Original Article

Evolution of viral RNA in a Chinese patient to interferon/ribavirin therapy for hepatitis C

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Objective: The combination of interferon (IFN) and ribavirin (RBV) is the standard therapy for hepatitis C virus (HCV) infection. HCV genotype 2a has proved more amenable to the therapy, but its efficacy is yet limited. This study aimed to investigate the mechanism of the poor response in a case of HCV genotype 2a infection.

Methods: We analyzed dynamic change of HCV RNA from a patient, infected with HCV genotype 2a, showing a poor virological response to IFN/RBV as judged 12 weeks after initiation of the therapy by HCV clone sequencing. Then we constructed subgenomic Japanese fulminant hepatitis-1 (JFH1) replicon and different chimeric replicons with humanized Gaussia luciferase gene. The chimeric replicons were derived from subgenomic JFH1 replicon, in which the NS5A region was replaced by the patient's sequence from the pre/post-treatment, and the chimeric replicons' susceptibility to IFN were evaluated by relative Gausia Luciferase activity.

Results: The pretreatment HCV sequences appeared almost uniform, and the quasispecies variation was further more simplified after 12 weeks of therapy. Besides, the quasispecies variation seemed to be more diversified in the NS5A, relatively, a region crucial for IFN response, and each of chimeric replicons exhibited distinct response to IFN.

Conclusions: During the course of the chronic infection, HCV population seems to be adapted to the patient's immunological system, and further to be selected by combination of IFN/RBV therapy, indicating quasispecies may completely eliminated by addition of other drugs with targets different from those of IFN. In addition, each different response of chimeric replicon to IFN is most likely related to amino acid changes in or near the IFN-sensitivity determining region (ISDR) of NS5A during chronic infection and IFN/RBV therapy.

Key Words: HCV-2a; IFN; poor response; JFH1; chimeric replicon

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Introduction

Hepatitis C virus (HCV) is a single stranded RNA virus of positive polarity belonging to the Flaviviridae family. The about 9.6 kb single-stranded viral RNA genome expresses a single polyprotein consisting of about 3,000 amino acids, which undergoes proteolytic cleavage with the help of host and virally-encoded proteases. This results in production of up to 10 different viral proteins, both structural and non-structural (NS) proteins.

HCV infection affects 170 million people worldwide (1). It is estimated that 20% of HCV-infected patients will develop cirrhosis, with the associated risks of developing liver failure and/or liver cancer (2,3). The primary aim of anti-HCV therapy is permanent eradication of the virus or a sustained viral response, finally to prevent development of cirrhosis and hepatocellular carcinoma. Currently,

the standard therapy for chronic HCV infection is the combination of pegylated interferon (PEG-IFN) α-2a or α-2b and ribavirin (RBV). The combination of both drugs has a significant effect on virological and histological responses, especially to the patient with HCV genotypes 2, reaching a sustained viral response rate in 80% of patients in randomized controlled trials (4-6).

However, even with genotype 2 HCV infection, the combination therapy for 24 weeks failed to eradicate the virus in about 20% of patients, although the reason is still unclear. To investigate the mechanism of poor response in a case of genotype 2a, we analyzed the HCV RNA sequences before and after standard therapy.

Materials and methods

Patient

One patient chronically infected with HCV-2a, enrolled in HCV clinical trial at Beijing Ditan Hospital, whose diagnosis had been made based on anti-HCV antibody detection, HCV genotype determination according to the method by Okamoto *et al.* (7) and clinical follow-up, was treated with PEG-IFN-2b (1.5 μg/kg body weight, once a week, subcutaneously) and RBV (600-800 mg daily, *per os*), according to a standard treatment protocol. The patient was confirmed negative for hepatitis B surface antigen and human immunodeficiency virus (HIV). Plasma samples were collected from the patient during and after 12 weeks of treatment. The quantification of serum HCV RNA titers was performed by reverse transcription-polymerase chain reaction (RT-PCR) with an internal RNA standard derived from the 5' noncoding region of HCV (Amplicor HCV Monitor test, version 2.0, Roche Diagnostics, Tokyo, Japan). The viral load before treatment (0 week) and during treatment (12 weeks) is 1.95×10^7 and 2.19×10^3 IU/mL, respectively.

The study protocol was approved by the Ethic Committee of Beijing Ditan Hospital, and written informed consent was obtained from the patient before the treatment.

Sequence analysis

RNA was extracted from 150 μL of plasma with ISOGEN-LS (Wako Corporation, Japan). For amplification of different region of the HCV genome, covering NS4B, NS5A and NS5B, the extracted RNA was reverse-transcribed and amplified using PrimeSTAR Max DNA polymerase (Takara Bio Inc., Japan). The sets of primers are: 8S [5'-AAACACATGTGGAACTTCATC-3'; sense nucleotide (nt) 5,639-5,659] with 8As (5'-GGAT GGATCTGTTAGCATGGAC-3'; sense nt 6,868-6,889), 9S (5'-ACTGGTATCATGACCACACG-3'; sense nt 6,416-6,435) with 9As (5'-GCGTCCTGTCAAAA GTTACC-3'; sense nt 7,823-7,842), and 10S (5'-GACT CCGTCGTGTGCTGCTC-3'; sense nt 7,649-7,668) with 10As (5'-CCTGGTCA TGGCCTCCGTGAA-3'; sense nt 8,681-8,701), and each pair of primers produces fragment 8, 9 and 10, covering NS4B, almost whole part of NS5A and NS5B, respectively. The PCR products were cloned using Mighty Cloning Reagent Set (Blunt End) (Takara Bio Inc., Japan) and sequenced.

Plasmid construction

pFK-Lu-JFH1 and pFK-Lu-JFH1/GND were generated from subgenomic replicon pSGR-JFH1 (AB114136.1) and pSGR-JFH1/GND (8) [both plasmids were kindly provided by Takaji Wakita (Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan)], respectively, in which *Neo* gene was replaced by humanized Gaussia luciferase (*hGLu*) gene. In brief, an HCV replicon expressing hGLu was constructed by replacing *Neo* gene of plasmid pSGR-JFH1 with the luciferase gene from *Gaussia princeps*. The T7 promoter and the HCV 5' untranslated region (UTR) were amplified by PCR using JFH1-*Eco*R I (5'-GGAATTCTAATACGACTCACTATAG-3') and JFH1-hGL (5'-ACTTTGACTCCCATTTTGGTTT TTCTTTGAGG-3') as primers and pSGR-JFH1 as a template, while the *hGLu* gene was amplified by PCR using hGL-ATG (5'-ATGGGAGTCAAAGTTCTGTTTGC-3') and hGL-T (5'-TTAGTCACCACCGGCCCCCT-3') as primers and pRNAi-GL as a template (Takara Bio Inc., Japan). These two PCR products were used as templates for second round PCR to combine the 5'UTR with *hGLu* gene. The second round PCR product was digested with *Eco*R I and ligated with the linearized pSGR-JFH1 by digesting with *Eco*R I and *Pme* I. The newly generated pSGR-Lu-JFH1 and pSGR-Lu-JFH1/GND were double digested with *Age* I and *Eco*R V, respectively, and put into linearized pFK with same restriction enzyme sites.

The next step was to generate chimeric replicon of pFK -Lu-JFH1/0-37, pFK-Lu-JFH1/12-26 and pFK-Lu-JFH1/12-29, using pFK-Lu-JFH1 as template. Briefly, both clones of fragment 9 (clone 0w-37, 12w-26 and 12w-29) and pFK-Lu-JFH1 were digested with restriction enzyme *Bae* I, each relevant fragment was purified by gel extraction

