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NS5A resistance – associated substitutions in chronic hepatitis C patients with direct acting antiviral treatment failure in Turkey

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ABSTRACT

Objectives: Chronic hepatitis C (CHC) is now a more curable disease with new direct acting antivirals (DAA). Although high sustained virologic response rates, failures still occur in DAA regimens. Our objective in this study was to characterize the real-life presence of clinically relevant resistance – associated substitutions (RASs) in the HCV NS5A gene in CHC patients whose DAA regimen has failed.
Methods: The study enrolled 53 CHC patients who experienced failure with DAA regimen as the prospective longitudinal cohort between 2017–2019. Genotypic resistance testing was performed via the viral population sequencing method and The Geno2pheno HCV tool was used for RAS analysis.
Results: The most frequent failure category was relapse (88%) followed by non-responder (12%). For a total of 36% of patients, RASs was detected in NS5A, Y93H was the most detected RAS in GT1b infected patients (89%).
Conclusions: This study establishes an HCV failure registry for Turkey in which samples were combined with clinical, virologic and molecular data of adult patients whose DAA therapy failed. RASs can occur in CHC patients with DAA treatment failures. Evaluation of RAS after DAA failure is very important before re-treatment is initiated to prevent virologic failure.

Introduction

Hepatitis C virus (HCV) is the etiologic agent of chronic hepatitis C (CHC) and a major cause of cirrhosis and liver cancer (Lee et al., 2012). Since 2013, CHC has become a more curable disease as a result of new direct acting antivirals (DAA) (Rodriguez-Torres et al., 2013). In June 2016, sofosbuvir (SOF) [nonstructural protein 5B (NS5B) polymerase inhibitor], the combination SOF and ledipasvir (LDV) [nonstructural protein 5A (NS5A) inhibitor], the combination of NS5A inhibitor ombitasvir (OBV), dasabuvir (DSV) [NS5B polymerase inhibitor] and paritepravir (PTV) [the nonstructural protein 3/4A (NS3/4A) inhibitor boosted with ritonavir (r) (3D) ± ribavirin (RBV)] started to be used on CHC patients in Turkey (Health Ministry, 2017). However, in 2017, the combination of an NS3/4A inhibitor glecaprevir, an NS5A inhibitor pibrentasvir (GLE/PIB), and sofosbuvir/velpatasvir/voxilaprevir (SOF/VEL/VOX) started to be used around the world (EMA, 2019; USD and HS,

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2019). Today, in Turkey, GLE/PIB has been approved for HCV-infected patients (Health Ministry, 2017).

With extremely high cure rates reported in clinical trials and in real-world data, modern DAAs can effectively cure the vast majority of patients infected with HCV. Sustained virologic response (SVR) rates of 94–100% have been reported with contemporary DAA regimens (Pecoraro et al., 2019; Kumada et al., 2018; Sarigul et al., 2019). Although a low rate of failure has been observed with current DAA regimens, failures still occur (Di Maio et al., 2017a). The reasons for failure of the DAA regimens are mostly patient factors such as nonadherence to treatment, drug interactions with HCV drugs, inappropriate treatment regimen, and inappropriate initial treatment duration (Aziz et al., 2018). Besides these, virologic factors - resistance-associated substitutions (RASs) can affect the response to the therapy in CHC patients with DAAs regimes. Viral failure can occur in 5–10% of patients (Wyles and Luetkemeyer, 2017; Bertolli et al., 2018). In chronically infected humans, HCV has high rates of replication dynamics and viral production with an error-prone RNA polymerase, providing a favorable environment for the emergence and enrichment of viral nucleotide substitutions that give resistance to certain drugs or classes of drugs, particularly under the pressure of drug selection (Wyles and Luetkemeyer, 2017).

In genotype 1a (GT1a) infection, baseline NS5A RASs have a 13% prevalence, whereas there is a 18% prevalence in GT1b infection and a 12–17% prevalence in GT3 infection (Zeuzem et al., 2017; Hezode et al., 2018). Patient characteristics, including the presence of cirrhosis in the patient and any previous HCV treatment regimens (based on non-NS5A inhibitor), can affect the clinical effect of NS5A RAS. After NS5A-based treatment failure, many individuals have HCV NS5A (75–90%) (Wyles et al., 2017). NS5A RASs persist in most individuals for more than 2 years (Lahser et al., 2018). When NS5A RAS is detected, either the duration of treatment is prolonged or ribavirin may be added to the treatment (Sarrazin et al., 2016). Consequently, the resistance test is the most important factor in the choice of the re-treatment method (Hayes et al., 2019).

HCV seroprevalence is about 0.9% in Turkey, whose total population is around 80 million (Data, 2019). HCV GT1b is the most prevalent genotype, accounting for 84% and GT1a is the second most prevalent genotype, with ratio of 6.6% of all infections among Turkish patients (Alagoz et al., 2014). In our country, approved regimens for GT1 - CHC patients are 3D + RBV in GT1a, and 3D in G1b non-cirrhotic naive patients for 12 weeks. For treatment experienced non-cirrhotic patients, LDV/SOF is given for 24 weeks or LDV/SOF + RBV for 12 weeks and a 3D regimen is approved similar to that of naive GT1 patients. In cirrhotic patients, both regimens are approved except for child-Pugh B and C patients (Health Ministry, 2017). Unfortunately, the accessibility to the treatment rate of HCV infection is very poor, but the probable treatment rate is 0.8% for CHC in Turkey (Dore et al., 2014). On the other hand, patients with HCV infection in our country can only be treated with DAA regimens once in their lives. If the patient does not respond to treatment, or experiences relapse after treatment or breakthrough, there is no possibility of being treated again.

In this study, we aimed to provide useful information for the clinicians the managing that the real-life presence of clinically relevant RASs in the NS5A gene in CHC patients whose DAA regimen has failed.

Material and methods

Patients

This work was designed as a prospective longitudinal cohort study, including 53 CHC patients whose DAA regimen failed. Cases has been followed after the cure of HCV RNA who under DAA regimen. Plasma samples have been analyzed only for RASs in the HCV NS5A gene region in the HCV RNA rebounded patients at the laboratory of the PCR Unit of the University of Kocaeli from July 2017 to September 2019. The case who HCV RNA cured has been excluded in the study. The therapy indication and DAA regimen choice were made by physicians according to the CHC guidelines and Local Health Implementation Guidelines (Health Ministry, 2017; Terrault et al., 2018; Lampertico et al., 2017). According to the Local Health Implementation Guideline of Turkey each patient can be treated once with DAA regime and biopsy application is mandatory as a rule before the treatment regime decision. The treatment duration and the dose of DAAs were chosen according to the same guidelines. Samples were taken from twenty-four clinics located in 18 cities in all regions of Turkey for the study: Marmara, Mediterranean, Central Anatolia, Aegean, Black Sea, Eastern Anatolia and Southeastern Anatolia regions.

After stopping the treatment at month 3, HCV RNA negative patients were accepted as SVR. Patients were identified as having relapsed when they showed a virologic reactivation after testing HCV-RNA negative at the end of treatment. Patients whose HCV RNA did not become negative during treatment were defined as non-responders. Patients whose HCV RNA was negative during DAA treatment and whose HCV RNA was positive before the end of treatment were defined as viral breakthrough (Terrault et al., 2018). Fibroscan, due to the limited number of presence in Turkey and the lack of social security coverage, histological scoring was made in the liver biopsy by the modified Ishak method according to the histological activity index (grade) and fibrosis (stage) (Ishak et al., 1995).

Plasma samples were collected from each patient in the process of recording the virologic failure and stored at −80 °C until the RAS analysis. The presence of anti-HCV antibody was tested through commercial ELISAs (Elecsys, Roche Diagnostics, Mannheim, Germany and Axsym, Abbott Laboratories, Abbott Park, IL, USA). To obtain the patients' samples and to ensure the anonymous use of their data in accordance with the Helsinki Declaration and international guidelines, written informed consent was obtained from all study patients. Ethical approval was obtained from the Kocaeli University Ethics Committee (KOU KAEK 12/2016).

HCV RNA isolation and quantification

HCV RNA was extracted by magnetic particle technologies [QIAsypmhony (Qiagen GmBH, Hilden, Germany), Abbott M2000 SP (Abbott Molecular Inc., Des Plaines, IL, USA), and COBAS Ampliprep (Roche Molecular Systems, Inc. Pleasanton, CA, USA) platforms] and quantificated by real-time PCR assays [artus HCV QS – RGQ kit (Qiagen GmBH, Hilden, Germany), Abbott RealTi*m*e HCV Amplification Kit (Abbott Molecular Inc., Des Plaines, IL, USA) and COBAS TaqMan HCV kit (Roche Molecular Systems, Inc. Pleasanton, CA, USA)], respectively.

HCV NS5A gene sequencing

For the amplification of the HCV NS5A gene region, specific primer pairs were designed against the HCV reference strain AF483269.1: HCV1a1b_NS5A_F1; 3'-TCCCCCACGCACTAYGTGCC-5' and HCV1a1b_NS5A_R2; 3'-GTGATRTTICCGCCCAT-5'. Protocols applied for the RT-PCR and cycle sequencing were as follows: cDNA synthesis at 48 °C for 30 min and denaturation at 95 °C for 15 min followed by 50 cycles consisting of the denaturation step at 95 °C for 1 min, annealing step at 55 °C for 1 min, extension at 72 °C for 1 minute, and a final step at 72 °C for 10 min. The extracted amplicon was approximately 938 bp and included all known RASs for the NS5A region. In the sequencing protocol, Hot Start DNA

polymerase (Finnzymes Oy, Vantaa, Finland) was used. For the purification of the PCR products, a High Pure PCR purification kit was used (Roche Diagnostics, Mannheim, Germany). Genotypic resistance testing was performed by the viral population sequencing method (NS5A amino acid position between 1935.–2237.). The cycle sequencing protocol was as follows: 35 cycles at 95 °C for 20 s, 50 °C for 25 s, and finally, 60 °C for 2 min. The final concentrations of the sequencing primers were 0.5 mM. Big Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey, United States), POP – 7 polymer and capillary (36 cm) were used in the sequencing process on an ABI PRISM 3130 platform (Applied Biosystems Inc., Foster City, California, United States).

Resistance-associated substitution detection

The Geno2pheno HCV tool (http://coreceptor.bioinf.mpi-inf.mpg.de/) was used for the RAS analysis. Gheno2pheno identifies the DAA susceptibility for HCV. It is a database specifically designed for rapid computer assisted virtual phenotyping, and accepts genome sequences (in FASTA format) as input for searching known mutations for both licensed and upcoming drugs. The reference strain for NS5A region in the Geno2pheno tool was HCV D90208.

Genotyping results of the HCV strains were provided by the submitter and mostly based on commercial methods. However, the HCV genotype was reassessed by the Geno2pheno-HCV tool. Also, we evaluated the RASs that were associated with DAA failure in the light of the 2018 EASL HCV guidelines. They were scored for the amino acid position 28., 30., 31., 32., 58., 92. and 93. of NS5A region for daclatasvir, elbasvir, ledipasvir, ombitasvir, pibrentasvir and velpatasvir.

Statistical analysis

Sociodemographic, virological and clinical variables were reported as absolute numbers, percentages and medians. Quantitative data processed with descriptive statistical techniques.

For the descriptive analysis, the frequencies and percentages of the qualitative variables and the means containing standard deviation and median with interquartile range of quantitative variables were calculated. All analyses were performed on Microsoft Excel 2017.

Results

The demographic, clinical and virologic data of 53 registered patients are summarized in Table 1. Thirty-three (75%) were males, and the median age was 31 years (range 24–77). All patients were domestic; those with cirrhosis (6 patients, 12%, 83% in Child A class and 17% in Child B class). A liver biopsy was carried out for 32 (62%) patients and the median score of fibrosis was 3 (Lee et al., 2012; Rodriguez-Torres et al., 2013; Health Ministry, 2017; EMA, 2019; USD and HS, 2019). Most of the patients were naive (69%) and 10% had pegylated-interferon experienced treatment. Only 2 (4%) patients had liver transplantation. The therapeutic regimen distribution for HCV genotypes is shown in Table 1.

After failed DAA therapy, the HCV genotype was re-evaluated in all patients and discordance in the genotyping results that was probably causing DAA failure was identified in 4 (8%) patients. The

Table 1
Baseline characteristics of the study population

Characteristic	Study group
Number of patients, *n*	53
Age, median year (range)	31 (24–77)
Gender, male, *n* (%)	39 (75)
Sampling, region/city of Turkey	Marmara; İstanbul, Kocaeli, Bursa Aegean; Çanakkale Mediterranean; Antalya, Hatay, Mersin, Adana, Isparta Southeastern Anatolia; Gaziantep Eastern Anatolia; Elazığ, Van Black Sea; Samsun, Ordu, Bartın, Amasya Central Anatolia; Ankara, Konya
HCV RNA load, baseline median IU/ml (range)	1.7+E6 (2.7+E4–4.0+E6)
ALT level, baseline median IU/ml (range)	54 (7–283)
Patient with cirrhosis, *n* (%)	6 (12)
Child A	5 (83)
Child B	1 (17)
Patient with liver transplantation, *n* (%)	2 (4)
Biopsy status, median score (range), *n* (%)	
Carried liver biopsy	32 (62)
Fibrosis	3 (1–5)
Treatment status, *n* (%)	
Naive patient	36 (69)
Pegylated-interferon experienced	16 (31)
Treatment choice on HCV genotype, *n* (%)	
GT 1a	6 (10)
Ombitasvir + paritaprevir/r + dasabuvir +ribavirin	5 (83)
Ledipasvir + sofosbuvir	1 (17)
GT 1b	42 (81)
Ledipasvir + sofosbuvir	7 (17)
Ledipasvir + sofosbuvir + ribavirin	9 (21)
Ombitasvir + paritaprevir/r + dasabuvir	26 (62)
GT 2	2 (4)
Sofosbuvir + ribavirin	2 (100)
GT 3	3 (5)
Sofosbuvir + ribavirin	1 (33)
Ledipasvir + sofosbuvir + ribavirin	2 (67)

Abbreviations: HCV RNA; hepatitis C virus RNA, ALT; alanine aminotransferase, GT; genotype.

Table 2
Demographic, clinical and virological characteristics of the four patients with misclassification of the HCV genotype, causing direct acting antiviral failure.

Patient	Age	Gender	Reported genotype	Identified genotype	DAA regimen	Duration of regimen, months	RAS in NS5A
1	26	M	1b	3	3D + RBV	12	ND
2	29	M	1b	3a	3D	12	ND
3	34	M	1b	1a	3D	12	ND
4	67	M	2	1b	SOF + RBV	24	ND

Abbreviations: M; male, DAA; direct acting antiviral, 3D; ombitasvir + paritaprevir/r + dasabuvir, RBV; ribavirin, SOF; sofosbuvir, RAS; resistance-associated substitution, ND; not detected.

Table 3
Description of failure during the direct acting antiviral treatment in HCV genotype 1a/b infected patients.

DAA regimen	Patient, *n* (%)	Treatment median time, week (range)	Breakthrough, *n* (%)	Non-response, *n* (%)	Relapse, *n* (%)
LDV + SOF	9 (18)	21 (8–24)	ND	1 (12)	8 (88)
LDV + SOF + RBV	10 (20)	15 (12–24)	ND	1 (10)	9 (90)
3D	25 (50)	12 (12–12)	ND	4 (17)	19 (83)
3D + RBV	4 (8)	12 (12–12)	ND	ND	4 (100)
Total	48 (100)	12 (12–12)	ND	6 (12)	42 (88)

Abbreviations: DAA; direct acting antiviral, LDV + SOF; ledipasvir + sofosbuvir, RBV; ribavirin, 3D; ombitasvir + paritaprevir/r + dasabuvir, ND; not determined.

Table 4
The most frequent treatment-selected resistance–associated substitutions in NS5A according to the treatment regimen.

DAA regimen	GT1a	GT1b
LDV + SOF	–	L28M, L31V, P58S, Y93H
LDV + SOF + RBV	–	L31M, P58S, Y93H
3D	–	L28M, Q30R, P32R, P58T, A92S, Y93H
3D + RBV	Q30H/R, H58C, Y93H	–

Abbreviations: LDV + SOF; ledipasvir + sofosbuvir, RBV; ribavirin, 3D; ombitasvir + paritaprevir/r + dasabuvir, GT; genotype.

characteristics of these patients are shown in Table 2. None of them had RASs in the NS5A region.

The remaining 49 patients had been treated with the optimal DAA regimen according to the guidelines. According to the DAA regime, the description of failure is shown in Table 3. The most frequent failure category with DAA was relapse (88%). Failure in the 3D regimen was highest with 24 (50%) patients, and 20 (83%) of the GT1a and GT1b patients relapsed. The number of patients in whom RASs was detected in NS5A was 19 (36%) and Y93H was the most detected RAS in GT1b patients (89%). The distribution of RASs within NS5A seen in genotypes 1a and 1b HCV patients is shown in Table 4.

Table 4 shows the most frequent treatment-selected RASs in NS5A according to the HCV treatment regimen. In treatment regime with 3D, L28M, Q30R, P32R, P58T, A92S, Y93H RASs were seen in GT1b HCV patients. L31 M, P58S, Y93H RASs were detected in GT1b HCV patients treated with LDV + SOF + RBV. L28M, L31V, P58S, Y93H were seen in patients infected with GT1b and treated with LDV + SOF. However, Q30H/R, H58C, Y93H were seen in patients infected with GT1a and treated with 3D + RBV.

Discussion

In spite of the high efficacy of DAA regimens, a failure rate of 5-15% is observed in HCV infected patients (Martínez et al., 2019; Wang et al., 2018). The failure is generally associated with the RASs. Thus far, a significant amount of real-life data have been studied on this subject. A total of 53 patients whose DAA regimen failed were analyzed in this study. The number of patients in which RASs were detected in NS5A was 36% and Y93H was the most detected RAS in GT1b infected patients (89%). When the DAA treatment of HCV patients is observed to fail, the presence of RASs should be considered (74.4%) (Starace et al., 2018). In the study of GT1 patients by Martinez et al., NS5A RASs were detected in 25.8% of the analyzed sequences (Martínez et al., 2019). In our study, 48 GT1 HCV infected patients failed after DAA treatment and 19 of them had RASs (39.5%). In addition, relapse was detected in 88% of 48 patients with failure, while the other 12% were non-responders. There were no breakthrough cases. Other studies have produced different results in the definition of failure during DAA treatment (Pawlotsky, 2016; Di Maio et al., 2017b).

First of all, treatment failure was the misclassification of the HCV genotype; four patients (8% of cases) were misclassified and all were treated with inappropriate DAA regimens. In studies of failure of DAA regimens in HCV patients, misclassifications were seen in 8–14.9% (Starace et al., 2018; Ceccherini et al., 2016). In cases where the cost of DAA regimens is high, misclassification of the HCV genotype and subtype, it is very important to avoid wrong treatment with the wrong regimens. In addition, the dynamics of RAS should be understood within the circulatory dynamics of HCV, which necessitates the correct typing of the HCV. Sequencing of HCV genome regions with phylogenetic analysis is considered the gold standard of HCV genotyping (Robertson et al., 1998). In countries with a high prevalence of GT1 infection, such as Turkey, it is important to type the HCV for clinical decisions (Alagoz et al., 2014). Therefore, the sequencing-based methods for HCV patients should be considered. Perhaps these findings of ours and similar studies would cause a strong feedback to the title of the typing of HCV diagnostic and treatment guidelines. In addition, correct sequencing methods and genotype and subtype determinations in treatment failures should be studied before resistance tests are performed.

The prevalence of RAS was found to be higher in our GT1b than in GT1a patients. Similarly, the prevalence of at least one major RAS in two or more HCV sites was more frequent in patients with GT1b patients and in patients who did not receive RBV. The high RAS prevalence in GT1b infected patients can be explained by the fact that most of the patients ($n = 26$, 62%) were treated with 3D according to the Turkish Ministry of Health rules of treatment regimen. (Health Ministry, 2017). In addition, our GT1a patients were treated with 3D + RBV according to the national guidelines. The effect of NS5A RAS is relative and can often be overcome by increasing the length of treatment and/or by adding RBV (Sarrazin et al., 2016). The clinical impact of baseline NS5A RASs varies by

HCV genotype and subtype and the largest impacts were shown in genotype 1a and 3 infections. In GT1a M28A/T/V, Q30E/H/K/R, L31M/V, Y93C/H/N and in GT1b L31I/M/V, Y93H are the key NS5A RASs (Wyles and Luetkemeyer, 2017). On the other hand, we published previous HCV drug resistance analysis results in Turkey (Şanlıdağ et al., 2017; Sarigul et al., 2017; Altunok et al., 2016; Ertem et al., 2015). These studies were mostly related to the HCV NS3 gene region. However, we do not have sufficient knowledge about NS5A gene region mutations. Our findings in this study may clarify this issue.

Regarding HCV treatment regimens, the prevalence of RAS in SOF-based regimens is less than that of 3D regimens. This result is consistent with the high resistance barrier of NS5B inhibitors and SOF failure reported in the presence of RAS (Coppola et al., 2016; Sarrazin, 2016). In contrast, the high prevalence of NS5A RAS is associated with a low barrier to the resistance of existing NS5A inhibitors (Sorbo et al., 2018). RASs tend to remain 1-2 years after treatment failure for the NS5A region. In this real-world study, the most common RAS (Y93H) was detected (88%) in the NS5A region in cases of failure, especially in GT1b treated with the optimal DAA regimen. (Sorbo et al., 2018). In particular, in GT1b failures, complex RAS patterns were observed more frequently (with A92S, L28M, L31M/V, Q30R, P32R, P58S/T, Y93H), while all the GT1a NS5A failures were only seen with H58C, Q30H/R, Y93H. These data appear important because NS5A inhibitors are the most popular components of DAA regimens. In previous studies with NS5A inhibitor containing DAA regimens, the amino acid substitutions that produce resistance to NS5A inhibitors have been shown to affect the SVR rate (Di Maio et al., 2017b; Tavares et al., 2018). On the other hand, in GT1a, a single RAS can provide high levels of resistance to most NS5A inhibitors, while in GT1b, there are only high levels of resistance to LDV (Lontok et al., 2015). Because NS5A region RASs tend to be persistent, retreatment strategies should involve a combination of triple or quadruple DAA regimens with high resistance barriers such as NS5B inhibitors as well as a combination of NS3 + NS5A inhibitors (Terrault et al., 2018).

In conclusion, this study established an HCV failure registry for Turkey in which samples were combined with clinical, virologic and molecular data of adult patients whose DAA therapy failed. RASs can occur in CHC patients with DAA treatment failures. Evaluation of RAS after DAA failure is important before re-treatment is initiated to prevent virologic failure.

Contribution of authors

Study design; Murat Sayan, Figen Sarıgül Yıldırım, Sıla Akhan.
Writing; Murat Sayan, Figen Sarıgül Yıldırım.
Data collection; All of authors.
Data analysis; Murat Sayan, Figen Sarıgül Yıldırım.
Patients providing; All of authors.

Conflict of interest

No competing interests exist.

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Ethical approval

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