Evidence for deleterious hepatitis C virus quasispecies mutation loads that differentiate the response patterns in IFN-based antiviral therapy

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Viral quasispecies (QS) have long been considered to affect the efficiency of hepatitis C virus (HCV) antiviral therapy, but a correlation between QS diversity and treatment outcomes has not been established conclusively. We previously measured HCV QS diversity by genome-wide quantification of high-resolution mutation load in HCV genotype 1a patients achieving a sustained virological response (1a/SVR) or a null response (1a/null). The current study extended this work into HCV 1a patients experiencing relapse (1a/relapse, n=19) and genotype 2b patients with SVR (2b/SVR, n=10). The mean mutation load per patient in 2b/SVR and 1a/relapse was similar, respectively, to 1a/SVR (517.6±174.3 vs 524±278.8 mutations, P=0.95) and 1a/null (829.2±282.8 vs 805.6±270.7 mutations, P=0.78). Notably, a deleterious mutation load, as indicated by the percentage of non-synonymous mutations, was highest in 2b/SVR (33.2±8.5 %) as compared with 1a/SVR (23.6±7.8 %, P=0.002), 1a/null (18.2±5.1 %, P=1.9×10⁻⁵) or 1a/relapse (17.8±5.3 %, P=1.8×10⁻⁶). In the 1a/relapse group, continuous virus evolution was observed with excessive accumulation of a deleterious load (17.8±5.3 % vs 35.4±12.9 %, P=3.5×10⁻⁶), supporting the functionality of Muller’s ratchet in a treatment-induced population bottleneck. Taken together, the magnitude of HCV mutation load, particularly the deleterious mutation load, provides an evolutionary explanation for the emergence of multiple response patterns as well as an overall high SVR rate in HCV genotype 2 patients. Augmentation of Muller’s ratchet represents a potential strategy to reduce or even eliminate viral relapse in HCV antiviral therapy.

INTRODUCTION

As with most RNA viruses, hepatitis C virus (HCV) presents a typical nature of viral quasispecies (QS), circulating as a heterogeneous population in infected individuals (Domingo et al., 2012). This is believed to be an essential factor modulating disease pathogenesis, mediating resistance to antiviral therapy and contributing to the difficulty in vaccine development. The intra-patient population heterogeneity of HCV, i.e. HCV QS, has thus been studied extensively for potential relevance to numerous clinical traits. However, it is difficult to reach a consensus in almost all HCV-associated clinical settings. For instance, slow disease progression or favourable response patterns in antiviral therapy appears to be correlated with either high (Farci et al., 2006; Li et al., 2010, 2011; Lyra et al., 2002; Qin et al., 2005; Sullivan et al., 2007) or low (Morishima et al., 2006; Wang et al., 2007) QS diversity or is independent of the HCV QS diversity (López-Labrador et al., 1999; Sandres et al., 2000). These discrepancies are hard to explain without the consideration of methodological shortcomings. One such shortcoming is the low resolution of mutation frequency of no more than 20 % in conventional methods to decipher viral QS diversity, such as gel-based shift assays or cloning/Sanger sequencing (Larder et al., 1993; Van Laethem et al., 1999). Under the
approach of cloning/Sanger sequencing, we have demonstrated that among-patient HCV QS diversity presents an exponential distribution pattern that may account for statistical ambiguity in comparative analysis (Fan et al., 2009). By combining long reverse transcription (RT)-PCR and pyrosequencing, we recently measured HCV QS diversity by direct counting of HCV genome-wide mutations (Wang et al., 2014). At 1% resolution of mutation frequency, the genome-wide HCV mutation load showed a normal distribution among HCV patients and was significantly different between two response patterns associated with IFN-based antiviral therapy: null and a sustained virological response (SVR) (Wang et al., 2014). Using this new index for HCV QS diversity, the current study extended our work to patients who were infected with HCV genotype 2 or relapsed from pegylated (peg)-IFN-α2a and ribavirin combination therapy. Our data provide a full scenario regarding the role of HCV QS in antiviral therapy, as well as interpretation in terms of evolutionary biology.

RESULTS

HCV mutation load among the response patterns in antiviral therapy

The 454 sequencing of 48 samples (Table 1) produced a total of 1,405,351 reads with a mean read length at 437 bp. After the quality control, 1,147,434 reads (81.6%) gave a mean base coverage of 1276.4 ± 570, which was deep enough for a saturated quantification of the HCV mutation load (Wang et al., 2014) (Fig. S1, available in the online Supplementary Material). Using our two-step protocol (Wang et al., 2014), 27,556 mutations were identified in the nearly full-length HCV coding region in the 48 samples. The mean mutation load per patient in the group of 1a/relapse_B (sampled at baseline) was 829.2 ± 282.8 mutations, which resembled the group 1a/null (805.6 ± 270.7) rather than the group 1a/SVR (524 ± 278.8 mutations) from our previous study (Wang et al., 2014).

Table 1. Overview of demographic, virological, disease status and genetic information of the 29 patients enrolled in the study

<table>
<thead>
<tr>
<th>Patients</th>
<th>Response</th>
<th>Age</th>
<th>Sex</th>
<th>Race</th>
<th>Weight</th>
<th>BMI</th>
<th>Genotype</th>
<th>HCV RNA</th>
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<td>2b</td>
<td>6.212 188</td>
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http://jgv.microbiologyresearch.org
et al., 2014). However, the mutation load at the time of viral relapse was significantly decreased in comparison with the baseline ($829.2 \pm 282.8$ vs $348.7 \pm 185$ mutations, respectively; $P = 3.8 \times 10^{-7}$) (Fig. 1a). Under the SVR pattern, the mutation load in HCV genotype 2b was similar to HCV 1a ($517.6 \pm 174.3$ vs $524 \pm 278.8$ mutations, $P = 0.95$) (Fig. 1a). However, the percentage non-synonymous mutation load was very different between these two groups ($33.2 \pm 8.5$ (2b) vs $23.6 \pm 7.8$ % (1a); $P = 0.002$) (Fig. 1d). Overall, the SVR of either HCV 2b or 1a had a much higher percentage non-synonymous mutation load than that from patients with null (18.2 ± 5.1 %) or relapse pattern (17.8 ± 5.3 %) (Fig. 1d). The highest percentage non-synonymous mutation load was found at the time of viral relapse in HCV 1a patients (35.4 ± 12.9 %) (Fig. 1d).

Of all four groups of patients (1a/SVR, 1a/null, 1a/relapse_B and 2b/SVR), 76 patients had IL28B genotypes available from the parent study (Lee et al., 2004). The HCV mutation load was lower in patients with the IL28B genotype CC ($n=29$) than in those with the genotypes CT and TT ($n=47$) (Fig. 2). However, the former had

![Fig. 1. Box and whisker plots showing response-based comparative analysis of HCV mutation load, including total mutation load (a), synonymous mutation load (b), non-synonymous mutation load (c) and the percentage of non-synonymous mutation load (d). Each group of data was represented by box (Second quartile, median and third quartile mutation loads) and whiskers (minimum and maximum mutation loads).](image1)

![Fig. 2. Comparison of the HCV mutation load between patients with IL28B genotype CC ($n=29$, dark grey bars) and genotype CT or TT ($n=47$, light grey bars). There was no statistical difference with regard to synonymous, non-synonymous or total HCV mutation load (left $y$-axis). However, the ratio of non-synonymous load to the total HCV mutation load was significantly higher in the genotype CC group than in the CT or TT group ($P=0.02$, right $y$-axis).](image2)
a percentage of non-synonymous mutation load significantly higher in comparison with the patients with IL-28B genotypes CT and TT (24.3 ± 10 vs 20 ± 6%, \( P=0.02 \)) (Fig. 2).

**Fig. 3.** (cont.)

Genome-wide structural comparison of HCV mutation loads

By including 36 665 mutations identified from 1a/SVR and 1a/null patients (Wang et al., 2014), we redefined
the distribution pattern of a total of 64,221 mutations with regard to their mutation frequencies in the HCV QS population. Again, a power-law distribution pattern was confirmed (Kolmogorov–Smirnov $K = 0.093$, $a = 1.85$, $P = 0.846$) (Fig. S2). On the curve, the low boundary of the number of mutations ($x_{\text{min}}$) was at 445, corresponding to a mutation frequency of 14.5% (Fig. S2). Based on this cut-off value of mutation frequency, genome-wide structural analysis was conducted in three comparisons. First, while the 1a/null and 1a/relapse_B had similar mutational loads ($805.6 \pm 270.7$ vs $829.2 \pm 282.8$ mutations, respectively; $P = 0.78$) (Fig. 1), sliding-window analysis revealed that major differences were located in the high-frequency synonymous load in which six domains achieved statistical significance (Fig. 3a). Secondly, under the SVR pattern, HCV 2b showed a much higher synonymous load, particularly in the low-frequency portion (Fig. 3b). Interestingly, the difference was found mainly in HCV non-structural genes except for NS5a (Fig. 3b). Finally, when comparing 1a/relapse_B with 1a/relapse_R (sampled at the time of viral relapse), the synonymous mutation load accounted for the major difference (Fig. 3c). At the time of viral relapse, the low-frequency synonymous load was shrunk dramatically throughout the entire HCV coding region (Fig. 3c). In the non-synonymous mutational load, a peak in the HCV hypervariable region 1 (HVR1), an 81-bp domain located at the 5’ end of the HCV E2 gene, was lost after viral relapse (Fig. 3c).

**Continuous evolution of HCV in patients relapsing after antiviral therapy**

In 19 patients who relapsed from peg-IFN/ribavirin therapy, phylogenetic analysis using nearly full-length HCV coding sequences confirmed the re-emergence of viral strains with continuous evolution (Fig. 4a). Comparing the consensus sequences derived at the baseline and at viral relapse, there were a total of 2527 substitutions with a mean of 133 substitutions per patient, ranging from 30 to 374. Of these, 14.6% resulted in amino acid changes, with the hot spot located in the HCV HVR1 (Fig. S3). However, there was no apparent difference with regard to the numbers of HVR1 variants reconstructed at baseline and at the time of viral relapse ($5.89 \pm 2.33$ vs $4.68 \pm 2.45$).
$P=0.12)$. On the neighbour-joining tree with all HVR1 variants ($n=201$), the HVR1 displayed either temporal or spatial topologies for individual patients (Fig. 4b).

**Lack of a correlation between treatment outcomes and HCV genomic domains**

Using nearly full-length genomic sequences, all 19 HCV strains in patients relapsed from antiviral therapy belonged to HCV 1a clade 1 in the phylogenetic analysis (data not shown). These sequences, together with 19 and 21 HCV 1a clade 1 strains from 1a/null and 1a/SVR patients, respectively, in our previous study (Wang et al., 2014), were analysed using phylogenetic approaches to explore the potential roles of any HCV genomic domains in the treatment outcomes. The results showed that no HCV domain or nearly full-length coding region had both an association index (AI) and parsimony score (PS) of less than 0.01, suggesting a lack of statistical correlation (Fig. 5).

**DISCUSSION**

Through the combination of long RT-PCR and next-generation sequencing, we have introduced high-resolution HCV genome-wide mutational load as a new index to quantify viral QS population diversity (Wang et al., 2014). The current study is an extension of our previous work by including 2b/SVR and 1a/relapse patients in the setting of HCV antiviral therapy. All four groups of patients (1a/SVR, 2b/
SVR, 1a/null and 1a/relapse) were chosen from the Hepatitis C Antiviral Long-term Treatment against Cirrhosis (HALT-C) trial (Lee et al., 2004) with compatible demographic factors and disease status (Table 1). Both the experimental method and the study design allowed the role of HCV genotypic heterogeneity in the antiviral therapy to be investigated in an unprecedented manner.

At the level of viral consensus sequences, the treatment outcomes (SVR, null and relapse) of IFN-based antiviral therapy did not correlate with the heterogeneity of any HCV domains (Fig. 5). Previous studies have suggested the existence of this kind of HCV domain, such as an IFN-sensitivity-determining region (Enomoto et al., 1996). As the phylogenetic approach did not require any reference sequences, methodological differences might explain the discrepancy between our findings and previous data. Indeed, so far, there is no explicit HCV target documented in IFN-mediated antiviral activity. In in vitro experiments, there are also no consistent mutational pathways found in the HCV genome in response to IFN treatment (Perales et al., 2013). Taken together, the genetic heterogeneity among HCV 1a strains has no apparent role in affecting the therapeutic efficiency of IFN.

Major findings come from the high-resolution quantification of HCV QS diversity. While the HCV mutation loads in the 2b/SVR and 1a/relapse_B groups were analogous, respectively, to those of the 1a/SVR and 1a/null groups, the percentage of non-synonymous mutation load presented an order of 2b/SVR > 1a/SVR > 1a/relapse_B or 1a/null (Fig. 1d). A high mutation load facilitates the evolution of viral populations in response to external pressure, such as antiviral therapy. Given its slightly deleterious nature (Eyre-Walker et al., 2002; Kimura, 1983), however, excessive non-synonymous mutations are detrimental to such an adaptive evolution (Peck, 1994). For the first time, our data thus provide an evolutionary interpretation as to why patients with HCV genotype 2 have overall high SVR rates in comparison with patients with HCV genotype 1a in IFN-based HCV antiviral therapy (Feld & Hoofnagle, 2005). As an index of deleterious mutation load, the magnitude of the percentage of non-synonymous mutation load also explains differential response patterns, at least in HCV 1a patients under antiviral therapy. It is worth noting that the percentage of non-synonymous mutation load correlated with IL28B genotype (Fig. 2), a documented host factor associated with treatment outcomes in IFN-based HCV antiviral therapy (Balagopal et al., 2010). IL28B-mediated inhibition of HCV replication dynamics contributes at least partially to a high deleterious mutation load in the HCV QS population (Zhang et al., 2011a). In addition, in each group of patients, presumptive deleterious mutation loads were not detected using consensus HCV sequences (Table 2), suggesting essential differences in viral QS population and consensus sequences in reflecting evolutionary forces.

Table 2. Selection pressures on the external and internal branches of the phylogenetic trees constructed with nearly full-length HCV coding sequences

<table>
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<th>Response pattern</th>
<th>Genotype</th>
<th>Number of patients</th>
<th>Number of codons</th>
<th>$d_{ns}/d_s$ ($\alpha$)</th>
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<td></td>
<td></td>
<td>Overall</td>
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<td>External</td>
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</table>
The survival of HCV depends solely on its continuous replication, as its genome does not become integrated into the host (Lindenbach & Rice, 2005). In patients relapsed from antiviral therapy, HCV might enter a population bottleneck in which the virus maintains a low-dynamic replication that is under the detection limit of current methods. Supporting this assertion, the low-frequency synonymous mutation load, closely relevant to viral replicative dynamics (Lemey et al., 2007), shrunk significantly at the time of viral relapse (Fig. 2c). During the bottleneck, however, HCV experienced continuous evolution, as evidenced by the phylogenetic trees based on the consensus sequences (Fig. 4a) and HVR1 QS variants (Fig. 4b). More importantly, such an evolution was accompanied by a remarkable increase in the percentage of non-synonymous mutation load (Fig. 1d). Pieced together, these data support the functionality of Muller’s ratchet in a treatment-induced viral population bottleneck (Chao, 1990). When a population bottleneck is long enough, Muller’s ratchet will eventually drive virus extinction through excessive accumulation of a deleterious mutation load. Indeed, the extension of treatment duration in either IFN-based or IFN-free HCV antiviral therapy does enhance the SVR rates (Pearlman et al., 2007; Wyles et al., 2015). Ribavirin might play a similar role by accelerating the accumulation of a deleterious mutation load (Ortega-Prieto et al., 2013). The high predictive value of a rapid virological response in SVR could also be explained as a result of early onset of the viral population bottleneck (Poordad et al., 2008).

Viral relapse remains an issue, even in the era of directly acting antivirals (DAAs) in HCV antiviral therapy (Pawlotsky, 2015). Our findings thus have important implications. If Muller’s ratchet also functions in the setting of DAA therapy, viral relapse could be eliminated by the modulation of treatment duration. Thus, a personalized treatment course of DAA therapy could benefit from measurement of pre-treatment HCV mutation load, monitoring the time of onset of the viral population bottleneck and novel methods capable of detecting low-dynamic HCV replication.

METHODS

Patient samples. The current study consisted of two groups of patients registered in the HALT-C trial (Lee et al., 2004). All patients received 48-week full-dose peg-IFN-x2a/ribavirin combination therapy (Lee et al., 2004). One group comprised 10 patients who were infected with HCV genotype 2b and achieved SVR to the treatment (2b/SVR). The other group had 19 patients infected with HCV genotype 2b, the same protocol was used to amplify a 9085 bp amplicon with the genotype 2b-specific primers QR2_2b_16 (5’-GCCCGAGGTTGACCAC-3’; positions 9310–9325) and WF33_2b (5’-ACGGCAAAAGGTCTCAGCCAT-3’; positions 65–85) for RT and the first round of PCR, and WF5_2b (5’-GATA- GTGTTCTGGGAACGG-3’; positions 138–159) and WR55_2b (5’-ATGCGGGCCCTCCCTCCTC-3’; positions 9203–9222) for the second round of PCR. Nucleotide numbering is according to HCV JCV-6 strain (GenBank accession no. AB047645). RT-PCR amplicons of the expected size were gel purified using a QIAEX II Gel Extraction kit (Qiagen) and subjected to library construction with a Rapid Library Preparation kit (Roche Applied Science), followed by 454 sequencing on a GS/FLX Titanium platform. After the quality control of sequencing reads, the genome-wide HCV mutation load was determined by a two-step protocol as described by Wang et al. (2014).

Structural analysis of the genome-wide HCV mutation load. By including data from previous studies, we first generated a histogram of mutational frequencies, followed by the determination of its distribution patterns (power law, log-normal, exponential and Poisson) using Clauset’s method (Clauset et al., 2009). On the selective pattern, the low-bound point, a lower cut-off (znl) for the scaling region, was calculated through the goodness-of-fit test based on the Kolmogorov–Smirnov statistic and likelihood ratios (Clauset et al., 2009). The value of znl then served as an artificial point to assign mutations into the low- or high-frequency category. Based on a mutation’s frequency (low or high) and nature (synonymous or non-synonymous), sliding-window analyses (windows size, 300 bp; overlap, 100 bp) were conducted over the HCV coding region from the core protein to NS5b. When necessary, statistical significance at each window was determined between the response patterns of IFN-based antiviral therapy.

Phylogenetic analysis. Multiple data analyses were performed using phylogenetic approaches. First, phylogenetic analysis was used to pursue the genetic origin of HCV QS variants that relapsed after the treatment cessation. Under the model of maximum composite likelihood, a neighbour-joining tree was constructed using nearly full-length HCV coding sequences assembled from the relapers in the MEGA program (version 6) (Tamura et al., 2011). Secondly, for each group of patients (Table 2), the potentially deleterious mutation load at the nearly full-length HCV genome level was estimated by a phylogenetic approach (Pybus et al., 2007). In doing so, the best-fit nucleotide substitution model for each group of HCV sequences was determined through JModelTest (Darriba et al., 2012). Selected models were used for the simulation of maximum-likelihood (ML) trees in PAUP* (version 4.0b) (Swofford, 2002). The ML trees then served as the template to compute the mean numbers of non-synonymous (dNS) and synonymous (dS) nucleotide substitutions per site (dNS/dS ratio) under the ‘one-ratio’ model (all branches have the same dNS/dS ratio) from the CODEML program in the PAML package (version 4) (Yang, 2007). Alternatively, the selection pressures on external and internal branches of each ML tree were quantified using the ‘two-ratio’ model, which allows a different dNS/dS for internal and
external branches. Finally, treatment outcomes (SVR, null and relapse) were examined to look for possible correlation with HCV domains, as described previously (Wang et al., 2014). Briefly, the nearly full-length HCV 1a coding region was scanned through a sliding window (size, 300 bp; overlap, 99 bp). Bayesian Markov chain Monte Carlo (MCMC) phylogenetic trees were simulated in the BEAST package under selective models from JModelTest as well as additional parameter settings, including a relaxed molecular clock, a Bayesian skyline coalescent prior and a total run of 10 million generations to reach relevant parameter convergence as estimated by Tracer (Drummond & Rambaut, 2007). The inferred MCMC trees, after discarding the first 10% trees as burn-in, were used as the input to estimate the strength of HCV 1a strain clustering in terms of response pattern with 1000 replications in BaTS (Parker et al., 2008). Based on empirical and simulated data, P values of both the AI and PS of less than 0.01 were considered as having statistical significance (Parker et al., 2008; Zhang et al., 2011b).

**Statistical analysis.** Except for those statistical analyses stated, comparisons were done with a two-tailed, unpaired Student’s t-test, χ² test or Fisher’s exact test. Where applicable, data are expressed as means ± SD. P < 0.05 was considered statistically significant.

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**REFERENCES**


