A Simple PCR-RFLP Method for Genotyping of *IFNL4* rs368234815 Polymorphism in Patients With Chronic Hepatitis C

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Laboratory Medicine 48:1:51-56

DOI: 10.1093/labmed/lmw060

ABSTRACT

Background: The *IFNL4* rs368234815 polymorphism plays a prominent role in spontaneous and treatment-induced clearance of hepatitis C virus (HCV) infection. This study aimed to develop a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method for assessment of rs368234815 polymorphism.

Methods: We genotyped the rs368234815 polymorphism in 87 patients with chronic HCV by PCR sequencing and PCR-RFLP methods, simultaneously.

Results: Genotyping of *IFNL4* rs368234815 via PCR-RFLP was concordant with PCR sequencing in all 87 individuals (100%). The analytical sensitivity and specificity of the developed PCR-RFLP method for genotyping of rs368234815 polymorphism were each 100%. Among these patients with chronic HCV, the frequency of rs368234815 TT/TT, TT/ΔG, and ΔG/ΔG were 44.8%, 37.9%, and 17.3%, respectively.

Conclusions: The PCR-RFLP method that we developed is accurate, rapid, inexpensive, and easy to perform for genotyping of the *IFNL4* rs368234815 polymorphism. This method can be used for clinical and research work.

Keywords: DNA sequencing, genetic polymorphism, hepatitis C, human IFNL4 protein, Polymerase Chain Reaction, restriction fragment length polymorphism

Worldwide, approximately 150 million people are infected with hepatitis C virus (HCV), and almost 500,000 people die annually from chronic HCV infection–related illnesses such as cirrhosis and hepatocellular carcinoma.[1] In recent years, HCV treatment was tied to combination therapy consisting of pegylated interferon (PEG-IFN) and ribavirin (RBV).[2-4] Recently, new antiviral agents known as direct-acting antivirals (DAAs) have been developed and introduced for treatment of HCV infection.[5,6] Although DAAs are more effective than PEG-IFN/RBV combination therapy, given these new treatments of HCV are not affordable and available in many countries, PEG-IFN and RBV are colloquially known to remain the alternative HCV treatment regimen.

Recently, studies[7-11] have shown that response to PEG-IFN/RBV combination therapy has been affected by host and virus genetic factors. Among the host genetic factors, rs368234815 (ss469415590) polymorphism within exon-1 of the interferon lambda 4 (*IFNL4*) gene (HGNC:44480) plays a prominent role in spontaneous and treatment-induced clearance of HCV infection.[9] The *IFNL4* gene, with a 2543 base-pair (bp) length and 5 exons, is located on chromosome 19q13.2. The rs368234815 polymorphism is classified as a deletion/insertion genetic variation (TT/ΔG). The *IFNL4* rs368234815-ΔG allele expresses the IFN-λ4 protein; however, the rs368234815-TT allele cannot

Abbreviations

HCV, hepatitis C virus; PEG-IFN, pegylated interferon; RBV, ribavirin; DAAs, direct-acting antivirals; IFNL4, interferon lambda 4; bp, base pair; PCR-RFLP, polymerase chain reaction–restriction fragment length polymorphism; RE, restriction endonuclease; IFNL2, interferon lambda 2; IFNL3, interferon lambda 3; RS, restriction site; SVR, sustained virologic response; SNPs, single nucleotide polymorphisms; LD, linkage disequilibrium; HRM, high resolution melting; TM, Melting Temperature

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Table 1. Primers for PCR Sequencing and PCR-RFLP Genotyping of the rs368234815 Polymorphism

Method	Primer Name	Sequence	GRCh38.p2 Primary Assembly No.	TM(°C)[a]	PCR Product Size (bp)
PCR sequencing	rs12-F	5′-GCGGAAGGAGCAGTTGCGCT-3′	39247951-39247970	66.08	745/746
	rs12-R	5′-GTGCCTTCACGCTCCGAGCA-3′	39248677-39248696	65.46	
PCR-RFLP	IFNL4-F	5′-GACGCAGGACCCCTTGGGACAGGA-3′	39248340-39248363	69.89	226/227
	IFNL4-R	5′-TCTGGGCCGCAGTGGCCGCGAGG-3′[b]	39248544-39248566	75.38	

TM, Melting Temperature; PCR, polymerase chain reaction; PCR-RFLP, polymerase chain reaction–restriction fragment length polymorphism; bp, base pair.
[a]TM calculation of primers was performed using the SantaLucia formula.[37]
[b]The underlined nucleotide represents mismatch, to abolish the nonspecific restriction site near the rs368234815 polymorphism.

express the functional IFN-λ4 protein as a result of frameshift polymorphism that introduces a premature stop codon that blocks the correct protein translation.[9] The results of the studies have shown that patients with the rs368234815-TT/TT genotype have a greater chance of favorable response to PEG-IFN/RBV treatment, compared with patients with rs368234815-TT/ΔG and -ΔG/ΔG genotypes. Similar results also have been observed in spontaneous clearance of HCV infection.[12] The results of previous studies[9,13-25] determined the *IFNL4* rs368234815 genotype using methods that require costly instruments and materials. Development of a simple, inexpensive, and rapid method for genotyping of *IFNL4* rs368234815 polymorphism might be helpful. The aim of this study was to develop a simple, inexpensive, and rapid polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method for genotyping of *IFNL4* rs368234815 polymorphism.

Material and Methods

Study Population

In this study, our cohort consisted of 87 patients with chronic HCV infection (70 males and 17 females), with a mean (SD) age of 30.6 (9.8) years, who were treated at Tehran Hepatitis Center and whose specimens were referred to the Armin Pathobiology Laboratory (both institutions are located in Tehran, Iran). The criteria for chronicity of HCV infection were having positive results for HCV antibodies and HCV RNA for a period of longer than 6 months. All study participants provided written informed consent; the study design was approved by the ethics committee of Baqiyatallah Research Center for Gastroenterology and Liver Diseases. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

IFNL4 Genotyping by PCR Sequencing

Genomic DNA was extracted from 200 μL of buffy coat fluid using the QIAamp DNA Blood Mini Kit (QIAGEN) according to manufacturer instructions. The direct DNA sequencing method for assessment of *IFNL4* rs368234815 polymorphism was described previously.[26] Briefly, we performed PCR reactions using Accupower PCR PreMix (Bioneer Corporation). We amplified 100 ng to 300 ng of genomic DNA using 10 pmol of rs12-F and rs12-R primer pairs (**Table 1**). The PCR temperature profile was as follows: 94 °C for 5 minutes, 35 cycles of 94 °C for 20 seconds, 66 °C for 20 seconds, and 72 °C for 20 seconds, followed by 72 °C for 5 minutes.

PCR products were sequenced using BigDye Terminator V3.1 Cycle Sequencing Kits (Applied Biosystems Inc.) in a 3130XL ABI Genetic Analyzer (Applied Biosystems Inc.) according to manufacturer instruction. Sequencing results were analyzed using Chromas Lite Version 2.1.1 (Technelysium).

IFNL4 Genotyping via PCR-RFLP

We used the IFNL4-F and IFNL4-R primers as a forward and reverse primer pair (**Table 1**). The PCR reactions were performed using Accupower PCR PreMix. The DNA amount and primer concentrations for the PCR mixture were the same as in the PCR sequencing method. The PCR temperature profile was as follows: 94 °C for 5 minutes, 40 cycles of 94 °C for 20 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds, followed by 72 °C for 5 minutes. For the RFLP analysis, the PCR product of rs368234815 was digested with 10 units of MspA1I (New England BioLabs) restriction endonuclease (RE) for more than 1 hour. The digested PCR products were separated on 3% agarose gel alongside the GeneRuler 100-bp DNA Ladder (Thermo Fisher

Scientific Inc.). The agarose gel was stained with the addition of 0.5 μL of DNA safe Stain (CinnaGen Co.) into each 100 mL of agarose gel. In each run of PCR-RFLP genotyping of rs368234815, as a control for enzymatic activity, we included a single rs368234815-ΔG/ΔG DNA specimen.

Validation Protocol

For the validation of the PCR-RFLP method for genotyping of *IFNL4* rs368234815, the PCR-RFLP genotyping results should be 100% concordant with the DNA sequencing results.

Results

IFNL4 Genomic Region and the PCR-RFLP Primer Design

The *IFNL4* gene (HGNC:44480) is located in the 24-Kb genomic region between interferon lambda 2 (*IFNL2*) and interferon lambda 3 (*IFNL3*) (**Figure 1**). This region of chromosome 19 contains few homologous sequences; designing of primers may be affected by this issue. To design the PCR-RFLP method, we assessed a 20-nucleotide region that contains *IFNL4* rs368234815 polymorphism at the middle of the sequence, using Gene Runner software (Version 5.1.06 Beta). We discovered that the rs368234815-ΔG allele could be digested with MspA1I; as a result, we chose this enzyme as our final target RE for digestion of the PCR product. Further, we selected a 1000-bp region (39248015 to 39249014 of GRCh38.p2 primary assembly) that contains the rs368234815 polymorphism at the middle of the sequence and assessed it with the nucleotide Basic Local Alignment Search Tool (BLAST).[27,28] Considering the high homology in this genomic region, we ran an alignment using MegaBLAST (highly similar sequences) and found 88% similarity between the selected 1000-bp region and the genomic region located at 39263982 to 39264945 of GRCh38.p2 primary assembly in the minus strand. In the next step, the selected 1000-bp region was assessed with Gene Runner software; we discovered that another restriction site (RS), which could be digested with MspA1I, is located 30 bp to 36 bp downstream of *IFNL4* rs368234815 polymorphism and can potentially affect the digestion step.

Finally, based on the aforementioned findings, we designed the PCR primers based on 2 goals. First, we aimed to give consideration to the homologous region; second, we aimed to abolish nonspecific RS, which is located downstream of the *IFNL4* rs368234815 polymorphism. We attempted to design the primers restricted to regions with the minimum homology. Further, in the reverse primer, we chose a conventional mismatched nucleotide near the 3′ end of the primer, to abolish the nonspecific RS (**Table 1**).

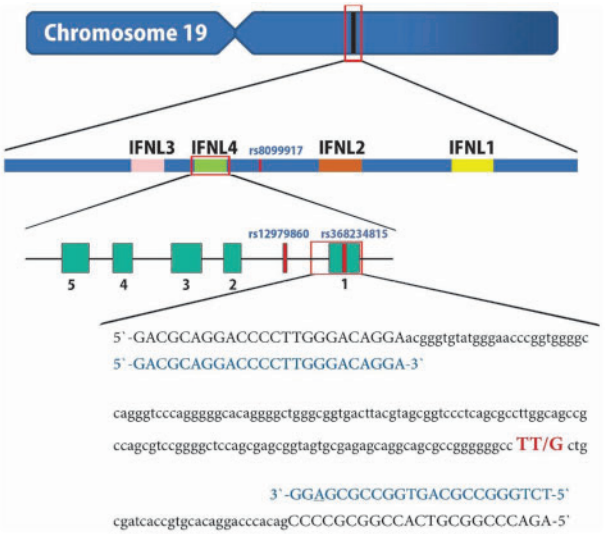

Figure 1

Chromosome 19, *IFNL4* gene, rs368234815 polymorphism and the primers for polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP).

PCR Sequencing Set-Up

The primers of PCR sequencing (rs12F and rs12R) amplify 745 bp/746 bp of the genomic DNA. In this fragment, rs368234815 (TT/ΔG) is located 564 bp/565 bp from the 5′ end of the forward primer. **Figure 2** shows the rs368234815-TT/TT, -TT/ΔG, and -ΔG/ΔG chromatograms.

PCR-RFLP Set-Up

We used the IFNL4-F and IFNL4-R primer pair for PCR-RFLP genotyping of *IFNL4* rs368234815, which amplified the 226 bp/227 bp DNA fragment (**Figure 1**). Digestion of this product with MspA1I in individuals with the ΔG/ΔG genotype showed 2 fragments of 175 bp and 51 bp; 3 fragments of 227, 175, and 51 bp in the TT/ΔG genotype; and a 227-bp fragment in the TT/TT genotype (**Figure 3**).

DOI: 10.1093/labmed/lmw060

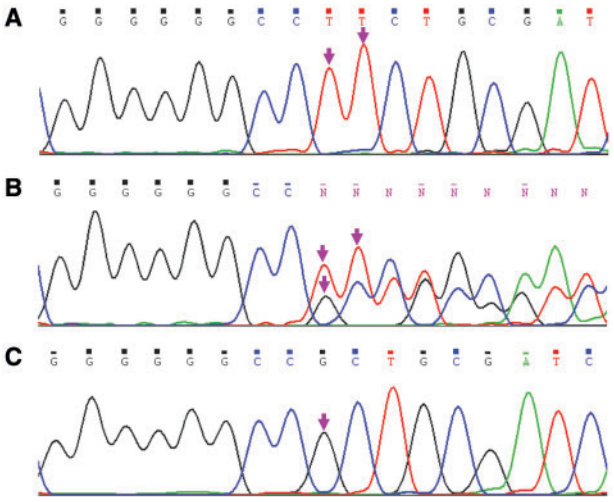

Figure 2

PCR sequencing chromatograms of rs368234815 polymorphism. **A**, Sequencing chromatogram of *IFNL4* rs368234815-TT/TT genotype. **B**, Sequencing chromatogram of *IFNL4* rs368234815-TT/ΔG genotype. **C**, Sequencing chromatogram of *IFNL4* rs368234815-ΔG/ΔG. Arrows show the polymorphic sites.

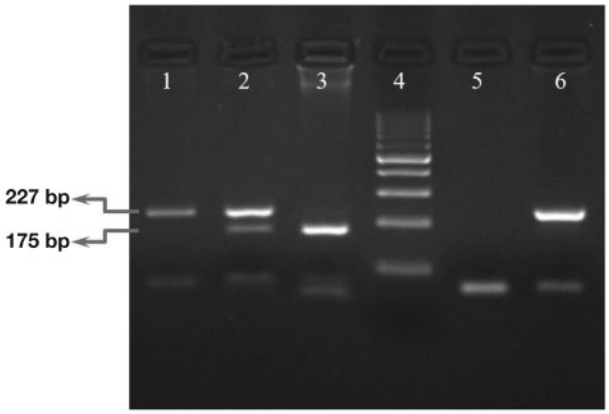

Figure 3

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) electrophoresis results of *IFNL4* rs368234815 polymorphism. Lanes 1, 2, and 3 were genotyped as TT/TT, TT/ΔG and ΔG/ΔG, respectively, for *IFNL4* rs368234815. Lane 4 was a 100-bp ladder. Lanes 5 and 6 were no template control and nondigested PCR product, respectively. For electrophoresis of digested polymerase chain reaction (PCR) products, 3% agarose gel was used.

Validation Results

The results of genotyping of the rs368234815 polymorphism by PCR-RFLP were concordant with those of PCR sequencing in all 87 individuals (100%) and showed the 100% analytical sensitivity and specificity of the PCR-RFLP method. In both methods, the frequencies of TT/TT, TT/ΔG, and ΔG/ΔG were 44.8%, 37.9%, and 17.3%, respectively.

Discussion

The final goal of HCV treatment is the eradication of the virus, which also can be determined by *sustained virologic response* (*SVR*), or a negative result for HCV RNA 6 months or longer after treatment termination.[2,4] Several genetic markers have been proposed as the predictors of SVR, including HCV and host genetic factors. In 2009, 3 genome-wide association studies[7,29,30] identified a genomic region located upstream of *IFNL3* (formerly *IL28B*) that contains several single-nucleotide polymorphisms (SNPs) associated with the rate of SVR to treatment with PEG-IFN/RBV. These markers are in strong linkage disequilibrium (LD) with each other. The results of further studies[31-33] have shown that these markers are also associated with spontaneous clearance of HCV infection. Among these markers, *IFNL3* rs12979860 polymorphism has attracted the most attention for testing as a pretreatment marker, for prediction of response to PEG-IFN/RBV combination therapy.[34] The studies attempted to identify the functional mechanisms linked to *IFNL3* SNPs, but those efforts failed.[35]

In 2013, sequencing of RNA specimens from primary hepatocytes that underwent treatment with *polyinosinic:polycytidylic acid* (a synthetic double-stranded RNA that mimics HCV infection) uncovered a novel gene called *IFNL4*, which is located upstream of the *IFNL3* gene.[9] *IFNL4* contains rs368234815 within exon-1 and rs12979860 within intron-1. These 2 polymorphisms are linked together in ethnic Asian and ethnic European populations; less LD was observed in ethnic Africans. Evaluations of African American patients with HCV infection indicated that rs368234815 may be the strongest host genetic factor for prediction of HCV treatment response.[12,36] Designing a simple, inexpensive, and rapid method for genotyping of the *IFNL4* rs368234815

polymorphism might be helpful for predicting response to treatment. Further, new studies, which will require the determination of *IFNL4* rs368234815 genotypes, can be easily performed using the PCR-RFLP method, even in local centers.

Until now, several studies[9,15,16,19,21-24] have genotyped *IFNL4* rs368234815 with probe-based assays, including the TaqMan and Invader assays.[13,20] Wu et al[25] genotyped rs368234815 using the mass spectrometry method. Keshvari et al[17] used the PCR sequencing method to genotype the *IFNL4* rs368234815 polymorphism. Galmozzi et al[14] and Lamoury et al[18] detected *IFNL4* rs368234815 genotype using the high-resolution melting (HRM) assay.

Overall, these methods have certain limitations, including dependence on instruments with sophisticated technology and possible lack of affordability by some laboratory centers. Probe-based and HRM assays can be used as high-throughput methods, and the testing procedures can be acceptably speedy for a large number of specimens. Although the present PCR-RFLP method is not acceptable as a high-throughput method, it can serve as a high-speed method for genotyping of a small number of specimens. The present method and almost all of the aforementioned methods require a control specimen for assessment of the validity of the testing procedures. In the present study, we used an *IFNL4* rs368234815-ΔG/ΔG specimen as the digestion control. Despite the advantages of probe-based assays, the probes involved are sensitive; synthesis of those probes requires instruments with sophisticated technology. The present study is primer-dependent; synthesis of the primers can be performed even by local providers. In the PCR sequencing method, the PCR step is primer-dependent; however, further steps, including the sequencing step, require instruments that feature sophisticated technology, which may be costly compared with the PCR-RFLP method.

In conclusion, the PCR-RFLP method that we have developed for genotyping of rs368234815 is accurate, inexpensive, rapid, and easy to perform. This method is comparable to the PCR sequencing method, which is the criterion-standard method for detecting genetic markers, and so it can be used for clinical and research work. **LM**

References

1. World Health Organization (WHO). Hepatitis C. Geneva, Switzerland: World Health Organization; 2015.
2. Fried MW, Shiffman ML, Reddy KR, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med.* 2002;347(13):975-982.
3. Manns MP, McHutchison JG, Gordon SC, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet.* 2001;358(9286):958-965.
4. McHutchison JG, Lawitz EJ, Shiffman ML, et al. Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. *N Engl J Med.* 2009;361(6):580-593.
5. Asselah T, Marcellin P. New direct-acting antivirals' combination for the treatment of chronic hepatitis C. *Liver Int.* 2011;31 (Suppl 1):68-77.
6. Schinazi R, Halfon P, Marcellin P, Asselah T. HCV direct-acting antiviral agents: the best interferon-free combinations. *Liver Int.* 2014;34 (Suppl 1): 69-78.
7. Ge D, Fellay J, Thompson AJ, et al. Genetic variation in *IL28B* predicts hepatitis C treatment-induced viral clearance. *Nature.* 2009;461(7262):399-401.
8. Pouryasin M, Keshvari M, Sharafi H, et al. The *ITPA* and *C20orf194* polymorphisms and hematological changes during treatment with pegylated-interferon plus ribavirin in patients with chronic hepatitis C. *Hepat Mon*. 2016;16(2):e35278. [10.5812/hepatmon.35278]
9. Prokunina-Olsson L, Muchmore B, Tang W, et al. A variant upstream of *IFNL3* (*IL28B*) creating a new interferon gene *IFNL4* is associated with impaired clearance of hepatitis C virus. *Nat Genet.* 2013;45(2):164-171.
10. Haj-Sheykholeslami A, Keshvari M, Sharafi H, Pouryasin A, Hemmati K, Mohammadzadehparjikolaei F. Interferon-λ polymorphisms and response to pegylated interferon in Iranian hepatitis C patients. *World J Gastroenterol.* 2015;21(29):8935-8942.
11. Behnava B, Sharafi H, Keshvari M, et al. The role of polymorphisms near the *IL28B* gene on response to peg-interferon and ribavirin in thalassemic patients with hepatitis C. *Hepat Mon.* 2016;16(1):e32703.
12. O'Brien TR, Prokunina-Olsson L, Donnelly RP. IFN-λ4: the paradoxical new member of the interferon lambda family. *J Interferon Cytokine Res*. 2014;34(11):829-838.
13. Fujino H, Imamura M, Nagaoki Y, et al. Predictive value of the $IFNL_4$ polymorphism on outcome of telaprevir, peginterferon, and ribavirin therapy for older patients with genotype 1b chronic hepatitis C. *J Gastroenterol.* 2014;49(12):1548-1556.
14. Galmozzi E, Facchetti F, Perbellini R, Aghemo A. High-resolution melting assay for genotyping of $IFNL_4$-associated dinucleotide variant rs368234815. *Clin Microbiol Infect*. 2014;20(11):O936-O938.
15. Gonzalez-Aldaco K, Rebello Pinho JR, Roman S, et al. Association with spontaneous hepatitis C viral clearance and genetic differentiation of *IL28B*/*IFNL4* haplotypes in populations from Mexico. *PLoS One*. 2016;11(1):e0146258. [10.1371/journal.pone.0146258]
16. Jouvin-Marche E, Macek Jilková Z, Thelu MA, et al. Lymphocytes degranulation in liver in hepatitis C virus carriers is associated with *IFNL4* polymorphisms and ALT levels. *J Infect Dis.* 2014;209(12):1907-1915.
17. Keshvari M, Pouryasin A, Behnava B, Sharafi H, Hajarizadeh B, Alavian SM. Letter: the rs12979860 and ss469415590 polymorphisms of IFNL4 gene are in strong linkage disequilibrium in Caucasian patients with chronic hepatitis C. *Aliment Pharmacol Ther.* 2014;39(3):343.

DOI: 10.1093/labmed/lmw060

18. Lamoury FMJ, Bartlett S, Jacka B, et al. Interferon λ 3 and 4 genotyping using high-resolution melt curve analysis suitable for multiple clinical sample types. *J Mol Diagn.* 2015;17(5):583-589.
19. Miyamura T, Kanda T, Nakamoto S, et al. IFNL4 ss469415590 variant is associated with treatment response in Japanese HCV genotype 1 infected individuals treated with IFN-including regimens. *Int J Hepatol.* 2014;2014:723868.
20. Nagaoki Y, Imamura M, Kawakami Y, et al. Interferon lambda 4 polymorphism affects on outcome of telaprevir, pegylated interferon and ribavirin combination therapy for chronic hepatitis C. *Hepatol Res.* 2014;44(14):E447-E454.
21. Real LM, Neukam K, Herrero R, et al. *IFNL4* ss469415590 variant shows similar performance to rs12979860 as predictor of response to treatment against hepatitis C virus genotype 1 or 4 in Caucasians. *PLoS One.* 2014;9(4):e95515.
22. Sperl J, Frankova S, Senkerikova R, Neroldova M, Hejda V, Volfova M, et al. Relevance of low viral load in haemodialysed patients with chronic hepatitis C virus infection. *World J Gastroenterol.* 2015;21(18):5496-5504.
23. Stättermayer AF, Strassl R, Maieron A, Rutter K, Stauber R, Strasser M. Polymorphisms of interferon-λ4 and *IL28B* – effects on treatment response to interferon/ribavirin in patients with chronic hepatitis C. *Aliment Pharmacol Ther.* 2014;39(1):104-111.
24. Wiegand SB, Heidrich B, Susser S, et al. Performance and value of IFN-lambda3 and IFN-lambda4 genotyping in patients with chronic hepatitis C (CHC) genotype 2/3 in a real world setting. *PLoS One.* 2015;10(12):e0145622.
25. Wu R, Chi X, Wang X, et al. *IFNL4* ss469415590 polymorphism contributes to treatment decisions in patients with chronic hepatitis C virus genotype 1b, but not 2a, infection. *Infect Genet Evol*. 2016;39:132-140.
26. Sharafi H, Pouryasin A, Alavian SM, et al. Development and validation of a simple, rapid and inexpensive PCR-RFLP method for genotyping of common IL28B polymorphisms: a useful pharmacogenetic tool for prediction of hepatitis C treatment response. *Hepat Mon.* 2012;12(3):190-195.
27. Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schäffer AA. Database indexing for production MegaBLAST searches. *Bioinformatics.* 2008;24(16):1757-1764.
28. Zhang Z, Schwartz S, Wagner L, Miller W. A greedy algorithm for aligning DNA sequences. *J Comput Biol.* 2000;7(1-2):203-214.
29. Suppiah V, Moldovan M, Ahlenstiel G, et al. *IL28B* is associated with response to chronic hepatitis C interferon-α and ribavirin therapy. *Nat Genet.* 2009;41(10):1100-1114.
30. Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of *IL28B* with response to pegylated interferon-α and ribavirin therapy for chronic hepatitis C. *Nat Genet.* 2009;41(10):1105-1119.
31. Thomas DL, Thio CL, Martin MP, et al. Genetic variation in *IL28B* and spontaneous clearance of hepatitis C virus. *Nature.* 2009;461(7265):798-801.
32. Rauch A, Kutalik Z, Descombes P, et al. Genetic variation in *IL28B* is associated with chronic hepatitis C and treatment failure: a genome-wide association study. *Gastroenterology.* 2010;138(4): 1338-1345.e1-7.
33. Sharafi H, Alavian SM, Behnava B, Pouryasin A, Keshvari M. The impact of *IFNL4* rs12979860 polymorphism on spontaneous clearance of hepatitis C; a case-control study. *Hepat Mon.* 2014;14(10):e22649.
34. Pacanowski M, Amur S, Zineh I. New genetic discoveries and treatment for hepatitis C. *JAMA.* 2012;307(18):1921-1922.
35. Keshvari M, Sharafi H. Interferon-λ genetic variations and hepatitis C: yet to be discovered. *Hepat Mon.* 2014;14(5):e19433.
36. Wu Q, Wang C, Chen EQ, Tang H, Li ZZ, Lei XZ. Interferon lambda 4 polymorphism predicts sustained viral response in hepatitis C virus patients irrespective of hepatitis C virus genotypes, ethnicity or treatment regimen: results from a meta-analysis. *Hepat Mon.* 2015;15(12): e32707.
37. SantaLucia J, Jr. A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proc Natl Acad Sci U S A*. 1998;95(4):1460-1465.

DOI: 10.1093/labmed/lmw060