted by HLA-DR15 and it is significant that another laboratory has identified, using a partial NS3 construct, the same epitope as immunodominant in HCV-infected, DR15<sup>+</sup> patients (11).

If the segment of NS3 including amino acids 358–375 is immunodominant or at least represents a T cell epitope recognized in vivo, we thought that immune recognition might
lead to sequence variations in the NS3358–375-encoding region of the HCV genome arising from immune selection. We sought to address this possibility by sequencing cloned viral isolates in the NS3358–375 region and determining whether there is any evidence for mutation driven by immune selection in vivo (manuscripts submitted). If a T1 epitope was being recognized in vivo, there might be evidence of localized mutation or escape variants as has been described for HIV and HBV (5,12,13). Previous studies aimed at identifying polymorphisms in HCV isolates have identified and tracked viral quasispecies (14) and examined their significance for cytotoxic T cell recognition (4), but none have tried to resolve the effect on T1 cells of specific HCV variants arising from selective pressure applied by the immune system in response to a specific epitope. We report here on a screening method of identifying viral variants that depends upon sequence-specific oligonucleotide probe hybridization (SSOPH), which allows us to determine the frequency of epitope-specific mutations with a high degree of statistical confidence and to concentrate on those naturally occurring variants that may have functional effects upon the HCV-infected host. Furthermore, we show that many of the variants identified in the NS3358–375 epitope represent so-called loss of function mutations consistent with viral escape. We view these studies as a bridge to understanding how interactions between HCV and its host lead to viral persistence or effective viral elimination.

Methods

Patient materials

Blood was collected over the course of 2 years from an asymptomatic, HCV viremic patient (B3019) with anti-HCV antibodies. Citrated plasma was frozen at −80°C for PCR testing for HCV genomic RNA, whereas peripheral blood mononuclear cells (PBMC), after dilution in tissue culture medium and isolation using Lymphocyte Separation Medium (Organon-Teknika), were stored in the vapor phase of a liquid nitrogen freezer. We have seen previously that the response phenotype of B3019 is typical of those measured in HCV antibody-positive, viremic hemophilic patients. Sample B3019.1 was obtained at ~12 months and B3019.3 at 28 months post-infection. Detection of viral RNA in serum or plasma samples was accomplished using a previously published set of PCR primers specific for the 5’ untranslated region of the HCV genome (15). This patient had no symptoms of HCV infection other than specific antibody and viremia.

Cell culture

PBMC at 5×10⁵/well were cultured with the chemically synthesized peptide NS3358–375 at indicated concentrations for 6 days in 96-well U-bottom trays. Tissue culture medium consisted of RPMI 1640 supplemented with 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 U/ml sodium heparin, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml gentamicin sulfate and 10% screened, pooled human plasma. Responses to peptide were compared to negative controls consisting of medium alone. Cultures were incubated at 37°C in 5% humidified CO₂ for 6 days, pulsed overnight with 1 µCi/well [³H]thymidine and harvested onto glass filters.

Radiolabel incorporation was measured by gas scintillation spectroscopy. Counts from triplicate cultures were averaged and presented as the mean ± SEM. Results from similar kinds of experiments have been presented in detail elsewhere (7,10).

SSOPH analyses

HCV RNA was extracted using RNAzol B (Tel-Test, Friendswood, TX) and converted to cDNA using MMLV reverse transcriptase (Gibco/BRL, Gaithersburg, MD) using hexadecyloxynucleotide primers (Pharmacia Biotech, Piscataway, NJ). A 790 base segment of NS3 corresponding to nucleotides 813–1602 was amplified by nested PCR using primers NS3-3A (5’-CGGACCTTTACCTGTCACG-3’) and NS3-2M (5’-CGCCCTCCCCAAATCCAAGATGG-3’) in the first round of 35 cycles, 2 µl of which was amplified in a second round of 35 cycles using the NS3-2M primer and NS3-3C (5’-CAAGTT- CTTGCCGAGCGCCG-3’). PCR was carried out in 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.1 mg/ml gelatin, 0.02% NP-40 and 5% DMSO with cycle times of 30 s at 94°C for denaturation, 60 s at 55°C for annealing and 60 s at 72°C for extension; an initial denaturation step of 94°C for 5 min and a final extension at 72°C for 7 min were also included. The 790 base PCR fragment was cloned using the T-vector cloning kit (Invitrogen, San Diego, CA) under condition specified by the manufacturer. Recombinant clones were identified by PCR amplification of the NS3 insert. Plasmid DNA was prepared from selected clones using a Wizard Plus Miniprep DNA purification kit (Promega, Madison, WI) according to the manufacturer’s specifications.

Probes were designed to cover nucleotides 1072–1125 of the NS3358–375 encoding region overlapping by 3–4 nucleotides at each end. Probes 1–3 covered nucleotides 1072–1091, 1087–1108 and 1105–1125 respectively. Probe 4 was designed to anneal to nucleotides 1194–1115 of variant clone 1-5, which had an A to G mutation at position 1106 of the coding region.

For SSOPH, ~0.1 µg of plasmid or PCR DNA was denatured and blotted onto nylon Hybond N+ membranes (Amersham, Chicago, IL). Digoxigenin-labeled 20mer probes were added at 10 pmol/ml in hybridization buffer for 40 min at 42°C. Membranes were washed twice in 2×SSC, 0.1% SDS buffer at room temperature for 5 min each, twice at 58°C for 10 min each and twice at 2×SSC at room temperature for 10 min each. The membranes were blocked with 0.1 M maleic acid, 0.5 M NaCl for 30 min. Anti-digoxigenin antibody conjugated with horseradish peroxidase (Boehringer Mannheim, Indianapolis, IN) was added at a concentration of 0.15 U/ml in the same buffer for 30 min at room temperature. Free antibody was washed out twice in 0.1 M maleic acid, 0.5 M NaCl, 0.3% Tween 20 at room temperature for 15 min each. After rinsing membranes in buffer containing 100 mM Tris, 100 mM NaCl, 50 mM MgCl₂ (pH 9.5), substrate consisting of PPD 4 methoxy-4-(3-phosphatophenyl)spiro(1,2-dioxetane-3,2’-adamantane) from Lifecodes (Detroit, MI) was added and the membranes were used to expose autoradiographic films (Kodak, Rochester, NY) under empirically determined conditions. The clones with negative or weak results were judged as candidate variants and flagged for automated sequencing.
DNA sequencing
To sequence selected clones with candidate variant NS3 inserts, plasmid DNA was sequenced in both directions using the HCV 4MF (5' - CCATCCTAACATCGAGGAGG-3') and HCV 3M (5' -TCTGAGTGACACAGTGTTGC-3') primers by dye termination cycle sequencing on an automated 373A instrument from Applied Biosystems (Foster City, CA). Sequencing results were analyzed and compared using the manufacturer's GeneWorks software (Intelligenetics, Mountain View, CA).

Peptides
Based on sequencing results within the region encoding the NS3 358–375 epitope, corresponding 18mer peptides were synthesized using standard Fmoc chemistry on a MilliGen 9050 PepSynthesizer. Crude peptides were then purified by HPLC. Peptide powder was dissolved in a drop of DMSO and adjusted to ~1 mg/ml with RPMI 1640 tissue culture medium as a stock solution. For bioassays, peptide antigens were diluted to indicated concentrations in supplemented tissue culture medium as above.

Results
Epitopes of NS3 that stimulate T cell proliferation
Overlapping, chemically synthesized peptides corresponding to the length of the HCV NS3 molecule were used to stimulate PBMC from donor B3019 in T cell proliferation assays. As we have observed previously, two distinct regions of the NS3 molecule, corresponding to amino acids NS3 274–298 and NS3 358–375, stimulate T cell proliferation strongly (Fig. 1). Such localized areas that induce proliferation also elicit production of IL-2, whereas other epitopes stimulate production of different cytokines including IFN-γ and IL-10 (7). Furthermore, the NS3 358–375 epitope has been identified by others as immunodominant in DR15+ patients like donor B3019 (11) and we have seen that the NS3 358–375 epitope is cryptic in terms of IL-2 production when it is presented in the context of the entire NS3 antigen (7). Because of the intensity of the immune response to NS3 358–375 in vitro, we wanted to determine if there was any evidence for sequence variations localized to the NS3 358–375 region, which might help to explain why this epitope stimulates different responses depending on the context in which it is presented to the immune system, and which could be consistent with in vivo recognition and immune selection leading to viral escape variants.

Identification of NS3 358–375 variants using SSOPH
To identify variant sequences within the NS3 358–375 region, a series of probes based on the wild-type sequence of the Hutchinson strain 1a plasmid (16) was designed (Fig. 2). Using such an approach, it should be possible to identify clones differing from the wild-type sequence by a failure to hybridize and allow us to focus on naturally occurring variants for subsequent sequencing analyses. Three overlapping, digoxigenin-labeled probes were hybridized with filters dotted with an array of control and randomly selected cDNA clones. A fourth probe, P4, with a single substitution at position 1106 was used as a control for sensitivity and specificity. Filters were developed by chemiluminescence using a horseradish peroxidase-labeled anti-digoxigenin antibody (Fig. 2). Using the wild-type probes, a number of point mutations were identified within the NS3 358–375 region as indicated by a failure of one of the probes to bind or as suggested by an attenuated signal. In this array, our positive control consisted of a known wild-type clone (position A1), the negative control was a clone that contained an irrelevant sequence in the same plasmid as the others (A2) and a specificity control was designed to detect a single A to G change at position 1106 (A3). Variants are highlighted in the figure with the corresponding clone sequence indicated, as determined by automated DNA sequencing (see below). Excellent concordance was achieved between the probe hybridization patterns and the positions of the sequence variations we observed. The only
exception was at position A9, where subsequent testing with altered hybridization and development conditions revealed the expected lower signal with the P3 probe relative to P1 and P2 (data not shown). Thus, because of a low frequency of false positives, the SSOPH strategy would at least allow us to determine both the minimal frequency of naturally occurring NS3 encoding region variants and also to focus our sequencing efforts efficiently.

Table 1. Analysis of NS3 mutations by SSOPH

<table>
<thead>
<tr>
<th></th>
<th>SSOP hybridization</th>
<th>cDNA sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>B3019.1</td>
<td>88</td>
<td>25</td>
</tr>
<tr>
<td>B3019.3</td>
<td>99</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td>187</td>
<td>44</td>
</tr>
</tbody>
</table>

Mutational frequency in NS3: 18%.

The NS3 region, 98 clones from an early isolate (B3019.1) and 102 clones from an isolate 2 years later (B3019.3) were hybridized with the wild-type probes (Fig. 2). All such candidate variants were sequenced and the data combined in Table 1. Taken together with our previous results, more than one in five failed to hybridize with at least one of the probes. The mutation in the earlier isolate (B3019.1) was slightly higher (21%) than that found in the later isolate (B3019.3, 15%). Sequencing of clones that failed to hybridize with at least one of the probes revealed wild-type sequence in only two clones, one from each isolate, giving a concordance rate of 94%. It should be emphasized that the observed
Table 2. Naturally occurring NS3<sub>358–375</sub> variant peptides

<table>
<thead>
<tr>
<th>Clone</th>
<th>NS3&lt;sub&gt;358–375&lt;/sub&gt;</th>
<th>Wild-type Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–40</td>
<td>A---------V-----------</td>
<td>V358A I366V Wt</td>
</tr>
<tr>
<td>1–11</td>
<td>G---------------------</td>
<td>V358G Wt</td>
</tr>
<tr>
<td>2–13, 2–15</td>
<td>R-----------------</td>
<td>K360R Wt</td>
</tr>
<tr>
<td>3–95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3–19</td>
<td>-----G---------------</td>
<td>R363G Wt</td>
</tr>
<tr>
<td>3–65</td>
<td>-----K---------------</td>
<td>R363K Wt</td>
</tr>
<tr>
<td>3–21</td>
<td>----V---------------</td>
<td>I366V Wt</td>
</tr>
<tr>
<td>3–28</td>
<td>---------TL---------</td>
<td>I366F F367L</td>
</tr>
<tr>
<td>1–33</td>
<td>---------R----------</td>
<td>C368R</td>
</tr>
<tr>
<td>1–42, 1–50</td>
<td>S--------------</td>
<td>C368S</td>
</tr>
<tr>
<td>1–5</td>
<td>------------------R-</td>
<td>H369R</td>
</tr>
<tr>
<td>1–14</td>
<td>------------------T-</td>
<td>S370T Wt</td>
</tr>
<tr>
<td>1–17, 3–50</td>
<td>P--------------</td>
<td>S370P</td>
</tr>
<tr>
<td>3–115</td>
<td>------------------E-</td>
<td>K371E</td>
</tr>
<tr>
<td>2–29</td>
<td>------------------R-</td>
<td>K372R Nt</td>
</tr>
<tr>
<td>3–15</td>
<td>------------------RR-</td>
<td>K372R K373R Wt</td>
</tr>
<tr>
<td>1–95, 3–22</td>
<td>R---------------</td>
<td>K373R</td>
</tr>
<tr>
<td>3–12</td>
<td>------------------B-</td>
<td>C374G NT</td>
</tr>
<tr>
<td>3–27</td>
<td>------------------R-</td>
<td>C374R NT</td>
</tr>
<tr>
<td>3–3</td>
<td>------------------G-</td>
<td>D375G</td>
</tr>
</tbody>
</table>

Nt, not tested.

Naturally occurring NS3<sub>358–375</sub> variant peptides

Clone NS3<sub>358–375</sub> Wild-type Response

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Effect of naturally occurring NS3<sub>358–375</sub> variants on T cell proliferation

To address the question of whether such naturally occurring sequence variants might have an effect on T cell responses, specifically substituted peptides were used to stimulate PBMC from donor B3019 (Table 2). Nineteen variants were identified with substitutions occurring at 12 of 18 positions within the NS3<sub>358–375</sub> epitope. We did not identify variants with changes at amino acids 359, 361, 362, 364, 365 and 367. The sequence relative to wild-type along with the clone number are shown, and variant peptides are indicated as xyyyz, with x representing the wild-type residue, yyy the position within the NS3 molecule and z the substituted amino acid. Three of these sequences were observed in separate HCV isolates and therefore must have been maintained in vivo; two of such variants stimulated responses below wild-type as discussed below. We also discovered numerous examples of the same mutation in distinct clones.

As summarized in Table 2, variant peptides were compared with wild-type peptide NS3<sub>358–375</sub> in T cell proliferation assays to determine the biological effect of such mutations in an immunodominant epitope. Responses to variant synthetic peptides could be classified in three categories (Fig. 3). Heteroclitic peptides C368S and D375G stimulated responses consistently higher than that of wild-type peptide in dose–response assays. Eight variant peptides stimulated proliferation comparable to that of wild-type NS3<sub>358–375</sub> and consisted mostly of conservatively substituted residues with the exception of R363G. Six peptides were markedly attenuated in their ability to stimulate peripheral T cells from donor B3019 and as such could represent escape variants. Of note were three double substitutions, two of which (V358A I366V and K372R...
We have seen previously, that NS3358–375 peptide will produce peptide, isolated from the context of the intact NS3 antigen. The NS3358–375 epitope may play a role in explaining our results, presented herein, suggest that localized mutation in represents a cryptic IL-2 epitope, the response to which which (I366T F367L) caused a much reduced proliferative K373R) stimulated T cells at wild-type levels and one of

582 Escape variants in HCV NS3 

Discussion

We have shown that naturally occurring mutations in an immunodominant epitope of HCV NS3 affect in vitro T cell responses. Close to 20% of the sequences amplified from viral RNA in the serum of a chronically infected patient had mutations in the NS3358–375 epitope. These ranged from point mutations resulting in single and double amino acid replacements to frameshift mutations and to truncated proteins. In testing the corresponding synthetic peptides resulting from point mutations, we found that almost half the stimulated responses were different from the native sequence, two of which were higher than wild-type while the rest produced much lower or negligible proliferative responses. The fact that essentially all of the changes in nucleotide sequence resulted in amino acid replacements suggests that such mutation is a product of in vivo selection. Coincident with this observation is the fact that the NS3358–375 wild-type sequence represents a Th1 epitope when recognized as a chemically synthesized peptide, isolated from the context of the intact NS3 antigen. We have seen previously, that NS3358–375 Peptide will produce high levels of IL-2 and IFN-γ, whereas intact NS3 protein stimulates large quantities of IFN-γ and IL-10, but essentially no IL-2 at the message level (7,10). Thus, NS3358–375 represents a cryptic IL-2 epitope, the response to which seems to be suppressed in the intact protein antigen. Our results, presented herein, suggest that localized mutation in the NS3358–375 epitope may play a role in explaining our previous observations.

We postulate active selection on the NS3358–375 epitope for a number of reasons. First, we see that virtually all of the clones with nucleotide substitutions would encode amino acid changes; several of these were seen in early and late isolates and therefore must represent stable viral lines. Among these, S370P and K373R were found in both early and late samples, and both stimulated peripheral T cells at least 10- to 100-fold less effectively than wild-type peptide. Further study should seek to determine whether later samples also contain these particular species and whether their frequency of representation increases as McMichael's laboratory has seen with HIV escape mutants (17). Second, because our laboratory has seen examples as well as multiple substitutions at the same position (e.g. S370P and S370T), along with the fact that we have previously seen clustered areas of mutation along the length of NS3 (manuscript submitted), all of these comprise evidence that the changes arise from in vivo selection.

The question of whether mutations in the NS3358–375 epitope arise from selection or stochastic processes is important. We believe this is not due to polymerase mis-incorporation errors because we calculate from parallel experiments with comparable starting amounts of plasmid DNA that random Taq errors are to be expected at a rate of $2.16 \times 10^{-5}$, which is well within the Taq error rate reported by others (18,19), which involve comparison of the ratios of synonymous and non-synonymous mutations over a given sequence; based on these calculations, we observe a 5-fold increase in non-synonymous mutations (replacements) in the NS3358–375-encoding region of the HCV genome significant at the $P < 0.001$ level (manuscript submitted). Analysis of functionally conserved or adjacent regions of NS3 showed fewer mutations than expected, also marginally significant. Taken together these results are consistent with a positive or over-dominant selection model for variation in the NS3358–375 epitope and we postulate that this is driven by immune mechanisms.

There is additional evidence that mutation in the NS3358–375 epitope is under immune selective pressure. In both the early and late isolates, we observed several examples of frameshift mutation due to deletion of the G at position 1085 and to insertion of a G after position 1086, and we raise the question as to whether it is merely coincidental that these mutations coincide with what we predict, based on the work of Diepolder et al. (11) and our own, to be close to the N-terminus of the minimal epitope included within the NS3358–375 peptide. We are currently in the process of defining the N- and C-terminal limits of this epitope by truncation analysis. We also predict, that while the results presented herein will apply to DRB*1501+ or 5–15% of HCV-infected patients, depending on genetic background, similar rules of epitope selection and presentation will pertain to patients with other HLA alleles. Of course, whether such variation in a single epitope actually affects virus survival remains for future investigation.

Other methods for identifying HCV quasispecies include SSCP, which is typically sensitive to changes in a few bases over a somewhat large segment of the HCV genome (14). Our approach enables us to screen variants from a large number of clones and to focus on those regions that may be immunologically important while avoiding complications with more extensive screening of HCV polymorphisms that seems to arise elsewhere throughout the genome. Certainly SSOOH can be technically difficult as well, but using wild-type probes, it facilitates efficient sequencing studies, and provides a simple and conservative means by which to estimate mutation rates in immunologically important epitopes. Based on our other studies and those presented herein, we suggest that the variability in HCV is more extensive than estimates produced using SSCP and, further, that the variability observed arises through immune mediated selection pressure.

Simple escape mutation is difficult to understand as applied to Th cells, which are primarily measured in proliferation assays such as ours. It is easier to see how viral escape mutants could evade antibody binding or cytolytic T cell recognition where the direct consequences of recognition include opsonization or death of the infected cell (4,17). Because CD4 T cells are generally presumed not to play a direct role in viral elimination, the consequences of escape mutations affecting Th cell recognition must be mediated by indirect means through relatively long range effects on other cells, e.g., B cells and CD8 killer T cells. Furthermore, both B
cells and killer T cells depend upon factors produced by Th cells that drive maturation of effector cell function. Therefore, viral escape mutants that cause a loss of CD4 T cell proliferation and presumably attenuated IL-2 production might be expected to lead to a failure in the amplification of an effective anti-viral immune response. Such a scheme does not exclude direct anti-viral effects of cytokines such as IFN-γ also secreted by Th cells. Further investigation is also critical to determine whether the escape mutants we observe can act as antagonists or partial agonists and thus alter the immune programming directed at epitope NS3_358–375.

An important caveat to this work is the extent to which escape mutation in a single immunodominant epitope influences persistence of virus. We have argued that this may be so because of the cryptic Tp1 nature of the NS3_358–375 epitope, but formal proof is lacking. For this reason, we have been careful to describe our results as antigenic escape variants rather than viral escape mutants as would seem to apply to parallel examples with HIV (17). We think it is more likely that our observations with NS3_358–375 are likely to apply to a number of epitopes scattered throughout the HCV structural and non-structural proteins, which in aggregate, may serve as some of the conducive factors that contribute to HCV chronicity despite immune recognition.

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Abbreviations
HCV hepatitis C virus
NS non-structural
PBMC peripheral blood mononuclear cell
SSOPH sequence-specific oligonucleotide probe hybridization

References