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Inosine Triphosphate Pyrophosphatase Gene Polymorphisms and Ribavirin-Induced Anemia in HCV Patients

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Abstract

In spite of the interferon (INF) redundancy in treating HCV, ribavirin (RBV) is still included with the new direct antiviral therapies. Ribavirin-induced hematological alterations had been referred to ITPA gene polymorphisms.

Objective: Evaluate ITPA gene polymorphisms (rs1127354 and rs7270101) with development of anemia in chronic hepatitis C (CHC) Egyptian patients during treatment with pegylated-interferon (Peg-IFN) plus ribavirin (RBV).

Methods: 100 Egyptian CHC patients treated with PEG-IFN/RBV were recruited, 55 patients developed anemia (Hb decline>2 g/dl), and other 45 would not developed anemia (Hb decline ≤ 2 g/dl) at week 12 throughout the treatment course. Routine laboratory investigations were done for all participates (HCV-Abs, HBs Ag, HCV-RNA levels, complete blood picture, liver and kidney function tests. Single nucleotide polymorphism (SNP) was done by ABI TaqMan allelic discrimination kit for ITPA polymorphisms (rs1127354 and rs7270101).

Results: CC and AA were the most prevalent genotypes of SNPs rs1127354 and rs7270101 respectively among two studied groups. rs1127354 polymorphism was associated with Hb-decline at week 12 of treatment and with rs7270101 polymorphism for predicting platelet decline during treatment. Lower levels of platelet decline were detected with CC rs1127354 and AA rs7270101.

Conclusion: While ITPA polymorphisms rs1127354 CC genotype carried a predilection of anemia occurrence in PEG-IFN/RBV HCV treated subjects, the minor allele rs1127354 AA plays a crucial role in their protection. Platelet decline was reported in both ITPA rs1127354 and rs7270101 polymorphisms. Screening for ITPA polymorphisms in Egyptian HCV patients would be of value in avoiding hematological disturbances and dose modulations in RBV-based therapies.

Keywords: Ribavirin; ITPA; Chronic hepatitis C

Introduction

In Egypt, HCV genotype 4 (GT4) accounts for approximately 90% of HCV infections in Egypt, with subtype 4a predominating [1]. The standard of care for treatment of HCV infection many years ago was combination therapy with pegylated-interferon (Peg-IFN) plus ribavirin (RBV) [2,3]. The combination therapy with PEG-IFN and RBV has a broad range of side-effects including hematological changes especially bone marrow suppression and hemolytic anemia [4].

More than 50% of the patients may have hemoglobin decline and hemolytic anemia is an important adverse effect associated with Peg-IFN and ribavirin for chronic hepatitis C and this leads the physicians for dose reduction, blood transfusion and even discontinuations of therapy [5,6]. The mechanism of anemia induction has not been fully elucidated *in vivo*. Therapy-induced anemia is caused by RBV-induced hemolysis, while bone marrow suppression is caused by protease inhibitors (PI) and Peg-IFN treatment [7].

New direct-acting antivirals (DAAs) have been recently developed to improve the response rate, particularly in patients infected with HCV genotypes 1 or 4, and they have less common adverse effects (EASL, 2014). The use DAAs with RBV is used in certain subgroups of patients. Thus, RBV continues to maintain an important role in HCV therapy even with the introduction of DAAs [8,9].

The genetic variation in Inosine Triphosphate Pyrophosphatase (ITPA) encoding inosine triphosphatase (ITPase) was a protective factor against hemolytic anemia in patients receiving Peg-IFN and RBV therapy [10,11]. The ITPase catalyses the conversion of inosine triphosphate (ITP) to inosine monophosphate (IMP), and pyrophosphate, so the ITP does not accumulate in normal cells. There are two single nucleotide polymorphisms (SNPs) in ITPA gene, located on chromosome 20 [12]. The first polymorphism concerns a missense variant in exon 2; a variant of *ITPA* 94C>A (rs1127354), the second concerns a splicing-altering single nucleotide polymorphism (SNP) in intron 2; IVS2+21 A>C (rs7270101) [7,12]. These two ITPA polymorphisms are associated with reduced ITPase activity resulting in accumulation of ITP in red blood cells (RBCs) and subsequent hemolysis [13,14].

RBV can be incorporated into erythrocytes, where it undergoes phosphorylation to its active forms through adenosine kinase. The RBV triphosphate conjugates cannot cross the erythrocyte cell membrane and accumulate in the intracellular compartment, causing oxidative damage and leading to haemolysis [8]. RBV causes the depletion of

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ATP which is followed by guanosine triphosphate (GTP) deficiency in RBCs and causes RBC lysis [15].

Accumulated ITP instead of RBV triphosphate in ITPase-deficient patients would play a great role for compensating the depletion of ATP, and prevention of oxidative reactions and RBC lysis [10]. The ITPase deficiency causes accumulation of ITP in RBCs that may compete with RBV triphosphate, thereby protecting from RBV-induced haemolysis [16-19]. Although ITPase deficiency is protective against anemia, it has been associated with a greater decline in platelet count during Peg-IFN and RBV therapy [20].

Patients and Methods

The current study was conducted on selected 100 chronic HCV Egyptian patients treated with pegylated interferon (PegIFN-a2b used at the weight-based dose of (1.5 µg/kg subcutaneous once a week) and ribavirin (15 mg/kg).

Inclusion criteria: Patients with HCV-RNA positive with elevated transaminases 2.5-fold, Hb level more than 10 g/dl and platelets more than 100,000/ul, WBCs count more than 4,000/ul, normal thyroid profile, normal kidney function tests.

Exclusion criteria: Autoimmune liver disease, liver cirrhosis, treatment duration of less than 12 weeks; patients with coinfection with hepatitis B virus or HIV.

The patients were evaluated at week 12 for the development of anemia (Hb decline>2 g/dl). Accordingly, they were divided into:

Group I: Fifty-five patients developed anemia (Hb decline>2 g/dl from base line level) at week 12 throughout the treatment course.

Group II: Forty-five would not developed anemia (Hb decline<2 g/dl from base line level) at week 12 throughout the treatment course.

All individuals were subjected to:

1. Clinical assessment including full history and clinical examination.

2. Abdominal ultrasonography

3. Routine laboratory investigations including: Hepatitis markers: Hepatitis markers for hepatitis B and C virus infection, as HBs Ag, HBc Ab, HBe Ag, HCV-Abs were done by The electro chemiluminescence immunoassay "ECLIA" using cobas e 411 analyzers (Roche-Germany); HCV-RNA levels, liver function and kidney function tests were done using Integra 400 Auto analyzer; serum alpha fetoprotein (AFP) by Elecsys 2010 auto analyzer, complete blood count (CBC) using by Sysmex XT-180*i* automated hematology analyzer; markers of autoimmune liver disease (ANA, ASMA, AMA, LKM) by Alegeria automated The electro-chemiluminescence immunoassay (ECLIA) and thyroid stimulating hormone (TSH) were done by ECLIA using cobase 411 analyzers (Roche-Germany).

Inosine Triphosphate Pyrophosphatase (ITPA) genotyping

Single nucleotide polymorphisms (SNP) for ITPA genotyping (rs1127354 and rs7270101) was performed by real-time polymerase chain reaction (PCR, ABI TaqMan allelic discrimination kit).

DNA extraction: Genomic DNA was extracted from 200 µL whole peripheral blood using QIAamp® DNA Blood Mini kits according to the manufacturer's instructions (QIAGEN, Inc., Hilden, Germany). The purity of DNA was determined by measuring optical density at WL of 260 nm/280 nm by spectrophotometer.

Real-time polymerase chain reaction: ITPA gene SNP (rs1127354) was determined by real-time detection polymerase chain reaction using the ABI TaqMan allelic discrimination kit (catalogue # 4351379, assay ID C_29168507_10, Applied Biosystems, Carlsbad, CA) and the ABI 7500 real time PCR System (Applied Biosystems, USA).

ITPA gene SNP (rs7270101) was determined by real-time detection polymerase chain reaction using the ABI TaqMan allelic discrimination kit (catalogue#4351379, assay ID C_27465000_10, Applied Biosystems, Carlsbad, CA) and the ABI 7500 HT Real Time PCR System (Applied Biosystems, USA). Figure 1 shows amplification curve for ITPA gene by real time PCR.

Statistical methods

The data collected were tabulated and statistically analyzed by statistical package of social sciences (SPSS, version 16) and statistical graphs were created by Med Calc (version 15.6.1). Quantitative data were expressed as median and range or interquartile range (IQR) and analyzed by applying Mann Whitely U test for non-normally distributed variables. Qualitative data were expressed as number and

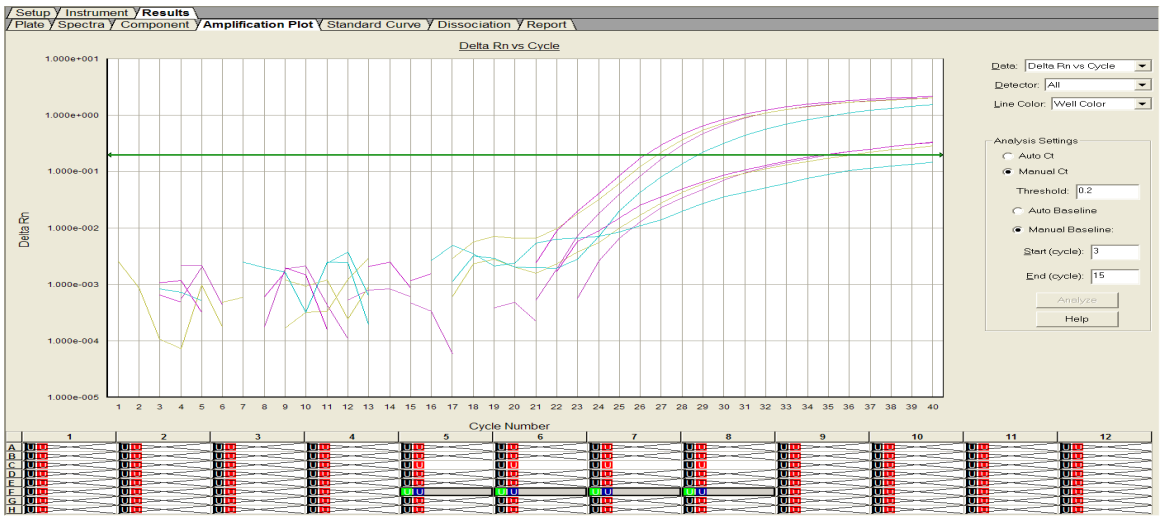

Figure 1: Amplification plot of ITPA polymorphism by real time PCR.

percentage (No.&%) and analyzed by applying Chi-square test. Fisher-exact test was used instead of Chi-Squared (χ^2) when the expected frequency in any one cell of the 2 × 2 contingency table is less than five. Hardy-Weinberg equilibrium was assessed for rs1127354 and rs7270101. Odds ratios (OR) for the relative risk and 95% CI in the univariate analysis was estimated by binary logistic regression. All these tests were used as tests of significance at P<0.05.

Results

Table 1 showed there was no statistical significant difference among anemic and non-anemic patients regarding age, gender, and base line of (WBCs count, Hb level, platelets count, WBCs count).

The most prevalent genotype among 100 HCV treated Egyptian patients was homozygous genotype AA (92%) of rs1127354, whereas heterozygous AC was detected in (8%) only and non-patient in our study harbored homozygous variants of minor alleles AA. The homozygous genotype CC of rs7270101 was detected in about 78% in HCV patients, followed by heterozygous genotype AC (22%) and no patients with minor alleles CC of genotype rs7270101 was detected Table 2. The minor allele frequency of rs1127354 and rs7270101 were (0.08 and 0.22, respectively), the distribution is consistent with Hardy Weinberg equilibrium (p=0.545 and p=1.00, respectively).

Characteristics	Baseline values		P-value
	Non-anemic (n=45)	Anemic (n=55)	
Gender (n (%))			
Male	31 (68.9)	37 (67.3)	0.863 NS, a
Female	14 (31.1)	18 (32.7)	
Age (years)			
Median (IQR)	46.00 (15.50)	50.00 (16.00)	0.355 NS, b
Range (min-max)	(28.00-64.00)	(25.00-63.00)	
WBCs count (10^3 cell/µL)			
Median (IQR)	4.60 (3.35)	5.30 (2.80)	0.419 NS, b
Range (min-max)	(2.20-11.70)	(2.10-10.00)	
Hb level (g/dL)			
Median (IQR)	13.20 (2.10)	13.8 (2.60)	0.361 NS, b
Range (min-max)	(10.20-17.00)	(11.10-16.30)	
Platelets count (10^3 cell/µL)			
Median (IQR)	220.00 (99.00)	211.00 (100.00)	0.202 NS, b
Range (min-max)	(125.00-395.00)	(99.00-360.00)	

BMI: Body Mass Index; IQR: Interquartile Range (Difference between 1st and 3rd Quartiles), a: Pearson Chi-Squared Test; b: Mann-Whitney U-Test.

Table 1: Baseline demographic data and hematologic parameters of the studied groups (n=100).

ITPA SNPs	ITPA genotyping (n (%))
rs1127354 (C/A)	
CC	92 (92.0)
AC	8 (8.0)
AA	0 (0.0)
Total C alleles	192 (96.0)
Total A alleles	8 (4.0)
rs7270101 (A/C)	
AA	78 (78.0)
AC	22 (22.0)
CC	0 (0.0)
Total A alleles	178 (89.0)
Total C alleles	22 (11.0)

Table 2: Distribution of genotype and allele frequencies of ITPA polymorphisms among 100 selected HCV infected patients.

ITPA		
SNP	Non-anemic (n=45)	Anemic (n=55)
rs1127354 (C/A)		
CC	38 (84.4%)	54 (98.2%)
CA	7 (15.6%)	1 (1.8%)
AA	0	0
rs7270101 (A/C)		
AA	35 (77.8%)	43 (78.2%)
AC	10 (22.2%)	12 (21.8%)
CC	0	0

Table 3: Distribution of ITPA Genotypes among anemic and non-anemic patients.

Variable	ITPA SNP (rs1127354)		P-value
	CC (n=92)	CA (n=8)	
Hb decline (Anemia) (n (%))			
Yes	54 (58.7)	1 (12.5)	0.021 a, s
No	38 (41.3)	7 (87.5)	--
Gender (n (%))			
Male	61 (66.3)	7 (87.5)	0.430 a, NS
Female	31 (33.7)	1 (12.5)	--
Baseline Hb (median (IQR))			
Male	14.6 (2.3)	13.5 (1.1)	0.511 b, NS
Female	13.0 (1.4)	--	0.250 b, NS
Hb at week 12 (median (IQR))			
Male	11.3 (2.2)	12 (1.1)	0.082 b, NS
Female	9.7 (2.9)	--	0.375 b, NS
Platelets decline			
Range (min-max)	80.0-41.0	12.0-133.0	<0.001 a, HS
Median (IQR)	15.0 (35.50)	99.00 (87.25)	
WBCs decline			
Range (min-max)	2.8-6.90	0.80-1.60	0.103 a, NS
Median (IQR)	1.00 (1.70)	0.20 (1.05)	

Note: a: Fisher-exact test; b: Mann-Whitney U-test, NS: Non-significant at P-value ≥ 0.05; S: Significant at P-value<0.05, IQR: Interquartile range (difference between 1st and 3rd quartiles).

Table 4: ITPA (rs1127354) genotype frequencies in relation to gender and hematological levels in treated HCV patients.

Table 3 showed that CC and AA were the most prevalent genotypes detected of SNP rs1127354 and rs7270101 respectively among the two studied groups. Regarding rs1127354 genotype, the CC homozygous genotype was detected in 54 (98.2%) of patients who developed anemia and in 84.4% of patients who did not develop anemia followed by heterozygous CA genotype was observed in 1.8% of patients who developed anemia and in 15.6% of patients who did not develop anemia at weak 12 throughout the course of treatment. As regard, rs7270101, the AA homozygous genotype was found in 78.2% of patients who developed anemia and in 77.8% of patients who did not develop anemia. While, heterozygous AC genotype of rs7270101 was observed in 21.8% of patients who developed anemia and 22.2% of patients who did not develop anemia at weak 12 throughout the course of treatment.

At week 12 throughout the course of treatment, hemoglobin decline (anemia) was observed in 54 out of 92 (58.7%) patients with homozygous CC genotype at rs1127354 and one patient (12.5%) with heterozygous CA genotype anemia was observed anemia, with a statistically significant increase (p=0.021) of Hb decline among CC compared to CA groups of rs1127354. While, no statistical significant differences (p>0.05) was detected between the CC or CA groups of

rs1127354 and each of gender and Hb level at baseline or at week 12 of treatment for male and female (Table 4) (Figure 2). There was a significant platelet decline (p<0.001) at homozygous CC compared to CA at rs1127354 with a median IQR of 1.7 in CC group and 1.05 in CA group. In contrast, no significant association between both CC or CA genotypes regarding the WBCs decline (p=0.103) Table 4.

At week 12 throughout the course of treatment, hemoglobin decline (anemia) was observed in in 43 out of 78 (55.1%) patients with homozygous AA genotype at rs7270101 and in 12 (54.5%) with heterozygous CA genotype, with a non-significant difference between two AA and AC at rs7270101 genotypes (p>0.05). In addition, no statistical significant differences (p>0.05) was detected between the AA or AC groups of rs7270101 and each of gender and Hb level at baseline or at week 12 of treatment for male and female (Table 5) (Figure 3). There was a statistical significant platelet decline (P-value=0.017) in group with homozygous AA (Median=14.5) compared to heterozygous AC (Median=33.5) at rs7270101. No significant association between both genotypes and WBCs levels (Table 5).

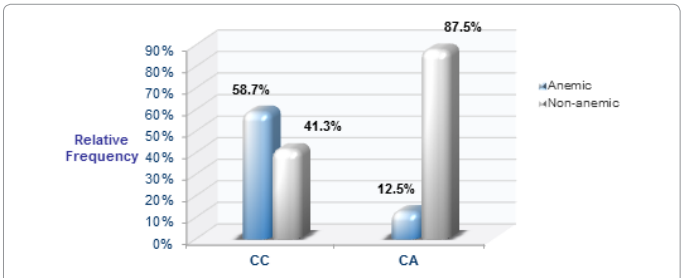

Figure 2: Distribution of anemia according to ITPA SNP (rs1127354) genotypes.

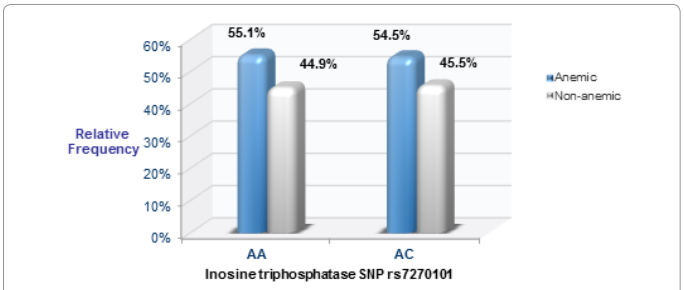

Figure 3: Distribution of anemia according to ITPA SNP (rs7270101) genotypes.

Variable	rs7270101		P-value
	AA (n= 78)	AC (n=22)	
Hb decline (Anemia) (n (%))			
Yes	43 (55.1)	12 (54.5)	0.961 a, NS
No	35 (44.9)	10 (45.5)	
Gender (n (%))			
Male	53 (67.9)	15 (68.2)	0.983 a, NS
Female	25(32.1)	7 (31.8)	
Baseline Hb ([median (IQR))			
Male	14.1 (2.3)	13.0 (2.8)	0.095 b, NS
Female	13.0 (1.8)	13.0 (1.4)	0.503 b, NS
Hb at week 12 (median (IQR))			
Male	11.7 (2.4)	11.0 (1.7)	0.162 b, NS
Female	9.7 (3.3)	11.3 (3.6)	0.656 b, NS
Platelets decline			
Range (min-max)	80.0-109	4.0-141	0.017 a, S
Median (IQR)	14.50 (30.25)	33.50 (54.25)	
WBCs decline			
Range (min-max)	2.8-6.90	0.80-5.50	0.790 a, NS
Median (IQR)	0.90 (1.60)	1.05 (1.55)	

a: Pearson chi-squared; b: Mann-Whitney U-test; NS: Non-significant at P-value ≥ 0.05; S: Significant at P-value<0.05; IQR: Interquartile range (difference between 1st and 3rd quartiles).

Table 5: ITPA (rs7270101) genotype frequencies in relation to gender and Hb levels in treated HCV patients.

Discussion

Clinical risk factors for severe RBV-induced anemia include impaired renal function, age, dose per body weight, female gender, baseline platelet levels, baseline hemoglobin levels, and haptoglobin phenotype [21,22]. In this context, the identification of successful predictors of RBV-induced anemia is of great value for preventing its toxicity. The functional single nucleotide polymorphisms (SNPs) in inosine triphosphatase (ITPA) gene locus rs1127354, which confers decreased ITPase activity, protected patients from the development of anemia early in Peg-IFN/RV treatment [11,16].

To our knowledge, the relationship between ITPA gene polymorphisms and hematologic changes followed treatment by Peg-IFN/RBV in chronic hepatitis C (CHC) Egyptian patients not fully elucidated. Therefore, this study was designed to evaluate the frequency and the association of ITPA gene polymorphisms with the development of hematologic changes namely Hb, WBCs and platelets after double therapy by Peg-IFN plus RBV in CHC Egyptian patients.

Conclusion

In this study, the frequency of ITPA gene polymorphisms, allelic distribution at rs7270101 and rs1127354 in all treated patients showed high rates of the genotypes AA (78%) and CC (92%) respectively. This was in agreement with Delvaux et al. [23] who reported that in Brazilian population the frequency of allelic distribution at rs7270101 and rs1127354 shows high rates of the genotypes AA (84%) and CC (94.3%), respectively. Also, Domingo et al. [24], D'Avolio et al. [25], Naggie et al. [26] Rau et al. [27] who demonstrated that the majority of European individuals carry AA and CC genotypes for rs7270101 and rs1127354, respectively. Also, Ochi et al. [28], Tanaka et al. [29], Kim et al. [18] reported that among Japanese and Korean populations, the CC genotype is also more prevalent at rs1127354, but they are monoallelic for the rs7270101 AA genotype. In contrast, Fellay et al. [11], showed that 48.4% of American individuals carried the AA genotype (rs7270101) and 47.6% had the CC genotype (rs1127354). This discrepancy in the results may be due to the different methods used in these studies and on the other hand, less patient numbers in some studies may contribute to this discrepancy.

At weak 12 throughout the course of treatment, our findings demonstrated that, the CC homozygous genotype of rs1127354 was detected in 98.2% of patients who developed anemia and in 77.8% of homozygous AA genotype of rs7270101. There was statistically significant difference of Hb decline between the CC and non-CC (CA) groups of rs1127354 throughout the treatment. Furthermore, the hemoglobin decline (>2 gm/dl) was observed in 54 out of 92 (58.7%) patients with homozygous CC genotype at rs1127354 and one patient (12.5%) with heterozygous CA genotype anemia was observed anemia, with a statistically significant increase (p<0.05) of Hb decline among CC compared to CA groups of rs1127354. However, Hb level at baseline (before therapy) was nearly the same between both

genotypes (CC and CA), suggesting the risk role of homozygous allele C of rs1127354 in development of anemia after Peg-IFN/RV therapy of HCV and the protective benefit of the minor allele A of rs1127354 against RBV-induced anemia. This was in agreement with Sakamoto et al. [16], Domingo et al. [24] and Thompson et al. [30] who reported that ITPA variants were strongly associated with protection against treatment-related anemia in patients with genotypes 2 and 3 HCV. This was also in agreement with Maan et al. [31] who reported that ITPase deficiency is associated with the protection against hemolytic anemia among Caucasian patients with chronic HCV infection who are treated with PegIFN and RBV [32-35].

Out of tune was the study of Aghemo et al. [36] as they argued on the relevance of ITPA polymorphism to anemia occurrence related to ribavirin therapy. These conflicting results might be justified by the small sized sample, with different grades of fibrosis, and variable SNPs studied.

Due to many previous supported hypothesis, the distribution of predicted ITPase activity according to the genotype at rs7270101 and rs1127354 indicated that most (73%) of our population exhibited 100% of predicted ITPase activity. A small number of patients (5%) presented ITPase activity equal to or below 30%, only (3%) patients presented ITPase activity equal to or below 10%. ITPase activity is known to increase the probability of the development of anemia. Additionally, most patients in our study exhibited the worst combination for both SNPs $AA_{rs7270101}$, $CC_{rs1127354}$ and $AC_{rs7270101}$, $CC_{rs1127354}$, which could explain why so many Egyptian patients developed RBV-induced anemia. This was in agreement with Delvaux et al. [23] also found that, most Brazilian patients carried the worst combination for both SNPs and developed RBV-induced anemia.

Concerning genotypes at rs7270101 in the ITPA gene studied in this work, however, baseline Hb level not significantly different between the AA or AC groups of rs7270101. The hemoglobin decline (>2 g/dl) was observed in 55.1% of patients with homozygous AA genotype at rs7270101 and in 54.5% of patients with heterozygous CA genotype at week 12 of treatment, with no significant difference between two AA and AC ($p>0.05$). This was in agreement with Domingo et al. [18], who reported that genotypes at rs7270101 in the ITPA gene were not associated with Hb decrease measured in any form. Also, Pouryasin et al. [37,38] reported that no association was found between rs7270101 and Hb-decline at week 4 of the combination therapy with PEG-IFN plus RBV. While Pineda-Tenor et al. [34] was against to the present study revealed that minor alleles of both rs1127354 and rs7270101 had the major role in preventing treatment-induced Hb-decline. This discrepancy may be due to the different methods used in these studies and different HCV genotypes in these studies.

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