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ORIGINAL ARTICLE

Naturally occurring NS5A and NS5B resistant associated substitutions in HCV and HCV/HIV patients in iranian population

Amitis Ramezani[a], Kazem Baesi[b], Mohammad Banifazl[c], Minoo Mohraz[d], Farzin Khorvash[e], Majid Yaran[f], Payam Tabarsi[g], Amir hosein Dalirrooyfard[d], Fatemeh Motevalli[b], Anahita Bavand[a], Arezoo Aghakhani[a,*]

[a] *Clinical Research Dept, Pasteur Institute of Iran, Tehran, Iran*
[b] *Hepatitis and AIDS Dept, Pasteur Institute of Iran, Tehran, Iran*
[c] *Iranian Society for Support of Patients with Infectious Disease, Tehran, Iran*
[d] *Iranian Research Center for HIV/AIDS, Iranian Institute for Reduction of High-Risk Behaviors, Tehran University of Medical Sciences, Tehran, Iran*
[e] *Nosocomial Infection Research Center, Isfahan University of Medical Sciences, Isfahan, Iran*
[f] *Infectious Diseases and Tropical Medicine Research Center, Isfahan University of Medical Sciences, Isfahan, Iran*
[g] *Clinical TB and Epidemiology Research Center, NRITLd, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

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Resistance associated substitution (RAS);
NS5A;
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Summary
Background: The introduction of direct acting antivirals (DAAs) for hepatitis C virus (HCV) treatment promises shorter treatment duration, higher cure rates and fewer side effects. Naturally, occurring Resistance Associated Substitutions (RASs) are major challenge to the success of the HCV antiviral therapy.
Aim: To determine the naturally occurring NS5A and NS5B RASs in Iranian HCV and HCV/human immunodeficiency virus (HIV) patients.
Methods: A total of 209 DAA-naïve chronic HCV patients including 104 HCV mono-infected and 105 HCV/HIV co-infected cases were enrolled. Amplification and Sanger population sequencing of NS5A and NS5B regions of HCV genome were carried out. The amino acid sequence diversity of the NS5A and NS5B regions were analyzed using geno2pheno HCV.

* Corresponding author at: Clinical Research Department, Pasteur Institute of Iran, No. 69, Pasteur avenue, Tehran, 13164, Iran.
E-mail addresses: amitisramezani@hotmail.com (A. Ramezani), kbaesi@gmail.com (K. Baesi), mohammadbanifazl@aol.com (M. Banifazl), minoomohraz@ams.ac.ir (M. Mohraz), khorvash@med.mui.ac.ir (F. Khorvash), Yaranmajid@yahoo.com (M. Yaran), Payamtabarsi@yahoo.com (P. Tabarsi), mehmanedanesh987@gmail.com (A.h. Dalirrooyfard), Fatemehmotevalli@gmail.com (F. Motevalli), anahita_bvd@yahoo.com (A. Bavand), araghakhani@hotmail.com (A. Aghakhani).

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Results: NS5A RASs were detected in 25.5% of HCV and 16.9% of HCV/HIV subjects. In HCV cases, clinically relevant RASs were L28M followed by M28Vand Q30H and Y93H/N. In HCV/HIV subjects, clinically relevant RASs were Y93H/N followed by L28M and P58T and M28V/T and Q30R. NS5B RASs were observed in 11.8% of HCV and 5.9% of HCV/HIV subjects. Clinically relevant substitutions were included V321A/I, C316Y, S282R and L159F. The major *S282T* mutation was not observed.
Conclusion: The emergence of RASs is a growing issue in the setting of current treatment with DAAs. Although currently, screening of RASs is recommended before specific DAA regimens, it should be consider in patients with therapeutic failure and in the cases of retreatment.

Introduction

Hepatitis C virus (HCV) is a serious public health concern which affects 3% of the world's population (130 million people) and one million new cases reported annually [1]. The global prevalence of HCV varies throughout the world [2]. It is estimated that the HCV prevalence in Africa is about 5.3% and in Eastern Mediterranean Region (EMRO) is 4.6% [3]. Middle East also shows high prevalence of HCV, but Iran shows the lowest prevalence of HCV infection in the Middle East (HCV prevalence less than 0.5%) [4].

Currently, HCV is classified into seven genotypes and several subtypes based on sequence differences. The geographic distribution of HCV genotypes is different worldwide. Globally HCV genotype 1 is the most prevalent genotype throughout the world followed by genotype 3 and 2, which accounts for 46%, 22% and 13% of all HCV infections respectively [5]. HCV genotypes 1 and 3 are the most common genotypes in Iran with some regional variations due to differing population backgrounds [1].

The HCV genome consists of 9.0 to 9.8 thousand nucleotides that encode structural and non-structural proteins. The structural proteins (Core and the envelope glycoproteins E1 and E2) form viral particles. These proteins are separated from the non-structural proteins by the short membrane peptide p7. The non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) are involved in viral polyprotein processing and viral replication. NS5A is a pleiotropic protein that has roles in viral replication and assembly, and complex interactions with cellular functions and NS5B acts as a RNA-dependent RNA polymerase (RdRp) which synthesizes RNA using an RNA template [6,7,8].

Since 2011, new medications named direct acting antivirals (DAA) which have the ability to directly inhibit specific HCV proteins that are important for HCV replication in hepatocytes were developed for HCV treatment [9]. DAAs treats HCV infections with shorter treatment duration, higher cure rates and fewer side effects. Currently, four classes of DAAs have been approved for HCV treatment which act on three therapeutic targets including non-structural NS3/4A protease inhibitors, NS5A replication complex inhibitors and nucleoside and non-nucleoside NS5B RNA dependent polymerase inhibitors [10,11]. In Iran current HCV treatment guidelines with DAAs described in Alavian et al. study [4].

Naturally occurring substitutions which account for various degrees of resistance to DAAs are called Resistance Associated Substitutions (RASs) that represent a major challenge to the success of the HCV antiviral therapy [4,12]. The RASs may occur naturally in HCV infected patients before starting DAA treatment and most of the patients with treatment failure harbor the viral isolates with RASs [4]. Two forms of RASs are defined as Drug-Class RAS and Drug-Specific RAS. Drug-class RASs are amino acid substitutions that decrease the susceptibility of a virus to any member of a drug class and may or may not lead to resistance to a specific drug in that class. Drug-specific RASs are amino acid substitutions that decrease the susceptibility of a virus to a specific drug [13].

Due to common routes of transmission, human immunodeficiency virus (HIV) and HCV co- infection is common. The presence of HIV changes the HCV progression and leads to more frequent HCV persistence after acute infection and increased rate of HCV infection progression to liver cirrhosis and hepatocellular carcinoma [14]. RAS were also reported in HIV/HCV co-infected patients [15,16]. However, a smaller body of data is present about the impact of HIV infection in the frequency of RASs in HCV infected cases.

Sofosbuvir (SOF) is a NS5B polymerase inhibitor which had shown a high genetic barrier in all studies. But, nevertheless few substitutions had been detected in NS5B with resistance to SOF [17–20]. The NS5A inhibitor resistant mutants are shown in 10-30% of HCV patients at baseline [21,22]. It seems that assessment of baseline NS5A and NS5B RASs can have a role in optimization of HCV treatment with DAAs in HCV patients with or without HIV co-infection. So, in this study, we evaluated naturally occurring NS5A and NS5B RASs in DAA-naive chronic HCV patients with or without HIV co-infection in Iranian population.

Material and methods

Study population

In this cross sectional study, a total of 209 DAA-naïve chronic HCV patients were enrolled from May 2017 to February 2018. 104 cases were HCV mono-infected and 105 subjects were HCV/HIV co-infected. HCV infection was confirmed by

positive results in HCV antibody (anti-HCV) and/or HCV-RNA tests. HIV infection was determined with positive HIV antibody (anti-HIV) testing, which was confirmed by Western blot assay. A questionnaire was used to collect demographic and clinical data. The project was approved by Pasteur Institute of Iran ethical committee. Informed consent was obtained from all subjects before participation.

Five ml of whole blood from each patient was collected in an ethylene diamine tetra-acetic acid (EDTA) containing sterile tube. Plasma was separated by centrifugation and stored at −70 °C until use.

HCV-RNA extraction

HCV-RNA was extracted from plasma samples using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocol.

NS5A and *NS5B* genes amplification

Reverse transcription polymerase chain reaction (RT-PCR) and first round PCR were performed using the PrimeScript One Step RT-PCR Kit Ver.2 (Takara Bio Inc. Shiga, Japan) for both *NS5A* and *NS5B* genes under the following thermal cycling conditions: 30 minutes (min) at 50 °C and 2 min at 94 °C, then 40 cycles of 30 seconds (sec) at 94 °C, 30 sec at 57 °C, 1 min at 72 °C and 5 min at 72 °C for the final extension.

Second round PCR for the *HCV NS5A* gene was performed using the following reaction mixture: 2 μL of DNA, 2.5 μL of 10X PCR buffer, 0.5 μL of 5 U Pfu, 1 μL of dNTP, 0.5 μL of each PCR primer (10 pM) and up to 25 μL of DEPC-treated water. The final amplified region covered the *HCV NS5A* gene from codon 1 to codon 122. The following primer pairs were used: outer sense primer (SEQ ID NO -1), 5′- GCGGTRCAGTGGATGAACAG -3′; outer antisense primer (SEQ ID NO -2), 5′-GCCCCYGTGATGTAATGG-3′; nested sense primer (SEQ ID NO -3), 5′- CRAGGGTCACKGCRCTGCTG-3′; nested antisense primer (SEQ ID NO -4), 5′- TGCYGGGTCCGGTGGTGTAC- 3′. Denaturation was done at 94 °C, annealing was done at 56 °C and extension at 72 °C. Each step of a cycle was carried out for 1 minute. The outer PCR consisted of 40 cycles, and the nested round consisted of 35 cycles. A final extension step at 72 °C was carried out for 2 minutes.

Second round PCR for the NS5B gene was performed using the following reaction mixture: 2 μL of DNA, 2.5 μL of 10X PCR buffer, 0.5 μL of 5 U Pfu, 1 μL of dNTP, 0.5 μL of each PCR primer (10 pM) and up to 25 μL of DEPC-treated water. The amplicon was a 598-bp region encoding amino acids 162 to 361 of the NS5B gene, and the following primer pairs were used: outer sense primer (RT-1), 5′-AGACACYACAACYCCAATTCCAAC-3′; outer antisense primer (RT -2), 5′- RTACCTCYTCCCCTTGTCGTC-3′; nested sense primer (RT -4), 5′-TGGCGAAGAACGAGGTGTTTTG-3′; nested antisense primer (RT -3), 5′- CCTGGTCATAGCCTCCGTGAAG - 3′. The PCR conditions were as follows: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 seconds (denaturation), 59 °C for 30 seconds (annealing), and 72 °C for 1 minute (extension). A final extension step at 72 °C was carried out for 4 minutes.

Both PCR products were visualized on a 2% agarose gel with gelRed staining.

Direct nucleotide sequencing and sequence analysis

The PCR products were purified using gel purification kit (FavorPrep™ GEL/ PCR Purification Kit, Ping-Tung, Taiwan) and sequenced in an automated DNA sequencer (Macrogen Inc. Seoul, South Korea) and those primers used in second round PCR.

The consensus sequences of each sample were aligned using the CLUSTAL W software, integrated within the MEGA software version 6. The amino acid sequence diversity of the NS5A and NS5B genes were analyzed using geno2pheno HCV (http://hcv.geno2pheno.org/index.php) [23]. The presence of previously identified NS5A and NS5B resistant associated Substitution were analyzed between positions 1-122 and 159-361 of the HCV-NS5A and HCV-NS5B proteins respectively.

Statistical Analysis

The Chi^2 and Fisher Exact tests were used with the SPSS 16 Package program for statistical analysis (Chicago, IL, USA). Data are presented as mean ± SD or, when indicated, as an absolute number and percentage.

Results

A total of 209 chronic HCV patients were enrolled in this study, including 104 HCV mono-infected (GT1a: $n = 26$, GT1b: $n = 3$, GT3a: $n = 75$) and 105 HCV/HIV co-infected (GT1a: $n = 23$, GT1b: $n = 3$, GT3a: $n = 79$) cases. At the time of this study, none of the enrolled patients had been treated with DAAs. All cases were HBsAg negative.

The mean age of HCV mono-infected patients was 41.63 ± 10.82 years. 94.2% of cases were male and 5.8% were female.

The mean age of HCV/HIV co-infected cases was 43.13 ± 7.65 years. 95.2% of patients were male and 4.8% were female. The mean CD4 count of HIV subjects was 409.9 ± 293.38 cells/mm^3. The most common routes of HIV transmission were intravenous drug use (71.6%), heterosexual contact (4.9%) and infected blood and blood products transfusion (2.9%). All of the patients received highly active antiretroviral therapy (HAART) and thus has a good immune profile.

The analysis of substitutions in the NS5A protein was performed on 177 patients (94 HCV mono-infected and 83 HCV/HIV co-infected cases) because 32 samples showed low quality sequences due to poor amplification. We failed to amplify the NS5B region in 48 patients due to low viral load, so this region was analyzed in 76 HCV mono-infected and 85 HCV/HIV co-infected cases.

The HCV sequences used in this study were deposited in GenBank under accession numbers MH562114-MH562322.

Table 1 Amino acid substitutions in NS5A region potentially associated with resistance to DAAs in HCV and HCV/HIV infected patients.

Amino acid residues	HCV mono-infected patients ($n=94$)			HCV/HIV co-infected patients ($n=83$)		
	GT1a ($n=22$)	GT1b ($n=3$)	GT3a ($n=69$)	GT1a ($n=24$)	GT1b ($n=7$)	GT3a ($n=52$)
L28	–	**M ([a]2, 66.7%)**	–	–	**M (1, 14.3%)**	–
M28	**V (1, 4.5%)**	–	–	**V (1, 4.2%)** **T (1, 4.2%)** L (1, 4.2%)	–	–
Q30	**H (1, 4.5%)**	–	–	**R (2, 8.4%)**	–	–
A30	P (2, 9%)	–	V (2, 3%) I (1, 1.5%) M (1, 1.5%) S (1, 1.5%) P (1, 1.5%) T (1, 1.5%)	–	–	S (1, 1.9%)
L31	-	–	P (1, 1.5%) I (1, 1.5%)	–	–	–
H58	Y (2, 9%) P (1, 4.5%)	–	–	–	–	–
P58	–	–	–	–	**T (1, 14.3%)**	–
A92	–	E (1, 33.3%)	–	–	–	–
Y93	Q (1, 4.5%)	–	T (3, 4.5%) **H (1, 1.5%)** **N (1; 1.5%)** D (1, 1.5%) R (1; 1.5%) F (1; 1.5%) G (1; 1.5%) P (1; 1.5%) S (1; 1.5%) V (1; 1.5%)	**N (1; 4.2%)**	**H (1; 14.3%)**	**H (3; 5.7%)** **N (1; 1.9%)** S (1; 1.9%) V (1; 1.9%)

HCV: hepatitis C virus; HIV: human immunodeficiency virus; DAA: direct acting antivirals.
Bold type represents the clinically relevant Resistance Associated Substitutions.
[a] Number and percentages of sequences with NS5A substitutions.

Analysis of natural occurring RASs in NS5A region of HCV

The analysis of natural occurring RASs in NS5A region of HCV was performed in 177 patients including 94 HCV mono-infected and 83 HCV/HIV co-infected cases. NS5A naturally resistance associated substitutions were analyzed between residues 1-122 in which mutations conferring resistance to the NS5A inhibitors including Daclatasvir (DCV), Elbasvir (EBR), Ledipasvir (LDV), Ombitasvir (OBV), Velpatasvir (VEL) and Pibrentasvir (PIB). Major sites associated with resistance to NS5A inhibitors including M28A/T/V, L28M/T, Q30 E/H/R/K, L31M/V/F, P58 S/A/L/T/H/D/P, H58 D/P and Y93 C/H/N were analyzed. Amino acid substitutions in NS5A region potentially associated with resistance to DAAs in HCV and HCV/HIV infected patients is shown in Table 1.

Overall, naturally occurring NS5A RASs were detected in 21.5% (38/177) of the amplified NS5A region sequences [21.7% (10/46) of GT1a, 40% (4/10) of GT1b and 19.8% (24/121) of GT3a infected patients]. Clinically relevant NS5A RASs were seen in 11% (5/46), 40% (4/10) and 5% (6/121) of GT1a, GT1b and GT3a infected cases respectively. The most commonly detected clinically relevant NS5A RASs in GT1a were Q30H/R and M28V/T each with the baseline prevalence of 6.5%, L28M in GT1b with baseline prevalence of 30% and Y93H/N in GT3a with prevalence of 3.3%.

NS5A RASs were seen in 25.5% (24/94) of HCV mono-infected and 16.9% (14/83) of HCV/HIV co-infected cases. There was no significant difference between the two groups regarding the frequency of NS5A naturally occurring RASs (*P*-value = 0.16). Frequency of HCV NS5A naturally resistance associated substitutions in HCV and HCV/HIV patients based on HCV subtypes were shown in Table 2.

NS5A RASs in HCV mono-infected patients

A total of 25.5% (24/94) of HCV mono-infected patients (22.7% of GT1a, 66.7% of GT1b, 24.6% of GT3a) showed natural occurring RASs in NS5A region (Table 2).

The most commonly detected clinically relevant NS5A RASs were L28M (GT1b) with a baseline prevalence of 66.7%, followed by M28V and Q30H (both in GT1a, each with

Table 2 Frequency of HCV NS5A naturally occurring Resistance Associated Substitutions (RASs) in HCV and HCV/HIV patients based on HCV subtypes.

HCV subtypes	NS5A RASs in HCV mono-infected patients	NS5A RASs in HCV/HIV co-infected patients	*P*-value
1a	5/22(22.7%)	5/24(20.8%)	0.87
1b	2/3(66.7%)	2/7(28.6%)	0.67
3a	17/69(24.6%)	7/52 (13.5%)	0.12
Total	24/94 (25.5%)	14/83(16.9%)	0.16

HCV: hepatitis C virus; HIV: human immunodeficiency virus; RASs: resistance associated substitutions.

Table 3 Frequency of HCV NS5B naturally occurring Resistance Associated Substitutions (RASs) in HCV and HCV/HIV patients based on HCV subtypes.

HCV subtypes	NS5B RASs in HCV mono-infected patients	NS5B RASs in HCV/HIV co-infected patients	*P*-value
1a	3/10(30%)	1/22(4.5%)	0.15
1b	1/3(33.3%)	0/7(0%)	0.64
3a	5/63(7.9%)	4/56(7.1%)	1.00
Total	9/76(11.8%)	5/85(5.9%)	0.18

HCV: hepatitis C virus; HIV: human immunodeficiency virus; RASs: resistance associated substitutions.

prevalence 4.5%) and Y93H/N (in GT3a each with baseline prevalence of 1.5%). Clinically relevant RASs were detected in 9.1%, 66.7% and 3% of GT1a, GT1b and GT3a infected cases respectively. We also found some other substitutions in NS5A region with limited clinical significance (Table 1).

Multiple amino acid substitutions in HCV mono-infected patients were observed at a frequency of 7.4% (7/94) in NS5A sequences including L28M + A92E in GT1b, Q30H + M58Y and A30P + Y93Q in GT1a, Y93D + Y93F, A30P + Y93 T and A30M + L31I + Y93T in GT3a.

NS5A RASs in HCV/HIV co-infected patients

The natural occurring NS5A RASs frequency in HCV/HIV co-infected patients was 16.9% (14/83) (20.8% of GT1a, 28.6% of GT1b, 13.5% of GT3a) (Table 2).

Clinically relevant detected RASs were Y93H/N (in GT1a, 1b and 3a) with a baseline prevalence of 26.1% (4.2% of GT1a, 14.3% of GT1b and 7.6% of GT3a) followed by L28M and P58T in GT1b, each in 14.3% of subjects and M28V/T and Q30R in GT1a that both were detected in 8.4% of cases. Clinically relevant RASs were detected in 21%, 42.9% and 7.6% of GT1a, GT1b and GT3a infected cases respectively. We also showed some other substitutions in NS5A region with limited clinical significance (Table 1).

In HCV/HIV co-infected patients, multiple amino acid substitutions were detected with a low frequency of 2.4% (2/83) which detected in one GT1a (M28V + Q30R) and one GT1b (Y93H + P58T) infected patients.

Analysis of natural occurring RASs in NS5B region of HCV

Natural occurring RASs in NS5B region of HCV were analyzed in 76 HCV mono-infected and 85 HCV/HIV co-infected cases. NS5B naturally resistant associated substitutions were analyzed between residues 159—361. Because Sofosbuvir (SOF) is the predominant available NS5B polymerase inhibitor [Nucleoside/nucleotide inhibitors (NI)] in Iran, we mainly investigated the positions which associated to resistance to SOF and other positions such as 368, 411, 414, 448, 553, 554 and 556 which are related to resistance to another NS5B polymerase inhibitor, Dasabuvir [non-nucleoside inhibitor (NNI)], not fully covered in this study.

Natural occurrence of RASs was detected in both HCV and HCV/HIV co-infected patients. Naturally, occurring NS5B RASs were detected in 8.7% (14/161) of the amplified NS5B region sequences. RASs were seen in 12.5% (4/32) of GT1a, 10% (1/10) of GT1b and 7.6% (9/119) of GT3a infected patients.

NS5B RASs were observed in 11.8% (9/76) of HCV and 5.9% (5/85) of HCV/HIV subjects. There was no significant difference between two groups regarding the frequency of natural occurring RASs in NS5B region of HCV (*P*-value = 0.18).

In HCV mono-infected cases RASs were detected in 30% of GT1a, 33.3% of GT1b and 7.9% of GT3a. In HCV/HIV co-infected cases RASs were observed in 4.5%, 0% and 7.1% of GT1a, 1b and 3a respectively. Frequency of HCV NS5B naturally resistance associated substitutions in HCV and HCV/HIV patients based on HCV subtypes were shown in Table 3.

Amino acid substitutions in NS5B region potentially associated with resistance to DAAs in HCV and HCV/HIV infected patients is shown in Table 4. The most common detected mutations were *V321A*/I/H/L/S in both HCV mono and co-infected groups followed by C316L/Y/G, S282 R/N and L159F/P.

The major *S282T* mutation inducing a high level resistance to SOF as well as other reported mutation which is clinically important in resistance to SOF (L320F/C) was not detected. Whereas, the polymorphisms V321A/I (in 1.6% of HCV infected and 3.6% of HCV/HIV co-infected patients), S282R (in 1.6% of HCV infected cases) and L159 F (in 33.3% of HCV infected subjects) that cause reduced susceptibility to SOF were found. C316Y substitution which causes

Table 4 Amino acid substitutions in NS5B region potentially associated with resistance to DAAs in HCV and HCV/HIV infected patients.

Amino acid HCV esidues	HCV mono-infected patients ($n=76$)			HCV/HIV co-infected patients ($n=85$)		
	GT1a ($n=10$)	GT1b ($n=3$)	GT3a ($n=63$)	GT1a ($n=22$)	GT1b ($n=7$)	GT3a ($n=56$)
V321	–	–	**A ([a]1, 1.6%)** T (2; 3.2%) S (1; 1.6%)	–	–	**A (1; 1.8%)** **I (1, 1.8%)** H (1; 1.8%) L (1; 1.8%)
S282	N (1; 10%)	–	**R (1; 1.6%)**	–	–	–
C316	**Y (1; 10%)** G (1; 10%)	–	–	L (1; 4.5%)	–	–
L159	P (1; 10%)	**F (1; 33.3%)**	–	–	–	–

HCV: hepatitis C virus; HIV: human immunodeficiency virus; DAA: direct acting antivirals.
Bold type represents clinically relevant Resistance Associated Substitutions.
[a] Number and percentages of sequences with NS5B substitutions.

resistance to Dasabuvir was also detected in one HCV GT1a mono-infected patient.

Double mutation (*C316Y/L159P*) was observed in one HCV-1a mono-infected subject. In one HCV-3a infected patient, both *NS5A* and *NS5B* mutations were seen (Y93S + V321A).

Discussion

To our knowledge, this study is the first study, which evaluated naturally occurring NS5A and NS5B RASs in DAA-naive chronic HCV patients with or without HIV co-infection in Iranian population.

With introducing DAAs in HCV treatment, the potential impact of natural occurring RASs on HCV treatment outcome is identified and current American Association for the Study of Liver Diseases (AASLD)/ Infectious Diseases Society of America (IDSA) HCV treatment guideline includes specific recommendations for evaluation of natural occurring RASs in clinical practice in order to choose an appropriate regimen [13].

Current data have shown that HCV/HIV co-infection has no effect on the outcome and course of HCV treatment. Indication of therapy, choice of medication, management and follow-up of patients are the same for HCV and HCV/HIV co-infected cases. However, careful evaluation of interactions between DAAs and HIV antiretroviral drugs should be considered before starting any treatment [5,27]. Our findings also showed no significant difference between HCV mono-infected and HCV/HIV co- infected cases regarding the frequency of naturally occurring NS5A and NS5B RASs. Our results also showed that the presence of HIV had no influence on RAS selection.

Overall, NS5A is the most important genomic region considered for the screening of naturally occurring RASs [28]. Recent studies have demonstrated that the prevalence of naturally occurring RASs in HCV infected patients range from 1%–80%, which results to decrease in SVR rates between 1% and 50% [29]. In our investigation, the overall NS5A RASs frequency was 21.5% which is in agreement with the published range in different studies [12,30,31].

The prevalence of NS5A RASs is dependent on viral genotype/subtype, geographic origin and population characteristics [32]. NS5A RAS have an important effect on patient response to treatment, especially in those infected with HCV-GT1a and HCV-GT3a [13,26]. We found naturally occurring NS5A RASs in 40%, 21.7% and 19.8% of GT1b, GT1a and GT3a DAA naïve HCV patients respectively. Sarrazin and Dietz et al. also reported a higher prevalence of naturally occurring NS5A RASs in GT1b than GT1a infected cases with percentages of 25% and 17.6% vs. 13% and 7.1% in each study respectively [32,33]. Paolucci et al. [34] also showed that NS5A RASs were more frequent in GT1b cases than GT1a (53.3% vs. 12.5%). In different studies, natural NS5A RASs were reported in 7.5%–23% of GT3 infected subjects [33,34]. These differences can be due to difference in geographic areas and studying population characteristics. Besides, the higher frequency of NS5A RAS in GT1b infected cases in our survey may be due to the low number of GT1b infected cases compared to GT1a and GT3a patients in this study (due to lower frequency of GT1b in our country), so it is not possible to make a definitive comparison of the NS5A RAS between subtypes.

In our survey, the most commonly detected clinically relevant NS5A RASs were Q30 H/R and M28 V/T in GT1a (6.5%), L28 M in GT1b (30%) and Y93 H/N in GT3a (3.3%) infected cases. GT1a Q30 H/R and M28 T variants show intermediate to high resistant to Daclatasvir, Ledipasvir, Elbasvir and Ombitasvir and intermediate to low resistance to Pibrentasvir and Velpatasvir (Q30H was also low level resistant to Ombitasvir) and M28 V variant show high level resistance to Ombitasvir and intermediate resistance to Ledipasvir and Velpatasvir. GT1b L28 M variant was low resistant variant. GT3a Y93 H/N variants confer high level resistance to Daclatasvir and Velpatasvir [29].

Several studies reported that the majority of GT1a NS5A RASs are at positions M28 T/V, Q30 H/E/R/K, L31 M/V, P32L and Y93 C/H/N which act as primary resistance variations. For GT1b, variants L31F/M/V, P32L and Y93 C/H/N act as primary resistance substitutions, and *L28M*, *L23F*, *R30Q* and *P58S* act as secondary resistance mutations [34,36,37,38]

which are in accordance to our results. In contrast to our findings, a recent study in Uruguayan genotype 1 HCV infected cases, did not find M28T/V, Q30 R/H or Y93H substitutions reported in our survey and worldwide [39]. Besides, we found the amino acid substitution P58T in a GT1b HCV/HIV co-infected patient. While this substitution alone confers minimal resistance to some NS5A inhibitors, it can increase viral resistance to NS5A inhibitors in combination with other NS5A RASs such as Y93H [35].

Multiple amino acid substitutions were detected in 7.4% and 2.4% of HCV mono-infected and co-infected cases respectively. Zeuzem et al. [35] reported that multiple NS5A RASs confer high levels of resistance compared to single RAS, but the clinical importance of this finding is variable because the impact of NS5A RASs to any DAA is multifactorial. It seems that combination of (Y93H + P58T), (M28V + Q30R) and (Q30H + H58Y) have some clinical significance.

Regarding NS5B, in this investigation, naturally occurring RASs were detected in 8.7% of the amplified NS5B region sequences which is comparable to previous reports [30]. Sofosbuvir is a potent inhibitor of the HCV NS5B polymerase with a high genetic barrier to resistance, so emergence of RASs is rare in patients who treated with NS5B included regimens. Dasabuvir, however, shows a low genetic barrier to resistance since the associated *C316N/Y* mutation tends to achieve a naturally occurring prevalence of 10%—36% in HCV infected patients with Dasabuvir in their regimen [12,28,40].The most common detected RAS in SOF treated patients were V321A/I, L159F, C316N/Y/R and L320F [28,35,41]. The S282T variant was initially associated with resistance in vitro [42] but it was not reported in recently published in patients' clinical trials [43]. In this study, the major *S282T* mutation as well as other reported mutation, which is clinically important in resistance to SOF like *L320F/C*, was not detected. This result is in agreement with previous studies from Iran and other countries [44,45,46].

In a study carried out by the FDA Division of Antiviral Products, the *NS5B* L159F mutant (especially in combination with L320F or C316N/Y) and V321A/I emerged in 2.2—4.4% of subjects who failed SOF treatment. In this study, the L159F variant was detected in a 33.3% of HCV GT1b mono-infected patients. NS5B sequences from HCV infected patients showed a prevalence of up to 34% for the *L159F* mutation in GT1b infected patients [40]. Also, V321A/I Substitution were detected in one GT3a HCV mono-infected (1.6%) and in two GT3a HCV/HIV co-infected (3.6%) cases. Double mutation (*C316Y/L159P*) was observed in one HCV-1a mono-infected subject. Dual-class RAS (Y93S + V321A) in one GT3a HCV/HIV co-infected patient were seen. It was reported that 10% of patients harbored RASs in 2 or more drug classes, with NS3/NS5A dual-class RAS being the most common, followed by NS3/NS5B RASs and NS5A/NS5B RASs [30].

There are several limitations in this study. First, the number of GT1b patients was limited, which may have led to relatively high prevalence of mutants in this subtype. Second, some NS5A and NS5B genes were unsuccessfully amplified. Third, Sanger sequencing, not Next-Generation Sequencing (NGS) was used in this study, which has ability to investigate the prevalence of < 20% of Substitution.

Conclusion

To our knowledge, this study is the first study, which evaluated naturally occurring NS5A and NS5B RASs in DAA-naive chronic HCV patients with or without HIV co-infection in Iranian population. Overall, naturally occurring NS5A and NS5B RASs were detected in 21.5% and 8.7% of our cases. There was not any significant difference between HCV mono-infected and HCV/HIV co- infected cases regarding the frequency of naturally occurring NS5A and NS5B RASs. The emergence of RAS is a growing issue in the setting of current treatment with DAAs. Although currently, screening of RAS is recommended before specific DAA regimens, it should be consider in patients with therapeutic failure and in the cases of retreatment. In the other hand screening of naturally occurring RASs may be useful as a strategy to overcome drug resistance in some cases and minimize the risk of treatment failure.

Uncited References

[24,25].

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of Pasteur Institute of Iran ethics committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Disclosure of interest

The authors declare that they have no competing interest.

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References

[1] Gower E, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection. J Hepatol 2014;61(1):45—57.

[2] Karbalaie Niya MH, Salman-Tabar S, Bokharaei-Salim F, Keyvani H. Prevalence of resistant associated variants (RAVs) in the naïve HCV patient candidate for direct acting antiviral (DAA) therapy. Microb Pathog 2017;105:166—70.

[3] Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J Hepatol 2006;45(4):529—38.
[4] Alavian SM, Hajarizadeh B, Bagheri Lankarani K, et al. Recommendations for the clinical management of hepatitis c in iran: a consensus-based national guideline. Hepat Mon 2016;16(8):40—959.
[5] Noble CF, Malta F, Lisboa-Neto G, et al. Natural occurrence of NS5B inhibitor resistance-associated variants in Brazilian patients infected with HCV or HCV and HIV. Arch Virol 2017;162(1):165—9.
[6] Lavanchy D. Evolving epidemiology of hepatitis C virus. Clin Microbiol Infect 2011;17(2):107—15.
[7] Daw MA, Dau AA. Hepatitis C virus in Arab world: a state of concern. Scientific World Journal 2012;2012 [719494].
[8] Alavian SM, Haghbin H. Relative Importance of Hepatitis B and C Viruses in Hepatocellular Carcinoma in EMRO Countries and the Middle East: A Systematic Review. Hepat Mon 2016;16(3):35106.
[9] Bacon BR, Khalid O. Triple therapy with boceprevir for HCV genotype 1 infection: phase III results in relapsers and nonresponders. Liver Int 2012;32:51—3.
[10] Vermehren J, Sarrazin C. New HCV therapies on the horizon. Clin Microbiol Infect 2011;17:122—34.
[11] Perales C, Quer J, Gregori J, Esteban JI, Domingo E. Resistance of hepatitis C virus to inhibitors: complexity and clinical implications. Viruses 2015;7:5746—66.
[12] Chen M, Ma Y, Chen H, et al. Multiple introduction and naturally occurring drug resistance of HCV among HIV-infected intravenous drug users in Yunnan: an origin of China's HIV/HCV epidemics. PLoS One 2015;10(11):142—543.
[13] Hepatitis C guidance: AASLD-IDSA recommendations for testing, managing, and treating adults infected with hepatitis C virus. *Hepatology* **62**, 2015, 932—954.
[14] Sulkowski MS, Thomas DL. Hepatitis C in the HIV-infected patient. Clin Liver Dis 2003;7:179—94.
[15] Jabara CB, Hu F, Mollan KR, et al. Hepatitis C Virus (HCV) NS3 sequence diversity and antiviral resistance-associated variant frequency in HCV/HIV coinfection. Antimicrob Agents Chemother 2014;58:6079—92.
[16] Piroth L. Direct-acting antivirals for hepatitis C virus infections in patients co-infected with human immunodeficiency virus. Clin Res Hepatol Gastroenterol 2011;35(2):75—83.
[17] Gane EJ, Stedman CA, Hyland RH, et al. Nucleotide polymerase inhibitor sofosbuvir plus ribavirin for hepatitis C. N Engl J Med 2013;368:34—44.
[18] Afdhal N, Zeuzem S, Kwo P, et al. Ledipasvir and sofosbuvir for untreated HCV genotype 1 infection. N Engl J Med 2014;370:1889—98.
[19] Afdhal N, Reddy KR, Nelson DR, et al. Ledipasvir and sofosbuvir for previously treated HCV genotype 1 infection. N Engl J Med 2014;370:1483—93.
[20] Kowdley KV, Gordon SC, Reddy KR, et al. Ledipasvir and sofosbuvir for 8 or 12 weeks for chronic HCV without cirrhosis. N Engl J Med 2014;370:1879—88.
[21] Namazee N, Sali S, Asadi S, Shafiei M, Behnava B, Alavian SM. Real response to therapy in chronic hepatitis C virus patients: a study from Iran. Hepat Mon 2012;12(9):6151.
[22] Sharafi H, Alavian SM. IL28B polymorphism, explanation for different responses to therapy in hepatitis C patients. Hepat Mon 2011;11(12):958—9.
[23] Kalaghatgi P, Sikorski AM, Knops E, et al. Geno2pheno[HCV] — a web-based interpretation system to support hepatitis C treatment decisions in the era of direct-acting antiviral agents. PLoS One 2016;11(5):155—869.
[24] Teraoka Y, Uchida T, Imamura M, et al. Prevalence of NS5A resistance associated variants in NS5A inhibitor treatment failures and an effective treatment for NS5A-P32 deleted hepatitis C virus in humanized mice. Biochem Biophys Res Commun 2018;500(2):152—7.
[25] Sagnelli E, Coppola N, Scolastico C, et al. Virologic and clinical expressions of reciprocal inhibitory effect of hepatitis B, C, and delta viruses in patients with chronic hepatitis. Hepatology 2000;32(5):1106—10.
[26] Pawlotsky JM. Hepatitis C virus resistance to direct-acting antiviral drugs in interferon-free regimens. Gastroenterology 2016;151(1):70—86.
[27] Schlabe S, Rockstroh JK. Advances in the treatment of HIV/HCV coinfection in adults. Expert Opin Pharmacother 2018;19(1):49—64.
[28] Brandão R, Marcelino R, Gonçalves F, et al. Characterization of NS5A and NS5B resistance-associated substitutions from genotype 1 hepatitis C virus infected patients in a portuguese cohort. Viruses 2018;10(5):223.
[29] Sorbo MC, Cento V, Di Maio VC, et al. Hepatitis C virus drug resistance associated substitutions and their clinical relevance: Update 2018. Drug Resist Update 2018;37:17—39.
[30] Wang GP, Terrault N, Reeves JD, et al. Prevalence and impact of baseline resistance-associated substitutions on the efficacy of ledipasvir/sofosbuvir or simeprevir/sofosbuvir against GT1 HCV infection. Sci Rep 2018;8(1):31—99.
[31] Patiño-Galindo JÁ, Salvatierra K, González-Candelas F, López-Labrador FX. Comprehensive screening for naturally occurring hepatitis C virus resistance to direct-acting antivirals in the NS3, NS5A, and NS5B genes in worldwide isolates of viral genotypes 1 to 6. Antimicrob Agents Chemother 2016;60(4):2402—16.
[32] Sarrazin C, Dvory-Sobol H, Svarovskaia ES. The prevalence and the effect of HCV NS5A resistance-associated variants in patients with compensated cirrhosis treated with ledipasvir/sofosbuvir- RBV. Proceedings of the 50th Annual Meeting of the European Association for the Study of the Liver 2015:22—6.
[33] Dietz J, Susser S, Berkowski C, Perner D, Zeuzem S, Sarrazin C. Consideration of viral resistance for optimization of direct antiviral therapy of Hepatitis C virus genotype 1-infected patients. PLoS One 2015;10:134—395.
[34] Paolucci S, Floria L, Mariana B, et al. Naturally occurring resistance mutations to inhibitors of HCV NS5A region and NS5B polymerase in DAA treatment-naïve patients. Virol J 2013;10:355.
[35] Zeuzem S, Mizokami M, Pianko S, et al. NS5A resistance-associated substitutions in patients with genotype 1 hepatitis C virus: prevalence and effect on treatment outcome. J Hepatol 2017;66(5):910—8.
[36] Fridell RA, Qiu D, Wang C, Valera L, Gao M. Resistance analysis of the HCV NS5A inhibitors BMS-790052, in the in vitro replicon system. Antimicrob Agents Chemother 2010;54:3641—50.
[37] Fridell RA, Wang C, Sun JH, et al. Genotypic and phenotypic analysis of variants resistant to hepatitis C virus non-structural protein NS5A replication complex inhibitor BMS-790052 in humans: In vitro and in vivo correlations. Hepatology 2011;54:1924—35.
[38] Sun D, Dai M, Shen S, Li C, Yan X. 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. Gene Expr 2018;18(1):63—9.
[39] Aldunate F, Echeverría N, Chiodi D, et al. Pretreatment hepatitis C Virus NS5A/NS5B resistance-associated substitutions in genotype 1 uruguayan infected patients. Dis Markers 2018;2018 [2514901].
[40] Alves R, Queiroz AT, Pessoa MG, et al. The presence of resistance mutations to protease and polymerase inhibitors in Hepatitis C virus sequences from the Los Alamos databank. J Viral Hepat 2013;20(6):414—21.

[41] Svarovskaia ES, Zeuzem S, Hedskog C. Prevalence of pre-treatment NS5A and NS5B resistance-associated variants and genetic variation within HCV subtypes across different countries/Gilead-study. Proceedings of the 50th Annual Meeting of the European Association for the Study of the Liver 2015:22—6.
[42] Ludmerer SW, Graham DJ, Boots E, et al. Replication fitness and NS5B drug sensitivity of diverse hepatitis C virus isolates characterized by using a transient replication assay. Antimicrob Agents Chemother 2005;49:2059—69.
[43] Andre-Garnier E, Ribeyrol O, Gournay J, et al. Emergence of HCV resistance associated variants in patients failing sofosbuvir-based regimens: an observational cohort. Antivir Ther 2016;21(7):611—9.
[44] Franco S, Casadella M, Noguera-Julian M, et al. No detection of the NS5B S282T mutation in treatment-naive genotype 1 HCV/HIV-1 co-infected patients using deep sequencing. J Clin Virol 2013;58:726—9.
[45] Castilho MC, Martins AN, Horbach IS, et al. Association of hepatitis C virus NS5B variants with resistance to new antiviral drugs among untreated patients. Mem Inst Oswaldo Cruz 2011;106:968—75.
[46] Karbalaie Niya MH, Salman-Tabar S, Bokharaei-Salim F, et al. Prevalence of resistant associated variants (RAVs) in the naïve HCV patient candidate for direct acting antiviral (DAA) therapy. Microb Pathog 2017;105:166—70.