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RESEARCH ARTICLE

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A facile PCR-RFLP method for genotyping of *ITPA* rs1127354 and rs7270101 polymorphisms

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Background: Inosine triphosphate pyrophosphatase (*ITPA*) gene single nucleotide polymorphisms (SNPs), rs1127354 and rs7270101, may cause a functional impairment in ITPase enzyme, resulting anemia protection in patients with chronic hepatitis C virus (HCV) infection undergoing ribavirin (RBV)-dependent regimens. The main purpose of this study was to provide and validate a simple, rapid, and inexpensive polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique for genotyping of *ITPA* rs1127354 and rs7270101 polymorphisms in chronic HCV-infected patients.

Methods: In the current study, 100 Iranian patients with chronic hepatitis C were examined and genotyped for *ITPA* rs1127354 and rs7270101 gene polymorphisms. To genotype rs1127354 and rs7270101 polymorphisms, PCR-RFLP technique and sequencing technique were performed on these samples. To validate the PCR-RFLP method, the PCR-RFLP genotyping results should be 100% concordant with the PCR-sequencing results.

Results: The rs1127354 and rs7270101 polymorphisms of *ITPA* gene were genotyped by PCR-RFLP technique and sequencing simultaneously, and the results of both techniques were 100% concordant in all 100 patients. Both PCR-RFLP and sequencing techniques indicated that the genotypic frequency of rs7270101 was 80% AA, 19% AC and 1% CC, and for rs1127354 was 79% CC, 20% CA and 1% AA, respectively.

Conclusion: We developed and validated a rapid and inexpensive PCR-RFLP technique for the detection of *ITPA* rs1127354 and rs7270101 gene polymorphisms.

KEYWORDS

hepatitis C virus, inosine triphosphate pyrophosphatase gene, polymerase chain reaction, restriction fragment length polymorphism, sequencing, single nucleotide polymorphisms

1 | INTRODUCTION

Inosine triphosphate pyrophosphatase (ITPase) is an enzyme encoded by the human *ITPA* gene (Gene ID: 3704).[1] This gene has 8 exons and 7 introns and is located on the short arm of human chromosome number 20, which, according to GRCh38.p2, is located from nucleotide 3208868 to nucleotide 3227449.[2] Two functional variants of *ITPA* gene, rs1127354 (NM_033453.3:c.94C>A), P32T (in exon 2) a missense variant and rs7270101 (NM_033453.3:c.124 + 21A>C) (in intron 2) a splice variant have been identified and associated with RBV-induced anemia.[3,4] ITPase promotes the hydrolysis of inosine triphosphate (ITP) and deoxy inosine triphosphate (dITP) to monophosphate and diphosphate nucleotides (IMP and IDP).[5] The *ITPA* rs1127354

and rs7270101 polymorphisms may result in reducing the activity of the ITPase enzyme and subsequent accumulation of ITP and protecting against adenosine triphosphate (ATP) reduction in erythrocytes.[6,7]

The rs1127354 and 7270101 polymorphisms have 3 genotypes CC, CA, and AA in which wild-type CC (rs1127354) and wild-type AA (rs7270101) follow by complete activity of ITPase; wild-type CC (rs1127354) and heterozygous AC (rs7270101) follow by 60% activity; heterozygous CA (rs1127354) and wild-type AA (rs7270101), wild-type CC (rs1127354) and homozygous CC (rs7270101) follow by 30%; heterozygous CA (rs1127354) and heterozygous AC (rs7270101) follow by 10% activity; and homozygous AA (rs1127354) and wild-type AA (rs7270101) follow by no activity to ITPase.[8,9] Mutated *ITPA* gene is considered as a symptom of benign enzyme disorder.[10,11] This benign disorder leads to the accumulation of ITP in the red blood cells (RBC). It has been determined that ITP can be used as an alternative to GTP in ATP biosynthesis. This will lead to the resistance of those carrying the *ITPA* variant to anemia and severe hemoglobin decrease following RBV-related treatments.[12] Ribavirin is known as a purine nucleoside analogue and one of the fundamental treatments of chronic hepatitis C.[4,13,14] The ITPase activity may lead to anemia induction via endogenous purines.[4] Hemolytic anemia, as one of the major side effects of pegylated-interferon/RBV therapy, affects 30% of chronic HCV-infected patients.[15-17] Interactions between RBV and intracellular concentrations of GTP and, as a result, ATP levels in RBCs lead to RBV-induced anemia.[18] Ribavirin decreases the ATP levels followed by GTP decline when enters to the leukocytes and ultimately causes oxidative stress and lysis of RBCs, and subsequently, hemolytic anemia occurs.[19] Studies showed that *ITPA* gene variations are determining factors for protection against RBV-induced anemia.[9,20] In 2014, Hai and colleagues investigated the relationship between *ITPA* genotypes and hepatitis C treatment outcomes. They asserted that *ITPA* rs1127354 is a predictive factor for determining sustained viral response (SVR) in patients with hepatitis C and prognosis factor of RBV post-treatment anemia.[21] Hence, we assessed the rs1127354 and rs7270101 genotyping for the detection of *ITPA* gene polymorphisms and the main purpose of our study was to establish a PCR-RFLP method as a rapid and cost-effective technique for genotyping of *ITPA* gene polymorphisms.

2 | MATERIAL AND METHODS

2.1 | Study population

In this study, 100 patients with chronic hepatitis C (93 males and 7 females) were examined. Patients having HCV antibody and HCV RNA for more than 6 months were considered as chronic HCV-infected patients. These patients were referred periodically to the Armin Pathobiology Laboratory (Tehran, Iran). According to sterile conditions, 5 mL of blood samples was collected and centrifuged at 1000 × *g* for 10 minutes. Buffy coat of samples was separated and transferred to 1.5-mL sterile tubes. The samples were quickly stored at −20°C and were kept frozen until further examinations. It should be noted that patients were negative for viral infections including HIV or other hepatitis viruses.

2.2 | *ITPA* Genotyping by PCR-RFLP

According to manufacturer's instructions, DNA of buffy coat from venous blood samples was extracted using QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The extracted DNA samples were stored at −20°C until later experiments.

The PCR products were performed using ready-to-use Taq DNA Polymerase Master Mix RED (Ampliqon, Denmark). The 10-100 ng of genomic DNA was amplified using 15 pmol of RFLP-F and RFLP-R primer pairs. The sequence of the specific primers of PCR-RFLP for the genotyping of the *ITPA* gene polymorphism and confirmation of the gene polymorphism are listed in Table 1. The thermal cycling conditions for PCR-RFLP were as follows: 94°C for 5 minutes followed by 35 cycles of 94°C for 20 seconds, 58°C for 20 seconds, 72°C for 20 seconds and the final extension step at 72°C for 5 minutes. The PCR amplicons were visualized under the UV light. In this study, MboII and XceI restriction enzymes were used to investigate rs7270101 and rs1127354 polymorphisms, respectively. The MboII and XceI enzymes are inactivated at 65°C during 20-minute incubation. The PCR products were digested using 10 units of MboII (5 U/μL) and 10 units of XceI (10 U/μL) for more than 1 hour for the detection of rs7270101 and rs1127354 polymorphisms, respectively. The digested PCR products were detected by 3% gel electrophoresis.

TABLE 1 The specific primers for genotyping of *ITPA* gene polymorphisms by PCR-RFLP and PCR sequencing

Molecular method	Nucleotide sequence (5′→3′)	TM(C°)	Amplicon size (bp)
PCR-RFLP	F-5′-AGATGGGCAGCAGAGTTATCG-3′	60	213
	R-5′-AAGACAGAGAAATCCAACCATCTTTTAAG*AA-3′	60	
PCR Sequencing	F-5′-GGAAGGGGCTGGCTTGCTGG-3′	66	353
	R-5′-CCGGCTCCCCCTGGTACTCC-3′	66	

TM, melting temperature; PCR, polymerase chain reaction; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; bp, base pair.
TM calculation of primers was performed using the Santa Lucia formula.
*The third nucleotide in 3′ end of the RFLP-R primer is mismatch nucleotide which is shown by an underline.

2.3 | *ITPA* genotyping via PCR sequencing

The amount of PCR materials including master mix, forward and reverse primers for sequencing as listed in Table 1, deionized water, and the DNA template was the same as the protocol for PCR-RFLP genotyping of *ITPA* polymorphisms. The samples were transferred to the thermal cycler with the following PCR program: 94°C for 5 minutes followed by 35 cycles of 94°C for 20 seconds, 60°C for 20 seconds, 72°C for 20 seconds, and the final extension step at 72°C for 5 minutes. The PCR products were detected using 2% of agarose gel according to the previous process. 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 instructions. Sequencing results were analyzed using Chromas Lite Version 2.1.1 (Technelysium).

2.4 | Validation protocol

To validate the genotyping methods of *ITPA* rs7270101 and rs1127354 polymorphisms, both techniques of PCR-RFLP and sequencing should be 100% concordant.

3 | RESULTS

3.1 | *ITPA* genomic region and the primer design

Primer-Blast was used to design the specific primers. For this purpose, the exact location of the *ITPA* gene polymorphisms was determined on the human chromosome 20. The location of rs1127354 and rs7270101 polymorphism was at the nucleotide position of 3213196 and 3213247 on chromosome number 20 based on GRCh38.p2. Both polymorphisms are located in close proximity to each other with a distance of 51 nucleotides. As depicted in Figure 1, rs1127354 polymorphism in the ancestral allele appears as C and the rs7270101 as A. It is noteworthy that the nucleotide sequence containing rs1127354 A allele is recognized by XceI restriction enzyme with recognition site of 5′-RCATGY-3′ and also MboII targets rs7270101 polymorphism and cuts at 5′-GAAGA-3′. We introduced a mutation in 3 nucleotides downstream of rs7270101 using a mismatch in RFLP-R primer as presented in Figure 1. With the mentioned change in the PCR product, the MboII restriction enzyme would digest the allele C of rs7270101, while the allele A will not be digested using MboII. To obtain better results in the PCR-RFLP technique, the reverse primer was selected slightly longer. The characteristics of primers are specified in Table 1.

3.2 | The PCR-RFLP results

Treatment with XceI restriction enzyme for rs1127354 and MboII restriction enzyme for rs7270101 was conducted for more than 1 hour. In rs1127354 polymorphism, fragments of digested PCR products as 213, 135, 78 bp (CA); 135, 78 bp (AA); and 213 (CC) were observed, and also in rs7270101 polymorphism, fragments of 213, 173, 40 bp (AC); 173, 40 bp (CC); and 213 (AA) were obvious. Figure 2 represents PCR-RFLP electrophoresis results.

3.3 | The PCR-sequencing results

The rs1127354 and rs7270101 polymorphisms were nucleotides of 208 and 259 from the 5′ end of the forward sequencing primer, respectively. Figure 3 represents chromatograms of the sequences and the given genotypes.

3.4 | Validation results

Based on PCR-RFLP method, genotypic frequency of rs7270101 polymorphism was as follows: 80% AA, 19% AC, and 1% CC and these results for rs1127354 polymorphism were as follows: 79% CC, 20% CA, and 1% AA. The both PCR-RFLP and sequencing results were 100% concordant.

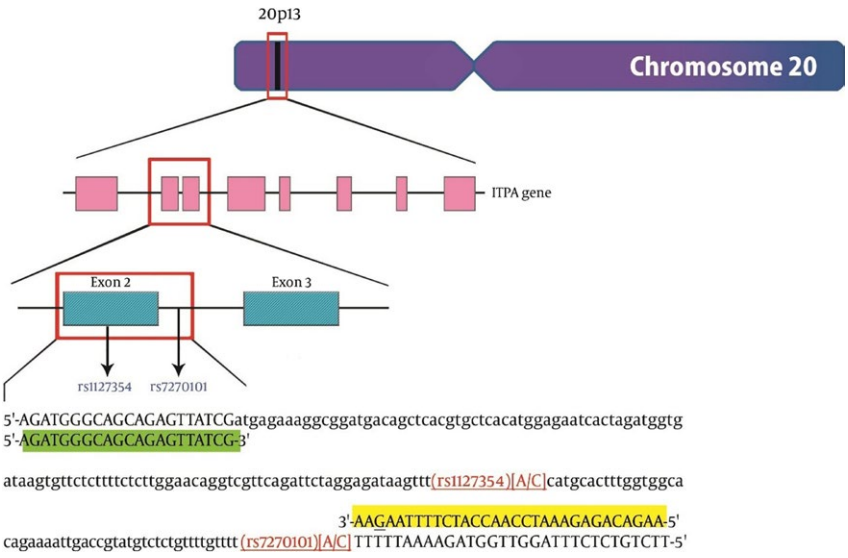

FIGURE 1 Chromosome 20, *ITPA* gene, rs1127354 and rs7270101 polymorphism, and the primers of PCR-RFLP. The third nucleotide in 3′ end of the reverse primer is mismatch nucleotide which is shown by an underline

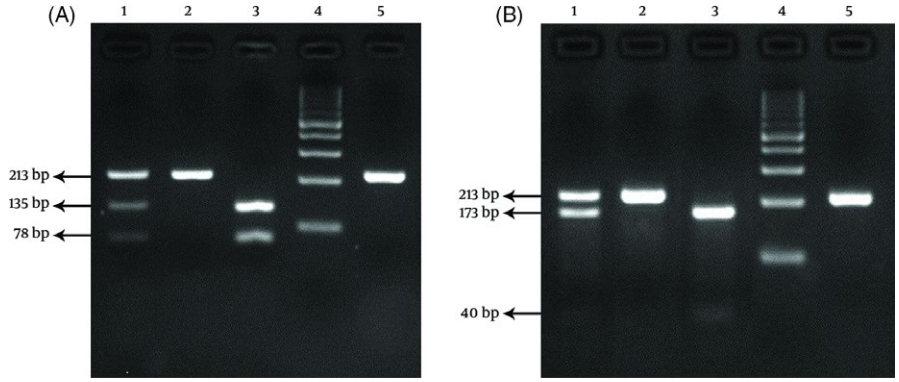

FIGURE 2 The result of PCR-RFLP products after digestion by the XceI and MboII enzymes. A, Gel electrophoresis results of PCR-RFLP products after digestion for rs1127354 by the XceI enzyme. Lane 1, 2 and 3 genotyped as CA, CC, and AA, respectively. Lane 4 indicates the 100-bp gene ruler. Lane 5 is non-digested PCR product. B, Gel electrophoresis results of PCR-RFLP products after digestion for rs7270101 by the MboII enzyme. Lane 1, 2, and 3 genotyped as AC, AA, and CC, respectively. The lane 4 indicates the 100-bp molecular gene ruler. Lane 5 is non-digested PCR product

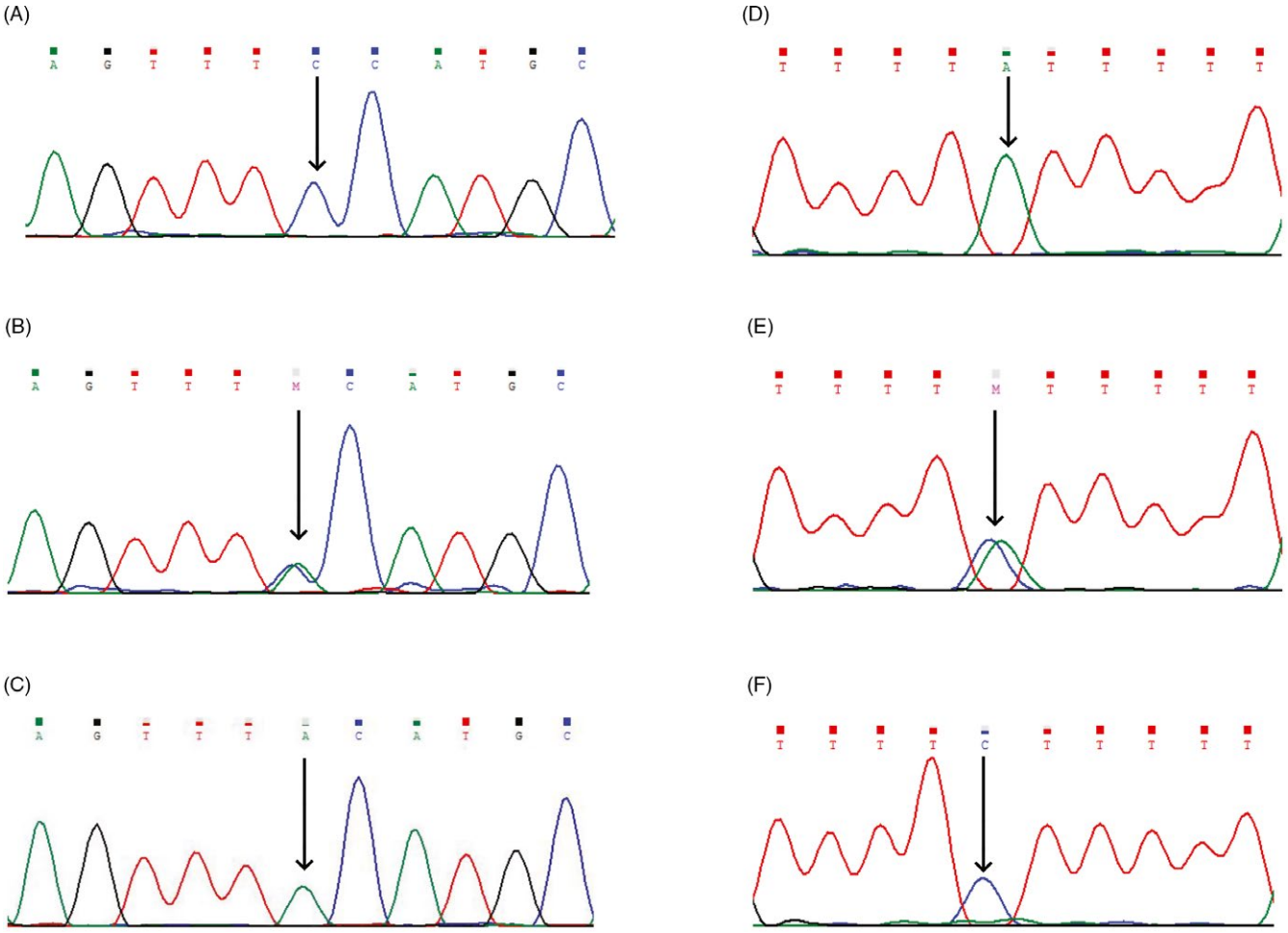

FIGURE 3 PCR-sequencing chromatograms of rs1127354 and rs7270101 polymorphisms. A, Sequencing chromatogram of *ITPA* rs1127354 CC genotype; B, sequencing chromatogram of *ITPA* rs1127354 CA genotype; C, sequencing chromatogram of *ITPA* rs1127354 AA genotype; D, sequencing chromatogram of *ITPA* rs7270101 AA genotype; E, sequencing chromatogram of *ITPA* rs7270101 AC. F, sequencing chromatogram of *ITPA* rs7270101 CC genotype. Arrows show the polymorphic sites

4 | DISCUSSION

In the present study, a total of 100 patients with chronic hepatitis C have been investigated whom 79 (79%) were wild-type homozygous (CC), 20 (20%) mutated heterozygous (CA), and one (1%) person mutated homozygous (AA) for rs1127354 of the *ITPA* gene and these results for rs7270101 were as follows: 80 (80%) wild-type homozygous AA, 19 (19%) mutated heterozygous AC, and 1 (1%) mutated homozygous CC.

Since now, several studies have been conducted in the field of genotypic frequencies of *ITPA* polymorphisms gene and the pharmacological effects of these polymorphisms on Peg-IFN/RBV post-treatment anemia.[22] In a study conducted by Fellay et al, 1280 patients with chronic hepatitis C infection from European-American, African-American, and Spanish populations were studied. Using microarray technique design to study the genomic-wide association study (GWAS), they found that individuals with mutated polymorphisms at rs1127354 and rs7270101 compared to those patients with wild-type homozygotes (wild type) for both of these polymorphisms experience fewer abnormalities and less decrease in hemoglobin levels after treatment with Peg-IFN/RBV. Genotypic frequency of rs1127354 was found as 1114 (87%) wild-type (CC), 156 (12.2%) heterozygote (CA), and 10 (0.8%) recessive homozygous. The results of the current study are quite consistent with Fellay's study and also other studies in other populations.[9]

In 2015, Chen et al[23] estimated the effect of *ITPA* polymorphism status on severe anemia and treatment responses to construct a clinically practical predictive index for severe anemia and their results showed that among 405 patients the CC genotype was present in 66.4% of patients and 33.6% had the AA or the CA allelic variants ("AA or CA" as a combo variable) at rs1127354. *ITPA* polymorphism can influence hemoglobin levels and sustained virological response (SVR) rates and also the incidence of RBV dose reduction in Sofosbuvir plus RBV treatment.[24,25] These studies all agree on the effect of rs1127354, as a recessive allele, which is a protective factor against post-treatment anemia.

In a study conducted by Domingo et al[26] on 73 Spanish patients co-infected with HCV-HIV undergoing Peg-IFN/RBV, it was found that the rs7270101 polymorphism does not affect hemoglobin decline in the first month of treatment. In the present study, from a total of 100 patients with chronic hepatitis C, 79 (79%) were wild-type homozygote, 20 (20%) were heterozygote, and one (1%) was homozygous for rs1127354 polymorphism of *ITPA* gene. These findings represent that the genotypic frequency of this polymorphism is relatively common in most populations around the world, and also, our study is perfectly consistent with the study of Fellay et al[9] and other studies conducted in other populations.

Studies have shown that the *ITPA* gene plays a prominent role in immunosuppressive treatments of multiple sclerosis (MS). In one study, analysis of the results indicated the correlation between *ITPA* gene polymorphisms and MS via PCR-RFLP technique.[27]

In the study of Sharafi et al[28] on IL28B polymorphism to evaluate the response to hepatitis C treatment, they showed that the PCR-RFLP technique was convenient to use and inexpensive. However, the method of sequencing is a standard and validated, but it is expensive and requires experienced technician.[29]

The PCR-RFLP technique used in this study to detect rs1127354 and rs7270101 polymorphisms of *ITPA* gene is up to date and also inexpensive compared to other techniques. Regarding the history of the previous research, we decided to perform PCR-RFLP and sequencing techniques to determine the rs1127354 and rs7270101 polymorphism of *ITPA* gene.[28]

In conclusion, we compared the two techniques to find the appropriate method. The result showed that all the 100 samples tested with PCR-RFLP technique and sequencing have exactly the same results. As sequencing method is a standard method, comparison with this method has shown that the PCR-RFLP is a reliable and acceptable technique and is proposed to reduce the costs in studies requiring an examination of the *ITPA* gene polymorphisms.

AUTHOR CONTRIBUTIONS

Seyed Ehsan Alavian, Heidar Sharafi, Seyed Moayed Alavian, Bita Behnava, Maryam Keshvari, and Ali Pouryasin designed the study; Seyed Moayed Alavian, Bita Behnava, and Maryam Keshvari contributed to the sample collection; Seyed Ehsan Alavian, Heidar Sharafi, and Mohammad Pouryasin performed the study; Paniz Shirmast, Seyed Ehsan Alavian, and Heidar Sharafi analyzed the data; and Paniz Shirmast, Seyed Moayed Alavian, Heidar Sharafi, Mohammad Pouryasin, and Ali Pouryasin drafted the manuscript.

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