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Analysis of Naturally Occurring Resistance-Associated Variants to NS3/4A Protein Inhibitors, NS5A Protein Inhibitors, and NS5B Polymerase Inhibitors in Patients With Chronic Hepatitis C

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The first NS3/4A hepatitis C virus (HCV) protease inhibitors telaprevir and boceprevir were approved in 2011, and both NS5A and NS5B polymerase inhibitors were launched. Recently, direct-acting antivirals (DAAs) have had a major impact on patients infected with HCV. HCV DAAs are highly effective antivirals with fewer side effects. DAAs have been developed for the treatment of HCV infection in combination with PEG-IFN-α/RBV as well as in IFN-free regimens. However, some drug resistance mutations occur when a single oral DAA is used for treatment, which indicates that there is a low-frequency drug resistance mutation in HCV patients before the application of antiviral drugs. Our research showed that natural resistance to HCV DAAs was found in treatment-naive CHC patients and that the drug resistance mutation rates differ in various HCV genotypes. Many challenges posed by natural resistance should be considered in the context of DAA therapies.

Key words: Viral hepatitis; Infectious liver diseases; Liver diseases

INTRODUCTION

Hepatitis C virus (HCV) infection is a frequent cause of progressive liver damage[1] and can lead to cirrhosis and hepatocellular carcinoma[2]. It is estimated that there are 170–200 million people with chronic HCV infection worldwide. The HCV genome is a 9.6-kb, positive-sense, single-stranded genome that includes three structural proteins (core, E1, and E2), the ion channel protein p7, and six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B)[3]. It possesses a high genetic diversity, and the differences in the nucleotide sequence are 30%–35% among different genotypes but are approximately 20%–20% among diverse subtypes. According to the methods of Simmonds and colleagues, HCV can be divided into 7 genotypes, and 67 subtypes have been characterized to date; however, in China, priority is given to genotypes 1b and 2a[4,5]. Pegylated interferon-α and ribavirin are approved as standard-of-care treatments, but only 50%–60% of patients achieve a sustained virologic response[6].

Direct-acting antivirals (DAAs) that can block viral production by directly inhibiting one or more steps of the HCV replication cycle are in various stages of clinical development. Three targets for DAAs (i.e., HCV NS3 protease, NS5B polymerase, and NS5A protein) are essential for virus replication[7]. However, HCV mutations that confer resistance to DAAs play an important role in treatment. Previous clinical trials have confirmed that the antiviral efficacies of DAAs are closely related to the genotype and subtype of HCV. Currently, direct sequencing is commonly used, which can detect drug-resistant mutation sites, based on the premise that quasi-species must be greater than 25%. To date, most of the drug resistance sites have been identified in vitro in phase I and phase II clinical trials. Guidelines suggest that the evaluation sequence of the NS5A resistance site is from 24 to 93, which can be used to guide the treatment, and they emphasize that this site contains more than 15% generation sequences before resistance mutations are considered. According to GenBank data, the global prevalence of DAA resistant-associated variants (RAVs) includes strains with at least one resistance mutation, and the highest RAV frequency occurred in Asia (74.1%), followed by Africa (71.9%), America (53.5%), and Europe (51.4%). The highest incidence of drug resistance is reported for genotype 6 (99%), followed by GT2 (87.9%),

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GT4 (85.5%), GT1a (56%), GT3 (50.0%), and GT1b (34.3%). The overall prevalence of RAVs to NS3 protease inhibitors is high and is followed by the prevalence of RAVs to NS5A inhibitors. The lowest prevalence of RAVs is associated with NS5B polymerase inhibitors. In our study, protease inhibitor-associated resistance mutation sites were detected in 38.20% of the patients with genotype 1b and in 100% of patients with genotype 2a. The variation of related resistance loci will directly affect the antiviral efficacy of DAAs. We showed that the rate of RAVs to NS5A protein inhibitor was 22.41% in GT1b, 100% in GT1a, and 5.12% in GT2a. In this study, 24 cases (23.08%) of HCV strains with wild-type NS5B sequences were observed, but a significantly higher number of variant strains (80 cases; 76.92%) was identified. The prevalence of wild-type strains with the NS5B sequence was significantly greater in GT2a than in GT1. Thus, different genotypes have different mutation incidences.

The aims of this study were to evaluate whether patients with chronic hepatitis C (CHC) infection who did not receive antiviral treatment in our country have naturally occurring DAA resistance and to assess the distribution of related mutation sites. A comparison of the clinical features of DAA-infected patients between those who do or do not have anti-HCV can lay a foundation for using DAAs to treat CHC domestically.

MATERIALS AND METHODS

Overall, 594 serum samples were obtained from DAA-naive patients with chronic HCV infection. These patients were treatment naive and were not infected with hepatitis B virus (HBV) or human immunodeficiency virus (HIV). Our experiment was divided into three parts. In total, 170 of the 314 samples were used for HCV NS3 sequencing, 104 of the 144 samples for NS5A, and 104 of the 136 samples for NS5B sequencing. This study was carried out in accordance with the management guidelines for CHC established by the Hepatology Association of the Chinese Medical Association and was approved by our local ethics committee.

Viral RNA was extracted from serum using a TIANamp Virus DNA/RNA Kit (TIANGEN Biotech, Beijing, P.R. China), and the viral RNA was then transcribed to cDNA. HCV NS3, NS5A, and NS5B sequences were amplified using nested RT-PCR.

Direct sequencing of PCR products was performed using an automatic sequencer (ABI PRISM 3100 genetic analyzer DNA Sequencer; Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were analyzed using the Bioedit software. The GenBank accession numbers were used for reference.

Sequences used to determine the HCV genotypes were as follows: NC_004102 (subtype 1a), D90208 (subtype 1b), AB047639 (subtype 2a), HQ639936 (subtype 6a), AB520610.1 (subtype 1a), HC J4-D10750 (subtype 1b), EU857431.1 (subtype 1b), HC J6-D00944 (subtype 2a), and KF676352.1 (subtype 2a).

Statistical Methods

Data were recorded in Excel, and the SPSS-17.0 software was used for statistical analysis. Statistical description was obtained using the frequency, and count data were evaluated using the chi-square test. Quantitative data are presented as the mean ± standard deviation. Student's *t*-test was used to compare parameters between two groups, and an ANOVA was used to analyze the parameters. The p values were calculated with two-tailed statistical analysis, and a value of $p<0.05$ was considered statistically significant.

RESULTS

The NS3A region was successfully amplified in 148 of 170 samples (87.06%). Among the 148 NS3A sequences obtained, 2 sequences were genotype 1a, 89 were genotype 1b, 55 were genotype 2a, and 2 were genotype 6a. The baseline characteristics of these patients are provided in Table 1.

Mutations V36L/M, Q41R, T54S, V55F, Q80L/R/G/K, A156S, and V170I were detected in the HCV NS3 serine protease region. The rate of resistance-associated mutations was different in various genotypes. The total rate of resistance-associated mutations in the NS3A region was 33.71% (30/89) for genotype 1b and 100% for genotype 2a; the differences between 1b and 2a were statistically significant ($p<0.001$). In the genotype 1b-infected population, 2.25% of the patients expressed substitution

Table 1. Baseline Characteristics of Patients With Genotypes 1b and 2a in the NS3 Region

Baseline Characteristics	1b ($n=89$)	2a ($n=55$)	p Value
Age (years)	45.89 ± 15.51	45.31 ± 16.61	0.505
Sex (male/female)	50/39	30/25	0.848
AST (U/L)	54.34 ± 55.96	53.55 ± 44.02	0.774
ALT (U/L)	71.02 ± 97.84	72.11 ± 82.27	0.965
TBIL (μmol/L)	18.45 ± 8.97	19.73 ± 17.78	0.166
HCV RNA ($\log_{10}$ copies/ml)	5.99 ± 0.91	6.02 ± 0.96	0.654

Table 2. Amino Acid Substitutions Conferring Resistance to HCV NS3 Protease Inhibitors

NS3 Protease Positions	HCV Genotypes			
	1a ($n=2$)	1b ($n=89$)	2a ($n=55$)	6a ($n=2$)
V36	–	V36L(2)	V36M(1)	–
F43	–	–	–	–
T54	–	T54S(2)	–	–
V55	–	–	–	–
Q80	–	Q80L/R(3/1)	–	Q80K(2)
R155	–	–	–	–
A156	–	–	A156S(2)	–
V158	–	–	–	–
D168	–	D168A(1)	–	–
V170	–	–	–	–

V36L, and 98.18% of the genotype 2a-infected patients had V36L. All of the patients with genotype 2a displayed Q80G substitutions, and Q80L/R/K mutations were detected in other genotypes. There was no statistical significance between HCV wild-type and mutant strains compared to baseline level. The amino acid substitutions conferring resistance to HCV NS3/4A protein inhibitors that were identified across the samples sequenced are summarized in Table 2.

The NS5A region was successfully amplified in 104 of the 144 samples by real-time PCR. Among the 104 sequences, 7 were genotype 1a, 58 were genotype 1b, and 39 were genotype 2a. Similarly, the NS5A region was successfully amplified by this method in 104 of the 136 samples, including 11 genotype 1a, 50 genotype 1b, and 43 genotype 2a. The baseline characteristics of these patients are shown in Tables 3 and 4. The total frequency of resistance mutations in genotypes 1a and 1b was significantly higher than that in genotype 2a.

We found that the mutation rate of NS5A protein inhibitor in genotype 1b was 22.41%, that in genotype 1a was 100%, and that in genotype 2a was 5.12%. These differences were statistically significant ($p<0.05$). There were 24 cases (23.08%) of wild-type strains with the NS5B region of HCV and 80 cases (76.92%) of variant strains, which was significantly higher; however, wild-type strains were significantly higher in genotype 2a than in genotype 1. In genotype 1a, amino acid substitutions conferring resistance to NS5A inhibitors (M28L) were detected in 4/7 (57.1%). However, upon analyzing the HCV NS5A sequences, this same site was found in 1/39 (2.56%) in genotype 2a patients. We found other resistance mutations in the genotype 1a nucleotide sequences: Q30R, H54Q ($n=5$; 71.4%) and Q30L ($n=1$; 14.3%). In genotype 1b, the resistance mutations P58S (3/58), A92T (1/58), and Y93H (9/58) were observed in the NS5A region. Thus, it is not difficult to suggest that Y93H ($n=9$; 15.5%) predominated over P58S ($n=3$; 5.2%) and A92T ($n=1$; 1.7%). The amino acid substitutions conferring resistance to HCV NS5A inhibitors and NS5B polymerase inhibitors are shown in Tables 5 and 6.

Among the 104 cases of amplified patients infected with the HCV virus, 19 (18.2%) had a mixture of virus variants carrying multiple NS5A resistance mutations, whereas 23 (22.1%) exhibited a mixture of strains with various NS5B resistance mutations. In detail, in the NS5A region of 13 patients carrying genotype 1b, four different mixtures were observed (Y54Q+Y93H, Y54Q+A92T, Y54Q+P58S, and Y54L+P58S). One patient with genotype 2a had F28L+Y93M mutations in the NS5A region. However, the NS5A nucleotide sequence within genotype 1a viruses had the most complex mutations; three different mixtures were observed (Q30R+H54Q, 0.98%; Q30L+H54Q, 0.96%; and M28L+Q30R+H54Q, 2.88%).

Table 3. Baseline Characteristics of Patients With Genotypes 1a, 1b, and 2a in the NS5A Region

Baseline Characteristics	1b ($n=58$)	2a ($n=39$)	1a ($n=7$)	p Value
Age (years)	44.05 ± 16.03	48.23 ± 15.79	32.00 ± 16.79	0.27
Sex (male/female)	32/26	20/19	5/2	0.61
AST (U/L)	55.87 ± 37.26	58.18 ± 35.79	39.5 ± 23.79	0.52
ALT (U/L)	67.79 ± 18.34	59.27 ± 37.29	39.57 ± 17.81	0.79
ALB (g/L)	43.71 ± 6.91	40.51 ± 5.39	40.94 ± 13.91	0.12
TBIL (μmol/L)	15.26 ± 10.48	16.96 ± 7.66	26.52 ± 17.02	0.68
HCV RNA (IU/ml)	9.88E+06	3.26E+06	1.42E+06	0.59

Table 4. Baseline Characteristics of Patients With Genotypes 1a, 1b, and 2a in the NS5B Region

Baseline Characteristics	1b ($n=50$)	2a ($n=43$)	1a ($n=11$)	p Value
Age (years)	46.08±16.32	47.03±16.49	43.45±17.55	0.80
Sex (male/female)	30/20	28/15	7/4	0.88
AST (U/L)	49.89±32.22	51.72±45.76	36.56±12.74	0.48
ALT (U/L)	58.37±46.75	52.90±0.62	42.36±21.49	0.57
ALB (g/L)	43.72±4.66	43.29±5.11	41.69±4.92	0.46
TBIL (μmol/L)	13.73±6.66	17.34±9.22	17.32±9.04	0.08
HCV RNA (IU/ml)	3.55E+06	3.45E+06	2.07E+06	0.83

The multiple drug resistance sites of NS5A protein are shown in Table 7.

In the NS5B region of eight patients with genotype 1b, seven different virus variant mixtures were observed (S282C+C316N, S282R+C316N, C316N+V321G, C316N+A421V, M414L+C316N, C289W+C316N, and C316N+L392I). In five patients with genotype 2a, two different mixtures were detected (L392I+V421A and Q414M+V421A+C316N+M289C). Among 10 patients carrying genotype 1b, 2 mixtures were detected (C316N+V421A and C316N+L392F+V421A). The highest incidence of NS5B resistance mutations occurred for C316N+A421V in HCV genotype 1a. In addition, combinations of multiple resistance variants in both the NS5A and NS5B genes of the same HCV strain were observed in 1/32 (3.1%) patients with HCV genotype 1a and 8/30 (26.6%) patients with HCV genotype 1b. Multiple drug resistance sites of NS5B polymerase are shown in Table 8.

DISCUSSION

In the past, the standard anti-HCV treatment has been pegylated interferon and ribavirin, but its sustained virologic response is low. With the development of DAAs, treatments for HCV infection are evolving rapidly. Telaprevir and boceprevir (N3/4A protease inhibitors) are the earliest DAAs to be used in combination with pegylated interferon-α and ribavirin for the treatment of chronic HCV genotype 1 infection. Simeprevir has been used in combination with pegylated interferon-α and ribavirin or in combination with sofosbuvir for genotypes 1 and 4. In our study, protease inhibitor-associated resistance mutation sites were detected in 38.20% of patients with genotype 1b and in 100% ($n=55$) of patients with genotype 2a. V36L was present in 2.25% of patients with genotype 1b and 98.18% with genotype 2a. The substitution V36L confers decreased sensitivity to telaprevir[8,9]. Of note, mutation Q80K was observed in two cases of patients with genotype 6a and is known to confer resistance to simeprevir. Additional studies have shown a high rate of Q80K mutation in patients with genotype 6a, so these patients were not suitable candidates for treatment with second-generation protease inhibitors. The substitutions R155 and A156 are known to confer a high level of resistance to all protease inhibitors[10–12]. However, we only observed substitution A156S, which can lead to resistance to telaprevir and boceprevir, in two individuals with genotype 2a. However, boceprevir and telaprevir have been reported to have several drug–drug interactions and are not approved for use together as combination therapy for HCV.

Some studies have described that the majority of resistance mutations are at positions M28T/V, Q30H/E/R/K, L31M/V, P32L, and Y93C/H/N, which act as primary resistance variations. For genotype 1b, variants L31F/M/V, P32L, and Y93C/H/N act as primary resistance substitutions, and L23F, R30Q, and P58S act as secondary resistance mutations[13–16]. The sites M28T, Q30E/H/R, and L31M/V conferred high levels of resistance, and these mutations are in the NS5A region associated with resistance to daclatasvir[17]. In patients infected with genotype 1a, we found M28L (57.1%) substitutions that confer very low-level resistance to daclatasvir, ledipasvir, and ombitasvir. The Q30R/L variation is an additional substitution associated with resistance to daclatasvir, ledipasvir, and ombitasvir. We also found two species of the Q30 mutation, and the incidence of Q30L was higher than Q30R, which was similar to a previous report. In addition, we found that at the baseline level, the prevalence of NS5A resistance mutations was higher in patients infected with genotype 1a than with genotype 1b.

Table 5. Amino Acid Substitutions Conferring Resistance to HCV NS5A Inhibitors in Direct-Acting Antiviral (DAA)-Naive Patients Infected With HCV Genotypes 1a, 2a, and 1b

	Genotypes		
NS5A Residues	1a ($n=7$)	1b ($n=58$)	2a ($n=39$)
M28	M28L(4)	–	–
F28	–	–	F28L(1)
Q30R	Q30R/L (5/1)	–	–
P58	–	P58S(3)	–
E62D	E62Q (5)	–	–
A92	–	A92T(1)	–
Y93	–	Y93H(9)	–
L31V/M+Y93H	–	–	–

Table 6. Amino Acid Substitutions Conferring Resistance to HCV NS5B Polymerase Inhibitors in DAA-Naive Patients Infected With HCV Genotypes 1a, 1b, and 2a

NS5B Residues	Genotypes		
	1a ($n=11$)	1b ($n=50$)	2a ($n=43$)
L159			
S282T		S282R/C(1/1)	
M289I/L		C289W(1)	M289K/C(1/2)
C316	C316N(11)	C316N (49)	C316Q/N(1/2)
L320			
V321		V321G(1)	
L392	L392F(1)	L392I(1)	L392I(16)
N411		441(deficiency 1)	
M414		M414L(1)	Q414M(2)
A421	V421A(9)	A421V(2)	V421A(6)

The Y93H mutation was observed in NS5A genotype 1b strains, which confers high-level resistance to daclatasvir, ledipasvir, and samatasvir. In our research, Y93N/C was not observed, although Y93H was detected in nine patients and was mainly concentrated in genotype 1b. Additionally, we detected the mutations Y93M and Y93T in one patient each. Moreover, the same site is mutated into other amino acids that have not been proven to confer resistance. At present, researchers have reported the prevalence of natural mutations conferring resistance to NS5A inhibitors. In Italy, Paolucci et al. showed that the incidence was 12.5% and 53.3% in patients infected with genotype 1a and genotype 1b, respectively[13]. Suzuki et al.[17] have already demonstrated that the rate of mutations L31M/V and Y93H conferring resistance to NS5A inhibitors reached 11.2% in treatment-naive individuals infected with HCV genotype 1b in Japan. In summary, we found that the total frequency of the Y93H mutation was 8.65%, of which the genotype 1b mutation rate was 15.51%, and the mutation rates in genotype 1a and genotype 2a were low. The rate of the Y93H mutation in genotype 1b has previously been reported at 4%–8%, with the incidence of the mutation in genotype 1a reported to be higher than that of genotype 1b. In the present research, we found substitutions involved in the resistance to HCV NS5A inhibitors for genotype 1b, and our results were similar to those previously reported.

Table 7. Multiple Drug Resistance Sites of NS5A Protein Inhibitor

NS5A Residues	No.	Genotype	Incidence
F28L+Y93M	1	2a	0.96%
Y54Q+Y93H	9	1b	8.65%
Y54Q+A92T	1	1b	0.96%
Y54Q+P58S	2	1b	1.92%
Y54L+P58S	1	1b	0.96%
Q30R+H54Q	1	1a	0.96%
Q30L+H54Q	1	1a	0.96%
M28L+Q30R+H54Q	3	1a	2.88%

Table 8. Multiple Drug Resistance Sites of NS5B Polymerase Inhibitors

NS5B Residues	No.	Genotype	Incidence
S282C+C316N	1	1b	0.96%
S282R+C316N	1	1b	0.96%
C316N +V321G	1	1b	0.96%
C316N+A421V	2	1b	1.92%
C316N+V421A	9	1a	8.65%
M414L+C316N	1	1b	0.96%
C289W+C316N	1	1b	0.96%
L392I +V421A	3	2a	2.88%
C316N+L392I	1	1b	0.96%
C316N+L392F+V421A	1	1a	0.96%
Q414M+V421A+C316N+M289C	2	2a	1.92%

The higher prevalence of mutations in genotype 1b is due to the presence of C316N, which confers low-level resistance to tegobuvir and HCV-796 in most genotype 1b strains[18]. In previous studies, the C316N site was found at baseline in individuals infected with HCV GT1b whose treatment failed and was observed in patients who were infected with GT1a and experienced relapse. However, more research needs to be done to reduce susceptibility to sofosbuvir. We found that a single C316N mutation emerged in 98% of subjects in genotype 1b and that it existed at 100% in genotype 1a. In contrast, this variation was present at a much lower rate of 4.65% in genotype 2a. The overall variation in genotypes 1b and 1a was significantly higher than in genotype 2a. Previous studies have confirmed the high prevalence of C316N in the NS5B region using direct sequencing and deep sequencing[19]. Our results were identical to previous reports. The Los Alamos database on Asian populations showed that the frequency of C316N reached 91.6%, which is more than the global mutation rate[20]. This may that suggest the prevalence of the mutation in a population is closely related to ethnicity. Our experiment is consistent with this conclusion. In vitro studies demonstrated that S282T is the signature resistance-associated mutation to sofosbuvir[21–23]. However, it is rarely identified in patients treated with NS5B NIs in clinical trials. In our study, S282T was not observed in any patient. In addition to the drug resistance mutations above, M414T is associated with reduced susceptibility to NS5B nonnucleoside polymerase inhibitors[24]. The M414T mutation was not detected in this study; however, M414L/M mutations were detected in three patients[20].

Combinations of mutations in the NS5A sequences were rare. It should be stressed that the Q54H+Y93H combination has already been reported to have direct resistance

to daclatasvir[25,26]; the other combinations have not been investigated. In this study, we found 19 cases (18.3%) of multiple resistance variants in the NS5A protein region. The highest incidence of Y54Q+Y93H was 8.65% in the HCV genotype 1b-infected patients. The frequency of multiple variant combinations in NS5B sequences was 21.15%, and the C316N+A421V combination was most frequent. Moreover, in our experiment, the frequency of multiple variant combinations in patients infected with HCV genotype 1b was lower in NS5A than in NS5B.

In conclusion, our comprehensive analysis of naturally occurring RAVs to DAAs indicates that RAVs are relatively common in treatment-naive patients. The variation of the related drug resistance sites will directly reduce the antiviral effect of DAAs. We not only found single drug resistance sites for different regions but also multiple drug resistance sites. It is necessary to detect baseline-level resistance before antiviral treatment, especially for patients who did not achieve SVR on a DAA-based therapy. Therefore, it is suggested that it is necessary to conduct gene sequencing before applying DAAs therapeutically. Furthermore, the European Society for the Study of the Liver (EASL 2017) emphasizes evaluating RAS at the baseline level can help optimize the treatment regimen, although it is not recommended for systemic drug resistance testing. Therefore, detecting the drug resistance loci is particularly important for optimizing individualized antiviral therapy.

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