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Original Article 27

Effect of Baseline Resistance-Associated Substitutions on Thalassemia Patients with Chronic HCV Infection: A Two-Year Follow-Up

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ABSTRACT

BACKGROUND

Direct-acting antivirals (DAAs) against hepatitis C virus (HCV) infection showed the presence of resistant-associated substitutions (RASs). The aim of the present study was to carry out a follow-up of patients with baseline RASs to report the impact of RASs on DAA therapy outcome.

METHODS

In a cohort study, we analyzed NS5A and NS5B RASs among nine thalassemia cases by baseline RASs. In a 2-year follow-up, we analyzed viral markers and biochemical and hematological parameters of the participants and their sustained virologic response (SVR). Statistical analyses were performed using SPSS software version 22.

RESULTS

RASs for HCV subtype 1a included M28V, L31M, and H58P. For subtype 1b: L28M, R30Q, S24F, and C316N. And for subtype 3a: C316S, and S24F. In patients with cirrhosis (n = 5), ALT (p = 0.001) and AST (p = 0.007) levels were significantly reduced after treatment, and creatinine level slightly increased (p = 0.025). However, no significant data was observed in non-cirrhotic patients following the treatment.

CONCLUSION

The present study did not show any adverse effects of DAA therapy among patients with thalassemia suffering from chronic HCV infection with baseline RASs. Furthermore, reduction in ferritin and liver stiffness levels after DAA therapy could show the efficacy of DAA in such patients.

KEYWORDS:
Direct acting antivirals (DAAs), Resistant associated substitutions (RASs), Chronic HCV infection

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INTRODUCTION

Globally, 71 million individuals suffer from chronic hepatitis C virus (HCV) infection with genotypes having specific geographical differences.[1] HCV lifecycle is associated with intra- and extrahepatocellular complications, such as cirrhosis and hepatocellular carcinoma (HCC).[2] However, recently, it has been shown that direct-acting antivirals (DAAs) have extensively been effective and can reduce the disease burden. It has been shown that DAAs results in a sustained virologic response (SVR) in more than 90% of the treated patients.[3]

Recently, wide range usage of DAAs against HCV has increased the risk of emerging resistant-associated variants (RAVs) and/or resistant associated substitutions (RASs). Although RASs usually lead to single or multiple antiviral drug resistance, their functions have been altered in unknown situations.[4,5] Moreover, there is some evidence that elucidates the co-occurrence of RASs, such as C316N in 1b subtype NS5B gene, which has co-occurring mutations, including L159F, A207T, and A218S. This phenomenon could enhance antiviral resistance activity and confer ultimate treatment failure.[6]

Although long-term follow-up studies show different and valuable data in different communities as well as long-term safety and treatment outcomes in anti-HCV drug recipients, so far, only a few studies have tracked patients with thalassemia suffering from HCV infection.[7,8] In addition, patients with advanced disease can be at the risk of developing liver disease and HCC even though their viral infection is inactive and may require long-term monitoring of their liver disease.[9,10]

Here, we aimed to report a long-term follow-up of patients with thalassemia, suffering from HCV infection, who underwent DAA antiviral therapy and had identified RASs before baseline treatment initiation.

MATERIALS AND METHODS

Patients and setting

The population of the present cohort study was patients with thalassemia suffering from HCV infection who were admitted for DAA therapy to the referral Firoozgar Hospital, Tehran, Iran affiliated to Iran University of Medical Sciences, from 2016 to 2019. They had related data sheets as patients with thalassemia and relevant data of suffering from chronic HCV infection identified by sophisticated expert hematologists, specialists in infectious diseases, and gastroenterologists. All participants underwent RAS diagnosis based on NS5A and NS5B using polymerase chain reaction (PCR)-sequencing method at the baseline of DAA treatment initiation. Inclusion criteria were having at least an identified RAS based on PCR-sequencing results and published literature, age > 18 years, and signing informed consent. Excluded patients were those who did not refer to the clinic for follow-up and testing, had previous or present co-infection with HBV and/or HIV, in an HCC stage of the disease, have bone marrow defects, underwent liver transplantation, defect creatinine clearance (< 30 mL/min/1.73 m^2), and history of severe heart disease.

Thalassemia was identified by a hemoglobin electrophoresis test and/or a confirmatory DNA test. Regular deferoxamine injections were received by the participants. HCV infection was identified by viral-specific antibody testing, liver enzymes (AST, ALT, ALK), and viral load calculation using molecular procedures. Transient elastography (FibroScan) was used for liver status evaluation, and > 14.6 kPa was defined as cirrhosis. DAA therapy combination, duration, and dosage were justified as routinely based on cirrhosis and non-cirrhosis conditions evaluated by FibroScan results in which patients with compensated cirrhosis lasts for 6 months and for others 3 months. Treatment follow-up was conducted following a conventional protocol for cirrhotic and non-cirrhotic patients at baseline, end of treatment, and months 6, 12, and 24 after treatment. SVR evaluation was performed based on cirrhosis and non-cirrhosis conditions after the end of treatment at months 6, 12, and 24.

Paraclinical tests and data collection

After 12 hours of fasting, 10 cc whole blood samples were collected in each time from the patients during the follow-up. EDTA containing vacuum tubes (Terumo Europe, Leuven, Belgium) and serum separator tubes (SST, tiger top tube) were used for sample collection. A BS200 auto-analyzer (Mindray, China) was used to measure alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, BUN, alkaline phosphatase (ALP), total and direct bilirubin, albumin, and ferritin. An automated cell counter (Sysmex K-4500,

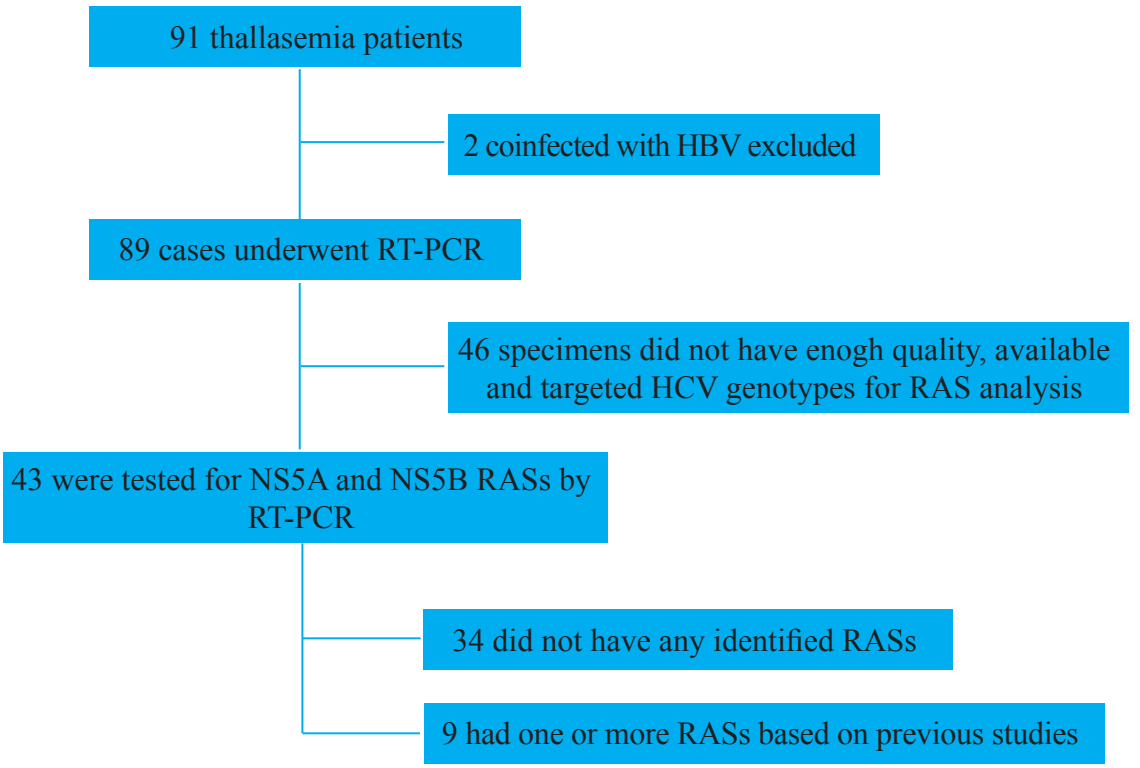

Fig.1: Flow diagram of included participants in the current study

Sysmex, Japan) was used to assess hemoglobin (Hb), platelet (PLT), and white blood cell count (WBC) as well as complete blood cell count (CBC). Also, other hematological tests, including prothrombin time (PT) and partial thromboplastin time (PTT), were performed. In addition, other demographic data were collected using a questionnaire and medical data repository, including age, sex, and splenectomy and treatment history.

Molecular tests

Sera were used for RNA extraction making use of High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the protocols. A nanodrop spectrophotometer (Thermo Scientific, Wilmington, MA) was used to assess the nucleic acid qualification. Also, a cDNA kit for reverse transcription (RT)–PCR (MBI Fermentas, Toronto, Canada) was utilized for cDNA synthesis. Viral load was quantified using RT-PCR (Amplicor HCV, Roche) according to the protocols. Additionally, PCR-RFLP or -Sequencing was carried out for HCV genotyping. Moreover, primers for targeting NS5A and NS5B amplification were designed using popular bioinformatics software. Furthermore, PCR was performed using an in-house protocol (not published), and the products were sequenced using ABI 3730xl sequencer after purification by High Pure PCR Product Purification Kit (Roche Diagnostic, Mannheim, Germany). Sequence analysis was performed via CLC Genomics workbench 5 software (CLC bio, Aarhus, Denmark) and aligned against reference sequences including NC_004102 for 1a, EU781825 for 1b, and NC_009824 for 3a subtypes obtained from GeneBank (https://www.ncbi.nlm.nih.gov/).

RESULTS

Briefly, out of 91 patients with thalassemia referred to the clinic, 89 passed the inclusion criteria, and 43 underwent RASs investigation using conventional RT-PCR. Overall, nine were identified with baseline RASs, which were used for further follow-up at baseline, at the end of treatment, and months 6, 12, and 24 after treatment (Figure 1).

Data analysis using bioinformatics software showed that there were some identified RASs at baseline, which for subtype 1a included M28V, L31M, and H58P, for subtype

Table 1: Characteristics of patients with Hepatitis C virus (HCV) identified by resistant associated substitution (RAS) before direct acting antiviral (DAA) therapy at baseline (n = 9)

HCV subtype	1a					1b			3a	Total
Patient No	10	42	6	32	39	9	65	80	46	9 cases
NS5A RAS	M28V	M28V, L31M	H58P	H58P	H58P	L28M, R30Q	R30Q	R30Q	S24F	10
NS5B RAS	-	-	-	-	-	-	C316N	C316S	-	2
Gender (F/M)	M	F	F	M	M	F	M	F	F	5.4
Age (y)	34	36	26	34	35	38	25	33	30	32.0 ± 4
Hb (g/dl)	8.3	9.9	6.2	8.5	10.4	9.9	12.8	7.3	9.2	9.0 ± 1.0
ALT(mg/dL)	14	154	145	30	72	210	88	13	47	85 ± 69
AST(mg/dL)	24	145	133	29	58	205	77	29	30	81 ± 65
Viral load ($\times 10^4$) (copy/dL)	82.8	19.2195	745.338	103.0	28.217	969.7455	360.623	37.8	166.639	279 ± 348
PLT ($\times 10^3$) mm^3	763	888	160	925	238	142	723	703	510	561 ± 310
WBC ($\times 10^3$) mm^3	13.8	24.9	3.9	2.3	3.85	2.4	10.5	7.45	19.6	9 ± 7
Thalassemia	Major	Major	Major	Major	Major	Major	Major	Major	Major	9
Treatment failure (IFN/RBV) (Y/N)	Yes	No*	Yes	Yes	No*	Yes	Yes	Yes	Yes	7.2
Liver stiffness (NC/C)	Cirrhosis	Non-cirrhotic	Cirrhosis	Non-cirrhotic	Non-cirrhotic	Cirrhosis	Cirrhosis	Non-cirrhotic	Cirrhosis	4.5

Key: *naïve patient; NC/C: Non-cirrhotic/Cirrhosis; Hb: hemoglobin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; PLT: platelet; WBC: white blood cell count; SD: standard deviation; IFN/RBV: Interferon/Ribavirin; NC/C: Non-cirrhosis/Cirrhosis

1b: L28M, R30Q, S24F, C316N, and for subtype 3a: C316S, and S24F (Table 1). RAS positive patients' demographic and laboratory analyses before DAA treatment are shown in Table 1.

Table 2 shows the results of some laboratory tests of nine patients at pre-treatment and immediately at the end of treatment, and also 6 months, 1 year, and 2 years after treatment. The results showed that a significant change occurred in the values of ALT ($p < 0.001$) and AST ($p < 0.001$) levels, where both ALT and AST critically reduced following the treatment.

We also analyzed the results in patients with and without cirrhosis separately. In five patients with cirrhosis, while the ALT ($p = 0.001$) and AST ($p = 0.007$) levels significantly reduced after treatment, the creatinine level slightly increased ($p = 0.025$). More details are reported in Table 3.

Table 4 shows the related results in four non-cirrhotic patients. Based on our results, no significant change was detected in these patients following the treatment.

We also analyzed liver stiffness before and after the treatment and found that in patients with cirrhosis, the means (range) was 25 (12-38) kPa and 14 (10-18) kPa, before and after treatment, respectively. This number was 6 (4-8) kPa both before and after the treatment in patients with cirrhosis.

DISCUSSION

As currently DAAs are widely recommended for HCV infection, baseline-resistant mutants and variants have become a critical issue. Here, we followed up the patients with thalassemia and HCV without a history of DAA and those who relapsed, and those who had DAA RASs prior to the treatment. In this regard, in a cohort of patients with thalassemia, nine cases with baseline RASs were followed up for 2 years after DAA therapy.

In a study of 293 patients infected with HCV genotype 3 and with baseline NS5A RASs (Y93H, A30K, L31M), SVR12 rates for sofosbuvir/velpatasvir (SOF/VEL) ± RBV were reported to be 95.9%.[11] They included 74 (25.3%) patients with cirrhosis, and only one relapse was observed among them, and there were no treatment-related adverse effects. They concluded that NS5A RASs did not have a significant impact on the SVR. Compared with our study results, we have found that NS5A RASs

Table 2: The results of laboratory tests in all patients at pre-treatment and immediately end of treatment, and also 6 months, 1 year, and 2 years after treatment

Variables	Mean ± SD					*p* value*
	Pre-treatment	End treatment	6 months	12 months	24 months	
WBC×1000 mm^3	18.08 ± 22.41	16.08 ± 23.80	18.45 ± 22.66	22.08 ± 24.81	22.39 ± 26.61	0.097
Hb (g/dl)	9.06 ± 1.90	9.63 ± 1.37	9.61 ± 1.01	9.49 ± 1.68	9.43 ± 1.58	0.863
ALT (mg/dL)	72.00 ± 53.29	28.78 ± 19.28	30.44 ± 17.57	26.67 ± 14.76	32.68 ± 20.72	< 0.001
AST(mg/dL)	70.56 ± 45.83	28.67 ± 8.69	30.33 ± 11.95	26.11 ± 6.68	30.60 ± 6.10	< 0.001
Cr (mg/dL)	0.60 ± 0.24	0.73 ± 0.16	0.81 ± 0.22	0.72 ± 0.16	0.68 ± 0.13	0.051
BUN (mg/dL)	21.86 ± 6.49	23.34 ± 9.99	22.11 ± 6.29	22.89 ± 7.82	22.93 ± 7.26	0.963
Ferritin (mg/dL)	2028.75 ± 1704.90	1390.01 ± 1153.60	2134.78 ± 1441.86	1212.56 ± 1446.39	1628.00 ± 1647.66	0.773
ALKP (mg/dL)	238.67 ± 82.61	237.00 ± 82.36	219.78 ± 83.79	212.56 ± 64.66	216.32 ± 94.94	0.326
Bili-T (mg/dL)	2.88 ± 1.69	2.69 ± 1.08	2.66 ± 1.25	2.50 ± 1.45	3.07 ± 1.24	0.693
Bili-D (mg/dL)	0.59 ± 0.38	0.65 ± 0.17	0.62 ± 0.24	0.60 ± 0.28	0.86 ± 0.46	0.216
PT (sec)	14.40 ± 1.80	14.34 ± 1.99	13.36 ± 1.26	14.29 ± 1.47	14.53 ± 2.29	0.665
PTT (sec)	40.47 ± 10.54	35.43 ± 3.94	35.69 ± 3.60	35.06 ± 3.89	33.88 ± 4.62	0.115
Plt×1000 mm^3	458.19 ± 327.44	526.03 ± 409.87	932.66 ± 1329.63	529.00 ± 302.07	553.55 ± 333.57	0.478
Albumin (mg/dL)	4.49 ± 0.47	4.49 ± 0.501	4.62 ± 0.30	4.36 ± 0.40	4.20 ± 0.47	0.069

Hb: hemoglobin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; PLT: platelet; WBC: white blood cell count; SD: standard deviation; Bili-T: Total Bilirubin; Bili-D: Direct Bilirubin; PT: prothrombin time; PTT: partial thrombin time; Cr: creatinine; WBC: white blood cell

*repeated measure test was utilized

Table 3: The results of laboratory tests in patients with cirrhosis at pre-treatment and immediately end of treatment, and also 6 months, 1 year and 2 years after treatment

Variables	Mean ± SD					*p* value*
	Pre-treatment	End treatment	6 months	12 months	24 months	
WBC × 1000 mm^3	10.35 ± 6.71	8.20 ± 5.11	10.62 ± 5.56	15.62 ± 14.02	11.35 ± 6.43	0.328
Hb (g/dL)	9.10 ± 2.38	10.02 ± 1.39	9.54 ± 0.93	10.04 ± 2.12	10.24 ± 1.57	0.773
ALT(mg/dL)	77.40 ± 49.67	34.80 ± 23.09	31.00 ± 17.73	26.40 ± 13.87	35.42 ± 22.73	0.001
AST(mg/dL)	72.40 ± 46.07	28.40 ± 10.21	29.40 ± 12.50	24.40 ± 8.44	32.88 ± 2.68	0.007
Cr (mg/dL)	0.48 ± 0.24	0.75 ± 0.20	0.78 ± 0.11	0.74 ± 0.05	0.67 ± 0.08	0.025
BUN (mg/dL)	20.74 ± 8.00	18.02 ± 3.73	19.20 ± 5.72	20.40 ± 7.70	19.48 ± 5.67	0.922
Ferritin (mg/dL)	2454.76 ± 2175.91	1483.22 ± 1258.91	2316.00 ± 1616.56	920.00 ± 1149.12	1857.20 ± 1646.08	0.239
ALKP (mg/dL)	277.20 ± 92.10	265.80 ± 97.68	237.20 ± 103.44	223.40 ± 68.48	254.58 ± 84.3	0.245
Bili-T (mg/dL)	2.68 ± 1.87	2.27 ± 0.60	2.26 ± 1.08	2.30 ± 1.23	2.94 ± 0.58	0.334
Bili-D (mg/dL)	0.67 ± 0.49	0.72 ± 0.18	0.62 ± 0.23	0.66 ± 0.38	1.10 ± 0.51	0.182
PT (sec)	15.08 ± 2.07	14.50 ± 2.29	13.60 ± 0.89	14.32 ± 0.86	13.24 ± 0.83	0.446
PTT (sec)	39.94 ± 7.19	36.40 ± 3.97	35.64 ± 2.04	35.70 ± 3.73	33.60 ± 5.32	0.411
Plt × 1000 mm^3	457.40 ± 300.7	357.80 ± 209.88	1043.39 ± 1831.29	416.400 ± 295.7	426.00 ± 290.89	0.424
Albumin (mg/dL)	4.62 ± 0.53	4.60 ± 0.66	4.76 ± 0.34	4.46 ± 0.49	4.42 ± 0.49	0.506

Hb: hemoglobin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; PLT: platelet; WBC: white blood cell count; SD: standard deviation; Bili-T: Total Bilirubin; Bili-D: Direct Bilirubin; PT: prothrombin time; PTT: partial thrombin time; Cr: creatinine; WBC: white blood cell

*repeated measure test was utilized

do not have adverse outcomes on the results of DAA therapy, and NS5B RASs were not found to have any impact on drug resistance. Additionally, our study showed that there was not any relapse identified associated with genotype, subtype, or RASs, which could prove the DAA safety and efficiency among patients with HCV.

In a cohort of patients with hemoglobinopathies,[12] DAA therapy during 12 weeks of follow-up were

Table 4: **The results of some laboratory tests in patients without cirrhosis at pre-treatment and immediately end of treatment, and also 6 months, 1 year, and 2 years after treatment**

Variables	Mean ± SD					*p* value*
	Pre-treatment	End treatment	6 months	12 months	24 months	
WBC × 1000 mm^3	27.75 ± 32.48	25.94 ± 35.24	28.24 ± 33.15	30.17 ± 34.97	36.19 ± 37.09	0.174
Hb (g/dL)	9.00 ± 1.42	9.15 ± 1.38	9.70 ± 1.23	8.80 ± 0.60	8.42 ± 0.98	0.120
ALT(mg/dL)	65.25 ± 64.61	21.25 ± 12.01	29.75 ± 20.07	27.00 ± 18.02	29.25 ± 20.68	0.183
AST(mg/dL)	68.25 ± 52.53	29.00 ± 7.87	31.50 ± 13.00	28.25 ± 3.59	27.75 ± 8.38	0.066
Cr (mg/dL)	0.75 ± 0.15	0.71 ± 0.14	0.84 ± 0.33	0.70 ± 0.25	0.70 ± 0.19	0.441
BUN (mg/dL)	23.25 ± 4.72	30.00 ± 11.89	25.75 ± 5.50	26.00 ± 7.79	27.25 ± 7.27	0.341
Ferritin (mg/dL)	1496.25 ± 870.55	1273.50 ± 1184.50	1908.25 ± 1391.55	1578.25 ± 1870.8	1341.50 ± 1851.93	0.806
ALKP (mg/dL)	190.50 ± 36.30	201.00 ± 47.51	198.00 ± 57.6	199.00 ± 66.76	168.50 ± 95.26	0.798
Bili-T (mg/dL)	3.12 ± 1.68	3.22 ± 1.40	3.15 ± 1.43	2.75 ± 1.86	3.24 ± 1.89	0.800
Bili-D (mg/dL)	0.48 ± 0.18	0.55 ± 0.10	0.63 ± 0.29	0.53 ± 0.05	0.56 ± 0.1	0.692
PT (sec)	13.55 ± 1.10	14.15 ± 1.88	13.05 ± 1.71	14.25 ± 2.18	16.15 ± 2.62	0.205
PTT (sec)	41.12 ± 15.05	34.22 ± 4.09	35.75 ± 5.32	34.25 ± 4.50	34.22 ± 4.35	0.509
Plt × 1000 mm^3	459.19 ± 406.63	736.32 ± 532.1	794.25 ± 44.38	669.75 ± 2 81.42	712.25 ± 351.35	0.583
Albumin (mg/dL)	4.32 ± 0.39	4.35 ± 0.19	4.45 ± 0.13	4.22 ± 0.26	3.92 ± 0.3	0.099

Hb: hemoglobin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; PLT: platelet; WBC: white blood cell count; SD: standard deviation; Bili-T: Total Bilirubin; Bili-D: Direct Bilirubin; PT: prothrombin time; PTT: partial thrombin time; Cr: creatinine; WBC: white blood cell

*repeated measure test was utilized

evaluated. The results of the study showed that of 139 patients, one did not achieve SVR, and five (3.6%) relapsed. Also, the serum ferritin levels reduction was seen at week 12. The researchers did not report any adverse effects of anti-viral therapy. Although ferritin level showed a fluctuating trend in our follow-up schedule, the overall levels showed a reduction in these patients regardless of genotype, subtype, RASs, and liver injury. This showed a less adverse effect and less possibility of relapse in HCV treatment with DAAs.

In another study on patients with thalassemia major,[13] it was found that the iron level was associated with liver fibrosis but not disease chronicity. Also, another study [14] reported the rate of liver fibrosis progression measured in a four-year follow-up study and found that median iron level was 8.7 mg/dL, and serum ferritin was 1615 g/L, and AST and ALT ratio were 1.5 and 2.5 times upper than the normal range, respectively. Our study showed that the liver stiffness was improved after two years of follow-up and for patients with cirrhosis it reduced drastically from a mean of 25 to 14 kPa (Figure 2). For patients without cirrhosis, no significant differences were found in the liver stiffness before and after the treatment. In other words, DAA therapy in patients with cirrhosis could have positive impacts on virus clearance as well as liver injury and healing of liver lesions.

Moreover, in another study, daclatasvir plus sofosbuvir (DCV + SOF) and daclatasvir plus sofosbuvir plus ribavirin (DCV + SOF + RBV) combination therapies were evaluated, and it was found that 91% of the patients (419/460) obtained SVR12.[15] Only one patient did not achieve SVR and 13 relapsed. SVR12 did not depend on HCV genotype or cirrhosis and liver transplant or HIV/HCV coinfection status. In comparison with our study results, we reported 100% SVR12 for non-cirrhotic and SVR24 for cirrhotic patients, and we did not have any relapse, nor did the patients achieve SVR. This finding showed the efficacy of DAA therapy regardless of the liver injury degree and other host and viral factors.

As DAA therapy achieves stable SVR in a majority of patients regardless of genotype, subtype liver disease status, etc., it could not prevent HCC occurrence or progression. Follow-up studies are recommended for patients surveillance.[16] The present study was an attempt to investigate the treatment outcome of the patients with RASs as a surveillance study, finding that DAA therapy had a satisfactory result on both patients with and without cirrhosis; this is while we followed up patients with

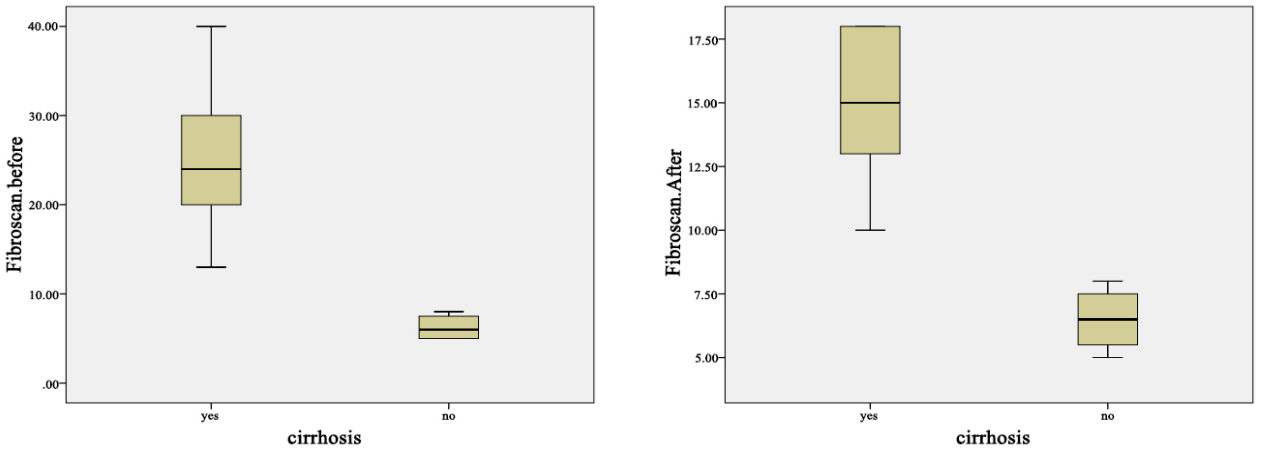

Fig.2: Fibroscan results of nine cases based on the treatment schedule at baseline (before) and after treatment

different genotypes, subtypes, and RASs frequencies.

Our study has some limitations, such as small sample size and limited population; thus further studies using a larger sample size and longer duration of the follow-up are suggested. Although we had 89 participants, due to strict inclusion criteria and using RASs molecular testing for NS5A and NS5B we had limited population. Iron levels could be another measure to investigate drug efficacy, which we could not take into account, and it is recommended for further studies. However, using regular deferoxamine as an iron-chelating agent could balance its levels as it reflects in ferritin levels, which did not have significant fluctuation during the study period. Another limitation of our study was just using SVR for patient follow-up, and other measures such as RVR or early viral response (EVR) were not calculated due to considering for fewer interventions and also because of a limited budget.

In conclusion, according to the present follow-up study, no adverse effects of DAA therapy and/or resistance to DAA therapy were found; however, some RASs were observed in these patients. It seems that NS5A and NS5B RASs do have significant impacts on the virus defense against DAAs. Also, DAAs could reduce liver injury as time passes. Additionally, ferritin levels could be adjusted and become lower after HCV treatment in patients with thalassemia.

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Ethics declaration:

All ethical issues were done according to the Helsinki declaration. Ethics was approved by the Ethics Committee of Iran University of Medical Sciences, Tehran, Iran by (code IR.IUMS.REC 1396.30299).

ETHICAL APPROVAL

There is nothing to be declared.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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