Complexity and Catalytic Efficiency of Hepatitis C Virus (HCV) NS3 and NS4A Protease Quasispecies Influence Responsiveness to Treatment with Pegylated Interferon plus Ribavirin in HCV/HIV-Coinfected Patients

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The role of the hepatitis C virus (HCV) NS3/4A protease in ablating the signaling pathway involved in the production of alpha/beta interferon (IFN-α/β) suggests a relationship between NS3/4A proteolytic activity and a patient’s response to IFN-based therapy. To identify viral factors associated with the HCV treatment response, we analyzed the pretreatment NS3/4A protease gene quasispecies composition of 56 HCV genotype 1–HIV-1-coinfected patients treated in our clinic with pegylated IFN (pegIFN) plus ribavirin (RBV). The catalytic efficiency of the dominant (i.e., the most abundant) quasispecies was also assayed for Cardif cleavage and correlated with treatment outcome. A total of 1,745 clones were isolated and sequenced. Significantly less nucleotide quasispecies heterogeneity and lower Shannon entropy values were detected within the responder group (P < 0.05). A correlation was also found between the efficiency of NS3/4A protease Cardif cleavage and therapy outcome. Proteases from sustained responder patients were more efficient at processing Cardif (mean ± standard error of the mean [SEM], 0.8960 ± 0.05568; n = 19) than proteases from nonresponders (mean ± SEM, 0.7269 ± 0.05306; n = 37; P < 0.05). Finally, the amino acid p distance (the proportion [p] of nucleotide sites at which two sequences being compared are different) was significantly shorter in patients with an interleukin-28B (IL-28B) risk allele (P < 0.01), suggesting that IL-28B risk allele carriers exert a lower positive selection pressure on the NS3/4A protease. NS3/4A protease efficiency in cleaving Cardif may be associated with the pegIFN-RBV treatment response, as shown in our cohort of HIV-HCV-coinfected patients. Greater NS3/4A nucleotide heterogeneity and higher Shannon entropy values in nonresponders suggest that less HCV quasispecies complexity may favor a better response to pegIFN-RBV.

Hepatitis C virus (HCV) nonstructural protein 3 (NS3) contains a serine protease that cleaves the virus-encoded polyprotein and inactivates cellular proteins required for innate immunity. HCV NS3/4A protease functions as an antagonist of virus-induced interferon (IFN) regulatory factor 3 activation and IFN-β expression through its ability to block retinoic acid-inducible gen I (RIG-I) and Toll-like receptor 3 signaling by cleaving caspase recruitment domain adaptor-inducing IFN-β (Cardif) and Toll/interleukin-1 (IL-1) receptor domain-containing adaptor-inducing IFN proteins, respectively. NS3/4A protease activity allows the virus to evade the cellular innate immune response, which may influence the subsequent development of adaptive immunity to HCV, virus persistence, and the response to IFN-based therapy (21).

HCV is the causal agent of chronic liver infection, which afflicts more than 170 million people worldwide (http://www.who.int/mediacentre/factsheets/fs164/en/), and one of the leading causes of liver cirrhosis and failure (9). The standard of care for patients with chronic hepatitis C is treatment with pegylated IFN-α (pegIFN-α) plus ribavirin (RBV). However, only 50 to 60% of the patients treated with pegIFN-α and RBV achieve a sustained virologic response (SVR) (18). In human immunodeficiency virus type 1 (HIV-1) patients coinfected with HCV, standard therapy elicits significantly lower rates of SVR. Among patients infected with HCV genotype 1 or 4, the SVR rate is only about 30% (5, 50). Therefore, a considerable effort has been made to develop markers associated with a better response to IFN-based therapies in HCV–HIV-1-coinfected patients.

Similar to other RNA viruses, one prominent feature of HCV is its genetic variability (30). Experimental evidence has demonstrated that HCV populations consist of a distribution of mutant genomes termed quasispecies (25). High mutation rates and the quasispecies dynamics of HCV are intimately related to both viral disease and antiviral treatment strategies (30). Several directly acting antiviral (DAA) agents for HCV infection are in phase 1 to 3 clinical trials (41). The most advanced compounds are inhibitors of the NS3/4A protease and include telaprevir and boceprevir, which are in phase 3 clinical development in combination with pegIFN-α and RBV. However, DAA therapies may be limited by the rapid selection of resistant virus unless administered in combination with...
peIFN-α and RBV. Thus, peIFN is likely to remain a basis of therapy in the near future.

We hypothesize that NS3/4A protease, by disrupting the signaling pathway involved in the production of IFN-α/β, could be implicated in the response to IFN-based therapy. Evasion of innate immune responses likely exerts a negative influence on the subsequent development of adaptive immunity to HCV and possibly contributes to virus persistence and resistance to therapy. We previously established the relationships between genotype, phenotype, and fitness within the NS3/4A quasispecies (13). In the present study, the impact of NS3/4A protease activity and quasispecies complexity on virus clearance after IFN-based therapy was examined in HCV–HIV-1-coinfected patients.

MATERIALS AND METHODS

Patients. A total of 56 samples from 56 HCV–HIV-1-coinfected patients at our HIV clinic were analyzed (Table 1). All patients included in this study were infected with HCV genotype 1 (38 with subtype 1a and 18 with subtype 1b) and our HIV clinic were analyzed (Table 1). All patients included in this study were Caucasians (13). In the present study, the impact of NS3/4A protease activity and quasispecies complexity on virus clearance after IFN-based therapy was examined in HCV–HIV-1-coinfected patients.

TABLE 1. Clinical characteristics of patients with chronic HIV-1–HCV coinfection treated with peIFN-α–RBV therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SVR</th>
<th>Treatment failure</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%) of samples</td>
<td>19 (34)</td>
<td>37 (66)</td>
<td>1.0000</td>
</tr>
<tr>
<td>Mean patient age, yr (range)</td>
<td>40.9 (32–53)</td>
<td>39.4 (23–50)</td>
<td>0.3680</td>
</tr>
<tr>
<td>No. (%) of patients with IL-28B risk allele rs12979860</td>
<td>2 (11)</td>
<td>24 (65)</td>
<td>0.0002</td>
</tr>
<tr>
<td>No. (%) of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>5 (26)</td>
<td>8 (22)</td>
<td>0.6936</td>
</tr>
<tr>
<td>Males</td>
<td>14 (74)</td>
<td>29 (78)</td>
<td></td>
</tr>
<tr>
<td>No. (%) with HCV subtype:</td>
<td></td>
<td></td>
<td>1.0000</td>
</tr>
<tr>
<td>1a</td>
<td>13 (68)</td>
<td>25 (68)</td>
<td></td>
</tr>
<tr>
<td>1b</td>
<td>6 (32)</td>
<td>12 (32)</td>
<td></td>
</tr>
<tr>
<td>Mean CD4+ cell count/μl (range)</td>
<td>553 (309–1186)</td>
<td>546 (265–1165)</td>
<td>0.7423</td>
</tr>
<tr>
<td>Mean ALT activity (U/liter) (range)</td>
<td>77.1 (13–208)</td>
<td>78.9 (15–239)</td>
<td>0.7033</td>
</tr>
<tr>
<td>Mean AST activity (U/liter) (range)</td>
<td>48.0 (19–89)</td>
<td>55.2 (24–157)</td>
<td>0.4516</td>
</tr>
<tr>
<td>Mean HCV RNA level (log10 IU/ml) (range)</td>
<td>5.75 (3.37–7.11)</td>
<td>6.25 (5.04–7.10)</td>
<td>0.0112</td>
</tr>
<tr>
<td>No. (%) of samples with undetectable HIV-1 RNA (&lt;50 copies/ml)</td>
<td>15 (79)</td>
<td>30 (81)</td>
<td>1.0000</td>
</tr>
</tbody>
</table>

* Age, Mann-Whitney U test; IL-28B genotype, Fisher’s exact test; gender, Fisher’s exact test; HCV subtype, Fisher’s exact test; CD4+ T cell count, Mann-Whitney U test; ALT (alanine aminotransferase) and AST (aspartate aminotransferase), Mann-Whitney U test; HCV RNA level, unpaired t test; undetectable HIV-1 RNA, Fisher’s exact test.
Shannon entropy (Sn) value was calculated as $S_n = -\sum p_i \log p_i$, where N is the total number of sequences analyzed and $p_i$ is the frequency of each sequence in the viral quasispecies. $S_n$ varies from 0 (no complexity) to 1 (maximum complexity) (51). To determine possible selective pressures, the proportion of synonymous substitutions to potential synonymous sites and the proportion of nonsynonymous substitutions to potential nonsynonymous sites were calculated by the SNAP software program (19). To estimate codon-specific selection pressures, we used the fixed-effects likelihood (FEL) method, which directly estimates nonsynonymous and synonymous substitution rates at each site (17). The HKY85 nucleotide substitution bias model was used with FEL.

**Genetic screen for determining the catalytic efficiency of HCV NS3/4A proteases.** The catalytic efficiency of the different HCV NS3/4A proteases was determined using a previously described bacteriophage lambda (λ)-based genetic screen (13, 26). A plasmid with the Cardif NS3/4A protease cleavage site, EREVPC/HRPS, was constructed (pcI.Cardif). The Cardif cleavage site was introduced by using pcI.HCVNS4B/NS5Aero (13) as a template and a PCR overlap extension protocol (37). Two fragments containing the Cardif cleavage site were amplified in separate amplifications. For amplification of the 5′ fragment, the cI19 oligonucleotide (sense; 5′-CCATTAACACAAGGACACCTT-3′, positions 19 to 39 of the λ cl repressor) (42) was used with an antisense oligonucleotide containing the Cardif cleavage site 4BSACARDIFR (5′-TGACGGTGGATGCGGCTACTAAGGGATGGCGCGCCTGAACAT-3′). For amplification of the 3′ fragment, cI697R (antisense; 5′-TTCAGGCCACTGCTAGCAT-3′, positions 679 to 699 of the λ cl repressor) was used with a sense oligonucleotide containing the Cardif cleavage site 4BSACARDIFF (5′-GAATGCCTGCATCGACCGTCACTAAGGGATGGCGCGCCTGAACAT-3′). The PCR mixture contained 20 pmol of each primer, 200 μM deoxynucleoside triphosphates, 2.5 mM MgCl2, PCR buffer (10 mM Tris-HCl, pH 8.3, and 50 mM KCl), and 0.5 U Taq polymerase (Promega) in a total reaction volume of 50 μl. Cycling parameters were 1 cycle of denaturation at 95°C for 2 min, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C, with a final extension step of 72°C for 7 min. The 5′ and 3′ PCR fragments were mixed with oligonucleotides cI19 and cI697R, and a second PCR was performed under the conditions described above. The resulting PCR products were digested with NsiI and HindIII and ligated to pcI.HCVNS4B/NS5Aero previously digested with NsiI and HindIII. Escherichia coli JM109 cells containing plasmid plasmid pcI.Cardif were then transformed with plasmid pHCVNS3<sub>1a</sub>Δ<sub>/12,13,14</sub> protease. Transformed cells were grown overnight at 30°C in the presence of 0.2% maltose-12.5 μg/ml of tetracycline–20 μg/ml of ampicillin, harvested by centrifugation, and resuspended to an optical density at 600 nm of 2.0/ml in 10 mM MgSO4. To induce the expression of HCV NS3/4A protease and response to pegIFN-RBV 1b viruses (32%, P = 1.0000, Fisher’s exact test). A significantly higher HCV RNA load was measured in patients who failed treatment (P = 0.0112, unpaired t test). Likewise, IL-28B risk allele rs12979860 was significantly associated with treatment failure (P = 0.0002, Fisher’s exact test). HBV infection was detected in only one sample (from patient 45). No significant differences were found between those who achieved SVR and those who did not with respect to sex, age, HIV-1 viral load, liver enzymes, or CD4 T cell count.

**HCV NS3/4A quasispecies diversity.** A total of 1,745 clones (an average of 31 clones per patient) were isolated, sequenced, and analyzed. Neighbor-joining phylogenetic reconstruction was performed for all NS3 protease nucleotide sequences to determine the evolutionary relationships of the different variants. Sequences from each individual produced a monophyletic group, which was supported by bootstrap analysis (data not shown). Similarly, sequences from subtype 1a and 1b viruses segregated separately. Similar numbers of clones per patient were obtained from those in the SVR and treatment failure groups (32 and 31, respectively, P = 0.8849, unpaired t test). Diversity was assessed by calculating intrasample genetic distances. Mean nucleotide p distances were higher in the group of patients who failed therapy than in the patient group with SVR (0.0127 and 0.0108, respectively; Table 2). However, these values were not significantly different (P = 0.2579, unpaired t test). A wide range of different nucleotide p distances was found within the two groups of patients (0.0034 to 0.0027 and 0.0027 to 0.0247, respectively), suggesting that different diversifying forces are acting in different individuals. Remarkably, we identified a positive relationship between the nucleotide p distance and patient viral load (P = 0.0205, linear regression; Fig. 1A). No significant difference was found between the amino acid p distance of therapy failures and SVRs (0.0072 and 0.0073, respectively, P = 0.9122, unpaired t test). Interestingly, the amino acid p distance was significantly shorter in patients with an IL-28B risk allele (0.0060 versus 0.0188, P = 0.0084, unpaired t test). This difference in the amino acid p distance was not observed at the nucleotide level (0.0136 versus 0.0150, P = 0.5172, unpaired t test). This result suggests that IL-28B risk allele carriers exert a lower positive selection pressure on the NS3/4A protease.

The synonymous-to-nonsynonymous (ds/dn) mutation ratio, a marker of selective pressure, was compared between patients with SVR and those who failed treatment (Table 2). The ds/dn ratio was greater than 1 in the 56 quasispecies analyzed, indicating a preponderance of genetic drift over selection within the coding region studied. However, different values were found for each quasispecies, ranging from 2.51 to 26.71, demonstrating that different selective constraints may be acting on different quasispecies. Patients who failed treatment exhibited a significantly higher mean ds/dn ratio (16.08) than SVRs (10.37) (P = 0.0383, unpaired t test; Table 2). Again, a significantly lower proportion of nonsynonymous mutations to potential nonsynonymous sites (ds) was calculated (0.0037 versus 0.0039, P = 0.7295, unpaired t test). Positive selective pressures were also evaluated using FEL, which directly estimated syn-
that lower treatment baseline NS3 protease quasispecies complexity is associated with SVR.

When sequences were grouped by subtype, a significantly higher Sn value was observed in subtype 1b amino acid quasispecies (0.4974 and 0.6104 for subtypes 1a and 1b, respectively, \(P = 0.0484\), unpaired \(t\) test). No significant differences were observed at the nucleotide level (0.9054 and 0.8616 for subtypes 1a and 1b, respectively, \(P = 0.2404\), unpaired \(t\) test).

**Catalytic efficiency of HCV NS3/4A protease and response to IFN-based therapy.** The catalytic efficiency of the dominant quasispecies of each type (Fig. 2) was assayed for Cardif cleavage using a bacteriophage \(\lambda\) genetic screen (13, 26). The enzymatic activities of variant proteases were evaluated by engineering the Cardif cleavage site in the \(\lambda\) cl repressor. The enzymatic activity was related to the activity of a patient protease variant carrying the inactivating substitution S139A. The distribution of log enzymatic activity values ranged over 2 orders of magnitude, which likely reflects the fact that mutations present in the different proteases affected their catalytic Cardif-cleaving efficiency (Fig. 3A). To further demonstrate the specificity of the genetic screen employed here, the assay was also performed in the presence of 25a, an inhibitor of the HCV NS3/4A protease (22). The enzymatic activity of one of the proteases that displayed a high efficiency level (sample 50) was inhibited by 25a (Fig. 3A). When the log enzymatic activities were grouped by patient response to IFN-based therapy, a significantly higher value was obtained in the SVR group (mean ± standard error of the mean [SEM], SVR versus treatment failure group, 0.8970 ± 0.0556 versus 0.7269 ± 0.0530; \(P = 0.0497\), unpaired \(t\) test; Fig. 3B). Remarkably, within the treatment failure group, some proteases had no Cardif-cleaving activity (samples 90 and 156; Fig. 3A) or very low activity (samples 51, 56, and 63). Proteases with low catalytic efficiency were not observed in the SVR group. Notably, the former low-activity proteases displayed good catalytic efficiency when tested with the HCV NS5A/NS5B cleavage site (data not shown). When proteases were grouped by IL-28B genotype, those obtained from patients carrying the IL-28B risk genotype displayed a lower mean log catalytic efficiency level (mean ± SEM, 0.7013 ± 0.0856 versus 0.8175 ± 0.0456).

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**Table 2. Comparison of HCV NS3 protease coding region quasispecies**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SVR ((n = 19))</th>
<th>Treatment failure ((n = 37))</th>
<th>(P) value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>p distance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleotides</td>
<td>0.0108 ± 0.0006 (0.0027–0.0247)b</td>
<td>0.0127 ± 0.0009 (0.0034–0.0027)b</td>
<td>0.2579</td>
</tr>
<tr>
<td>Amino acids</td>
<td>0.0073 ± 0.0008 (0.0020–0.0169)</td>
<td>0.0072 ± 0.0006 (0.0030–0.0159)</td>
<td>0.9122</td>
</tr>
<tr>
<td>% Heterogeneity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleotides</td>
<td>74.17 ± 5.33 (33.33–100)</td>
<td>85.48 ± 2.04 (52.27–100)</td>
<td>0.0208</td>
</tr>
<tr>
<td>Amino acids</td>
<td>37.88 ± 2.93 (16.67–61.90)</td>
<td>43.00 ± 3.02 (17.31–80.00)</td>
<td>0.2825</td>
</tr>
<tr>
<td>ds</td>
<td>0.0320 ± 0.0056 (0.0046–0.0096)</td>
<td>0.0412 ± 0.0032 (0.0076–0.0856)</td>
<td>0.1311</td>
</tr>
<tr>
<td>dn</td>
<td>0.0032 ± 0.0003 (0.0009–0.0077)</td>
<td>0.0031 ± 0.0003 (0.0007–0.0073)</td>
<td>0.9145</td>
</tr>
<tr>
<td>ds/dn ratio</td>
<td>10.37 ± 1.66 (2.51–26.71)</td>
<td>16.08 ± 1.72 (3.48–23.11)</td>
<td>0.0383</td>
</tr>
<tr>
<td>Sn value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleotides</td>
<td>0.8278 ± 0.0416 (0.4830–1.000)</td>
<td>0.9188 ± 0.0136 (0.6942–1.000)</td>
<td>0.0252</td>
</tr>
<tr>
<td>Amino acids</td>
<td>0.4973 ± 0.0439 (0.1978–0.8062)</td>
<td>0.5525 ± 0.0339 (0.2484–0.8945)</td>
<td>0.3343</td>
</tr>
</tbody>
</table>

*a* Unpaired \(t\) test.

*b* Values are mean ± SEM (range).
However, this difference was not significant (P = 0.2027, unpaired t test). Overall, these results suggest that NS3/4A protease Cardif-cleaving efficiency may be associated with the pegIFN-RBV treatment response in HIV-HCV-coinfected patients.

Next, the relationship between protease enzymatic activity and sequence conservation was investigated. The protease Cardif-cleaving activity was compared to how conserved the protease amino acid sequences were relative to amino acid frequencies in a reference database that included 307 sequences of subtype 1a (consensus 1a in Fig. 2A) and 328 sequences of subtype 1b (consensus 1b in Fig. 2B) (19). A positive linear relationship was found between the conservation of sequences relative to the site-specific database frequencies and their relative catalytic efficiency (r² = 0.1151, P = 0.0105, linear regression; Fig. 4). This result indicates that selected or random deleterious mutations are imprinted in HCV NS3 protease sequences. In contrast, no correlation was found between protease activity and patient HCV viral load (r² = 0.0008, P = 0.8284, linear regression).

Finally, when protease catalytic efficiencies were grouped by subtype, a significantly higher activity level was observed in 1b samples (mean ± SEM, 1b versus 1a, 0.9119 ± 0.0564 versus 0.7238 ± 0.0517; P = 0.0307, unpaired t test). Dominant quasispecies were also grouped by subtype (Fig. 2). Noticeably, subtype 1b virus nucleotide and amino acid p distances were significantly greater than those of 1a viruses (0.0694 and 0.0872 for nucleotide subtypes 1a and 1b, respectively, P < 0.0001; 0.0240 and 0.0272 for amino acid subtypes 1a and 1b, respectively, P = 0.0074, unpaired t test), indicating greater nucleotide and amino intersample diversification among 1b dominant quasispecies.

FIG. 1. (A) Quasispecies nucleotide p distance as a function of the HCV viral load. (B) Quasispecies nucleotide heterogeneity as a function of the HCV viral load. (C) Quasispecies nucleotide Sn value as a function of the HCV viral load. In panels A, B, and C, a significant positive linear correlation was found.

HOWEVER, TREATMENT-INDUCED CLEARANCE OF HCV INFECTION CAN BE AFFECTED BY VARIOUS HOST AND VIRAL FACTORS. BECAUSE A SIGNIFICANT NUMBER OF PATIENTS FAIL TO RESPOND TO CURRENT IFN-BASED THERAPY OR HAVE SIGNIFICANT SIDE EFFECTS, PREDICTING THE TREATMENT RESPONSE IS OF MAJOR INTEREST. IN ADDITION, PATIENTS WITH HIV-1 COINFECTION, PARTICULARLY THOSE INFECTED WITH HCV GENOTYPE 1, HAVE SIGNIFICANTLY LOWER RATES OF TREATMENT RESPONSE THAN HCV-MONINFECTED PATIENTS. SIGNIFICANT FACTORS ASSOCIATED WITH SVR ARE THE BASELINE HCV LOAD AND GENOTYPE. DESPITE HCV QUASISPECIES AND TREATMENT RESPONSE BEING A CONTROVERSIAL TOPIC, HCV QUASISPECIES COMPLEXITY BEFORE THERAPY HAS BEEN INDEPENDENTLY ASSOCIATED WITH TREATMENT RESPONSE (1, 7, 8, 10, 29, 31, 36, 43, 45). HOWEVER, ALTHOUGH PRIOR STUDIES HAVE DEMONSTRATED CORRELATIONS IN THE GENETIC COMPLEXITY OF HCV HYPERVARIABLE REGION 1 (HVR1) AT THE BASELINE AND NONRESPONSE TO IFN-BASED THERAPY (31), OTHER STUDIES HAVE FOUND THAT HVR1 GENETIC COMPLEXITY OR HETEROGENEITY DID NOT CORRELATE WITH HCV SUSCEPTIBILITY TO IFN-BASED THERAPY (23, 38). GENETIC STUDIES RECENTLY IDENTIFIED SEVERAL SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) IN AND NEAR IL-28B (WHICH ENCODES IFN-\( \lambda \)) THAT ARE STRONGLY ASSOCIATED WITH HCV CLEARANCE AND SVR AFTER IFN-BASED THERAPY (15, 35, 46, 49). THIS ASSOCIATION IS HIGHLY SIGNIFICANT FOR GENOTYPE 1-INFECTED PATIENTS AND MORE CONTROVERSIAL FOR NON-GENOTYPE 1 INFECTIONS (27, 28, 34, 35). IMPORTANTLY, HIV-1 INFECTION DOES NOT AFFECT THE ASSOCIATION BETWEEN IL-28B SNPs AND HCV CLEARANCE (2, 32, 34). HOWEVER, THE IL-28B GENOTYPE DOES NOT HAVE A POSITIVE PREDICTIVE VALUE OF 100% FOR SVR AND CANNOT BE USED AS THE ONLY PREDICTOR OF THE IFN-BASED TREATMENT RESPONSE (4).

In this study, low NS3 protease nucleotide quasispecies complexity and high NS3/4A protease Cardif-cleaving efficiency were significantly associated with a successful response to pegIFN-\( \alpha \)-RBV treatment. These findings provide support for a role for the NS3/4A protease in IFN-based therapies. Though the correlation between low protease Cardif-cleaving efficiency and treatment failure is counterintuitive, this result reflects the fact that sensitivity to exogenous IFN is inversely associated with levels of IFN-stimulated genes (ISGs) (39).
Moreover, individuals with IL-28B genotypes associated with SVR have lower pretreatment levels of hepatic ISG expression than individuals carrying the risk genotypes (16). Activation of the endogenous IFN system in HCV-infected patients may hamper the response to IFN-based therapy, most likely by inducing a refractory state of the IFN signaling pathway (39). Nevertheless, alternative interpretations of our findings cannot be discarded because there is a wide variation in the ISG...
expression and induction levels among patients during IFN-based therapy. A recent study has demonstrated that NS3/4A-mediated Cardif cleavage is an important, but not unique, determinant of activation of the IFN system in the livers of patients with chronic hepatitis C (6). We also observed significantly lower protease activity in 1a samples than in 1b samples. Similarly, subtype 1a has been associated with a weaker response to IFN-based therapy than subtype 1b (20).

Another important correlation found in this study is the positive relationship between the catalytic efficiency of the NS3/4A protease and its amino acid conservation. The precise conservation of some NS3 protease segments implies that certain amino acid changes affect enzyme viability. Indeed, mutations associated with drug resistance and CTL escape have been shown to have an impact on HCV fitness (44). Yet, little is known about the distribution of catalytic efficiency among NS3 protease mutations at the population level. Our results demonstrate how positive selection processes, random drift, or purifying negative selection of insufficiently fit variants can imprint HCV NS3 protease sequences and, as a consequence, modulate HCV fitness. Importantly, some mutations can affect the capability of the protease to process Cardif but not its

FIG. 2—Continued.

FIG. 3. Comparative catalytic efficiencies of 56 master HCV NS3/4A proteases based on Cardif cleavage. The catalytic efficiency of each protease variant was compared to that of a patient protease variant carrying the inactivating substitution S139A (0.0). White bars correspond to patients who failed therapy. Black bars correspond to patients with SVR. One gray bar corresponds to sample 50, which was tested in the presence of 20 μM 25a, an inhibitor of the HCV NS3/4A protease (22). Three independent replicates were performed for each sample. Error bars correspond to standard deviations. Catalytic efficiency distribution of 37 master NS3/4A proteases from treatment failure patients (squares) compared to the distribution of 19 proteases from patients with SVR (triangles). The horizontal continuous lines represent the mean values. NR, nonresponders.
ability to cleave viral polyprotein. This finding has implications not only for HCV fitness at the population level but also for therapeutic strategies targeting NS3/4A protease and aimed at limiting viral replication.

This study extends and confirms recent studies in which HCV quasispecies (43), in particular, NS3 quasispecies (8), complexity before treatment was identified as an independent predictor of SVR. A wide range of nucleotide and amino acid quasispecies diversity was observed in both patient groups, those with SVR and those who failed treatment. However, no significant differences were detected between the groups. A positive correlation was found between quasispecies nucleotide diversity and sample viral load, indicating that both high NS3 quasispecies diversity and a heavy viral load are signaling in the same direction: treatment failure.

To the best of our knowledge, this is the first study to investigate the relationship between HCV quasispecies diversity and the host IL-28B genotype. Our results provide compelling evidence of less NS3 protease amino acid quasispecies diversification in patients with an IL-28B risk allele and suggest that IL-28B risk allele carriers exert less positive selection pressure on the NS3/4 protease. Whether the lower amino acid quasispecies diversification in patients with an IL-28B risk allele is restricted to the NS3 protease coding region or affects other viral genomic regions remains to be elucidated. Lower NS3 protease amino acid quasispecies complexity (heterogeneity and Sn value) was also detected in IL-28B risk allele carriers, but this difference was not significant (data not shown). Taking into account the diversification of nucleotide quasispecies, which is essentially almost identical in the two groups of IL-28B genotype carriers, a tendency for less NS3 protease amino acid quasispecies diversification in patients carrying the IL-28B risk allele is clear. Determination of the IL-28B genotype in longitudinal HCV quasispecies previously reported in studies may provide critical insights into the relationship between IL-28B and early spontaneous virus clearance (12), IFN-associated viral clearance (8, 11, 31), HCV emergence following liver transplantation (24), and the association with progression to end-stage liver disease (33).

PI mutations observed in our study include V36A, Q41R, F43S, T54A, V55A, Q80R/K, R155K/T, A156T/V, D168G/N/E, and V170A. Three PI mutations (V36M, V55A, and Q80K) were found as dominant variants. The substitutions V36M and V55A were identified in one sample, whereas the substitution Q80K was detected in four patient samples. Remarkably, one sample (number 140) had two dominant resistance mutations: V36M and Q80K. The V36M mutation confers low-to-moderate resistance to telaprevir, boceprevir, and nablaprevir and higher resistance to telaprevir in conjunction with a R155K or A156T mutation (40). The V55A mutation confers low resistance to boceprevir (47). The Q80K mutation is associated with significantly reduced susceptibility to TMC-434350 and low-level resistance to vaniprevir and danoprevir but wild-type susceptibility to telaprevir and boceprevir (3). The other mutations associated with resistance were observed only as low-level variants, and most of them were detected in one clone per sample; only mutations Q41R and D168G were found in two clones of the same sample. The mutations R155K/T and A156T/V, which are associated with resistance to multiple PIs (41), including telaprevir and boceprevir, were also detected in our study samples. The A1565/T/V mutations are associated with high resistance to telaprevir and boceprevir (40, 47). These findings expand upon those of Chary et al. (8), confirming that the mutations R155K/T and A156T/V are minority variants in PI-naive patients. Using an allele-specific PCR protocol that detects the A156S/T/V substitutions in at least 0.05 to 0.5% of the viral population, we observed that 65% of PI-naive patients had at least one minor resistance variant at this NS3 position (S. Franco et al., unpublished data). Nevertheless, whether drug-resistant mutants present at low levels are associated with an increased risk of PI therapy failure remains to be elucidated.

Our study has some limitations that are worth noting. First, although our conclusions were supported statistically, they had narrow significance. Our results were likely limited by the small sample size, particularly by the small group of patients with SVR. Nevertheless, our results are in agreement with previous work and show a consistent trend. Second, the in vivo approach used to measure the capability of the protease to cleave Cardif only partially mimics what happens in vivo. However, the finding that defective or highly deleterious proteases that cleave Cardif were observed only in patients who failed therapy strongly supports the hypothesis of a role for the NS3/4A protease in the response to IFN-based therapies. Future work should include an evaluation of the pretreatment hepatic activation of endogenous IFN and whether it is related to the catalytic efficiency of the HCV NS3/4A protease in vivo.

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