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Evaluating Drug Resistant Mutations to HCV NS3 Protease Inhibitors in Iranian Naïve Patients

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Abstract

Hepatitis C virus (HCV) is an important causative agent of acute and chronic hepatitis. The non-structural protein 3 (NS3) of HCV retains two enzymatic domains which are essential for the virus life cycle. The serine protease inhibitors have developed to improve the responses of HCV-infected patients that have an effective impact on NS3. Nonetheless, drug-resistant variants are the prominent obstruction toward therapeutic success. Sixty-eight Iranian patients infected with HCV genotypes 1a and 3a and diagnosed with chronic active hepatitis were examined. Plasma viral RNA was used to amplify and sequence the HCV NS3 gene; also, HCV viral load, molecular genotyping, and the ALT test were determined for all samples. The sequencing results were used to be analyzed by several reliable bioinformatics tools to determine the physicochemical properties, B cell epitopes, post-modification changes and secondary/tertiary structures; and evaluate the interactions with four drugs. Our results showed that 45% of patients were 1a genotype, the rest of them belonged to 3a genotype, and 70% of patients had abnormal ALT and AST levels. Several substitutions were observed in codons I52M, S102A, L132I, and S166A in 3a genotype and 40, 153 and 91 in 1a genotype. Interactions between references and sample sequences with available drugs showed that different genotypes or common mutations could not have any striking effect on the energy value of the interaction. This study displayed resistance mutations and genetic polymorphisms of NS3 region that are crucial in determining the efficiency of protease inhibitor class of drugs in Iranian HCV infected patients.

Keywords HCV · NS3 · Drug resistance · Bioinformatics

Abbreviations

HCV	Hepatitis C virus
NS3	Non-structural protein 3
HCC	Hepatocellular carcinoma
IHN	Iran hepatitis network
NS	Nonstructural
PIs	Protease inhibitors
RDRP	RNA-dependent RNA polymerase
HIV	Human immunodeficiency virus
HBsAg	Hepatitis B surface antigen
aa	Amino acid

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Introduction

A report by WHO in 2017 described around 71 million people (1.1% of world population) have chronic hepatitis C virus (HCV) globally, which is a leading cause of chronic liver and disease and hepatocellular carcinoma (HCC); it is considered as a serious health-care problem all over the world (World Health Organization 2017; Hashempoor et al. 2018; Borhani et al. 2017).

The genome of this virus is a single-stranded RNA includes four structural proteins (C, E1, E2, and p7) and

six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Tan 2006).

Previously, the standard therapy for chronic hepatitis C patients was a combination of pegylated interferon-α (PEG-IFN)-α and ribavirin which was an indirect antiviral treatment without any direct target on HCV protein or RNA element (Asselah et al. 2010; Palumbo 2011; Chung et al. 2008; Alborzi et al. 2017). In 2016, Iran Hepatitis Network (IHN) prepared recommendations for the Clinical Management of Hepatitis C in Iran, in which in HCV genotype 1 and 4, Daclatasvir/Sofosbuvir and Ledipasvir/Sofosbuvir were recommended. In HCV genotype 2, Sofosbuvir plus Ribavirin and in HCV genotype 3, Daclatasvir/Sofosbuvir was recommended (Alavian et al. 2016).

Protease inhibitors (PIs) hinder NS3 protease activity by attaching to the activation site and surrounding motifs which lead to limitation of viral replication both in vitro and in vivo via blocking enzyme activity and functional properties (Xue et al. 2013; Afrasiabi et al. 2015; Romano et al. 2010).

189 amino acids from the N-terminal part of the NS3 protein are responsible for NS3 protease activity that is vital for replication as well as a target for antiviral agents(Tan 2006; Natarajan 2010). Recently, two main protease inhibitors, i.e. telaprevir, and boceprevir, are approved and used in combination with PEG-IFN-α and ribavirin that play a significant role in suppression of viral replication in chronic patients (Coppola et al. 2014; Saadoun et al. 2013; Wilby et al. 2012).

Prior to the widespread use of NS3 protease drugs in developing countries, screening of specific natural mutations in NS3 protease should be warranted based on their cost-effectiveness and susceptibility. Effective PIs resistant mutations have been characterized in different studies and their impacts on treatment outcomes have also been delineated recently, in addition, genetic barrier to resistance, fitness of resistant viral populations and drug exposure are the major parameters influence HCV resistance (López-Labrador et al. 2008; Romano et al. 2010).

Recently, bioinformatics analysis have progressed significantly in order to identify several tasks, namely gene identification, gene functional annotation, and analysis of phylogenetic relationships (Dehghani et al. 2017, 2019a; b, c; Moattari et al. 2015).

This research aimed to compare and identify mutations between HCV Iranian patients in 1a and 3a HCV genotypes. Besides, we used several trustworthy software to investigate the features of NS3 region and find the effects of mutations on the function of this region, physicochemical properties, B-cell and T-cell epitopes, and secondary and tertiary structures. In addition, evaluating the interaction between NS3 protein and four protease inhibitors were estimated to determine the effect of mutations on the function of drugs.

Materials and Methods

Study Population

Sixty-eight patients were enrolled in this study which was conducted at a referral university hospital (Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran).

The sample size of this study was calculated with alpha = 0.05 and power of 0.8 (80%), and the inclusion criteria were: being IFN-naïve CHC patients infected with genotype 1a and 3a with the negative antibody against human immunodeficiency virus (HIV) and hepatitis B (HBV) surface antigen (HBsAg).

All patients showed the chronic active hepatitis by histological tests and results of polymerase chain reaction (PCR) for HCV RNA, and none of them were at cirrhosis stage. Several criteria were considered to exclude the patients including being co-infected with HBV or HIV types 1 and 2; liver disease of origin other than HCV infection; and infection with other genotypes of HCV (1b, 2a, 3a, 4).

All the subjects provided informed consent and agreed that their samples be used for research. Patients' codes were used instead of names in the study databases for patient privacy. The study was approved by the Ethics Committee of Shiraz University of Medical Silences.

HCV Viral Load

In order to determine the viral load, we carried out reverse transcription (RT), followed by quantitative PCR (Stratec Biomedical, Birkenfeld, Germany).

Molecular Genotyping

INNO-LiPA HCV II kit (Innogenetics, Ghent, Belgium) was used for genotyping; the test was carried out according to the manufacture's instruction.

ALT Test

The ALT and AST levels in patients before treatment, monthly during treatment, at the end of treatment, and 6 months after the end of treatment were defined by Randox reagents.

PCR and Sequencing

Viral RNA was extracted from 200 µl serum sample positive for HCV-1a and HCV-3a by High pure viral RNA kit (Invitek) according to the manufacturer's instructions and

followed by cDNA synthesis using MMLV reverse transcriptase and random hexamer primers.

Amplification of NS3 protease catalytic domain performed using the primers listed in Table 1 as well as amplification condition which was shown in Table 2. All primers used in this study were designed by authors.

The amplified products were analyzed on 1% agarose gel, purified by using commercial gel extraction kit (QiagenGmbH, Hilden, Germany) and subsequently sequenced with the second-round PCR primers (ABI PRISM 3100 automated sequencer, Applied Biosystems, Foster City, CA, USA).

Amino Acid Changing

The CLC sequence viewer version Beta (QIAGEN) was employed to analyze the mutations in all sequences, also to translate sequences and edit them. In this study, we used two sequences as the reference sequences for 1a and 3a genotypes (1a: AFS60311, and 3a: D28917).

Physico-chemical Properties

"Expasy's ProtParam" (http://expasy.org/tools/protparam.html) (Gasteiger et al. 2005) was used to estimate all properties of NS3 protein including theoretical isoelectric point (pI), extinction coefficient, instability index, molecular weight, the total number of positive and negative residues, aliphatic index, and grand average hydropathy (GRAVY).

Post-modification Changes

NetPhosK (http://www.cbs.dtu.dk/services/NetPhosK/) (Blom et al. 2004) was used to determine kinase specific phosphorylation sites in eukaryotic proteins. DISPHOS (http://www.dabi.temple.edu/disphos/pred.html) (Iakoucheva et al. 2004) and NetPhos (http://www.cbs.dtu.dk/services/NetPhos/) (Blom et al. 1999) were used to predict serine, threonine, and tyrosine phosphorylation sites in eukaryotic proteins. N-glycosylation sites were predicted using NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) (Gupta et al. 2017) and GlycoEP (http://www.imtech.res.in/raghava/glycoep/submit.html) (Chauhan et al. 2013).

Table 2 The PCR thermal condition used in this study

	Temperature (°C)	Time	Cycles
Initial denaturation	94	5 min	1
Denaturation	94	50 s	15
Annealing	60	45 s	
Extension	72	45 s	
Denaturation	94	50 s	15
Annealing	57	45 s	
Extension	72	45 s	
Denaturation	94	50 s	10
Annealing	54	45 s	
Extension	72	45 s	
Final extension	72	5 min	1

B-Cell Epitopes Prediction

ABCpred software (http://www.imtech.res.in/raghava/abcpred/) (Saha 2006) and imtech (http://www.imtech.res.in/raghava/bcepred) (Saha and Raghava 2004) B-cell epitopes prediction were performed.

Table 1 The list of primers which was used in this study

	Primers	PCR products length
3a genotype		
Outer pair		
Forward	TTGCGGAGATATTCTTTGC(3333-3351)	701
Reverse	CCTACTTGATAGCTCTGTGGG(4034-4014)	
Inner pair		
Forward	GCCGTGAGGTGTTGTTGG(3377-3394)	622
Reverse	TGAATTGTCAGAGAAAGATGG(3999-3979)	
1a genotype		
Outer pair		
Forward	CAAATGGAGACCAAGCTCATCAC(3273-3295)	794
Reverse	AGCCGGGACCTTNGTGCT(4067-4050)	
Inner pair		
Forward	GCGTGCGGTGACATCATC(3315-3332)	743
Reverse	CTTNGTGCTCTTACCGCTGC(4058-4039)	

Secondary and Tertiary Structures

SOPMA software was used for calculating the secondary structure at (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) (Geourjon and Deleage 1995). The results were established by Phyre server (Kelley and Sternberg 2009) at (http://www.sbg.bio.ic.ac.uk/phyre). "I-TASSER" (Roy et al. 2010) at (http://zhanglab.ccmb.med.umich.edu/I-TASSER), "Phyre2" program (Kelley and Sternberg 2009) at (http://www.sbg.bio.ic.ac.uk/~phyre2/html), and "(PS)2-v2" (Chen et al. 2006) Served at (http://ps2v2.life.nctu.edu.tw) were utilized to predict the tertiary structure of the selected sequences. All predicted 3D structures were evaluated for the stereochemistry, reliability, and quality by "Qmean" (Benkert et al. 2008) at (http://swissmodel.expasy.org/qmean/cgi/index.cgi) and "Rammpage" at (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php).

In Silico Docking

Further refinement of the retrieved targeted compounds was done by Discovery Studio and NS3 proteins were docked with some HCV Protease Inhibitor Drugs by using the HEX docking software. The parameters used for the docking process were: 1. Correlation type: shape + electrostatics, 2. FFT Mode: 3D, 3. Post Processing: MM Energies, 4. Grid Dimension: 0.6, 5. Receptor range: 180, 6. Ligand range: 180, 7. Twist range: 360, 8. Distance range: 40.

Statistical Analysis

All statistical analyses were performed using the statistical package for the social sciences (SPSS version 15, USA). We used the Shapiro–Wilk test to assess normal distribution, the Pearson χ^2 test for categorical data and ANOVA for comparison of the mean values. A p value of less than 0.05 was considered as statistically significant.

Table 4 NS3 Amino Acid substitutions in 1a and 3a subtypes, the numbers indicated the number of patients who had the mutations, the most prevalent mutations were bolded

Mutations	HCV 1a	HCV 3a	Mutations	HCV 1a	HCV 3a
I 3 V	2		Q 80 K	1	
A 7 T		2	**S 91 A**	**16**	
L 14 F		3	P 96 A	2	
V 33 I	2		A 98 T		14
A 39 T	2		A 98 S		2
A 39 S	1		**S 102 A**		**29**
T 40 A	**15**		S 102 V		1
T 40 G	1		S 102 T		1
T 40 S	1		D 110 E		2
T 47 S		1	S 125 G	2	
I 52 M		**32**	L 127 I		4
V 55 I	2		**L 132 I**		**17**
I 64 L	5		S 147 A		2
G 66 S		2	A 147 S	2	
A 67 V		9	**L 153 I**	**16**	
A 67 I		1	**S 166 A**		**30**
Q 80 L	3		S 176 N		2

Results

Clinical Analysis

A total of 68 subjects were enrolled in this cohort study. There were 31 (45.6%) patients with HCV1a and 37 (54.4%) with HCV 3a infection. Our results indicated that 64.6%, 73.8% and 35.9% of patients had an abnormal level of ALT, AST, and FBS at the baseline, respectively. Glucose abnormality was observed in 25 (36.8%) patients, but it had no significant association with sex (0.352), age ($p = 0.151$), or HCV genotype ($p = 0.805$). Moreover, the glucose abnormality had no impacts on NS3 substitutions ($p > 0.05$). The pre-treatment characteristics of the patients are summarized in Table 3.

Table 3 Pre-treatment characteristics of HCV-infected patients

Parameters	Genotype 1a	Genotype 3a	Total
Sex (male); n (%)	24 (77.4%)	30 (81.1%)	54 (79.4%)
Age (year); mean ± SD	38 ± 13	40 ± 13	39 ± 13
ALT (U/L); mean ± SD	58 ± 38	71 ± 45	64 ± 42
AST (U/L); mean ± SD	64 ± 44	91 ± 56	78 ± 52
FBS (mg/dL); mean ± SD	95 ± 14	95 ± 29	95 ± 23
HCV Load (IU/mL); mean ± SD	$43.3 \times 10^5 \pm 73.3 \times 10^5$	$20.8 \times 10^5 \pm 37.1 \times 10^5$	$34.1 \times 10^5 \pm 61.6 \times 10^5$

Amino Acid Variations

The patients' sequences in comparison with reference isolates presented numerous amino acid (aa) residues substitution, as shown in Table 4.

The most prevalent mutations in 1a genotype were T40A (48.3%), S91A (51.6%), and L153I (51.6%); for 3a genotypes, and were I52M (86.4%), S102A(78.3%), L132I(45.9%), and S166A(81%) substitutions.

"Protparam" Results

PI analysis results showed that NS3 peptide is an acidic peptide. The estimated half-life expected the stability of peptide in mammalian reticulocytes, yeast, and Escherichia coli.

The instability index, an estimate of the stability of a protein in a test tube, indicated that it was a stable peptide.

Aliphatic index, a positive factor for the increase of thermostability of proteins, indicated that this peptide was a thermo-stable peptide. GRAVY is a hydropathicity index and positive score showed that this peptide was a hydrophobic peptide (Table 5).

Post-translational Modification Analysis

Prediction of serine, threonine, and tyrosine phosphorylation sites by "DISPHOS", "NetPhos" showed several positions for 1a genotype and 3a genotype; also, the results showed the remarkable impact of mutations in these regions (Table 6).

The outcomes of glycosylation site prediction for all sequences of 1a and 3a genotypes by using "NetNGlyc" and "GlycoEP" could not show any reliable sites. Disulfide bonds prediction by Dianna revealed 3 bonds for 1a genotype and two bonds for 3a; in some cases, mutations in three regions (aa 48, 51, and 147) affected these bonds and omitted them (Table 7).

B-Cell Epitopes Prediction

The combination of all predictions in 1a samples showed three potentials B-cell epitopes (21–32, 114–125, and

Table 5 The protparam results of HCV NS3 protein

protparam	NS3 1A	NS3 3A
Molecular weight	19072.8 Dalton	18635.3 Dalton
Theoretical pI	9.12	9.33
Estimated half-life	4.4 h (mammalian reticulocytes, in vitro)	4.4 h (mammalian reticulocytes, in vitro)
	> 20 h (yeast, in vivo)	> 20 h (yeast, in vivo)
	> 10 h (Escherichia coli, in vivo)	> 10 h (Escherichia coli, in vivo)
Instability index	36.34 stable	38.17 stable
Aliphatic index	88.28	88.33
(GRAVY)	0.02	0.091

Table 6 Several phosphorylation sites were predicted by DISPHOS and NetPhos tools (M stands for mutation)

Genotypes	Phosphorylation sites										
NS3 1a Reference	10	22	66	95	105	122	125	128	133	138	178
Samples	M 9 (1)	+	+	M 95 (1)	+	M 122 (1)	M 125 (2)	+	+	+	M 178 (1),M 180 (1)
NS3 3a Reference	10	22	93	105	122	128	138	139			
Samples	+	+	+	+	+	+	+				

+ The position was conserve among all samples

M Mutation in a codon which omitted the Phosphorylation sites

Table 7 Disulfide bonds of NS3 protein for 1a and 3a genotypes, showing one similar bond in both (M stands for mutation)

	NS3 1a			NS3 3a	
Reference	16–47	52–159	99–145	97–159	99–145
Samples	M 48 (1) M 51 (1)	M 48 (1) M 51 (1)	M 48 (1) M 51 (1)	M 147 (2)	

+ The position was conserve among all samples

M Mutation in a codon which omitted the Disulfide bonds

Table 8 B-cell epitopes of NS3 protein for 1a and 3a genotypes almost in both groups, having three similar regions (M stands for mutation)

	NS3 1A			NS3 3A			
Reference	21–32	114–125	135–145	20–28	113–125	133–143	96–105
Samples	+	+	+	+	+	+	M 98 (16) M 102 (20)

+ The position was conserve among all samples

M Mutation in a codon which omitted the B-cell epitopes

Table 9 Analysis of the secondary structure of NS3 in 1a and 3a genotypes. The majority of secondary structures for both genotypes were extended strand and random coil

Sopma	NS3 1A	NS3 3A
Alpha helix (Hh)	46 (25.56%)	34 (18.89%)
Extended strand (Ee)	51 (28.33%)	57 (31.67%)
Beta turn (Tt)	17 (9.44%)	26 (14.44%)
Random coil (Cc)	66 (36.67%)	63 (35.00%)

135–145). In 3a genotype, 4 regions were predicted as the potential B-cell epitopes (20–28, 113–125, 133–143 and 96–105). In addition, mutations in aa98 and aa102 were common mutations in sequences that had effective outcomes on B-cell epitopes prediction (Table 8).

Secondary and Tertiary Structures Prediction

Secondary structure prediction by SOMPA software is summarized in Table 9. Also, prediction of the transmembrane (TM) secondary structure features is displayed in Fig. 1.SOMPA showed the majority of NS3 protein contains Random coil and Extended strand in both 1a and 3a genotypes. The tertiary structures provided by Itasser illustrated in Fig. 2.

Docking Results

NS3 region of 1a reference sequence and the sequence with major mutations docking with Telaprevir, Boceprevir, Simeprevir, and ciluprevir produced energy values for 1a reference sequence as −280, −250, −130, and −257 respectively and energy values for 3a reference sequence as −242, −226, −154, and −280 (Table 10). By considering the most prevalent mutations, energy values showed insignificant changes, showing that mutations did have a striking effect on the interaction between drugs and NS3 protein.

Docking analysis showed some potential position in contraction between NS3 and drugs, showing that no mutation was located near to the binding site. Figures 3 and 4 showed the binding sites for the 4 selected drugs and genotype 1a and 3a NS3 proteins.

Discussion

The introduction of protease inhibitors constitutes a major discovery in the treatment of chronic Hepatitis C. Telaprevir and Boceprevir are inhibitors recommended for clinical practice that affects the serine protease catalytic site(Butt and Kanwal 2011; Patel et al. 2011). Ciluprevir is a drug used to treat HCV and has a tremendous potential to inhibit HCV genotypes especially 1a and 1b. Simeprevir is an orally-administered and is the potent NS3 protease inhibitor for use in combined treatment regimens against chronic HCV infection. It has been determined that this drug has

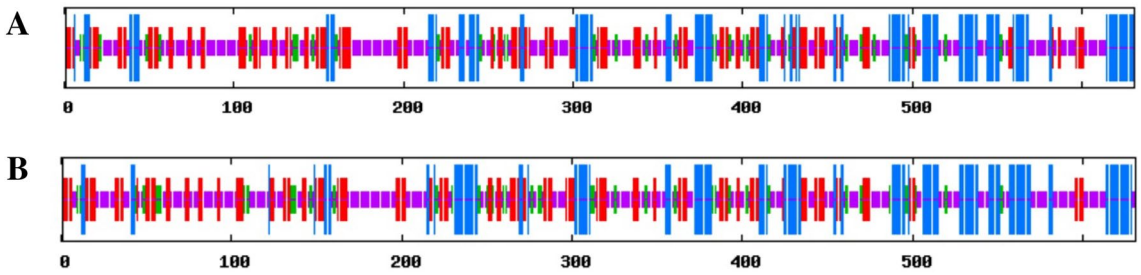

Fig. 1 The results of secondary structure prediction by SOMPA, **a** stands for genotype 1a and **b** for 1b. Blue, red, green, and purple stand for alpha helix, extended stand, beta turn, and random coil, respectively. The accuracy of prediction is shown by the length of a line (Color figure online)

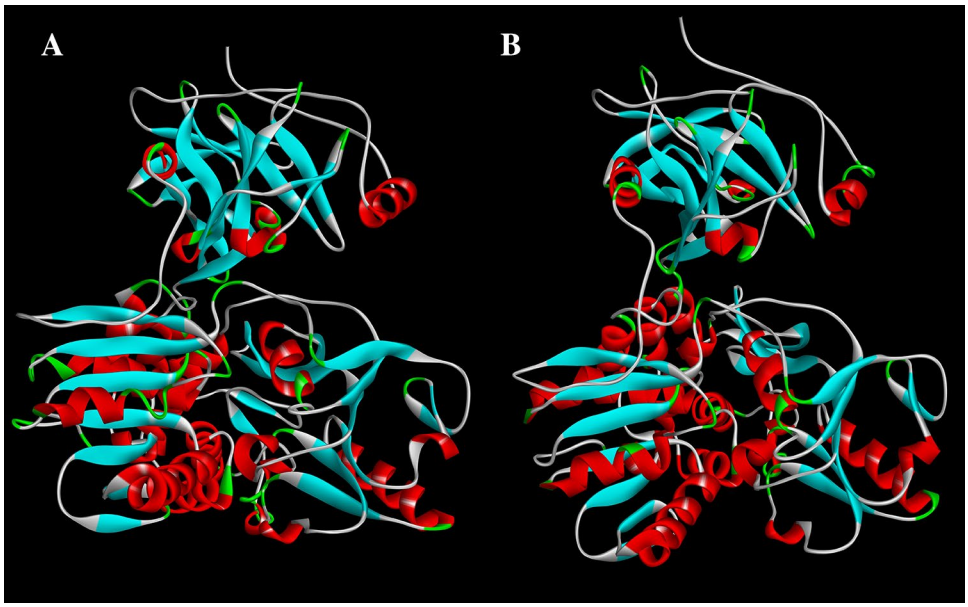

Fig. 2 The 3D structures of 1a genotype NS3 protein (**a**), 3a genotype NS3 protein (**b**)

Table 10 Docking results by Hex

	Telaprevir (*E* value)	Boceprevir (*E* value)	Simeprevir (*E* value)	Ciluprevir (*E* value)
1a				
T40A	− 265	− 226	− 117	− 267
S91A	− 248	− 246	− 120	− 276
L153I	− 279	− 238	− 121	− 257
Ref	− 280	− 250	− 130	− 284
Codons in interactions	238–242, 253, 364, 398–404, 419–423	125, 129, 160–165	75–77, 82–84	253,258,273,256–276, 501–503,548,551–552
3a				
I52M	− 278	− 236	− 124	− 275
S102A	− 230	− 209	− 126	− 257
L132I	− 278	− 235	− 124	− 280
S166A	− 226	− 218	− 129	− 257
Ref	− 242	− 226	− 154	− 280
Codons in interaction	232–238,397–402,418–421,467,469	232–238,256–257,363,396–397,402,418–421	367,425,461–465,473	212,236–241,363,397–402,406–408,418–422,467

There was no significant difference between 1a and 3a genotypes; in all protease inhibitors the energy values remained unchanged by considering the most prevalent mutations, and positions near the binding site were summarized

E value: total binding energy

potent antiviral activity against all HCV genotypes(Sanford 2015; Rajagopalan et al. 2017).

Studies showed that resistant variants against protease inhibitors are responsible for viral resistance in infected patients and are an obstacle to achieving SVR(Perales et al. 2015; Zeminian et al. 2013). Therefore, an assessment of the presence of such variants in patients who have not been treated with this class of drugs is mostly vital; besides, determining the frequency of resistance-associated substitution can be a good prediction of efficiency of PIs in countries, such an Iran, in which the PIs have not been introduced yet.

The present study showed that 80% of all sequences at least had a single mutation in comparison with reference sequences which is inconsistent with the results of previous studies (Lin et al. 2014; Kirst et al. 2013; Svarovskaia

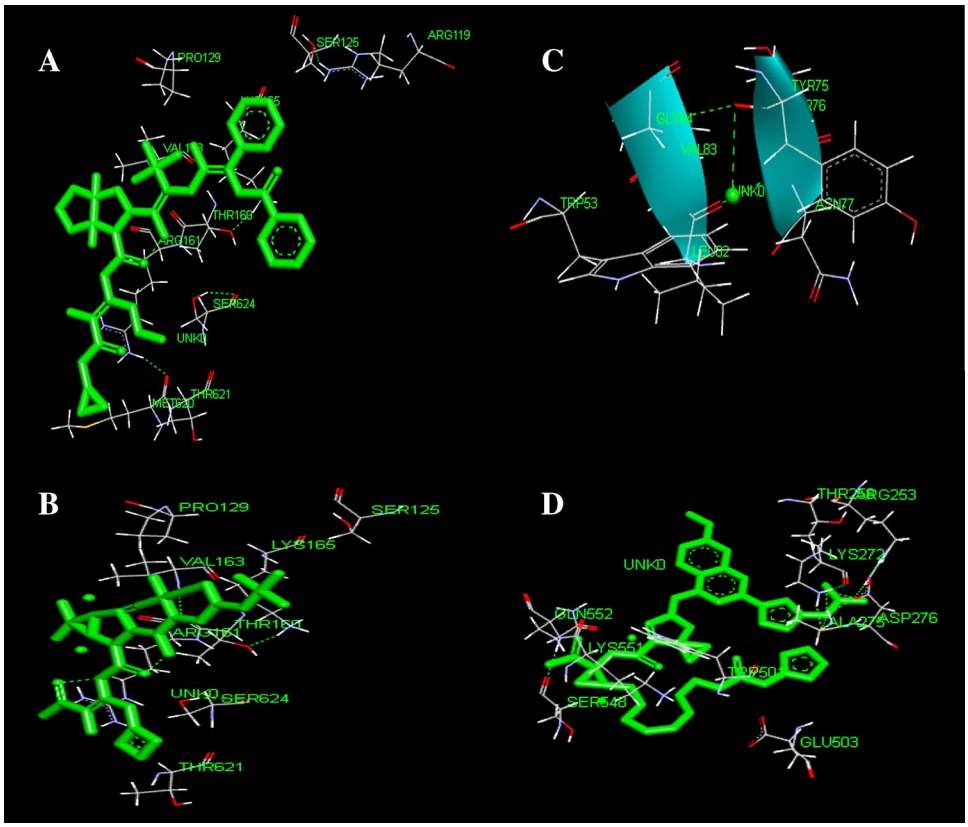

Fig. 3 Binding sites for NS3 genotype 1a and four selected drugs; **a** NS3- Telaprevir, **b** NS3- Boceprevir, **c** NS3- Simeprevir, **d** NS3- Ciluprevir

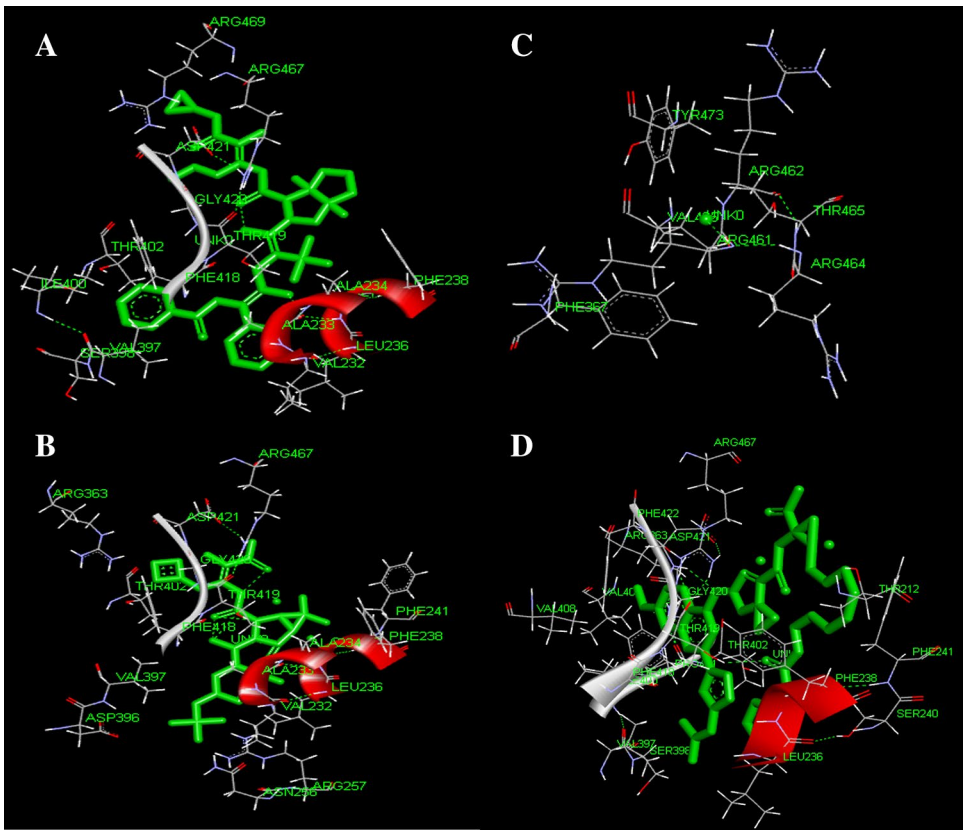

Fig. 4 Binding sites for NS3 genotype 3a and four selected drugs; **a** NS3- Telaprevir, **b** NS3- Boceprevir, **c** NS3- Simeprevir, **d** NS3- Ciluprevir

et al. 2012; Raj et al. 2017). However, when only individual frequencies of a mutation are considered, including 40, 52, 91, 102, 132, 153, and 166, the results are similar to those reported in numerous studies (Zeminian et al. 2013; Hedegaard et al. 2017; Kumthip and Maneekarn 2015; Susser et al. 2009).

Mutations in codons 52 and 153 have previously been reported in patients who had not been treated with protease inhibitors(Zeminian et al. 2013). The mutation in codon 55 that was found in two sequences has previously been shown to be associated with resistance to boceprevir and telaprevir in patients undergoing treatment (Zeuzem et al. 2005; Tong et al. 2008).

Substitution 55, which had a frequency of 6.4%, was previously reported at a frequency of 18.9% in HCV subtype 1a variants (Zeminian et al. 2013).

The mutation 153 alone does not appear to totally compromise the action of the drugs, but when combined with other substitutions, it may inhibit the drug function (Zeminian et al. 2013).

Remarkably, there was a higher occurrence of substitutions in codons 40, 91, 153, 52, 102, 132, and 166. The substitutions T40A and S91A involve changes in the amino acid's electrical charge and position 91 should be studied further because of its close proximity to the residues in the catalytic triad (Zeminian et al. 2013).

The substitution L153I does not lead to a change in the amino acid's electrical charge. However, based on its high frequency in the population studied, this substitution may be a regional genetic polymorphism (Zeminian et al. 2013).

Luísa Hoffmann et al. examined HCV-NS3 protease variants at baseline and at 4 weeks under triple therapy with telaprevir, pegylated interferon, and ribavirin and analyzed the presence of variants in HCV-NS3 protease region from peripheral blood samples of 16 patients infected with HCV-1 by using next-generation sequencing. They found nine PI-resistance-associated variants (V36A, T54S, V55I, Q80K, Q80R, V107I, I132V, D168E, and M175L) in HCV-NS3 of 10 patients. In comparison with our results, some regions such as 55, 80, and 132 were similar, but because the number of patients in Hoffman's study was very low, the comparison between the prevalence rates was not reasonable (Hoffmann et al. 2015).

Svarovskaia et al. (2012) evaluated the presence of DRMs in HCV-infected patients treated with the HCV protease inhibitors GS- 9256 or GS-9451 as monotherapy using deep sequencing in 137 longitudinal samples from 45 patients. No NS3 DRMs that confer resistance to GS-9256 and GS-9451 (R155K, A156T, and D168V/E) were observed in 33 baseline samples. Regarding our results, we could not find these mutations in our sequences, so the mentioned inhibitors may have an efficient effect on Iranian patients(Svarovskaia et al. 2012).

In 2013, 37 patients infected with HCV genotype 1 were examined by Zeminian et al. and they determined that a large number of substitutions were observed in codons 153, 40 and 91; in addition, the resistant variants T54A, T54S, V55A, R155K, and A156T were also reported (Zeminian et al. 2013). This study showed that resistance mutations and genetic polymorphisms were present in the NS3 region of HCV in patients who had not been treated with protease inhibitors; this was important in determining the efficiency of PIs in Brazilian patients. Our results were similar to Zeminian's study as both reports illustrated various akin mutations that can be related to the same efficiency of PIs in the two study patients. As to the number of viral loads, our results did not show any significant difference; also, the prevalence of protease inhibitor-resistant mutations was not different among genotypes. This is in contrast with the study of Nishiya et al. (2014) that analyzed 202 donors who returned for counseling from 2007 to 2010 and presented enzyme immunoassay and immunoblot-reactive results (Nishiya et al. 2014). They realized the mean viral load of genotype 1 was significantly higher than that of the genotype 3 isolates; also, protease inhibitor-resistant variants were detected in 12.8% of the sequenced samples belonging to genotype 1, and a higher frequency was observed among subtype 1a (20%) in comparison to 1b (8%).

By using high-density pyrosequencing, Kirst et al. analyzed HCV NS3 gene segments from 20 subjects with chronic HCV infection, including 12 subjects before and after liver transplantation (Kirst et al. 2013). Bioinformatics analysis revealed that Q80 substitution was a dominant variant in 40% of the subjects, whereas other substitutions circulated at low levels within a population. This result was contrary to those of the present study as the mutation in aa 80 occurred in around 1% of 1a subtype sequences that showed different mutation patterns.

Costantino et al. in Italy conducted a study on 152 naïve patients (84% Italian and 16% immigrants from various countries) infected with different HCV genotypes (21,1a; 21, 1b; 2, 2a; 60, 2c; 22, 3a; 25, 4d and 1, 4 k) (Costantino et al. 2015). Sequence analysis showed that mutations V36L and M175L in the NS3 protease region were observed in 100% of patients infected with subtype 2c and 4 and the polymorphism C316 N/H in NS5B region was associated with resistance to sofosbuvir.

In the present study, we could not find any mutations in aa36 and 175 and this is because of different subtypes of HCV in the two countries.

Direct sequencing of HCV NS3/4A protease in 156 HCV naïve patient to PIs who were infected with different genotypes in Paolucci et al.'s study showed aa substitutions associated with HCV PI resistance 10.8% of sequences (Paolucci et al. 2012). Mutations V36L, T54S, V55A/I, and Q80K/L were observed in 29% of patients with genotype 1a, and

V55F, Q80L/N and M175L in 10% of patients with genotype 1b. The mutation V158M was found in 3% of patients with genotype 2, D168Q was present in 100% of patients with genotype 3 and D168E was observed in 13% of patients with genotype 4. Substitutions in aa 55 and 80 were common in Paolucci's study and the present report; however, the prevalence of the mutation in aa 80 was around 1% and that of aa 55 was about 6.4%; both were lower than the prevalence reported in Paolucci's study.

Mozhgan Afrasiabi in 2015 analyzed the clonal-sequencing of NS3 gene sequences in 7 HCV-infected patients referred to the central liver center, south of Iran(Afrasiabi et al. 2015). Phylogenetic analysis of the reference and amplified sequences demonstrated the high similarity of all sequences with genotype 1. Interestingly, crucial protease resistant mutations were detected in V36 and R155 positions in one patient's sequence.

According to our data, the prevalence of the mentioned mutations in Afrasiabi's study was zero; in our sequences, this difference may be rooted in different regions where the samples were selected or different methods or the low number of samples in her study.

Data analysis showed that I52 M mutation was significantly higher in patients with high ALT ($p=0.006$) and all those who had the Q80L mutation were 40 years old and older ($p=0.034$). Considering our data, it could be predicted that PIs can be a good choice for treatment of Iranian HCV infected patients as the prevalence of influential resistance mutations, Q80L/N, V55A/I and I52M, against PIs had a low frequency in the enrolled patients. Besides, it seems the presence of I52M and Q80L mutations can have adverse effects on physiological conditions related to the outcome of therapy. S91A mutation occurred more in people younger than 40 years old ($p=0.001$). Results revealed that patients infected with 3a genotype had a significantly higher AST level than 1a genotype ($p=0.024$). Also the percentage of abnormal ALT in patients infected with 3a genotype was higher than 1a ($p=0.03$). It can be concluded that Iranian patients infected with HCV genotype 3a are more susceptible to liver damage compared with those infected with HCV genotype1a.

Generally, the NS3 protein had disparate B-cell epitope positions; regarding the humeral response, this protein can induce a strong immune response. In 1994, Khudyakov found some potential B-cell epitopes on NS3 region and suggested that this region contained very strong antigenic epitopes to induce humoral immune system (Khudyakov et al. 1995). Mondelli confirmed the application of these epitopes to diagnose anti-HCV positive sera (Cerino and Mondelli 1991).

Our results indicated 3 potential B-cell epitopes (21–32,114–125, and 135–145) for 1a genotype as well four potential epitopes (20–28, 113–125, 133–143, and 96–105) for 3a genotype.

Commonly, the position of the second structures, random coil, beta turn, sheets, etc., and the proportion of each one in the final structure were different in 1a and 3a genotypes. However, random coil had the dominant portion in secondary structures in both genotypes. Among different tools to construct tertiary structure for NS3 1a and 3a, itasser structures were more reliable and gained higher scores by Qmean and ramachanran plot.

Protein phosphorylation is a vital regulator of varied intracellular processes, protein activity, localization and protein–protein interactions. Various phosphorylation positions were found including 6 positions which were similar in both genotypes (10, 22, 105, 122, 128, and 138); also, there were several mutations which led to the omission of some phosphorylation sites in NS3 sequences.

Disulfide bonds play an indispensable role in stabilizing NS3 protein; 3 bonds in 1a genotype and two bonds in 3a genotype were founded, mutations in aa 48, 51, and 147 omitted some bonds that can decrease the stability of the NS3 protein.

Docking analysis showed different amino acids in 1a and 3a sequences forming the binding sites for selected drugs. As shown in Table 10 and Figs. 3 and 4, the most prevalent mutations which occurred in remote regions can describe the reason why mutations could not affect the interactions between NS3 and drugs.

All mentioned drugs are the protease inhibitors and their structure well-defined, among which ciluprevir was the first NS3/4A inhibitor applied in clinical studies (BILN 2061) and was an orally bioavailable, and peptidomimetic. Although the development of ciluprevir was stopped because of cardiotoxicity observed in animal models.

Telaprevir and Boceprevir were recommended for use in clinical practice, Telaprevir combined with peginterferon plus ribavirin significantly enhanced rates of sustained virologic response in patients, furthermore, a combination of boceprevir a peginterferon–ribavirin can increase the rates of sustained virologic response in previously untreated patients with chronic HCV genotype 1 infection. Finally, many studies suggested the application of simeprevir to increase sustained virologic response after 48 weeks.

Interaction of patients' samples and references in docking analysis had great energy values for Telaprevir, Boceprevir, and Ciluprevir determined the steric compatibility and pharmacological properties; therefore, these PIs can be considered as efficient HCV treatment in our population.

However, docking analysis for Semiprevir showed the lower energy value for NS3 in comparison with other drugs. From this result, it can be inferred that Semiprevir could not be a good drug option for HCV treatment.

In comparison between reference sequences and sequences with major mutations, no significant difference was recognized, showing that mutations may not have a significant effect on PIs drugs function that can lead to the encouragement of health care systems to use them more frequently.

Conclusion

Selecting a treatment regimen is contributed to different factors including high efficacy, high tolerability, pan-genotype activity, short treatment duration, and availability which have been approved for mentioned pharmaceutical drugs. However, the efficacy should be examined frequently in every population that was evaluated in the present study which, by using docking analysis, showed that NS3 protein was able to interact strongly with selected PIs drugs and mutations had no significant effect on the interaction between drugs and NS3 protein. Therefore, Telaprevir, Boceprevir, and Ciluprevir can be favorable candidates for Iranian HCV infected patients and it highly suggests to add protease inhibitors in the treatment regimen.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with animals performed by any of the authors.

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