BRIEF REPORT

Natural Variability of NS3 Protease in Patients Infected with Genotype 4 Hepatitis C Virus (HCV): Implications for Antiviral Treatment Using Specifically Targeted Antiviral Therapy for HCV

S. Akhavan,[1] A. Schnuriger,[1] P. Lebray,[2] Y. Benhamou,[2] T. Poynard,[2] and V. Thibault[1]

[1]Department of Virology, Hôpital Pitié-Salpêtrière, Assistance Publique–Hôpitaux de Paris, and Université Pierre et Marie Curie Equipe de Recherche 1 Paris 6 University, and [2]Department of Hepato-Gastroenterology, Hôpital Pitié-Salpêtrière, Assistance Publique–Hôpitaux de Paris, and Université Pierre et Marie Curie Paris 6 University, Centre National de la Recherche Scientifique Unité Mixte de Recherche 8149, Paris, France

To analyze the genetic diversity of the NS3 gene in patients infected with hepatitis C virus (HCV) genotype 4 (HCV-4) and to assess the possible effects of the gene polymorphism (or variability) on drug susceptibility, 43 NS3 gene sequences were determined for 53 selected patients with HCV-4 infection. NS3 sequencing, like NS5B sequencing, allowed correct subtype determination. Most residues that were located within the catalytic triad or the NS4-binding region or that were involved in metal binding were highly conserved and identical to those found in HCV genotype 1. Compared with HCV genotype 1, all HCV-4 NS3 protein presented V36L and C16T residue changes that could potentially reduce antiprotease activity. The efficacy of antiprotease in HCV-4–infected patients remains to be proven in large clinical trials.

The prevalence of hepatitis C and the genotype distribution of hepatitis C virus (HCV) vary largely according to geographic localization but, also, according to risk factors. Although large clinical trials have documented the clinical response to conventional pegylated interferon and ribavirin treatment for HCV genotypes 1 (HCV-1), 2 , and 3, sparse data are available for patients infected with HCV genotype 4 (HCV-4). Indeed, the reported response rates for patients infected with HCV-4 are somewhat controversial, and this genotype is usually considered to be of intermediate difficulty to treat, between "easy-to-treat" genotypes 2 and 3 and the more problematic HCV-1. In Egypt and the Middle East, high sustained virologic response (SVR) rates (67%–70%) have been reported in patients treated with pegylated interferon plus ribavirin for 48 weeks [1]. By contrast, in Europe, a lower SVR rate (32%) has been observed [2]. Because of the unsatisfactory treatment efficacy noted for this genotype, specifically targeted treatments against the viral protease (NS3) or polymerase (NS5) are eagerly awaited.

Although HCV-4 strains are found in 15% of all HCV-infected patients treated in our hospital, and although they have become more prevalent over the past decade, few studies have focused on HCV-4 NS3 genetic diversity. The largest study, which used NS3 sequences from public databases, was based on only 14 available sequences [3]. Therefore, we conducted the present study to expand our information about the genetic diversity of the HCV-4 NS3-encoding gene and to analyze the natural diversity of the NS3 protein regarding a possible influence on drug susceptibility. The availability of sequence data from NS5B and NS3 genes from the same strains allowed us to better assess the type and subtype imprint of NS3, particularly in different HCV-4 subtypes.

Methods. A total of 53 HCV-4–infected patients who were referred to our laboratory for HCV genotyping before initiation of anti-HCV treatment were included in the study. Genotyping was prospectively performed using NS5B sequencing [4]. A new method to amplify HCV-4 genomes from nucleotides 3358–4256 (H77 numbering) was developed using specific polymerase chain reaction (PCR) primers NS3-4FF (5′-TGGGCAATGARATCTTGTTCGG-3′) and NS3-4RR (5′-GCARCCRCCATCAGCCAGGAA-3′) and was applied retrospectively on stored frozen plasma samples. In brief, reverse transcription and PCR were performed in a single tube that contained 10 μL of RNA, 2 μL of Superscript III RT/Platinum Taq mix (Invitrogen), and 15 pmol of primers. The cycling conditions were 55°C for 30 min, 95°C for 5 min, and 41 cycles (95°C for 30 s, annealing at 3 different temperatures between 54°C and 50°C for 30 s, and elongation at 70°C for 1 min).

Bidirectional DNA sequencing was performed using the Big Dye Sequencing Kit (version 3.1; Applied Biosystems) with prim-

Received 19 December 2008; accepted 18 March 2009; electronically published 9 July 2009.

Potential conflicts of interest: none reported.

Presented in part: European Association for the Study of the Liver 2008 meeting, Paris, 14–16 February 2008 (poster 57).

Financial support: Action Coordonnée 11–VHC from Agence Nationale de Recherche sur le SIDA et les Hepatites.

Reprints or correspondence: Vincent Thibault, Virology Lab. CERVI, GH Pitié Salpêtrière, 83 Blvd. de l'Hôpital, F-75651 Paris Cedex 13, France (vincent.thibault@psl.aphp.fr).

The Journal of Infectious Diseases 2009; 200:524–7

0022-1899/2009/20004-0007$15.00
DOI: 10.1086/600893

ers NS3-4FF and NS3-4RR on an Applied Biosystems 3100 Sequence Analyzer (Applied Biosystems). Sequence analysis was performed using SeqScape software (version 2.5; Applied Biosystems). Full-length NS3 protease amino acids 1–181 were deduced from the nucleotide sequences and analyzed for particular signatures. From 53 selected samples, 43 HCV-4 NS3 complete sequences could be generated. Ten sequences that covered only part of the protease coding region were omitted to guarantee homogeneous analyses.

Overall, 70 HCV-4 sequences, including 27 available HCV-4 reference sequences (European HCV database), were analyzed and were also compared with 87 available HCV-1 GenBank NS3 sequences. Phylogenetic analyses were conducted using Mega version 4 software (Mega4) [5]. Figure 1 was generated using the WebLogo 3 tool interface [6]. The study protocol was approved by the institutional review board at Hôpital Pitié-Salpêtrière Comité de Protection des Personnes, Ile de France. Sequences are referenced in GenBank (accession nos. FJ775734–FJ775776 for NS3 and FJ795689–FJ795721 for NS5B).

Results. For the purpose of this study, we designed a new PCR approach that was based on 2 specific primers to specifically amplify the NS3-encoding gene of HCV-4 samples. Because of the high genomic variability between strains in the vicinity of this region, the primers were chosen specifically for HCV-4 strains and do not perform well for other genotypes. The 898-nucleotide fragment that was obtained covers the entire coding region of the serine protease domain of NS3 and was subjected to direct sequencing by use of the same primers.

Samples addressed to our laboratory for genotype determination before treatment initiation were arbitrarily chosen to cover most of the HCV-4 subtypes encountered in our practice. As shown in figure 2, and on the basis of NS5B gene sequencing and phylogenic analysis, most of the samples were found to be genotype 4a (19 [44%] of 43 samples), 4d (12 [28%] of 43 samples), and 4f (5 [12%] of 43 samples), but uncommon subtypes (h, k, l, m, p, and r) were also included. Our amplification strategy was successful for most samples; however, for 53 amplified products, only 43 workable and complete sequences (81%) were obtained. Failure of our approach was possibly the result of reduced PCR efficacy associated with either a lower viral load in some samples or hypothetical primer mismatches. Of note, for reasons of quality and to minimize any bias in our phylogenic analyses, incomplete or ambiguous sequences were excluded.

The mean genetic diversity of NS3 calculated using the maximum composite likelihood method was slightly higher among HCV-4 sequences (7%) than among HCV-1 sequences (5.7%). Although the diversity of NS3 was not as high as that of NS5 (16.7% for HCV-4 and 19.6% for HCV-1), phylogenic analyses performed on NS3 nucleotide sequences could classify the different subtypes comparably to those based on NS5B. Among all patients analyzed, a discrepancy between the subtype obtained by NS3 and NS5B sequencing was observed for 3 samples (4a, 4r, and 4p); this finding is possibly explained by a paucity of NS3 sequencing data for these subtypes. Indeed, all subtypes could not be attributed with enough confidence when attribution was based solely on the NS3 phylogenic tree generated with HCV-4 GenBank reference sequences (figure 2*A*). Of note, for 3 strains (NS5-3, NS5-32, and NS5-35), a subtype also could not be firmly attributed according to the NS5B phylogenic tree (figure 2*B*).

Amino acid comparison between HCV-1 and HCV-4 protein sequences identified 29 differences (figure 1). Of those differences, 15 were present only in HCV-4 strains and were never present in available HCV-1 sequences. Of note, amino acid changes at positions 16 and 36 that have been described to

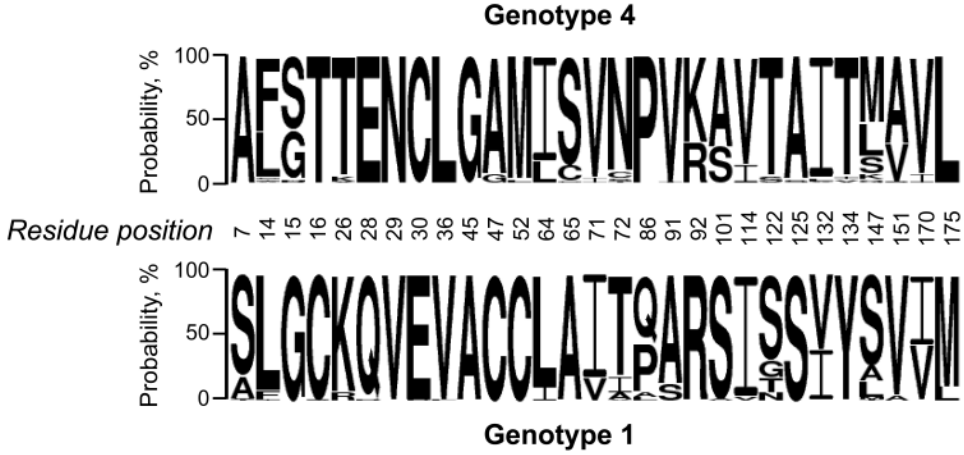

Figure 1. Amino acid signature of the hepatitis C virus (HCV) genotype 4 NS3 protease domain ($n = 70$), compared with that of the HCV genotype 1 NS3 protease domain ($n = 87$). Each graph shows the percentage of each indicated residue in genotype 1 and 4 NS3 sequences. Only positions that were significantly different between genotype 4 and 1 alignments are shown. Letter size is proportionate to the probability at which each residue is found in each genotype alignment.

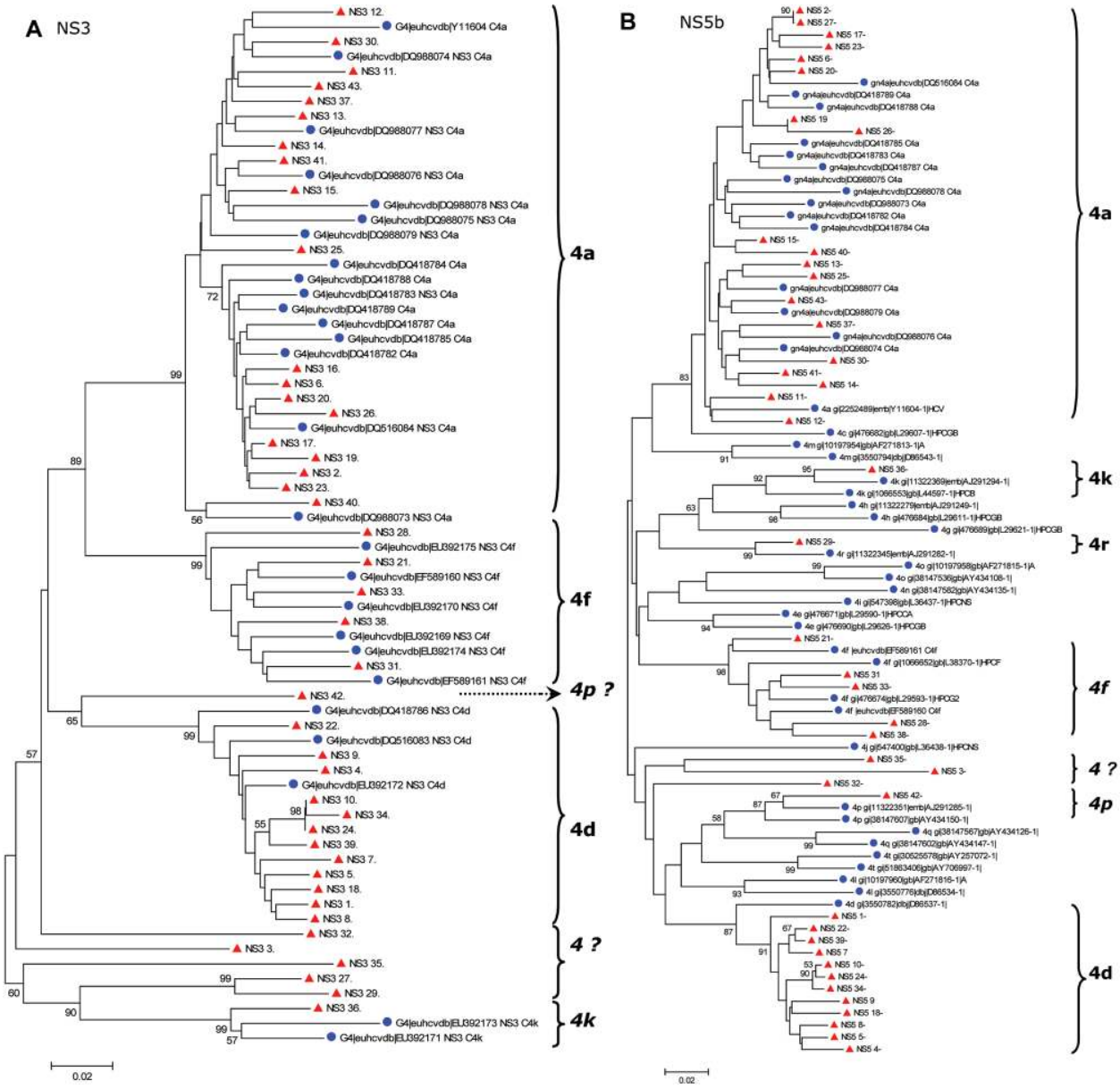

Figure 2. Phylogenic analysis of 70 hepatitis C virus (HCV) genotype 4 NS3 *(A)* sequences with a length of 498 nucleotides and 87 NS5 *(B)* sequences with a length of 210 nucleotides. The evolutionary distances were computed using the Jukes-Cantor model and the neighbor-joining method. The optimal tree is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. *Red triangles,* new sequences generated from patients from the present study; *blue circles,* reference sequences available in GenBank.

confer decreased susceptibility to antiproteases were almost exclusively present in HCV-4 samples. A change at cysteine position 16 has been shown to confer decreased susceptibility to ACH-806, and position V36 seems to be a critical residue for the antiviral efficacy of both telaprevir and boceprevir. Other positions reported as modifying susceptibility to antiproteases were not different between HCV-1 and HCV-4 sequences. Moreover, residues implicated in the catalytic triad, NS4, or metal binding also were not affected, with the exception of position L64I, an NS4-binding site.

Discussion. Although major achievements in hepatitis C treatment have been accomplished during the past decade, the future therapeutic approach will certainly rely on specifically targeted antiviral therapies (STAT-C) against essential viral proteins, especially for subjects who had no response to or had experienced relapses after receipt of current combination treatment with pegylated interferon and ribavirin [7]. Indeed, these new molecules have shown very good potency in in vitro assays, particularly against HCV-1 strains. Interestingly, because of genotype-dependent polymorphisms, the efficacy of antiproteases

is highly genotype dependent [8, 9]. In HCV-1–infected patients, phase 2 and 3 clinical trials evaluating these new protease-targeting compounds evidenced rapid emergence of resistant variants carrying amino acid changes in NS3. Recent data have also demonstrated the presence of a naturally occurring resistant variant, even in treatment-naive subjects [3, 10, 11]. Thus, it seemed interesting to document the prevalence of potentially resistant variants not only in patient candidates for STAT-C or in patients receiving treatment but, also, in the overall population.

Few data are available regarding the susceptibility of HCV-4 to these new compounds, and our objective was to study the NS3 sequences of patients infected with HCV-4. Our main finding is that positions known to confer decreased susceptibility to recent antiproteases were somewhat different in HCV-1 and HCV-4 sequences. Indeed, in HCV-4, all sequences presented with a V36L residue that has been shown to confer decreased susceptibility to telaprevir [12]. By contrast, L36 was present in only 1 (1.1%) of our 87 selected HCV-1 strains and recently was reported in 6 (1.7%) of 362 treatment-naive patients infected with HCV-1a [11]. Of note, a leucine at position 36 is also predominant in HCV genotypes 2, 3, and 5 [3]. The clinical consequences of this naturally occurring change in HCV-4 strains are, to date, unknown, and they will remain so until large therapeutic trials in HCV-4–infected patients are conducted. Indeed, using in vitro either the replicon system or enzymatic activity measurement, Zhou et al [12] reported a slight 2- to 3-fold increase in the telaprevir half maximum effective concentration (EC_{50}) on V36L variant, compared with the wild-type virus. Also, a change in V36A or V36M has been shown to decrease by a factor of 2–3 boceprevir EC_{50} [13]. Therefore, we may speculate that the low-level resistance to antiprotease conferred by V36L may have only little influence on overall treatment efficacy in patients infected with HCV-4. A C16T change present in all HCV-4 strains could also have consequences in terms of ACH-806 activity, because other residues (S, A, and M) have been shown to induce a >10-fold reduction in susceptibility to this molecule [14]. Confirmation, both in vitro and in vivo, will be necessary to clearly measure the influence of these changes on drug susceptibility. Although the number of tested samples is limited, we did not find any residue change at other positions associated with resistance.

Variability of the NS3 gene in HCV-4 is similar to that noted for other genotype [15]. As expected, HCV-4 NS3 nucleotide sequences showed a significant subtype distribution comparable to that seen on corresponding NS5B sequences, and NS3 sequencing allowed correct determination of the subtype (mostly 4a, 4c, and 4f) of our samples. However, the relatively lower genetic variability of the NS3 gene, compared with that of the NS5B gene, may decrease the reliability of subtyping performed using this region of the genome. By contrast, with the exception of 1 position, residues located within the catalytic triad or the NS4-binding region or involved in metal binding are conserved and identical to those found in HCV-1.

The findings of the present study suggest that the efficiency of drugs affected by a change at position 36 and, possibly, position 16 of the NS3 protein may be reduced in patients infected with HCV-4. In vitro studies are now necessary to appraise the real potency of STAT-C on HCV-4 strains, because many patients infected with this up-growing type throughout the world will require first- or second-line treatment. Large clinical trials to better document the efficacy of STAT-C in patients infected with HCV-4 are also eagerly awaited.

References

1. Kamal SM, El Kamary SS, Shardell MD, et al. Pegylated interferon alpha-2b plus ribavirin in patients with genotype 4 chronic hepatitis C: the role of rapid and early virologic response. Hepatology **2007**; 46: 1732–40.
2. Roulot D, Bourcier V, Grando V, et al. Epidemiological characteristics and response to peginterferon plus ribavirin treatment of hepatitis C virus genotype 4 infection. J Viral Hepat **2007**; 14:460–7.
3. López-Labrador FX, Moya A, Gonzàlez-Candelas F. Mapping natural polymorphisms of hepatitis C virus NS3/4A protease and antiviral resistance to inhibitors in worldwide isolates. Antivir Ther **2008**; 13: 481–94.
4. Laperche S, Lunel F, Izopet J, et al. Comparison of hepatitis C virus NS5b and 5′ noncoding gene sequencing methods in a multicenter study. J Clin Microbiol **2005**; 43:733–9.
5. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol **2007**; 24:1596–9.
6. WebLogo 3. Available at: http://weblogo.threeplusone.com. Accessed 8 June 2009.
7. Cholongitas E, Papatheodoridis GV. Review article: novel therapeutic options for chronic hepatitis C. Aliment Pharmacol Ther **2008**; 27: 866–84.
8. Reiser M, Hinrichsen H, Benhamou Y, et al. Antiviral efficacy of NS3-serine protease inhibitor BILN-2061 in patients with chronic genotype 2 and 3 hepatitis C. Hepatology **2005**; 41:832–5.
9. Tong X, Guo Z, Wright-Minogue J, et al. Impact of naturally occurring variants of HCV protease on the binding of different classes of protease inhibitors. Biochemistry **2006**; 45:1353–61.
10. Bartels DJ, Zhou Y, Zhang EZ, et al. Natural prevalence of hepatitis C virus variants with decreased sensitivity to NS3.4A protease inhibitors in treatment-naive subjects. J Infect Dis **2008**; 198:800–7.
11. Kuntzen T, Timm J, Berical A, et al. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. Hepatology **2008**; 48:1769–78.
12. Zhou Y, Bartels DJ, Hanzelka BL, et al. Phenotypic characterization of resistant Val36 variants of hepatitis C virus NS3-4A serine protease. Antimicrob Agents Chemother **2008**; 52:110–20.
13. Tong X, Bogen S, Chase R, et al. Characterization of resistance mutations against HCV ketoamide protease inhibitors. Antiviral Res **2008**; 77: 177–85.
14. Yang W, Zhao Y, Fabrycki J, et al. Selection of replicon variants resistant to ACH-806, a novel hepatitis C virus inhibitor with no cross-resistance to NS3 protease and NS5B polymerase inhibitors. Antimicrob Agents Chemother **2008**; 52:2043–52.
15. Simmonds P, Bukh J, Combet C, et al. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology **2005**; 42:962–73.