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Prevalence of S282T mutation in different genotypes of hepatitis C virus from DAA-treated naïve Chinese patients who were chronically infected with HCV

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Abstract. – OBJECTIVE: Although direct-acting antiviral agents (DAAs) for treating hepatitis C virus (HCV) infection have not yet been approved for clinical application at present in China, the development trend is irresistible. DAAs-containing therapeutic regimens have been approved and others are also under development worldwide. In vitro studies have shown that S282T mutation in the NS5B region of HCV is involved in DAAs resistance. The aim of this study was to investigate naturally occurring resistance mutation of S282T in different genotypes of HCV from DAA-treated naïve Chinese patients who were chronically infected with HCV.

PATIENTS AND METHODS: 250 Chinese patients chronically infected with HCV were enrolled in this study. All subjects were naïve to DAAs. Direct sequencing of HCV NS5B region was performed in all enrolled patients.

RESULTS: 70.4% (176/250) cases were infected with HCV genotype 1b, 19.2% (47/250) were 2a, 4.0% (11/250) were 6a, 3.6% (10/250) were 3b, 1.6% (4/250) were 1a and 1.2% (3/250) were 3a. Genotype 4, 5 and 7 were not observed. The S282T mutation was not found in any of the cases.

CONCLUSIONS: The results showed that the S282T mutation was not prevalent in DAA-treated naïve Chinese patients who were chronically infected with HCV.

Key Words

Hepatitis C virus, S282T, Genotype, NS5B region, DAAs.

Introduction

About 115 million persons have a history of HCV infection and 80 million have been chronically infected[1]. In China, HCV emerges as one of the most commonly reported pathogen (ranked as the forth) and represents the leading cause of death and morbidity[2]. At present, peginterferon and ribavirin (PegIFN/RBV) are applied as the standard of care for the treatment of HCV infection before DAAs are approved for clinical use in China[3]. This therapeutic regimen is associated with many side effects in at least 10% of patients[4,5]. Moreover, only half of the patients infected with genotype 1 present a sustained virologic response. However, about 60-80% of patients with genotype 2 or genotype 3 exhibit the response[6,7].

DAAs offer favorable outcomes with fewer adverse reactions[8-10]. However, the emergent of drug resistance severely affects the therapeutic efficacy and now the Q80K mutation testing is recommended for patients with HCV genotype 1a infection who receive Simeprevir treatment at baseline[11]. Sofosbuvir (SOF) is the first-in-kind nucleotide analog inhibitor that targets the HCV NS5B polymerase and presents pan-genotypic antiviral activity with a high barrier to resistance[12,13]. SOF eliminates hepatitis C in around 80% of people and was approved by FDA as Sovaldi for the treatment of chronic HCV infection[14]. S282T substitution in the NS5B region of HCV is the primary mutation selected by SOF *in vitro* studies[15]. Although drug resistance mutation is frequently observed as it appears after administration of an antiviral drug, it may pre-exist naturally in treatment-naïve patients due to the inherent high genetic diversity of RNA viruses[16-18]. To date, there are fewer researches investigating the prevalence of S282T mutation in Chinese patients infected with HCV. The aim of this study was to investigate the naturally occurring resistance mutation of S282T in different genotypes of hepatitis C virus from DAA-treated naïve Chinese patients who were chronically infected with HCV.

Patients and Methods

Patients

250 patients in our hospital between the 2013 and the 2016 were included in the study. 76% (190/250) of the subjects were treated with Pe-

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gIFN/RBV, while none had ever been treated with DAAs for hepatitis C. The viral load of everyone was higher than 1000 IU/mL by a highly sensitive Real-Time PCR assay. For NS5B sequencing, 3-5 mL EDTA-anticoagulated peripheral blood plasma was prospectively collected from each patient. The NS5B region was sequenced to further subtype HCV strains and identify the base mutation at position 282. Data were analyzed with the ClustalW tool. The study was approved by the Ethics Committee of Renmin Hospital of Wuhan University. All patients enrolled in this study had written the informed consents after a detailed explanation.

Nucleic Acid Extraction and Reverse Transcription

Viral RNA was extracted from 140 μL plasma using the QIAamp Viral RNA Mini KIT (Qiagen, Hilden, Germany) in accordance with the instructions. The ratio of 260/280 was used to assess the purity of RNA. A ratio of 2.0 was generally considered as "pure" for RNA. Viral RNA was stored at -80°C. Reverse transcription of first strand complementary DNA (cDNA) was synthesized with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) from HCV RNA according to the manufacturers' instructions. In detail, 0.1 ng-5 μg of HCV RNA, 1 μL of random hexamer primer and nuclease-free water were mixed together with a volume up to 12 μL in an RNase-free tube, centrifuged briefly and incubated for 5 min at 65°C in a thermocycler, and then chilled on ice for 1 min. The following components in the indicated order were added to the tube: 4 μL of 5X reaction buffer, 1 μL of RiboLock RNase inhibitor (20 U/μL), 2 μL of 10 mM dNTP mix, 1 μL of RevertAid M-MuLV RT (200 U/μL), mixed gently and centrifuged briefly, and incubated for 5 min at 25°C, 60 min at 42°C, and 5 min at 70°C.

Amplification of the HCV NS5B Region and Sequencing

2 μL HCV cDNA template was used for the PCR amplification, which was performed as follows: 95°C for 5 min; 95°C for 30 s, 56°C for 30 s, 72°C for 60 s, 35 cycles; 72°C for 10 min, 4°C for storage. Two pairs of primers designed as previously described were used both in PCR and sequencing, spanning NS5B region from 116th to 346th amino acid, were as follows: F1 5'-TTAACCACATCMRCTCCGTGTG-3', R1 5'-GTCAAAGCAGCGGGTRTCRTA-3'; F2 5'-ACACCCGCTGYTTYGACTC-3', R2 5'-GTACCTGGTCATAGCYTCCGTRAA-3'[18]. Degenerate base symbol: M, A and C; R, A and G; Y, C and T. The amplification products were electrophoresed on a 2.0% agarose gel in Tris base-acetic acid-EDTA (TAE) buffer solution and purified by AxyPrep DNA Gel Extraction Kit (Corning Inc., Corning, NY, USA). ABI BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) was used for sequencing in a system consisting of 5 μL of purified DNA. 1 μL of Nuclease-Free Water, 1 μL of 2.5X BigDye, and 1.5 μL of 5X BigDye buffer, which were then incubated as follows: 98°C for 2 min; 96°C for 10 s, 50°C for 5 s, 60°C for 4 min, 25 cycles; 4°C for storage. Sodium acetate-Ethanol precipitation was used for purifying the reaction products. 10 μL of highly deionized formamide was added to the products and analyzed with an automatic sequencer (ABI PRISM 3500Dx genetic analyzer DNA Sequencer, Applied Biosystems, Foster City, CA, USA). All acquired sequences were verified by Bi-directional sequencing and assembled together for further analysis.

HCV Genotyping

Phylogenetic analysis was performed for HCV genotyping. All nucleotide sequences from HCV strains were aligned using ClustalW embedded in MEGA7.0.14 software with confirmed references of different subtypes available in the NCBI reference sequence database (https://www.ncbi.nlm.nih.gov/refseq/). A Neighbor Joining tree was constructed with MEGA7.0.14 using the Neighbor Joining method. P-distance was used as an evolutionary model and the reliability of branch was assessed by bootstrap analysis with 1000 replicates. Bootstrap values of 60% were used as the cut off point for cluster analysis. The GenBank accession numbers for reference sequences to determine the HCV genotypes were as follows: EF_407419 (subtype 1a), NC_004102 (subtype 1a), M_62321 (subtype 1a), EU_781827 (subtype 1b), M_58335 (subtype 1b), HQ_639944 (subtype 2a), AY_746460 (subtype 2a), AB_030907 (subtype 2b), AB_661382 (subtype 2b), AF_046866 (subtype 3a), JN_714194 (subtype 3a), D_49374 (subtype 3b), JQ_065709 (subtype 3b), NC_009825.1 (subtype 4), NC_009826.1 (subtype 5), DQ_480513 (subtype 6a), Y_12083 (subtype 6a), D_84262 (subtype 6b), NC_030791.1 (subtype 7).

S282T Analysis

Reference sequence NC_004102 was used as a coordinate sequence for the amino acid position 282 of HCV NS5B region. All other nucleotide sequences were aligned with this reference sequence using the ClustalW method which is embedded in the Mega 7.0.14 package (Figure 1).

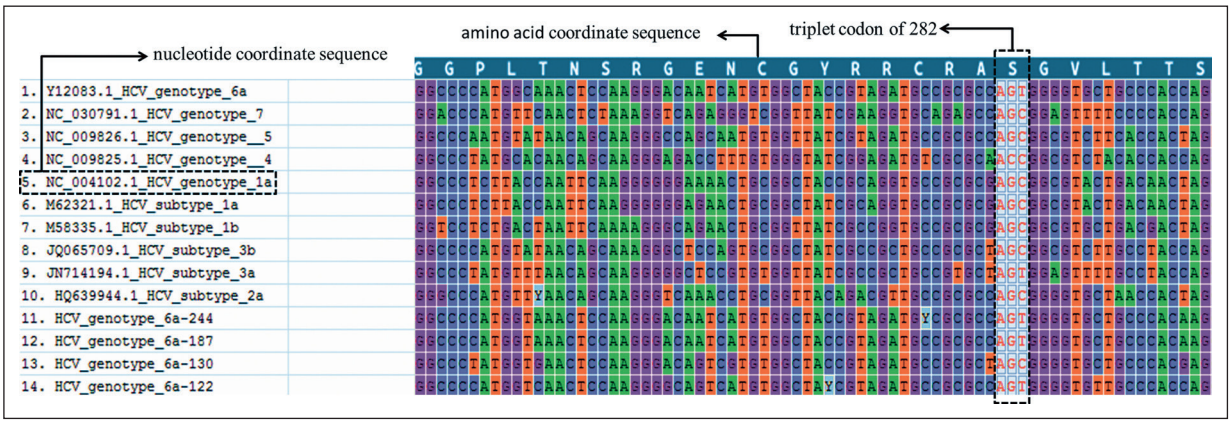

Figure 1. Analysis of triplet codon of amino acid position 282 of HCV NS5B region. Reference sequence NC_004102 was served as nucleotide coordinate sequence, and the expression protein was served as amino acid coordinate sequence.

Results

Two hundred and fifty patients were enrolled in this study. The clinical and virological characteristics of the patients were provided in Table I. 8 out of 250 (3.2%) patients were co-infected with HBV and did not receive treatment with a nucleotide analogue, while 96.8% (242/250) were HCV mono-infected. 76% (190/250) patients were naïve to PegIFN and RBV, while none had ever been treated with DAAs such as Simeprevir or Sofosbuvir.

HCV genotypes 1b (70.4%) and 2a (19.2%) accounted for the majority of the patients who were chronically infected with HCV in China, followed by genotype 6a (4.4%), 3b (3.6%), 1a (1.6%), and 3a (1.2%). Genotype 4, 5, and 7 were not observed in Chinese patients chronically infected with HCV in this work (Figure 2). The HCV genotype for 8 patients co-infected with HBV were 1b (87.5%) and 3b (12.5%).

Table I. Patients' characteristics.

Characteristics	No. of patients (n, %)
Male	142 (56.8)
Female	108 (43.2)
Age	47.2±16.3
HCV viral load, $\log_{10}$ (IU/mL)	4.7±1.6
No. of HBV/HCV co-infected patients	8 (3.2)
No. of patients naïve to Peg IFN/RBV	190 (76.0)

Sequencing analysis showed that the S282T mutation of HCV NS5B region was not found in any of the cases in this study, which contained treatment-naïve, PegIFN/RBV-experienced, HBV co-infected and HCV mono-infected patients. A mutation at the position 282 (S282R) condoned by AGA was found in one PegIFN/RBV-experienced, mono-infected with genotype 1b hepatitis C patient. AGA was different from that previously correlated with resistance (S282T) (Figure 3).

In addition, codon usage bias at amino acid position 282 of HCV NS5B region was observed at frequencies ranging from 0 to 100% with significant variations between different genotypes. Position 282 coded by AGT was mainly observed in 100% of genotype 3a and 90% of 6a, while one genotype 1b patient was observed to have this type. But other genotypes were not observed to have any isolates. The result suggested that, in genotypes 1a, 1b, 2a and 3b, condon AGC played a major role at position 282 (Table II).

Discussion

At present, populations are monitored to discover the baseline resistance mutations to anti-HCV DAAs, which is considered as beneficial reference for further therapeutic and policy decisions. A research[19] hypotesized that mutations played an important role in resistance to NS5B inhibitors, and highlighted the requirement to supervise resistant mutations induced by novel drugs.

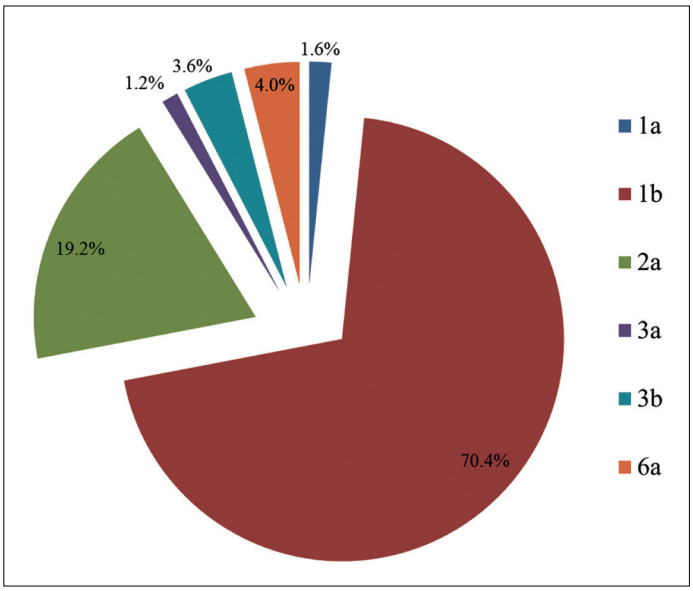

Figure 2. Distribution of HCV genotypes in DAA-treated naïve Chinese patients

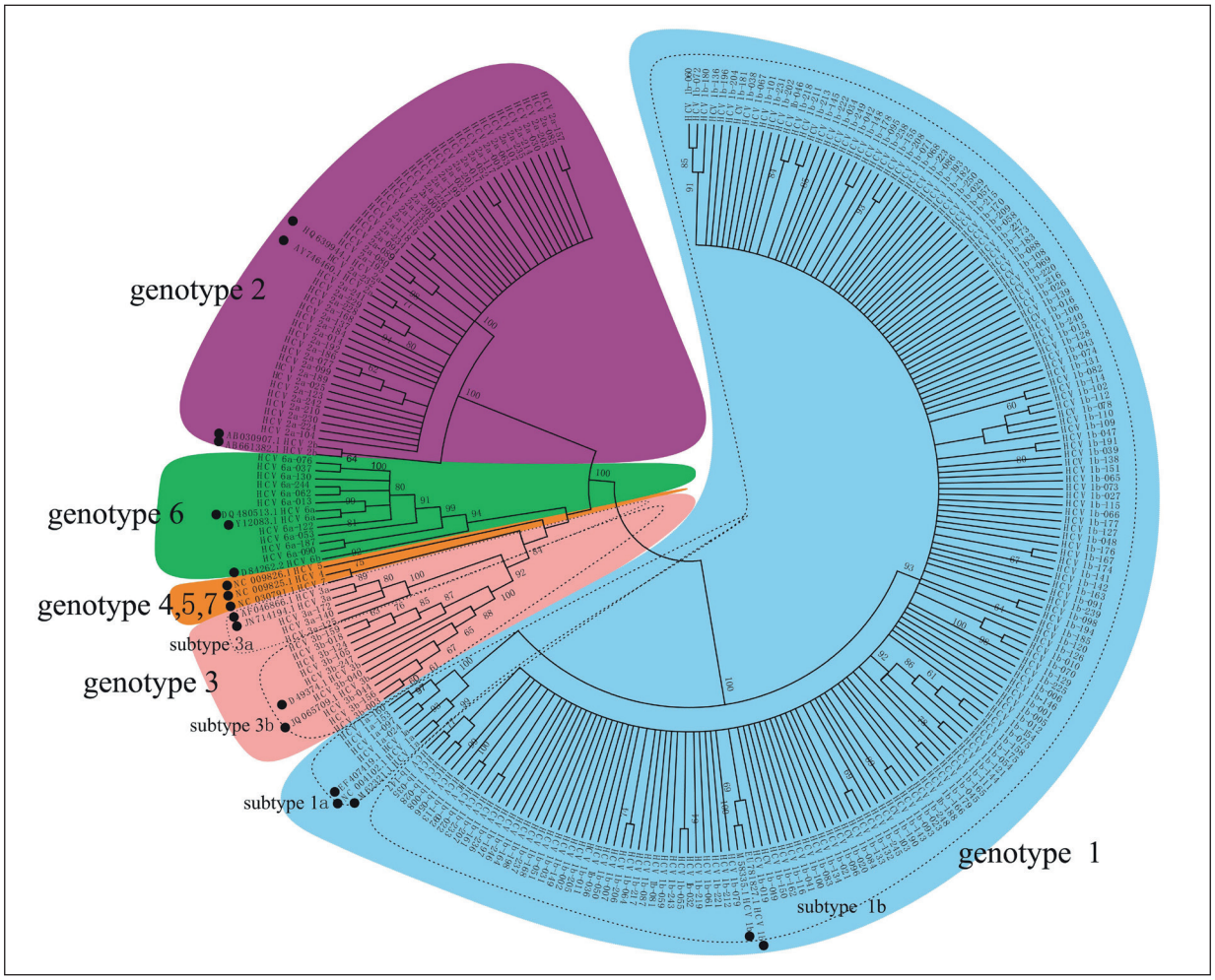

Figure 3. Phylogenetic analysis of the different sequences of NS5B region. Black circles ●, available reference sequences; white circles ○, HBV co-infected patients; Black stars ★, S282R mutation patients.

Table II. Condon usage bias at amino acid position 282 between different genotypes.

Genotypes	Codon of 282	
	AGT	AGC
1a	0	4 (100%)
1b	1 (0.57%)	174 (99.43%)
2a	0	48 (100%)
3a	3 (100%)	0
3b	0	9 (100%)
6a	9 (90%)	1 (10%)

An *in vitro* study[15] indicated that the S282T substitution in the NS5B gene, was the primary mutation selected by SOF and resulted in 9.5 fold resistance to SOF. In this paper, the S282T mutation in the HCV NS5B polymerase-coding region was analyzed in 250 patients with genotypes 1a, 1b, 2a, 3a, 3b, 6a, and 6b. Sequencing analysis showed that the S282T mutation was not present in any of the isolates. To our knowledge, the occurrence of this amino acid change has been previously described only in one patient infected with HCV genotype 2b who received SOF monotherapy for 12 weeks during relapse in phase III clinical trials of SOF[20]. To date, S282T mutation in the NS5B region has not been detected in clinical patients in any other studies[21-24]. This might suggest that this variant does not pre-exist therapy because of the lack of its natural occurrence. Fitness analysis showed that S282T conferred a high fitness cost[25]. It could partially contribute to the infrequent development of this mutation *in vivo*.

An S282R (AGC>CGG) mutation of controversial virological or clinical relevance was detected in a patient with HCV genotype 1b who received PegIFN/RBV. It was found that mutation S282R and S282G decreased the fitness of HCV to the generation of replication competent-viruses, indicating that viruses harboring S282R or S282G have potent ability to survive[24,26]. Interestingly, Donaldson et al[21] detected S282R mutation in a patient who experienced treatment failure with sofosbuvir-containing therapeutic regimen. Although in cell culture this variant was not associated with reducing susceptibility against sofosbuvir, affection of the interaction of the inhibitor with the NS5B polymerase was still predicted by structural bioinformatics[24]. Viruses with this substitution, although relatively infrequent, may reduce the effectiveness of SOF and impact future treatment options.

Relevant condon usage bias at amino acid position 282 was found among the different genotypes and subtypes. In this investigation, synonymous codons AGT and AGC coded arginine simultaneously, but were not used in equal frequencies among different genotypes. Reports[27,28] have shown that codon usage bias and synonymous mutations are under weak selection or evolutionary pressure in various organisms. Codon usage bias plays an important role in shaping gene expression and cellular function via its effects on diverse processes, ranging from RNA transcribing and processing to protein translation and protein folding[29,30]. A study[31] on HAV showed that condon usage biases were attributed to the co-evolution of genome composition, which controlled translation kinetics and even the ability to escape the antiviral cell responses. We provide a reference for more extensive analysis of codon usage bias on the evolution of HCV and drug-resistant mechanism, which may facilitate the personalized approach against chronic infection of hepatitis C[32]. Due to the low frequencies within HCV quasispecies and the methodological restrictions, whether certain variants below the detection limits of available assays exist remains unknown. Next generation sequencing technologies and further studies are required to better evaluate the role of all variants and condon usage biases in modulating resistance or susceptibility to HCV drugs.

Conclusions

Our data demonstrates that S282T mutation was not prevalent in DAA-treated naïve Chinese patients who suffered from chronic HCV infection. This provides a therapeutic basis in clinical practice.

Conflict of Interests:

The Authors declare that they have no conflict of interests.

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