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▌ ORIGINAL ARTICLE ▐

Comparison of Three Different Methods for Detection of IL28 rs12979860 Polymorphisms as a Predictor of Treatment Outcome in Patients with Hepatitis C Virus

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Abstract
Objectives: This study aimed to evaluate the specificity, sensitivity, cost, and turn-around time of three methods of gene polymorphism analysis and to study the relationship between IL28B rs12979860 and SVR rate to pegIFN-α/RVB therapy among patients with chronic hepatitis C.
Methods: A total of 100 samples from chronic hepatitis C patients were analyzed in parallel using the three methods: direct sequencing, real-time polymerase chain reaction (PCR), amplification refractory mutation system (ARMS)-PCR.
Results: The different profiles for IL28B rs12979860 alleles (CC, CT, and TT) obtained with PCR-RFLP, ARMS-PCR, and direct sequencing were consistent among the three methods. Prevalence of rs12979860 genotypes CC, CT and TT in HCV genotype 1a was 10(19.6%), 35(68.6%), and six (11.8%), respectively, and in HCV genotype 31, it was 13(26.5%), 31(63.3%), and five (10.2%), respectively. No significant difference was seen between rs12979860 genotype and HCV genotype ($p = 0.710$).
Conclusion: Screening by ARMS − PCR SNOP detection represents the most efficient and reliable method to determine HCV polymorphisms in routine clinical practice.

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1. Introduction

Hepatitis C virus (HCV) infection is one of the major causes of chronic liver disease. The long-term impact of HCV infection is variable, ranging from minimal histological changes to extensive fibrosis and cirrhosis with or without hepatocellular carcinoma (HCC). The number of chronically infected persons worldwide is estimated to be 160 million (2.35% of the world population), but most are unaware of their infection [1].

The current treatment standard of pegylated interferon (pegIFN)-α and ribavirin (RBV) produces only a 50% response rate in genotype 1 and 80% response rate in genotype 3 individuals [2]. In addition, combination therapy is expensive and many patients cannot tolerate the serious side effects that may prevent completion of the treatment course. Although age, sex, host genetics, body mass index (BMI), HCV genotype, and HCV viral load are associated with treatment response, finding a marker that predicts treatment outcome has been difficult. Therefore it is important to identify the factors that affect the response to treatment [3].

Several genome-wide association studies have reported that the rs12979860 single nucleotide polymorphism (SNP) in intron 1 of interferon λ (IFNL)4 gene is strongly associated with sustained virological response (SVR) [4,5]. Previously, rs12979860 SNP was recognized as a polymorphism of IL28B gene [5].

IFNλ genes located on chromosome 19 belong to the family of type III interferon. Different *IFNλ* genes are recognized, including *IFNλ1* (IL29), *IFNλ2* (IL28A), *IFNλ3* (IL28B), and *IFNλ4*, which have all been shown to possess antiviral activity *in vivo* and *in vitro* [6]. Many studies have demonstrated that the rs12979860 C allele favors response to antiviral treatment of chronic HCV infection [7,8].

Several methods, such as direct DNA sequencing, real-time polymerase chain reaction (PCR), amplification refractory mutation system (ARMS)-PCR, PCR-restriction fragment length polymorphism (RFLP), and allele-specific PCR can be used for genotyping SNPs, such as IL28B alleles [9].

This study had two aims: to evaluate the specificity, sensitivity, cost, and turn-around time of three methods of gene polymorphism analysis and to study the relationship between IL28B rs12979860 and SVR rate to pegIFN-α/RVB therapy among patients with chronic hepatitis C (CHC).

2. Materials and methods

2.1. Patients

The study examined outcomes in 100 patients with CHC in two provinces of Iran, including Tehran (Digestive Disease Research Institute) and Kerman (Virology Laboratory of the Besat Specialist Clinic)

from December 2011 to June 2013. The exclusion criteria included having other hepatitis viruses, using immunosuppressive drugs (organ transplantation), and having any diseases that suppress the immune system (human immunodeficiency virus).

2.2. Treatment of patients

All the patients were treated with 180 mg/wk PegIFNα-2a and 1,000−1,200 mg/d RBV. The patients with chronic HCV genotypes 1 and 3 were treated for 48 weeks and 24 weeks, respectively [10]. The viral load (HCV RNA) in blood was measured at the end of the treatment and 6 months later. The absence of detectable HCV RNA in blood for at least 24 weeks after discontinuing treatment was defined as an SVR.

2.3. Sample collection

Blood samples (8 mL) from HCV patients were collected in EDTA tubes. Plasma was separated and aliquoted. Then, it was stored at −80°C until analysis. Peripheral blood mononuclear cells were isolated using Ficoll (Ficoll-Paque PLUS; GE Healthcare) and stored at −20°C.

2.4. RNA and DNA extraction

HCV RNA was extracted from 200-μL plasma samples using a high pure RNA isolation kit (Roche, Germany). Purified RNA was stored at −80°C for later analysis. IL28B DNA was extracted from 200-μL peripheral blood mononuclear cell samples using a high pure PCR template preparation kit (Roche). Purified DNA was used directly or stored at 2−8°C or 15−25°C for later analysis. DNA and RNA concentrations were detected by calculating the absorbance ratio of optical density at 260/280 nm using Thermo Scientific NanoDrop-2000 model (Wilmington, DE, USA).

2.5. Virological testing

HCV genotyping and viral loading from plasma samples were carried out using the AmpliSens HCV-1/2/3-FEP PCR kit and AmpliSens HCV-Monitor-FRT (InterLabService Ltd., Moscow, Russia).

2.6. Laboratory procedures

Liver enzyme function tests were carried out for all the patients. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were measured on the Selectra E Clinical Chemistry analyzer (Vital Scientific, The Netherlands).

2.7. PCR-RFLP

The IL28B polymorphism (rs12979860) was detected following PCR-RFLP amplification using forward primer: 5′-CTCTGCACAGTCTGGGATTCCT-3′ and reverse primer: 5′-CTGAGGGACCGCTACG-TAAGTC-3′. The amplification reaction mixture consisted of 50 ng genomic DNA, forward and reverse

primers (0.15μM of each), different deoxynucleotides (0.2mM of each), Ex Taq DNA Polymerase (1.5 U) (Takara Korea Biomedical Inc.), and PCR buffer (10×) containing 1.5mM MgCl$_2$. First, PCR was performed by denaturing the samples (10 minutes at 95°C), followed by 35 cycles including 95°C for 45 seconds, 64°C for 45 seconds, 72°C for 30 seconds, and final extension at 72°C for 10 minutes. Electrophoresis of the PCR products was carried out on the agarose gels (2%). For RFLP analysis, the PCR product (390 bp) of rs12979860 was digested with 5 U Bsh1236I (*Bst*UI) with Tango buffer (Fermentas, Vilnius, Lithuania) for 3 hours. The digestion products were visualized on a 3% agarose gel alongside the GeneRuler Ultra Low Range DNA Ladder 10−300 bp (Fermentas; Figure 1).

2.8. ARMS-PCR

The IL28B polymorphism (rs12979860) was detected following ARMS-PCR amplification using rs12979860 (T allele) forward primer: 5′-GGAGCTCCCC-GAAGGCGT-3′ and reverse primer: 5′-CGAGTGTCTGGGCCGCAG-3′ and the rs12979860 (C allele) forward primer: 5′-GAGCTCCCC-GAAGGCGC-3′ and reverse primer: 5′-GCTGCCAAGGCGCTGAGG-3′. Forward primers were bound to mutant allele and reverse primers were

bound to another region; so, different fragment lengths were obtained. PCR was done in a single tube containing 25 μL reaction buffer with the following components: genomic DNA (50 ng), forward and reverse primer (0.15μM of 4 primers), different deoxynucleotides (0.2mM of each), Ex Taq DNA Polymerase (1.5 U; Takara Korea Biomedical Inc.), and PCR buffer (10×) containing 1.5mM MgCl$_2$. Cycling parameters were initial denaturation at 95°C for 10 minutes, followed by 35 cycles including 95°C for 45 seconds, 66°C for 45 seconds, 72°C for 45 seconds, and final extension at 72°C for 10 minutes. Electrophoresis of the PCR products was done on 2% agarose gels (Figure 2).

2.9. PCR sequencing

Direct sequencing was performed by the Sanger method (Source BioScience Launches New Illumina MiSeq Next Generation DNA Sequencing Service) with the PCR primers described above. The raw sequencing data was analyzed using CLC Main Workbench (CLC Bio, Aarhus, Denmark).

2.10. Ethics statement

The current study was approved by the Ethical Committee of the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. Informed consent was also obtained directly from each patient.
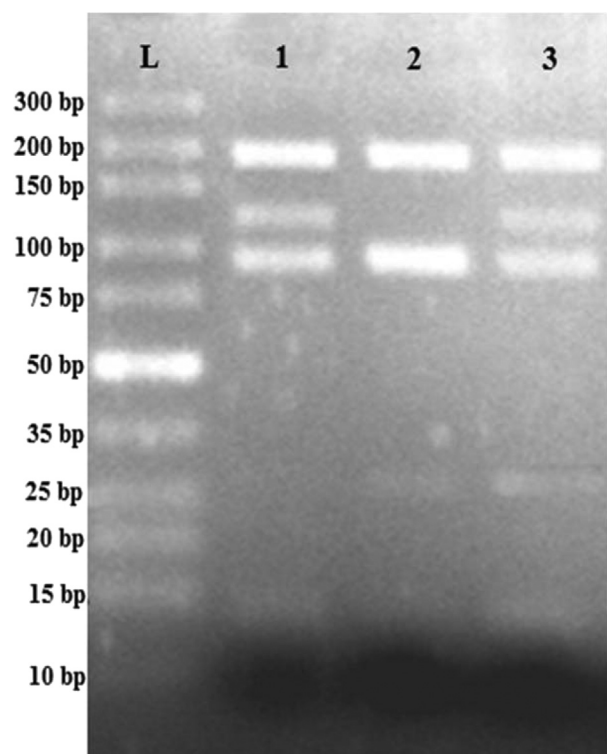
Figure 1. IL28B rs12979860 RFLP-PCR genotyping. Lane L, DNA marker; Lane No. 1 is genotype TT (89 bp, 118 bp, and 183 bp), Lane No. 2 is genotype CC (25 bp, 89 bp and 183 bp), and Lane No. 3 is genotype CT (25 bp, 89 bp, 118 bp, and 183 bp).
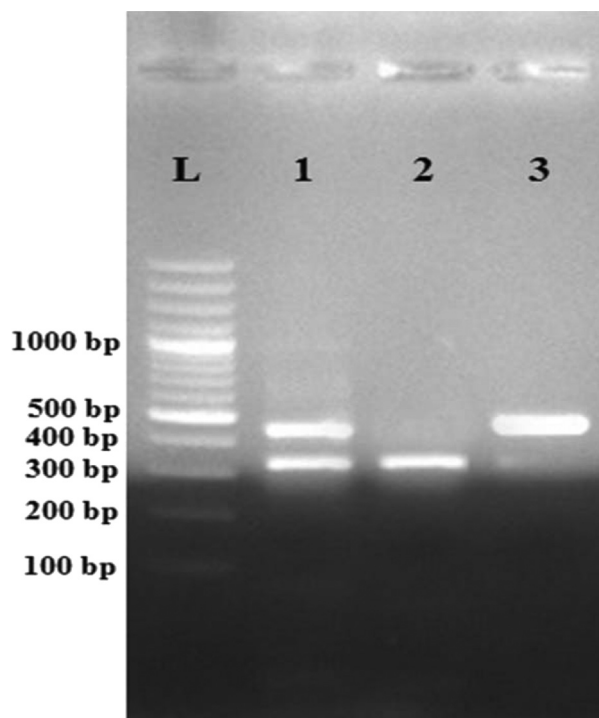
Figure 2. IL28B rs12979860 ARMS-PCR genotyping. Lane L. DNA marker; Lane No. 1 is genotype CT (326 bp and 443 bp), Lane No 2 is genotype CC (326 bp), and Lane No. 2 is genotype TT (443 bp).

Table 1. Characterization, clinical, and virological properties of Iranian patients with CHC before anti-viral therapy with pegylated interferon-α2a/ribavirin.

Variables	Patients			
HCV genotype	1a	3a	Total	p
No. (%)	51 (51)	49 (49)	100 (100)	
Age (y)	52.8 ± 4.7	51.7 ± 4.6	52.2 ± 4.6	0.259
Laboratory parameters				
AST (IU/L)	261.3 ± 203.8	217.3 ± 191.6	239.3 ± 197.7	0.310
ALT (IU/L)	274.0 ± 230.1	227.2 ± 228.3	250.6 ± 229.2	0.269
ALP (IU/L)	692.3 ± 487.7	618.7 ± 397.6	655.5 ± 442.6	0.411
HCV viral load (IU/mL)	$4.9 \times 10^6 \pm 2.5 \times 10^6$	$3.7 \times 10^6 \pm 2.2 \times 10^6$	$4.3 \times 10^6 \pm 2.4 \times 10^6$	0.018

Continuous parameters are presented as mean ± standard deviation. ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; HCV = hepatitis C virus.

3. Results

3.1. Patient characteristics

One hundred patients (84 male and 16 female) with chronic HCV infection were followed up in this study. There were 51 patients with HCV genotype 1a infection and 49 patients with HCV genotype 3a infection. The mean age of the patients was 52.2 ± 4.6 years (range, 41−61 years). Pretreatment clinical and virological characterizations including ALT, AST, (ALP), HCV genotype, and HCV viral load are shown in Table 1. There were no significant differences between HCV genotypes and age, ALT, AST and ALP levels, although there was a significant difference from HCV viral load ($p = 0.018$).

3.2. Comparison of three different methods for detection of IL28B rs12979860

A total of 100 samples were analyzed in parallel using the three methods. The different profiles for IL28B rs12979860 alleles (CC, CT, and TT) obtained with PCR-RFLP, ARMS-PCR, and direct sequencing were consistent among the three methods (Table 2).

3.3. Prevalence of rs12979860 genotype according to HCV genotypes

In the current study, prevalence of rs12979860 genotypes CC, CT and TT in HCV genotype 1a was 10 (19.6%), 35 (68.6%), and six (11.8%), respectively, and in HCV genotype 3a, it was 13 (26.5%), 31 (63.3%), and five (10.2%), respectively (Table 3). No significant difference was seen between rs12979860 genotype and HCV genotype ($p = 0.710$).

3.4. SVR rates according to rs12979860 genotypes

The SVR rate in patients with HCV genotype 1a and 3a infection was 18 (35.3%) and 38 (77.5%), respectively. SVR rate in patients with HCV genotype 1a infection was significantly less than in patients with HCV genotype 3a infection ($p < 0.001$). Among the HCV genotype 1a and 3a groups, 33 (64.7%) and 11 (22.45%) patients were non-responders (non-SVR; Table 4).

Among 18 (35.3%) patients with HCV genotype 1a infection, frequency of rs12979860 CC, CT, and TT genotypes in SVR patients was six, 11, and one, respectively, while in non-SVR patients, the frequency was four, 24, and five, respectively. CC genotype of rs12979860 was significantly associated with SVR compared to non-CC genotype ($p = 0.032$; Table 3).

For 38 patients with HCV genotype 3a infection, frequency of rs12979860 CC, CT, and TT genotypes in SVR patients was 12, 23, and three, respectively. By contrast, in non-SVR patients, the frequency was one, eight, and two, respectively. Two (CC and CT) genotypes of rs12979860 were significantly associated with SVR ($p = 0.032$; Table 3).

Multiple logistic regression analysis identified two independent factors that were significantly associated with SVR: IL-28B genotype (rs 12979860 CC vs. TT

Table 2. Analysis of IL28B rs12979860 polymorphisms in 100 patients with chronic hepatitis C virus genotype 1a and 3a using three different methods.

Method	IL28B locus rs12979860			Cost	Turnaround time
	CC (%)	TT (%)	CT (%)		
PCR-RFLP	23 (23)	11 (11)	66 (66)	US$4.70	6 h 45 min
ARMS-PCR	23 (23)	11 (11)	66 (66)	US$3.10	2 h 40 min
Direct sequencing	23 (23)	11 (11)	66 (66)	US$16.20	2 d

ARMS = amplification refractory mutation system; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism.

Table 3. Relationship between baseline demographic, clinical, virological characteristics and sustained virological responders with rs12979860 IL28B polymorphisms.

IL28B	rs12979860			
Variable	CC	CT	TT	p
HCV genotype (1a/3a)	10/13	35/31	6/5	0.710
Baseline viral load (IU/mL)	$3.3 \times 10^6 \pm 1.7 \times 10^6$	$4.6 \times 10^6 \pm 2.5 \times 10^6$	$4.5 \times 10^6 \pm 2.4 \times 10^6$	0.127
AST (IU/L)	243.4 ± 240.1	253.0 ± 183.3	239.7 ± 206.9	0.661
ALT (IU/L)	248.8 ± 245.9	262.2 ± 217.3	251.1 ± 213.3	0.689
ALP (IU/L)	586.4 ± 360.7	558.7 ± 481.6	656.3 ± 356.0	0.569
SVR	18	34	4	0.032*
Non-SVR	5	32	7	

*Statistically significant. ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; HCV = hepatitis C virus; SVR = sustained virological response.

and CT; odds ratio 7.86 and 4.08, respectively), and HCV genotypes (odds ratio, 7.42; Table 5).

4. Discussion

For SNP genotyping, Genome-Wide human SNP array, Genotyping Bead Chip, and the Invador method are used in the genome-wide association study stage [11]. DNA sequencing, TaqMan 5′ allelic discrimination method [12,13], and ARMS-PCR with or without tetraprimer [14,15] are used in the screening stage.

Several SNP genotyping methods have developed in recent years and many strong methods are currently available. The demand for SNP genotyping is great, however, and no one method is able to meet the needs of all studies using SNPs. Despite the considerable gains over the past decade, new methods must be developed to lower the cost and increase the speed of SNP detection [16].

In the present study, PCR-RFLP, ARMS-PCR, and sequencing were effective and specific in determining the IL28B rs12979860 genotypes. The electrophoresis pattern of the genotyping samples is shown in Figures 1 and 2. IL28B rs12979860 genotypes were successfully determined in all patients using the three methods. Among 100 patients with chronic HCV-1a and 3a infection, CC, CT, and TT genotypes accounted for 23% (23/100), 66% (66/100), and 11% (11/100), respectively.

Besides gene sequencing, other methods with high sensitivity, such as PCR-RFLP, ARMS-PCR SNP detection have been utilized for gene mutation detection. As for costs, excluding sample extraction that is common in all three methods, a single IL28B polymorphism determination was estimated to cost US$4.70 when performed using PCR-RFLP, US$3.10 using ARMS-PCR, and US$16.20 using sequencing. Turnaround time was 6 hours 45 minutes when performing PCR-RFLP, 2 hours 40 minutes when performing ARMS-PCR and 2 days for sequencing. Both PCR-RFLP and ARMS-PCR showed higher sensitivity with respect to direct sequencing.

Several studies have used PCR-RFLP for determining rs12979860 polymorphism. Nakamoto et al [17], Sharafi et al [18], and Irman et al [8] have utilized PCR-RFLP for detecting rs12979860. They have reported that this method is valid, fast, simple, and cost-effective for genotyping common IL28B SNPs and can be readily adopted by any molecular diagnostic laboratory with PCR-RFLP capability, and will be clinically beneficial in predicting treatment response in patients with HCV genotype 1 infection.

Thus, PCR-RFLP for detecting IL28B rs12979860 genotype appeared to be suitable for epidemiological investigations; however, two limitations of this technology might misdiagnose genotypes in the case of enzyme incomplete digestions of products and it requires a large amount of sample DNA [19].

Table 4. Relationship between virus genotype and SVR.

Patient features	SVR	Non-SVR	Total	p
No. of patients (%)	56	44	100	—
HCV genotype				
1a	18 (35.3)	33 (64.7)	51 (100)	< 0.001*
3a	38 (77.5)	11 (22.5)	49 (100)	

*Statistically significant. Data are presented as n (%). HCV = hepatitis C virus; SVR = sustained virological response.

Table 5. Results of multivariate regression analyses of the factors associated with sustained virological response in patients with HCV infection.

Characteristic	p	OR	95% CI
HCV genotype	<0.001[†]	7.42	2.605−21.105
Baseline viral load (IU/mL)	0.327	1.00	1.000−1.000
IL28 (CC)[†]	0.067	1	
IL28(TT)	0.030[b]	7.86	1.225−50.511
IL28(CT)	0.048[b]	4.08	1.010−16.524

[†]Statistically significant. CI = confidence interval; HCV = hepatitis C virus; OR = odds ratio.

In this study, we developed a reliable, fast, simple, inexpensive, sensitive, and specific ARMS-PCR method for genotyping of IL28B rs12979860 SNPs that is more cost-effective than PCR-RFLP and sequencing.

Galmozzi et al [14] have developed tetra-primer ARMS-PCR. This method was rapid, fast, inexpensive and accurate for detection of rs12979860 polymorphism, without the need for any special equipment, thus improving the accessibility to SNP genotyping for all minimally equipped laboratories.

ARMS-PCR is better than PCR-RFLP and sequencing considered one of the most cost effective, and easy to use. By contrast, ARMS-PCR SNP detection, which uses sequence-specific primers and specific targets, shows high sensitivity, low costs, and reduced turnaround time and specificity.

In the current study, frequency of rs12979860 genotypes CC, CT, and TT in HCV genotype 1a was 10 (19.6%), 35 (68.6%), and six (11.8%), respectively, and in HCV genotype 3a, it was 13 (26.5%), 31 (63.3%), and five (10.2%), respectively. The results of the present study indicate that the IL-28B rs12979860 CT genotype is the commonest genotype in patients with CHC. These results were consistent [20] and inconsistent [18] with other studies.

Different distributions of IL28B rs12979860 genotypes (protective rs12979860 CC genotype) among Caucasians, Asians, and Africans describe different rates of SVR in these populations. Previous studies have confirmed that rs12979860 CC allele has different frequencies in the worldwide population: CC genotype frequency in African populations is 23−55%, 52−80% in European populations, 75−98% in Southwest Asian populations, and 90−100% in East Asian populations [21].

Alternatively, various SVR rates might be in part described by distribution of different rs12979860 alleles in different HCV genotypes. In the current study, a lower frequency of CC genotype was found in the HCV-1a group in comparison with the HCV-3a group. In addition, it was demonstrated that the rs12979860 CC genotype was associated with SVR in HCV genotypes 1 and 3. The relationship between IL28B rs12979860 genotype and pegIFN-α/RBV treatment has been described in different studies [5,22,23]. The present study showed that CC allele in HCV genotype 1a and CC/CT alleles in HCV genotype 3a had an important role in infection control, and SVR rate in the CC group was higher than in the TT group among Iranian patients with CHC 1/3. Sharafi et al [18] found that CC and CT alleles in rs12979860 responded to treatment better than homozygous TT allele. The effect of rs12979860 polymorphism on response to pegIFN-α/RBV treatment in patients infected with HCV-1 has been extensively studied. Patients that carried the CC allele had SVR rates > 80%; so, this genotype is considered to be favorable. By contrast, patients with the TT allele has SVR rates < 40%; so, it is considered unfavorable. It is

clear which genotyping of the rs12979860 polymorphism is a strong predictor of the clinical outcome for HCV-1 patients [8], but also for HCV-3 to a lesser extent [24,25].

In the current study, SVR rates in patients with CC, CT, and TT (IL28B rs12979860) were 18, 34, and four, respectively. An association was found between SVR rates and IL28B polymorphisms. Understanding the associations between various IL28B polymorphisms and SVR after treatment with pegIFN-α/RBV is a major topic, because there is a debate over whether all untreated patients should receive triple therapy, given the increased costs and possible adverse effects. In countries such as Iran, the analysis of strategies based on IL28B rs12979860 and SVR could be useful in terms of cost-effectiveness. This analysis could also provide benefits related to adverse effects, treatment tolerance, and induction of viral drug resistance [26].

In conclusion, all three methods are suitable for determination of IL28B rs12979860 but the ARMS assay described here was rapid, inexpensive, sensitive, and accurate for detecting rs12979860 alleles in patients with CHC. Thus, in our opinion, screening by ARMS-PCR SNP detection represents the most efficient and reliable method to determine HCV polymorphisms in routine clinical practice. We also demonstrated that CC and CT alleles in HCV-3a and CC allele in HCV-1a were significantly associated with response to pegIFN-α/RBV treatment. The present results may help identify patients for whom therapy might be successful.

Conflict of interest

Acknowledgments

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