Original article

Effect of drug resistance mutations on antiviral agents in HCV patients

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Background: Gene polymorphism of HCV is an important cause of drug resistance to direct-acting antivirals (DAAs).
Methods: Nested PCR assays were performed to amplify the HCV viral regions of NS3, NS5A and NS5B.
Results: Major resistant mutation A156S was found in 18.33% of patients with HCV-1b and 64.28% of patients with HCV-2a. HCV-6a patients had a Q80K mutation rate of 95.45%, while the mutation rate of V170I was up to 100%. Mutation frequency varied with the different genotypes of HCV. The proportion of four resistance mutations (M36L, Q80K, A156S, V170I) in different groups were statistically significant (P<0.05). Resistant mutation Q30R was detected in 116 (72.5%) samples with HCV-1b and -6a, L31M was found in 16 patients, including 12 with HCV-2a and 4 with HCV-6a, H58P was discovered in 42.5% (68/160) of patients with the genotypes Q30R, L31M and H58P; Y93C was found in 9individuals with only HCV-2a. In HCV NS5B sequences, only a few resistant variants were detected, including C316N and S282T.
Conclusions: Naturally occurring dominant resistance mutations to HCV DAAs pre-existed in treatment-naive patients in China. Mutation frequency and characteristics varied with the HCV genotype.

Introduction

Chronic hepatitis C (CHC) caused by HCV infection is not only a serious liver disease threatening human health but also an important cause of cirrhosis, hepatocellular carcinoma, hepatic failure and liver transplantation at end stage liver disease [1]. The number of chronically HCV-infected people is 170 million [2] and increases by about 35,000 new cases a year. In China, there are about 38 million patients with CHC; a few individual areas have a high incidence of mainly subtype-1b infection, and the detection rate of HCV-6a infection is gradually rising in the south and southwest of China. CHC is a growing global health problem and poses a serious health burden.

The treatment of CHC has made great progress in the past decade and therapy with the combination of pegylated interferon-α (PEG-IFN-α) plus ribavirin (RBV) as the standard of care (SOC) for patients with CHC is recommended. However, different HCV genotypes and gene subtypes have obvious differences in therapeutic response. In Europe and the United States, patients infected with HCV genotypes-2 and -3, using the standard joint treatment plan of 48 weeks, can achieve a 70–80% sustained viral response (SVR), but patients infected with HCV genotype-1 can achieve no more than 50% [3]. In China, HCV genotype-1 patients can achieve an SVR rate of about 50–70% obtained with SOC treatment of 48 weeks; curative effect is better than in the Caucasian population. In spite of this, there are still some patients who cannot obtain an SVR or who can't complete the treatment as a result of being unable to tolerate PEG-IFN-α. There are also some patients who fail to complete the course of long-term PEG-IFN-α plus RBV antiviral treatment due to adverse reactions, such as a serious decline in white blood cell count, depression or autoimmune diseases. Some patients have IFN contraindications before treatment, such as combination with autoimmune diseases or hepatitis C after kidney transplantation. In this situation, it is particularly important to develop more effective antiretroviral drugs. Direct-acting antivirals (DAAs), also known as specifically targeted antiviral therapies for hepatitis C (STAT-C), have a specific, targeted role in HCV infection for different HCV non-structural protein loci. DAAs

mainly refer to protease inhibitors (PIs) and polymerase inhibitors, including NS3/4A PIs, NS5A protein inhibitors, nucleoside and non-nucleoside NS5B polymerase inhibitors. Telaprevir and boceprevir, which belong to the NS3/4A PI class, were formally approved by the US FDA in 2011. However, the use of DAA monotherapy in the treatment of HCV infection is complicated by the selection of some drug resistance mutations in HCV as therapeutic targets, and this then leads to treatment failure [4–6]. Accordingly, DAAs should be combined with PEG-IFN-α and RBV to cure patients with HCV genotype-1 infection. It is possible to achieve an SVR rate above 80% and to prevent HCV resistant mutations. In addition, there are still many DAAs under development, in research and in clinical trials. Previous studies have reported that primary drug resistance has been confirmed that can exist before the treatment of HCV with PIs and polymerase inhibitors [7]. At present, the research has reported that single amino acid mutations in HCV NS3/4A, NS5A and NS5B can lead to viral drug resistance. Some variants can even be detected in patients who have not been treated with DAAs and these variants can cause primary drug resistance [8]. Studies of pre-existing resistance of the NS3/4A PIs, which are mainly concentrated in patients with HCV genotype-1b, are more frequent but only a few studies have concentrated on the variety of HCV genotypes which are less frequent. We did not see the major HCV variation of the NS3/4A PIs occur, whereas the second major mutation occurred to a certain extent. However, different countries and regions have different mutation rates due to certain differences in ethnic groups: fluctuations of 0.54% to 3.3%. In NS5A PIs and NS5B PIs, the current research is still largely focused on drug development. The clinical trials and *in vitro* and *in vivo* drug resistance tests or studies are relatively few.

In early telaprevir and boceprevir monotherapy in clinical trials, drug resistance-related mutations rapidly emerge in the first 15 days of the treatment [6,9]. This finding strongly suggests that variants related to drug resistance already actually exist in the viral pool of quasispecies before initial antiviral treatment. In fact, by a direct sequencing analysis method, it has been confirmed that mutations associated with drug resistance to PIs (such as amino acid substitution in V36, T54, V55, Q80, R155, D168 and V170) pre-existed in patients without antiviral treatment at a different frequency [8,10,11]. Variations of M28V Q30R/H, Q54H and Y93H found in the NS5A region and mutations associated with drug resistance of non-nucleoside polymerase inhibitors, such as S282T/R, C316Y/F/S, M423T/I and V499A, were also detected in untreated patients. However, mutations related to drug resistance of NS5B polymerase inhibitors of the nucleoside class were mainly discovered in an *in vitro* test and have not been found in untreated patients [12–15]. Recently, with more sensitive second-generation sequencing methods, variants related to NS3, NS5A and NS5B drug resistance can be detected in HCV quasispecies in patients before the initial treatment, at different mutation frequencies, which can be as high as >90%. However, the main or secondary definition of pre-existing resistance variation remains to be further elucidated.

At present, there is no relevant research regarding drug-resistant mutations of HCV NS3/4A, NS5A or NS5B in China. This study was performed mainly through the analysis of patients with CHC before antiviral treatment in the southern region of China, with many HCV genotypes. We performed real-time PCR (RT-PCR) and examined the direct sequencing for relevant HCV gene sequences, including three regions associated with DAA treatment (NS3/4A, NS5A and NS5B). We also analysed the existence of the primary drug resistance mutation from these three regions. Meanwhile, we investigated the correlation between variation and HCV genotypes. The results of the study will provide the basis for further exploration of HCV resistance, perfecting individualized treatment based on genotype and assessing whether you need to detect pre-existing resistance before DAA treatment in the future.

Methods

Chemicals and reagents

Omega viral RNA kit, Toyobo ReverTra Ace-α reverse transcription kit, 10×PCR buffer, DEPC H_2O, dNTP mix (2 mM), rTaq enzymes and primers were purchased from TaKaRa Bio Inc. (Shiga, Japan).

Clinical samples

A total of 1,395 HCV patients were admitted into the Third Affiliated Hospital of Sun Yat-sen University between 2009 and 2012. Of these, 817 cases have been genotyped, among which 412 cases were HCV-1b, 115 cases were HCV-2a, 240 cases were HCV-6a, 32 cases were HCV-3a and 18 cases were HCV-3b. From them, 184 patients were selected, and the number of patients who had HCV-1b, HCV-2a and HCV-6a were 74, 15 and 95, respectively. None of the patients used DAA drugs or antiviral therapy with the combination of IFN and RBV. The diagnosis of HCV was according to guidelines on the prevention and treatment of hepatitis C approved by the American Association for the Study of Liver Diseases. All the study protocols were approved by ethics committees of the Third Affiliated Hospital of Sun Yat-sen University and informed consent was obtained from all patients.

RNA extraction, reverse transcription and quantification

RNA was isolated from the first RNA-positive serum sample obtained from each patient using 500 µl serum and an RNAiso™ Plus extraction kit (Takara, Dalian, China). The HCV RNA was quantified by detecting the light absorption value using the trace nucleic acid analyzer (Thermo, Carlsbad, CA, USA) at a wavelength of 260 nm. HCV RNA was eluted in 10 µl of Tris-EDTA (TE) buffer and was subsequently transcribed into cDNA using the ReverTra Ace-α-reverse transcription kit (Toyobo, Shanghai, China). This cDNA was used as the input for two separate PCR assays targeting the HCV core and NS5B regions.

Genotyping methods

5 ml of peripheral blood was taken from HCV patients and RNA was extracted using Omega Viral RNA kit (Tiangen, Beijing, China). The obtained cDNA was then synthesized from RNA with Toyobo ReverTra Ace-α reverse transcription kit (Tiangen). HCV genotyping was performed using our method as previously described [16]. In brief, nested PCR was utilized to amplify the conserved fragments of HCV core and NS5B genes. The obtained HCV core and NS5B genes were sequenced and compared with the existing HCV sequence to identify the genotype. The primers used for HCV core and NS5B are listed in Additional file 1. According to the gene sequences of HCV NS3, HCV NS5A, HCV NS5B in GenBank, we designed the specific nested PCR primers for HCV-1b, HCV-2a and HCV-6a. The primers were listed in Additional file 1. The PCR reaction was carried out with a Thermal Cycler S1000 PCR machine (Thermo) and the reaction conditions were reported in our previous study [16]. Clustal X was used to perform sequence alignment.

Sequence alignment and analysis

The gene sequence was compared using the Clustal X program. The NS4A/B, NS5A and NS5B mutations were analysed and compared with the mutations reported in previous studies [4–8]. The comparison was performed according to the output peak chart produced by ABI 3730xl DNA Sequencer (ABI, Carlsbad, CA, USA).

Statistical analysis

SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was employed to perform statistical analysis, and two-tailed Student's *t*-test, non-parametric Mann–Whitney U test and one-way AVOVA analysis were adopted to determine the statistical difference, and $P<0.05$ was considered to be significant.

Results

Clinical characteristics

The corresponding clinical characteristics are given in Table 1. There is no significant difference in demographic characteristics (for example, age and gender). Additionally, no significant difference was observed for other important parameters, including the activity of alanine transaminase (ALT), albumin (ALB), platelets (PLT) and HCV RNA level ($P>0.05$).

NS3/4A protein protease resistance related variations

As shown in Table 2, the success rate of amplification of NS3 was 88.0% (162/184), including 81.1% for HCV-1b (60/74), 93.3% for HCV-2a (14/15) and 92.6% for HCV-6a (88/95). The mutation rate was 38.3% (23/60), 100% (14/14) and 100% (88/88) for HCV-1b, HCV-2a and HCV-6a, respectively.

Among the cases successfully amplified, 266 mutation sites were found in 125 cases (77.2%, 125/162). There were 20 cases with the main mutation A156S (12.3%, 20/162), among which 11 cases had HCV-1b and 9 cases had HCV-2a. With regard to the secondary mutation, there were 4 cases with T54S and 84 cases with Q80K, 2 cases with D168E and 1 case with D168Y. The mutant frequency at the same site was different in the patients with different HCV genotypes. The frequency of V36L was 100% (14/14) in HCV-2a and 4.6% in HCV-6a (4/88). The frequency of Q80G was 100% in HCV-2a and 2.3% in HCV-6a. The frequency of V170I was 16.7% in HCV-1b, 100% in HCV-2a and 98.9% in HCV-6a. Only two cases with the mutation Q80L were found in HCV-1b patients. However, the previous reports showed that the Q80L

Table 1. Characteristics of the study population

Characteristic	Gt-1b	Gt-2a	Gt-6a	F	*P*-value
n	74	15	95	–	–
Mean age, years ±SD	38.34 ±13.34	32.07 ±15.82	33.48 ±14.11	2.96	>0.05
Male, %	59.5	46.7	61.1	1.11[a]	>0.05
Mean ALT, U/l ±SD	70.07 ±48.96	80.77 ±103.01	89 ±84.80	1.25	>0.05
Mean ALB, g/l ±SD	44.62 ±3.42	45.76 ±2.63	43.64 ±3.49	2.79	>0.05
Mean PLT, $\times 10^9$/l ±SD	192.54 ±70.45	178.58 ±61.18	206.32 ±83.75	1.00	>0.05
Mean HCV RNA, log IU/ml ±SD	7.02 ±0.71	6.82 ±0.66	6.98 ±0.66	0.57	>0.05

[a]Difference between groups. ALB, albumin; ALT, alanine transaminase; F, the value of the analysis of variance; Gt, genotype; PLT, platelets.

Table 2. The results of analyses of NS3/4A and NS5A protein protease-resistance related variations

Protease position	Gt-1b	Gt-2a	Gt-1a
NS3/4A	*n*=60	*n*=14	*n*=88
V36A/M	–	V36L (14)	V36L (4)
T54S/A	T54S (4)	–	–
V55A	–	–	–
Q80R/K	Q80L (2)	Q80G (14)	Q80K (84)
R155K/T/Q	–	–	–
A156S	A156S (11)	A156S (9)	–
D168A/E/G/H/T/Y	D168Y (1)	–	D168E (2)
V170A/T	V170I (10)	V170I (14)	V170I (87)
NS5A	*n*=59	*n*=13	*n*=88
M28T	M28L (58)	M28L (1)	M28L (75)
M28T	–	M28F (9)	M28F (12)
Q30E/R	Q30R (34)	–	Q30R (82)
Q30E/R	–	Q30K (12)	Q30R (4)
L31M/V	L31L/M (1)	L31M (12)	L31M (4)
H54Y	H54Q (49)	–	H54Q (1)
H54Y	–	H54T (11)	H54T (3)
H54Y	–	–	H54S (1)
H58P	H58P (51)	H58P (11)	H58P (6)
H58P	H58T (2)	H58T (2)	H58T (82)
H58P	H58S (4)	–	–
H58P	H58R (2)	–	–
Y93C/N	–	Y93C (9)	–
Y93C/N	Y93H (3)	–	–
Y93C/N	Y93T (1)	–	Y93T (41)
Y93C/N	Y93A (12)	–	Y93A (42)

Values in brackets represent number of cases. Gt, genotype.

mutation was not correlated with the drug-resistant properties.

Correlation between HCV NS3 mutation and clinical characteristics, liver function and HCV level

As shown in Table 3, there was no significant difference between the patients with and without HCV mutation ($P>0.05$) for clinical characteristics, ALT, ALB and PLT ($P<0.05$).

Analysis of drug-resistant mutations of NS5A

For HCV NS5A, the success rate of amplification was 87.0% (160/184), among which 79.7% were for HCV-1b (59/74), 86.7% were for HCV-2a (13/15) and 92.6% were for HCV-6a (88/95). The mutation rate was 100% (59/59), 100% (13/13) and 100% (88/88) for HCV-1b, HCV-2a and HCV-6a, respectively.

More mutation sites were also detected. A total of 116 cases (72.5%, 116/160) with Q30R mutation were found, including 82 HCV-6a (93.2%, 82/88) and 34 HCV-1b (57.6%, 34/59). There were 16 patients with L31M, including 12 HCV-2a (92.3%, 12/13) and 4 HCV-6a (4.6%, 4/88); 68 patients (42.5%, 68/160) with H58P were also found, including 51 HCV-1b (86.4%, 51/59), 11 HCV-2a (84.6%, 11/13) and 6 HCV-6a (6.8%, 6/88). Only nine cases were detected with Y93C and all the patients had HCV-2a. Only three cases were detected with Y93H and all the patients had HCV-1b.

A total of 160 patients had two or more than two mutation sites. However, none of the patients had the mutation types of L31M+Y93H and L31V+Y93H, which are highly resistant to BMS-790052 (Table 2).

Analysis of drug-resistant mutations of NS5B

Due to the great difficulty in amplifying the fragments of NS5B, the fragments of NS5B were divided into three and then amplification was performed. The first fragment contained A15 and S96. The second fragment contained C223, S282, C316, V321, S365 and S368. The third fragment contained M414, L419, M423, Y448, I482 and V494.

The success rate of amplification for the first fragment was 90.8% (167/184), including 93.2% HCV-1b (69/74), 66.7% HCV-2a (10/15) and 92.6% HCV-6a (88/95). Among the successfully amplified samples, the mutation rate was 0%, 20% and 3.4% for HCV-1b, HCV-2a and HCV-6a, respectively.

For the amplification of the second fragment, the success rate was 84.2% (155/184), including 81.1% HCV-1b (60/74), 86.7% HCV-2a (13/15) and 86.3% HCV-6a (82/95). Among the successfully amplified samples, the mutation rate was 100%, 0% and 23.2% for HCV-1b, HCV-2a and HCV-6a, respectively.

For the amplification of the third fragment, the success rate was 34.8% (64/184), including 68.9% HCV-1b (51/74), 60.0% HCV-2a (9/15) and 4.2% HCV-6a (4/95). Among the successfully amplified samples, the mutation rate was 47.1%, 100% and 100% for HCV-1b, HCV-2a and HCV-6a, respectively.

For the drug-resistant mutation, only two cases with A15G mutation were found in HCV-6a patients. The mutation S96T was found in one patient with HCV-6a. A total of 17 cases with S282T were detected in patients with HCV-6a (Tables 4 and 5).

Discussion

HCV genotypes mainly affect the antiviral therapeutic efficacy and course of treatment of IFN plus RBV [17]. However, there is no difference in clinical indicators in HCV infection with different genotypes, indicating that the majority of genotypes will not affect the progress of CHC. HCV NS3 has been reported to be a multi-functional antiviral target exhibiting large gene polymorphisms. Some of the variations were detected, and the main sites (R155 and A156) show less variation, whereas the second sites (V36, T54, Q80, D168, V170) change more frequently. Our recent study [16] showed

Table 3. Characteristics of patients with or without HCV genomes harbouring drug resistance mutations

Characteristic	Mutation	Wild-type	*P*-value
n	125	37	–
Mean age, years ±SD	38.38 ±12.71	34.22 ±14.53	>0.05
Male, %	64.86	58.40	>0.05
Mean ALT, U/l ±SD	71.22 ±46.89	85.25 ±84.88	>0.05
Mean ALB, g/l ±SD	44.27 ±3.55	44.06 ±3.32	>0.05
Mean PLT, $\times 10^9$/l ±SD	184.08 ±65.51	203.06 ±79.01	>0.05
Mean HCV RNA, $\log_{10}$ IU/ml ±SD	7.10 ±0.72	6.98 ±0.68	>0.05

ALB, albumin; ALT, alanine transaminase; PLT, platelets.

Table 4. Resistance profiles of nucleoside/nucleotide analogue inhibitors of HCV RdRp in clinical development

Protease position	Gt-1b	Gt-2a	Gt-6a
Case number	*n*=69	*n*=10	*n*=88
A15G	–	–	A15G (2)
A15G	–	A15S (2)	–
S96T	–	–	S96T (1)
Case number	*n*=60	*n*=13	*n*=82
C223H	–	–	–
S282T	–	–	S282T (17)
V321I	–	–	–

Values in brackets represent number of cases. Gt, genotype; RdRp, RNA-dependent RNA polymerase.

Table 5. Resistance profiles of non-nucleoside inhibitors of HCV RdRp in clinical development

Protease position	Gt-1b	Gt-2a	Gt-6a
Case number	*n*=60	*n*=13	*n*=82
C316Y/N	C316N (60)	–	–
S365T/A	S365A (2)	–	–
S365T/A	–	–	S365F (1)
S365T/A	–	–	S365P (1)
S368T	–	–	S368A (1)
S368T	–	–	S368L (1)
Case number	*n*=51	*n*=9	*n*=4
M414T/I/V/L	M414L (3)	M414Q (9)	M414Q (2)
L419M/V	–	L419I (9)	L419I (4)
M423T/I/V	M423I (1)	–	–
Y448C/H	Y448H (1)	–	–
I482L/V/T	I482T (3)	I482L (9)	I482L (4)
I482L/V/T	I482V (1)	–	–
V494S/Q/L/A/T	V494L (2)	V494A (9)	V494A (2)
V494S/Q/L/A/T	–	–	V494C (1)
P495S/Q/L/A/T	P495S (3)	P495R (1)	–
P496A/S	P496T (3)	–	–
V499A	V499A (8)	V499A (9)	V499A (4)
V499A	V499T (1)	–	–
V499A	V499I (2)	–	–

Values in brackets represent number of cases. Gt, genotype; RdRp, RNA-dependent RNA polymerase.

the existence of NS3/4A PIs in Chinese untreated patients with CHC before DAA treatment. Different mutation rates were observed in different HCV genotypes. The patients with HCV-2a and HCV-6a exhibited more mutations, with two or more mutation points at the same time. Additionally, the rate of single amino acid substitution in the NS3 varied with different HCV genotypes. In contrast to the results obtained from previous studies [18,19], the main mutations resulting in high levels of resistance were not detected, such as R155 and A156. A156T variation might make HCV patients highly resistant to some PIs. Although A156T was not observed in the present study, another variation, A156S, was found in a total of 20 cases. Apart from that, the existence of A156S was associated with high resistance, some invalid variations were found, including Q80L and Q80G, and it has not yet been shown that these mutations can lead to drug resistance.

NS5A PIs, a new type of DAA, interfered with the HCV replication cycle mainly through directly inhibiting NS5A. Although the mechanism of action is still not very clear, it may be as an NS3 protease cofactor to enhance the combination of NS3 and HCV RNA plus endoplasmic reticulum protease. The study evaluated natural mutation frequency of the HCV NS5A related to NS5A PI drug resistance in patients not using the antiviral therapy of PEG-IFN-α plus RBV, and also not experiencing any DAA drug anti-HCV treatment. The resistant mutation of NS5A PIs was more frequent in patients with HCV-1a and HCV-1b but less frequent in patients with HCV-2a and HCV-6a. Some studies have reported that a pre-existing resistance variation of NS5A PI could affect response to these drugs in CHC patients with HCV-1. Some reports have shown that the amino acid mutations are mainly at positions 28, 30, 31 and 93 in patients with HCV-1a, and in 31 and 93 in patients with HCV-1b [12]. According to Dennis Hernandez's research [20], variations in locus such as 28, 30, 31, 92 and 93 were found in HCV-3 infection, which can affect the therapeutic efficacy of NS5A PIs. It is interesting to note that these naturally occurring viruses may lead to different treatment responses of NS5A PIs to other different HCV genotypes. For instance, as we discovered in patients with HCV-1a in previous clinical trials, if Y93H variations occur, it can lead to HCV with moderate resistance to BMS-790052. If L31M and Q30E mutations appear, it will result in HCV with a higher degree of drug resistance to BMS-790052; however, in patients with HCV-1b, single aberrance of L31 or Y93 loci only causes minimal resistance to BMS-790052. A higher degree of drug resistance to BMS-790052 would happen in the case of L31V+Y93H or L31M+Y93H joint resistance. In this study, L31M+Y93H joint variations didn't occur in patients. When we analysed 635 cases of NS5A sequence in the Los Alamos HCV

database, we found that 0.6% of the sequences showed L31M+Y93H variations in 155 cases of HCV-1b NS5A sequence. The incidence rate of L31M+Y93H joint mutation was 7% in patients with HCV-1b and 13% in patients with HCV-4, which is significantly higher than the results of this study and Los Alamos HCV database analysis results. Due to the different regions and different subtypes of HCV-1b infection, there may be a large difference in NS5A region.

The HCV NS5B is the last non-structural gene sequence of HCV, and it is located in the end part of the genome of the HCV virus. The variation of NS5B amino acid sequence can influence DAA activity and genetic barrier to resistance.

In all of the HCV genotypes, HCV NS5B polymerase has 65% homology, thus it may become the antiviral target. There is not much clinical data regarding nucleoside NS5B PI monotherapy, so it is not clear whether there were differences in antiviral activity in the different genotypes. Previous research has shown that DAAs seem to have the same antiviral effectiveness in different genotypes. A report showed no significant difference in NM283, R1626 and R7128 to nucleoside NS5B PIs in the treatment of HCV-1a and HCV-1b patients [20]. Variation of NS5B active sites may cause the polymerase functional damage. A study has been reported where the nucleoside NS5B polymerase inhibitor R1626 and RG7128 (mericitabine) were used for the drug-resistant mutants in a single drug therapy study [20]. Similar to previous research results [21–23], there were very few drug-resistant mutation genes to the nucleoside NS5B PI. The variation in HCV-1b and HCV-2 is very small (1–2 samples) but was detected in 17 cases of S282T in 17 cases of HCV-6 type A patients (20.73%).

Although few mutations were found, we still noted the existence of S282T in HCV-6a in the present study. The mutation was found in *in vitro* tests and many of the Phase III and Phase II clinical trial drugs have good application prospects for induced resistance. At present, the majority of Phase II clinical trials in HCV were performed on genotype-1b patients, and less Phase II clinical research was performed on genotype-6a patients. However it is not clear whether the treatment of patients with genotype-6a triggers the drug resistance and treatment response.

In conclusion, in patients with CHC who had never used DAA therapy, we investigated the existence of some variation and detected the presence of resistance-associated mutations in China. The mutations are not universal, therefore, we speculate that DAA therapy may not be used in the future. However, the detection rate of secondary mutations was very high, which may cause the low resistance. Because of the combined mutation potential and rich polymorphism characteristics of HCV, whether these secondary mutations could affect a greater number of patients also needs to be further studied with regard to DAA drugs in the future.

At the same time, for different types of DAAs, the different HCV genotypes are characterized by different drug resistance mutations. HCV-2a and HCV-6a mutations exist commonly in HCV genes and the HCV-1b mutation is relatively less common. Therefore, this study suggests that the future application of DAAs for anti-HCV therapy should be carried out after the genotyping of the HCV gene. According to HCV genotype results, the doctors could decide whether to perform pre-existing DAA drug resistance tests or not in clinical practice.

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Disclosure statement

The authors declare no competing interests.

Additional file

Additional file 1: Supplementary data can be found at http://www.intmedpress.com/uploads/documents/3258_Liu_Addfile1.pdf

References

1. Lalezari J, Box T, O'Riordan W, *et al*. IDX184 in combination with pegylated interferon-alpha2a and ribavirin for 2 weeks in treatment-naive patients with chronic hepatitis C. *Antivir Ther* 2013; **18:**755–764.
2. Lauer GM, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; **345:**41–52.
3. Hadziyannis SJ, Sette HJ, Morgan TR, *et al*. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140:**346–355.
4. Hézode C, Forestier N, Dusheiko G, *et al*. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N Engl J Med* 2009; **360:**1839–1850.
5. Guedj J, Pang PS, Denning J, *et al*. Analysis of hepatitis C viral kinetics during administration of two nucleotide analogues: sofobuvir (GS-7977) and GS-0938. *Antivir Ther* 2014; **19:**211–220.
6. Sarrazin C, Kieffer TL, Bartels D, *et al*. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. *Gastroenterology* 2007; **132:**1767–1777.
7. Franco S, Parera M, Aparicio E, *et al*. Genetic and catalytic efficiency structure of an HCV protease quasispecies. *Hepatology* 2007; **45:**899–910.
8. Kuntzen T, Timm J, Berical A, *et al*. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. *Hepatology* 2008; **48:**1769–1778.

9. Sarrazin C, Rouzier R, Wagner F, *et al*. SCH 503034, a novel hepatitis C virus protease inhibitor, plus pegylated interferon alpha-2b for genotype 1 nonresponders. *Gastroenterology* 2007; **132:**1270–1278.
10. Bartels DJ, Zhou Y, Zhang EZ, *et al*. Natural prevalence of hepatitis C virus variants with decreased sensitivity to NS3.4A protease inhibitors in treatment-naive subjects. *J Infect Dis* 2008; **198:**800–807.
11. Vicenti I, Rosi A, Saladini F, *et al*. Naturally occurring hepatitis C virus (HCV) NS3/4A protease inhibitor resistance-related mutations in HCV genotype 1-infected subjects in Italy. *J Antimicrob Chemother* 2012; **67:**984–987.
12. Fridell RA, Wang C, Sun JH, *et al*. Genotypic and phenotypic analysis of variants resistant to hepatitis C virus nonstructural protein 5A replication complex inhibitor BMS-790052 in humans: *in vitro* and *in vivo* correlations. *Hepatology* 2011; **54:**1924–1935.
13. Gaudieri S, Rauch A, Pfafferott K, *et al*. Hepatitis C virus drug resistance and immune-driven adaptations: relevance to new antiviral therapy. *Hepatology* 2009; **49:**1069–1082.
14. Sun SC, Bae A, Qi X, *et al*. Natural variation in drug susceptibility to HCV polymerase inhibitors in treatment-naive HCV patient isolates. *J Viral Hepat* 2011; **18:**861–870.
15. Arnaud C, Pradat P, Spaziante M, *et al*. Pretreatment predictive factors for hepatitis C therapy outcome: relevance of anti-EIE2 antibodies compared to IP-10 and IL28B genotypes. *Antivir Ther* 2013; **18:**1027–1032.
16. Cai Q, Zhao Z, Liu Y, *et al*. Comparison of three different HCV genotyping methods: core, NS5B sequence analysis and line probe assay. *Int J Mol Med* 2013; **31:**347–352.
17. Taylor MW, Tsukahara T, Brodsky L, *et al*. Changes in gene expression during pegylated interferon and ribavirin therapy of chronic hepatitis C virus distinguish responders from nonresponders to antiviral therapy. *J Virol* 2007; **81:**3391–3401.
18. Vallet S, Viron F, Henquell C, *et al*. NS3 protease polymorphism and natural resistance to protease inhibitors in French patients infected with HCV genotypes 1–5. *Antivir Ther* 2011; **16:**1093–1102.
19. Paolucci S, Fiorina L, Piralla A, *et al*. Naturally occurring mutations to HCV protease inhibitors in treatment-naive patients. *Virol J* 2012; **9:**245.
20. Hernandez D, Zhou N, Ueland J, *et al*. Natural prevalence of NSSA polymorphisms in subjects infected with hepatitis C virus genotype 3 and their effects on the antiviral activity of NSSA inhibitor. *J Clin Virol* 2013; **57:**13–18.
21. Roberts SK, Cooksley G, Dore GJ, *et al*. Robust antiviral activity of R1626, a novel nucleoside analog: a randomized, placebo-controlled study in patients with chronic hepatitis C. *Hepatology* 2008; **48:**398–406.
22. Maimone S, Musolino C, Squadrito G, *et al*. NS3 genetic variability in HCV genotype-1b isolates from liver specimens and blood samples of treatment-native patients with chronic hepatitis C. *Antivir Ther* 2013; **18:**131–134.
23. Kuntzen T, Timm J, Berical A, *et al*. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. *Hepatology* 2008; **48:**1769–1778.
24. Lampe E, Lewis-Ximenez L, Espirito-Santo MP, *et al*. Genetic diversity of HCV in Brazil. *Antivir Ther* 2013; **18:**435–444.

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