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Viral sequence evolution in Chinese genotype 1b chronic hepatitis C patients experiencing unsuccessful interferon treatment

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ABSTRACT

The efficiencies of IFN-α based therapy in chronic genotype 1b HCV patients are still unsatisfied to date. The mechanisms underlining treatment failure remain unclear and controversial. To investigate HCV sequence evolution in unsuccessfully treated genotype 1b patients before, during and after the therapy, full-length open-reading-frame of HCV genomes at week 0, week 48 and year 5 in one breakthrough and one nonresponse patients were amplified by reverse transcription (RT)-nested-PCR and sequenced. Mutations were scored and analyzed according to their locations in the HCV genome. HCV sequences in the breakthrough patient displayed significantly more mutations during the one-year therapy than that in the nonresponse patient, with p7 and NS2 encoding regions having the highest mutation rates. Most of the mutations selected during the therapy phase in the breakthrough patient were maintained and few new mutations arose in the four-year post-therapy phase, suggesting these mutations might not compromise viral fitness. Altogether our data suggest that mutations occurred during the therapy phase in the breakthrough patient are likely driven by the action of interferon and ribavirin, and these mutations may have important effects on the responses to interferon based therapy in genotype 1b HCV patients.

1. Introduction

Chronic hepatitis C virus (HCV) infection is a major public health burden and a leading cause of chronic liver disease including chronic hepatitis, liver cirrhosis, end-stage liver disease and hepatocellular carcinoma (HCC), and it is one of the most common indications for liver transplantation worldwide (Ghany et al., 2009). The World Health Organization reports that over 3% of the global population with approximately 180 million individuals is estimated to be infected with HCV. The prevalence was estimated to be 1.6% in the United States (Armstrong et al., 2006), and about 1% in Europe (Sy and Jamal, 2006), but over 3.2% with more than 41 million people infected in China (Xia et al., 1996). In nearly 85% of the cases, the disease progresses into chronicity (Ghany et al., 2009).

Currently, there is no vaccine to prevent HCV infection, and the only available treatment regimens based on interferon alpha (IFN-α) or pegylated (peg)-IFN-α plus ribavirin for 24–48 weeks lead to a sustained virological response (SVR) in about 39.8–40.9% of patients infected by genotype 1 HCV (McHutchison et al., 2009), which is the prevalent HCV genotype in the United States, Europe, and China (Ghany et al., 2009; Lu et al., 2005), while 75–80% for genotypes 2 and 3 (Feld and Hoofnagle, 2005). Treatment failure occurs in the following forms: nonresponse (serum HCV RNA level declines less than 2 log between baseline and week 12, and remains above the detection limit throughout treatment), breakthrough (serum HCV RNA level drops below the detection limit, but rebounds while on therapy), and relapse (serum HCV RNA level drops below the detection limit, but rebounds after the therapy is discontinued) (Dieterich et al., 2009). Unfortunately, molecular mechanisms underlying the failure of the interferon based therapy for chronic hepatitis C (CHC) patients are still unclear. Host factors have been suggested to contribute to the resistance to the

Abbreviations: HCV, hepatitis C virus; CHC, chronic hepatitis C; SVR, sustained virological response; IFN-α, interferon alpha; ORF, open reading frame; HCC, hepatocellular carcinoma.

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interferon based therapy (Gao et al., 2004; Ge et al., 2009; Sarasin-Filipowicz et al., 2008; Suppiah et al., 2009; Tanaka et al., 2009). Recent studies showed that single nucleotide polymorphisms (SNPs) (rs12979860, rs12980275, and rs8099917) near the IL28B gene encoding IFN-λ-3 on chromosome 19 are strongly associated with the clinical outcome of the interferon based therapy, although the molecular mechanisms behind these observations remain elusive (Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009). On the other hand, the strong correlation of SVR rate with HCV genotypes suggests that HCV genomes (viral factors) must also be critical determinants for the outcome of the interferon therapy.

Although it has been suggested that multiple HCV proteins are associated with interferon resistance (Wohnsland et al., 2007), including NS5A (ISDR; PKR-BD; V3; V4 (Jain et al., 2009); IRRDR (El-Shamy et al., 2008)), E2 (PePHD; HVR1; HVR2; HVR3 (Troesch et al., 2006)), NS3/NS4A, and Core (Wohnsland et al., 2007), none of these studies reached a definitive consensus, and there are lots of contradictions between *in vivo* and *in vitro* results (Aus dem Siepen et al., 2005; Brillet et al., 2007; Gale et al., 1998; Gaudy et al., 2005; Jardim et al., 2009; Tsai et al., 2008). Moreover, most of these results were derived from the comparison of the HCV genomic sequences between the SVR patients and the patients who did not achieve SVR, therefore, it is difficult to rule out the contributions of genetic background and host factors of different patients to the outcome of the therapy. Breakthrough patients who initially respond to the interferon based therapy well and the HCV RNA level decreases to the undetectable level, but have reappearance of HCV RNA in serum during the therapy may represent a more unbiased subject to investigate whether the evolution of viral genomic sequences within a single patient play any roles in interferon resistance. Since interferon resistant HCV quasispecies might be selected during the therapy (Cuevas et al., 2008; Enomoto et al., 1995; Kuntzen et al., 2007; Xu et al., 2008), the analysis of the viral genomic sequences before, during and after the therapy may help understand the roles of viral determinants of interferon resistance.

The present study aimed to investigate the evolution of HCV genomic sequences in CHC patients with genotype 1b during the interferon therapy. We compared the full-length HCV ORF sequences before, during and after the therapy, as well as the mutation types, in one breakthrough patient and one nonresponse patient. We found that the HCV genomic sequences in the breakthrough patient displayed significantly more mutations during the 1-year therapy than that in the nonresponse patient, with p7 and NS2 encoding regions having the highest mutation rates. The HCV sequence analysis of a 4-year post-therapy follow-up revealed that a vast majority of mutations selected during the therapy phase in the breakthrough patient were maintained while very few new mutations arose during the 4-year post-therapy span, suggesting the selection of the mutations during the therapy phase may be driven by the action of interferon and ribavirin and these mutations likely did not compromise viral fitness. Our data provide additional evidence to demonstrate that viral determinants may contribute to interferon resistance during interferon therapy of CHC patients.

2. Methods

2.1. Patients and clinical treatment

A total of 19 CHC patients (11 males and 8 females) with genotype 1b who received the treatment of conventional IFN-α plus Ribavirin from August 2003 to July 2004 at the *Department of Infectious Diseases of Ruijin Hospital, Shanghai Jiaotong University School of Medicine*, were included in this study. The patients, all Chinese Han ethnicity, were treatment-naïve prior to IFN-α and Ribavirin, and were free of human immunodeficiency virus (HIV) infection and other concomitant liver diseases, such as hepatitis B virus (HBV) or other hepatotropic virus infections, alcohol abuse, autoimmune hepatitis and hereditary liver diseases. The study had been approved by the *Ethics Committee of Shanghai Ruijin Hospital* in accordance with the Helsinki Declaration, and written informed consents were obtained from all patients. They received conventional IFN-α three times a week at a dose of 6 million units subcutaneously, and ribavirin was administered orally twice a day to a total dose of 1000 mg (body weight <75 kg) or 1200 mg (body weight ≥ 75 kg) for 48 weeks and were followed up at least 6 months after completion of one-year therapy. Two cases (body weight <75 kg) were selected retrospectively for another four-year follow-up after termination of therapy: one was a breakthrough patient (patient 002), and the other was a nonresponse patient (patient 007). No dose modification was performed in the two patients during anti-viral treatment. Serum samples were collected at multiple time points during and after therapy, and were stored at -80 °C. Samples, collected at week 0 (baseline), week 12, week 48 and year 5, were used for HCV sequences analyzing in this study.

2.2. HCV RNA quantification and genotyping of CHC patients

HCV RNA quantification was performed using a one-step quantitative HCV RT-PCR kit (PG Biotech, Shenzhen, China) with the standard-curve of Taqman probe method, and the detection limit was 1000 copies/ml. HCV genotyping was performed using the HCV genotyping gene chip kit (Realchip Biotech, Ningbo, China) according to the manufacturer's instructions.

2.3. HCV RNA extraction, RT-PCR, full-length HCV ORF amplification and sequencing

Total RNA was extracted from 140 μl of serum samples, using QIAamp Viral RNA mini kit (QIAGEN, Hilden, Germany), and dissolved in 60 μl of elution buffer and stored at -80 °C until use. The sequencing of HCV genomes was performed following a previously published protocol with some modification (Yao and Tavis, 2005). In brief, HCV RNA was reverse-transcribed (RT) into cDNA using TaKaRa RNA LA PCR™ Kit (AMV) Ver.1.1 (TaKaRa, Dalian, China) with HCV specific primers. Nested-PCR primers spanning the full-length HCV ORF sequences used in this study were listed in Supplementary Table S1. All PCR fragments were purified using the AxyPrep DNA Gel Extraction Kit (Axygen, Hangzhou, China) and then sequenced. Consensus sequences for the full-length HCV ORF were obtained by aligning overlapping sequences of PCR products as described previously (Yao and Tavis, 2005). A total of 7 full-length HCV genotype 1b sequences generated in this study had been deposited in GenBank under the accession numbers from GU451218 to GU451224.

2.4. Sequence analysis

Vector NTI Advance Version 10.3 (Invitrogen, Carlsbad, CA, USA) and BioEdit Sequence Alignment Editor Version 7.0.9.9 (Ibis Biosciences, Carlsbad, CA, USA) (Hall, 1999) were used to edit, assemble and align sequences. To insure integrity of the sequence data, appropriate precautions were taken as recommended (Learn et al., 1996; Yao and Tavis, 2005). Amino acid sequences were deduced from nucleotide sequences. Mutations were rechecked through the chromatogram. If there were two peaks at the same residue, HCV RNA would be PCR amplified again and resequenced. By evaluation of numerous overlapping sequence fragments, only clear changes were identified as mutations. Mutation rates at different time points were calculated at nucleotide and amino acid levels in the 10 viral protein encoding regions, respectively. The