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(Research Paper)

The rate of IFNL3 and MTTP genes polymorphism in β-thalassemia major patients infected with hepatitis C virus treated with ledipasvir-sofosbuvir

Hussein Sattar Abbood
Department of Cell and Molecular Biology & Microbiology, Faculty of Biological Science and Technology, University of Isfahan, Isfahan, Iran.
hussainss501@gmail.com

Majid Bouzari [1]
Department of Cell and Molecular Biology & Microbiology, Faculty of Biological Science and Technology, University of Isfahan, Isfahan, Iran.
bouzari@sci.ui.ac.ir

Abbas Soleimani-Delfan
Department of Cell and Molecular Biology & Microbiology, Faculty of Biological Science and Technology, University of Isfahan, Isfahan, Iran.
asdelfan2003@gmail.com

Hussam Sami Awayid
Middle Technical University, Department of Medical Laboratory, Institute of Technical-Suwaira, Iraq.
husamshaft@gmail.com

Abstract

The main goal of the present study was to investigate the prevalence of interferon lambda 3 (IFNL3) and microsomal triglyceride transfer protein (MTTP) encoding gene polymorphism among thalassemia patients infected with HCV. The frequency of IFNL3 and MTTP encoding gene polymorphism in 79 thalassemia patients infected with HCV and their correlation with the treatment outcome using ledipasvir/sofosbuvir was investigated. Single nucleotide polymorphism detection confirmed the GT, TT, and GG (rs1800591) polymorphism of the synthesized MTTP gene fragment and AT, AA, and TT (rs8113007) and TG, TT, and GG (rs8099917) polymorphisms of the synthesized IFNL3 gene fragment. GG was the most frequent allele of rs1800591 in both males and females followed by GT allele which was 11.39% in males and 19% in females. The TT allele of rs8099917 was the most prevalent (37% in females and 25% in males), and the GG allele was the least frequent. Also, the AT allele was more prevalent than the AA and TT alleles of rs8113007. No significant correlation was observed between the SNPs detected and the treatment outcome. The GG allele of the MTTP gene was the most effective allele involved in decreasing the ALT, AST, and ALP enzymes. For IFNL3 (rs8099917) and IFNL3 (rs8113007), the TT and AT alleles were the most effective alleles for the ALP enzyme levels, respectively. It seems that there is a correlation between the frequent alleles detected and liver enzymes.

Keywords: Hepatitis C virus, Thalassemia, Single nucleotide polymorphism (SNP), IFNL3, MTTP, Ledipasvir-sofosbuvir.

[1] Corresponding Author

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1. Introduction

Chronic hepatitis C virus (HCV) infection is one of the most prevalent viral diseases across the world. Based on the WHO report, globally, an estimated 58 million people have chronic HCV infection, with about 1.5 million new infections occurring per year. However, HCV infection prevalence varies geographically (0.6%-10%), and the number of deaths attributable to HCV-related infection is high (1,2). HCV may progress toward cirrhosis and hepatocellular carcinoma. Furthermore, extrahepatic manifestations such as type II 'mixed' cryoglobulinemia (MC) and non- Hodgkin lymphoma (NHL) may be associated with HCV infection and exhibit immune-mediated pathogenesis in HCV-infected individuals (3). Viral transmission occurs mainly through receiving blood and blood products, like plasma from infected persons. The risk of HCV infection in patients with particular diseases, such as thalassemia and hemophilia are significantly high. This risk is due to the possibility of blood transfusion from donors with HCV infection (4, 5). IFNL3, also known as interferon λ-3, is an important cytokine that helps defend the body against viral infections. It is involved in the clearance of HCV from the blood and the success of antiviral therapy against HCV (6). During the last two decades, HCV therapy has developed from interferon-α (IFNα) to combination therapy with ribavirin (RBV), followed by a combination of pegylated-IFN (PEG-IFN) and RBV therapy, known as pegylated-interferon-ribavirin (PEG-IFN-RBV) therapy, in chronic hepatitis C cases. The use of ledipasvir/sofosbuvir in chronic hepatitis C treatment has been approved in several countries. High sustained virological response (SVR) rates have been reported after 12 weeks (7). The main goal of these treatment strategies is to enhance the SVR. The effectiveness of ledipasvir/sofosbuvir (Harvoni®; Gilead Sciences, Inc., Foster City, CA, USA) in the treatment of HCV infections has been well established (8). However, the impact of genetic polymorphisms in the IFNL3 and MTTP genes on the efficacy of ledipasvir/sofosbuvir remains uncertain. In addition to IFNL3, other factors, including MTTP and superoxide dismutase 2 (SOD2), may be involved in the body's response to HCV infection and its side effects, like fibrosis (9). MTTP is most prevalent in the endoplasmic reticulum of liver tissue, where it catalyzes the transport of phospholipids, triglycerides and, cholesteryl esters between phospholipid surfaces (10). Because HCV infection exerts MTTP accumulation in the liver by downregulation of the ATP-dependent RNA helicase, it is concluded that HCV infection affects the expression of genes involved in fatty acid metabolism (11). An association has been detected between the IFNL3 single nucleotide polymorphism (SNP) and an elevated SVR following antiviral therapy, as reported in a number of publications (12). The rs4803217 SNP emerges as a noteworthy and potent determinant in the prognosis of HCV genotype 1 infection in CHC (Chronic Hepatitis C) patients undergoing treatment with PEG-IFN-α (Pegylated Interferon-Alpha) and RBV (13). The analysis of 12 SNPs in 740 patients for IFNL3/4 indicated that distinct SNPs, including rs12979860-CC, rs8109886-CC, and rs8099917-TT, serve as predictive markers for SVR, with rs12979860-CC demonstrating a particularly potent effect (14). Although various studies have delved into the valuable research on the impact of MTTP and IFNL3 polymorphisms on the treatment of HCV infection using antiviral drugs such as RBV, the presence of polymorphisms in the IFNL3 gene, and the A/T (rs8113007) and G/T (rs8099917) polymorphisms of the synthesized IFNL3 gene fragment on the effectiveness of treatment with ledipasvir/sofosbuvir remains ambiguous. Furthermore, the influence of MTTP polymorphisms on HCV treatment and viral clearance when combined with ledipasvir/ sofosbuvir remains unclear. Despite developments in blood screening methods to decrease the risk of transfusion-transmitted infections, blood-borne hepatitis C remains an important challenge in patients with thalassemia (4). Recently, Khudhair *et al*. (2020) detected the prevalence of HCV among a total of 1650 Iraqi individuals, including hemodialysis and thalassemia patients, blood donors, and medical staff. The highest frequency was recorded among thalassemia patients (15). The importance of host genetic variation for spontaneous clearance and treatment response in HCV-infected individuals has been confirmed by genome wide-scale studies (16, 17). The aim of this study was to investigate the prevalence of IFNL3 and MTTP encoding gene polymorphism among thalassemia patients infected with HCV and its possible correlation with SVR when ledipasvir/ sofosbuvir is used for the treatment in Wasit Province, Iraq.

2. Materials and Methods

2.1. Sample collection

This cross-sectional study was carried out following the Helsinki Declaration (Ethical Principles for Medical Research Involving Human Subjects), and was approved by the University of Isfahan ethics committee (IR.UI.REC.1402.033) and the Ministry of Health of Iraq. Informed consent were obtained from all patients. Blood samples were collected from 2019 to 2021 in the Wasit province of Iraq. A total of 79 samples were collected (48 females and 31 males). Serum concentrations of the liver enzymes including alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP), were measured at baseline and ≥12 weeks after the end of drug therapy. Cut points for enzyme levels were considered as ALT ≤ 45 UL^{-1}, AST ≤ 45 UL^{-1}, and ALP 35-85 UL^{-1} (Reference range from Thalassemia Center of Wasit, Iraq). All patients received a fixed dose of a combination tablet of 90 mg ledipasvir/400 mg sofosbuvir, administered orally once daily for 12 weeks. Also, demographic data were recorded and provided in Table 1.

Table 1. Demographic data of the patients tested in Wasit province,Iraq.

Cases	Sex	Age	Thalassemia	HCV			IFNL3 GENE ID: 282617		MTTP GENE ID: 4547	Treatment		Liver Enzymesc Before Treatment			Liver Enzymes After Treatment		
				History	Infection	Viral load	rs: 8113007	rs: 8099917	rs: 1800591	VIT C,D+ ZINCa	HARVONYb	SGPT	SGOT	ALP	SGPT	SGOT	ALP
							AA	TT	GG								
							A>T	T>G	G>T								
1	F	24	Positive	2019	Positive	Detectable	AA	TT	GT	VIT C,D+ ZINC	Harvony	97	67	141	90	55	99
2	F	28	Positive	2021	Positive	Detectable	AT	TG	TT	VIT C,D+ ZINC	Harvony	119	70	133	110	62	105
3	F	29	Positive	2020	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	74	41	116	60	40	90
4	M	32	Positive	2021	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	67	45	93	130	95	120
5	M	19	Positive	2019	Positive	Detectable	AT	TG	GG	VIT C,D+ ZINC	Harvony	139	94	131	80	82	98
6	F	23	Positive	2021	Positive	Detectable	AT	TT	GG	VIT C,D+ ZINC	Harvony	70	89	168	82	80	210
7	M	20	Positive	2020	Positive	Detectable	AT	TT	GT	VIT C,D+ ZINC	Harvony	89	86	101	68	70	99
8	F	29	Positive	2020	Positive	Detectable	TT	TG	GG	VIT C,D+ ZINC	Harvony	86	80	118	70	88	110
9	F	26	Positive	2019	Positive	Detectable	AA	TT	TT	VIT C,D+ ZINC	Harvony	77	117	216	80	82	104
10	F	21	Positive	2019	Positive	Detectable	AT	TT	GT	VIT C,D+ ZINC	Harvony	56	77	285	58	56	98
11	F	30	Positive	2021	Positive	Detectable	AT	TT	GG	VIT C,D+ ZINC	Harvony	23	27	113	45	40	85
12	F	18	Positive	2021	Positive	Detectable	AA	TT	TT	VIT C,D+ ZINC	Harvony	141	114	111	40	42	90
13	F	24	Positive	2019	Positive	Detectable	AT	TG	GG	VIT C,D+ ZINC	Harvony	32	42	104	42	43	85
14	M	19	Positive	2019	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	138	103	109	22	24	80
15	M	28	Positive	2020	Positive	Detectable	TT	TG	GT	VIT C,D+ ZINC	Harvony	16	14	232	20	22	82
16	M	31	Positive	2021	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	59	50	160	55	56	80
17	M	25	Positive	2021	Positive	Detectable	AT	TG	GG	VIT C,D+ ZINC	Harvony	64	60	109	40	35	85
18	M	20	Positive	2021	Positive	Detectable	AT	TT	GG	VIT C,D+ ZINC	Harvony	76	60	77	70	60	105
19	F	27	Positive	2019	Positive	Detectable	AT	TG	TT	VIT C,D+ ZINC	Harvony	193	92	219	80	72	110
20	F	32	Positive	2019	Positive	Detectable	AT	GG	GT	VIT C,D+ ZINC	Harvony	111	85	393	92	93	120
21	F	22	Positive	2019	Positive	Detectable	AT	TG	GG	VIT C,D+ ZINC	Harvony	42	67	146	85	86	105
22	M	29	Positive	2020	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	28	26	144	42	43	90
23	M	19	Positive	2020	Positive	Detectable	AT	TT	GT	VIT C,D+ ZINC	Harvony	82	74	130	110	114	92
24	F	24	Positive	2021	Positive	Detectable	AT	TT	GT	VIT C,D+ ZINC	Harvony	112	140	360	88	86	105
25	M	21	Positive	2021	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	18	12	206	22	42	85
26	F	30	Positive	2021	Positive	Detectable	AT	TT	GG	VIT C,D+ ZINC	Harvony	72	100	216	40	32	80
27	F	23	Positive	2019	Positive	Detectable	TT	GG	GT	VIT C,D+ ZINC	Harvony	86	85	154	90	98	105
28	F	28	Positive	2019	Positive	Detectable	AT	TG	GT	VIT C,D+ ZINC	Harvony	92	90	164	77	67	103
29	F	19	Positive	2021	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	85	92	166	70	72	110
30	M	24	Positive	2021	Positive	Detectable	AT	TG	GT	VIT C,D+ ZINC	Harvony	92	102	180	80	83	98
31	F	28	Positive	2019	Positive	Detectable	AT	TT	GT	VIT C,D+ ZINC	Harvony	81	72	128	80	82	110
32	F	22	Positive	2020	Positive	Detectable	AA	TT	GT	VIT C,D+ ZINC	Harvony	60	58	103	90	70	98
33	M	26	Positive	2021	Positive	Detectable	AT	TT	GT	VIT C,D+ ZINC	Harvony	52	53	90	80	82	90
34	F	18	Positive	2019	Positive	Detectable	AT	TG	GT	VIT C,D+ ZINC	Harvony	68	79	112	45	46	85
35	F	32	Positive	2019	Positive	Detectable	AT	TT	GG	VIT C,D+ ZINC	Harvony	42	46	101	33	32	80
36	F	23	Positive	2019	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	67	70	108	36	34	82
37	F	29	Positive	2021	Positive	Detectable	AT	TG	GT	VIT C,D+ ZINC	Harvony	40	45	85	37	38	81
38	F	20	Positive	2021	Positive	Detectable	AT	TG	GG	VIT C,D+ ZINC	Harvony	68	79	99	45	44	85
39	M	25	Positive	2020	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	110	118	201	86	88	97
40	F	30	Positive	2019	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	88	67	160	40	42	80
41	F	19	Positive	2019	Positive	Detectable	TT	TG	TT	VIT C,D+ ZINC	Harvony	120	122	210	36	31	90

42	M	21	Positive	2020	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	68	60	100	21	22	73
43	M	23	Positive	2021	Positive	Detectable	AT	TG	GT	VIT C,D+ ZINC	Harvony	130	122	200	15	16	72
44	F	26	Positive	2021	Positive	Detectable	AT	TT	TT	VIT C,D+ ZINC	Harvony	91	82	104	18	20	70
45	F	28	Positive	2019	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	69	70	154	16	14	80
46	M	32	Positive	2019	Positive	Detectable	TT	TG	GG	VIT C,D+ ZINC	Harvony	88	56	120	21	22	83
47	F	19	Positive	2019	Positive	Detectable	TT	GG	GG	VIT C,D+ ZINC	Harvony	45	44	85	80	82	110
48	M	22	Positive	2021	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	67	42	91	88	86	120
49	M	25	Positive	2021	Positive	Detectable	AT	TT	GT	VIT C,D+ ZINC	Harvony	120	110	199	96	92	110
50	F	27	Positive	2020	Positive	Detectable	AT	TT	GG	VIT C,D+ ZINC	Harvony	155	140	198	110	112	120
51	M	30	Positive	2021	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	104	108	122	40	42	86
52	F	29	Positive	2021	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	86	87	100	40	33	80
53	M	20	Positive	2019	Positive	Detectable	TT	TT	GG	VIT C,D+ ZINC	Harvony	88	78	110	36	31	92
54	F	18	Positive	2019	Positive	Detectable	TT	GG	GT	VIT C,D+ ZINC	Harvony	66	52	90	37	22	100
55	F	31	Positive	2019	Positive	Detectable	AA	TT	TT	VIT C,D+ ZINC	Harvony	96	92	105	26	27	85
56	F	21	Positive	2020	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	111	112	122	66	71	86
57	F	28	Positive	2021	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	100	105	200	22	21	70
58	M	24	Positive	2021	Positive	Detectable	AT	TT	GG	VIT C,D+ ZINC	Harvony	105	106	150	21	22	80
59	M	26	Positive	2021	Positive	Detectable	AT	TG	TT	VIT C,D+ ZINC	Harvony	91	82	112	85	84	100
60	F	19	Positive	2019	Positive	Detectable	AT	TT	GT	VIT C,D+ ZINC	Harvony	120	130	200	70	62	98
61	M	23	Positive	2019	Positive	Detectable	AT	TG	GG	VIT C,D+ ZINC	Harvony	46	45	88	110	112	120
62	F	22	Positive	2021	Positive	Detectable	AT	TG	GG	VIT C,D+ ZINC	Harvony	88	76	105	40	32	80
63	M	19	Positive	2021	Positive	Detectable	AT	TT	TT	VIT C,D+ ZINC	Harvony	67	77	104	40	22	81
64	M	23	Positive	2021	Positive	Detectable	AA	TT	GT	VIT C,D+ ZINC	Harvony	210	200	254	16	22	82
65	F	25	Positive	2021	Positive	Detectable	AA	TT	TT	VIT C,D+ ZINC	Harvony	86	87	105	56	46	77
66	F	28	Positive	2020	Positive	Detectable	AT	TG	TT	VIT C,D+ ZINC	Harvony	46	42	90	36	26	70
67	M	30	Positive	2019	Positive	Detectable	TT	TG	GT	VIT C,D+ ZINC	Harvony	67	77	108	31	32	60
68	F	18	Positive	2019	Positive	Detectable	AA	TT	GT	VIT C,D+ ZINC	Harvony	55	56	89	37	38	60
69	M	21	Positive	2019	Positive	Detectable	AT	TG	TT	VIT C,D+ ZINC	Harvony	54	49	85	40	32	66
70	M	29	Positive	2021	Positive	Detectable	AT	TG	TT	VIT C,D+ ZINC	Harvony	45	46	89	40	42	62
71	F	20	Positive	2021	Positive	Detectable	AA	TT	GT	VIT C,D+ ZINC	Harvony	110	112	160	88	72	110
72	M	24	Positive	2019	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	67	80	120	45	46	89
73	F	27	Positive	2019	Positive	Detectable	AT	TT	TT	VIT C,D+ ZINC	Harvony	79	86	107	40	42	88
74	M	31	Positive	2020	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	88	80	112	31	32	87
75	F	18	Positive	2021	Positive	Detectable	AA	TT	GG	VIT C,D+ ZINC	Harvony	44	42	87	45	44	90
76	F	22	Positive	2019	Positive	Detectable	TT	TG	GG	VIT C,D+ ZINC	Harvony	133	120	155	96	86	110
77	F	26	Positive	2021	Positive	Detectable	AT	TG	GG	VIT C,D+ ZINC	Harvony	210	222	180	120	123	160
78	F	30	Positive	2020	Positive	Detectable	AT	TG	GT	VIT C,D+ ZINC	Harvony	222	234	199	110	112	160
79	F	21	Positive	2019	Positive	Detectable	AA	TT	TT	VIT C,D+ ZINC	Harvony	224	250	288	110	115	170

F: female; M: male; a: Vit. C 1000mg +Vit. D 6000 IU + Zinc 50mg, one tablet daily for one month; b: 90mg/400mg ledipasvir/ sofosbuvir, one tablet daily for 12 weeks; c: SGPT normal values < 45 U/L, SGOT normal values < 45 U/L, AL P normal values 35-85 U/L.

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2.2. DNA extraction and polymerase chain reaction (PCR)

A blood sample of 2 ml was withdrawn from the participants and placed in a sterile tube containing ethylenediaminetetraacetic acid (EDTA) under aseptic conditions and used for genomic DNA purification using ReliaPrep™ Blood gDNA Miniprep System Kit (Promega, USA). The quality and quantity of the DNA samples were assessed using 1.5% agarose gel electrophoresis and the Quantus™ Fluorometer (Promega, USA). PCR was performed using the extracted genomic DNA, primers, and GoTaq Green Master Mix (Promega, USA) and nuclease-free water (Promega, USA) in a final volume of 25 µl. The primer sequences used are listed in Table 2.

Table 2. The primers used for PCR in the current study

Primers	Sequence (5′ -3′)	Annealing (°C)	Product (bp)
MTTP-F	AGTTTCACACATAAGGACAATCATCTA	58	109
MTTP-R	GGATTTAAATTTAAACTGTTAATTCATATCAC		
IFNL3-F	CATCCCACTTCTGGAACAAATC	60	400
IFNL3-R	GTATCAACCCCACCTCAAATTATC		

Amplification reaction was performed using thermal cycler (Thermo Fisher Scientific, USA). It included a cycle of initial denaturation at 95 °C for 5 min, and then 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for MTTP and 60 °C for IFNL3 for 30 s, and extension at 72 °C for 30 s, followed by a cycle of final extension at 72 °C for 7 min. PCR products were assessed using 1.5% agarose gel electrophoresis and the Quantus™ Fluorometer. Finally, PCR products were sequenced by Sanger sequencing using ABI3730XL, an automated DNA sequencer (Macrogen, South Korea). The nucleotide sequences were analyzed using Geneious software version 11.1 (Biomatters, Auckland, NZ) for sequence alignments and SNP allele detection. Serum HCV RNA was quantified using a Hepatitis C (HCV RNA) PCR Kit (sensitivity, 13 IU/ml), SACACE BIOTECHNOLOGIES, Italy.

2.3. Statistical analyses

The obtained data were analyzed using GraphPad Prism version 8.3.0 with paired t test and Fisher's exact test.

3. Results

SVR refers to the condition in which the serum hepatitis C virus RNA becomes undetectable 12-14 weeks after completing treatment. The results of treatment in male and female thalassemia patients infected with HCV and treated with ledipasvir/sofosbuvir are shown in Table 3.

Table 3. The results of the treatment of the thalassemia patients infected with HCV and treated with ledipasvir/ sofosbuvir in males and females

Patients	SVR	NSVR
Total	0.0375%	99.9625%
Female	0.416%	99.95833
Male	0.343%	99.65%

SVR: sustained virological response; NSVR: non-sustained virological response

In PCR amplification, the entire 79 purified genomic DNAs with MTTP-F/MTTP-R and IFNL3-F/IFNL3-R primers, yielded fragments of 109 bp and 400 bp, respectively. The ethidium bromide-stained agarose gel (1.5%) electrophoresis of the PCR products of the MTTP and IFNL3

encoding genes is shown in Figures 1 and 2. The PCR products were sequenced and submitted to GenBank with accession numbers OQ980532-OQ980610. The allele frequencies of different SNPs detected in HCV-infected thalassemia patients treated with ledipasvir/ sofosbuvir and their association with SVR are shown in Table 4. GG allele was the most frequent allele of rs1800591 in both males (23%) and females (28%), followed by GT allele which was 11.39% in males and 19% in females. Also, the TT allele of rs8099917 was the most prevalent (37% in females and 25% in males), and the GG allele was the least frequent. Also, the AT allele was more prevalent than the AA and TT alleles of rs8113007. The presence of TT or GG polymorphisms only resulted in a 1.23% and 2.56% SVR, respectively in 3 patients. Also, our results revealed that ALT, AST, and ALP levels significantly decreased (P<0.001) after treatment (Table 5).

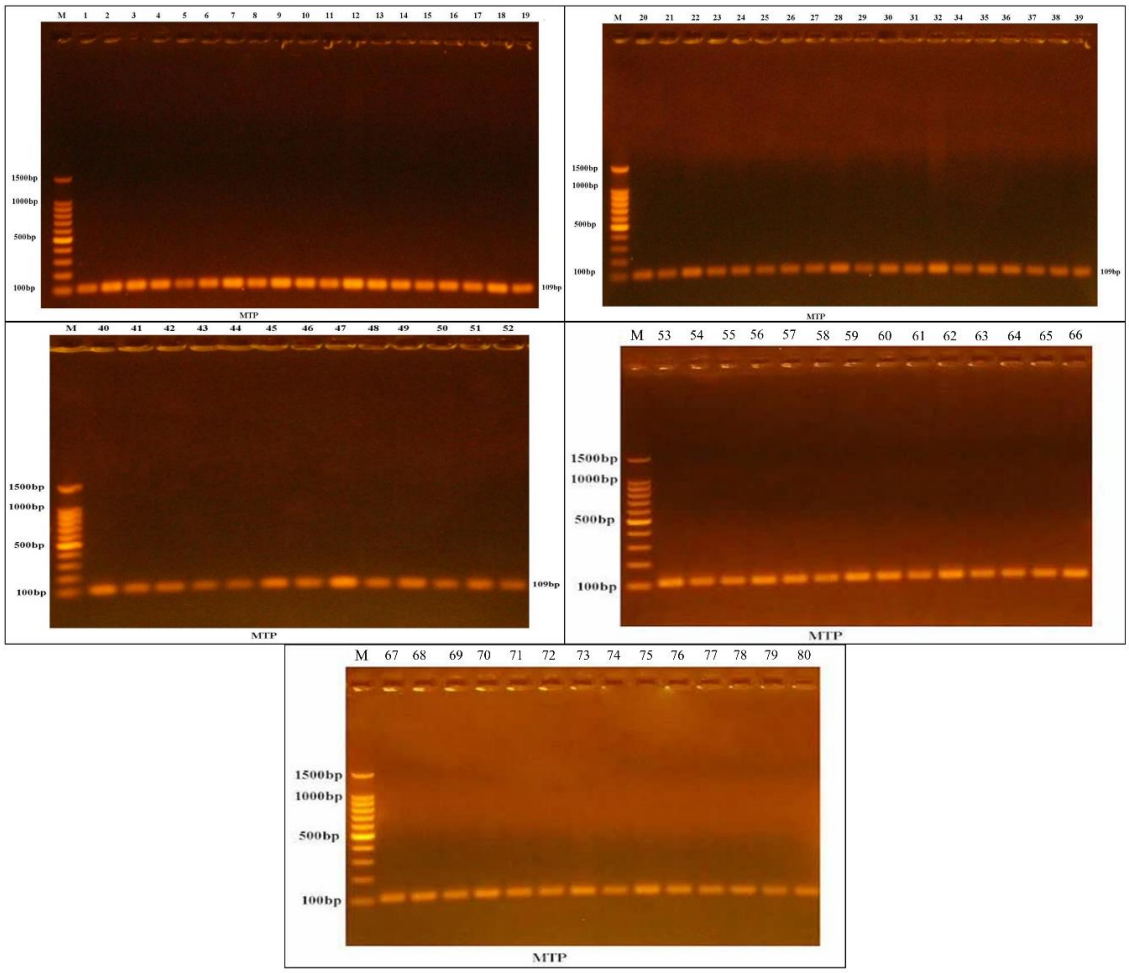

Figure 1: Agarose gel electrophoresis of the PCR products (109 bp) of the MTTP gene from HCV-infected thalassemia patients. M: 100 bp ladder marker (ExcelBand 100 bp DNA Ladder; Cosmo Bio, Tokyo, Japan).

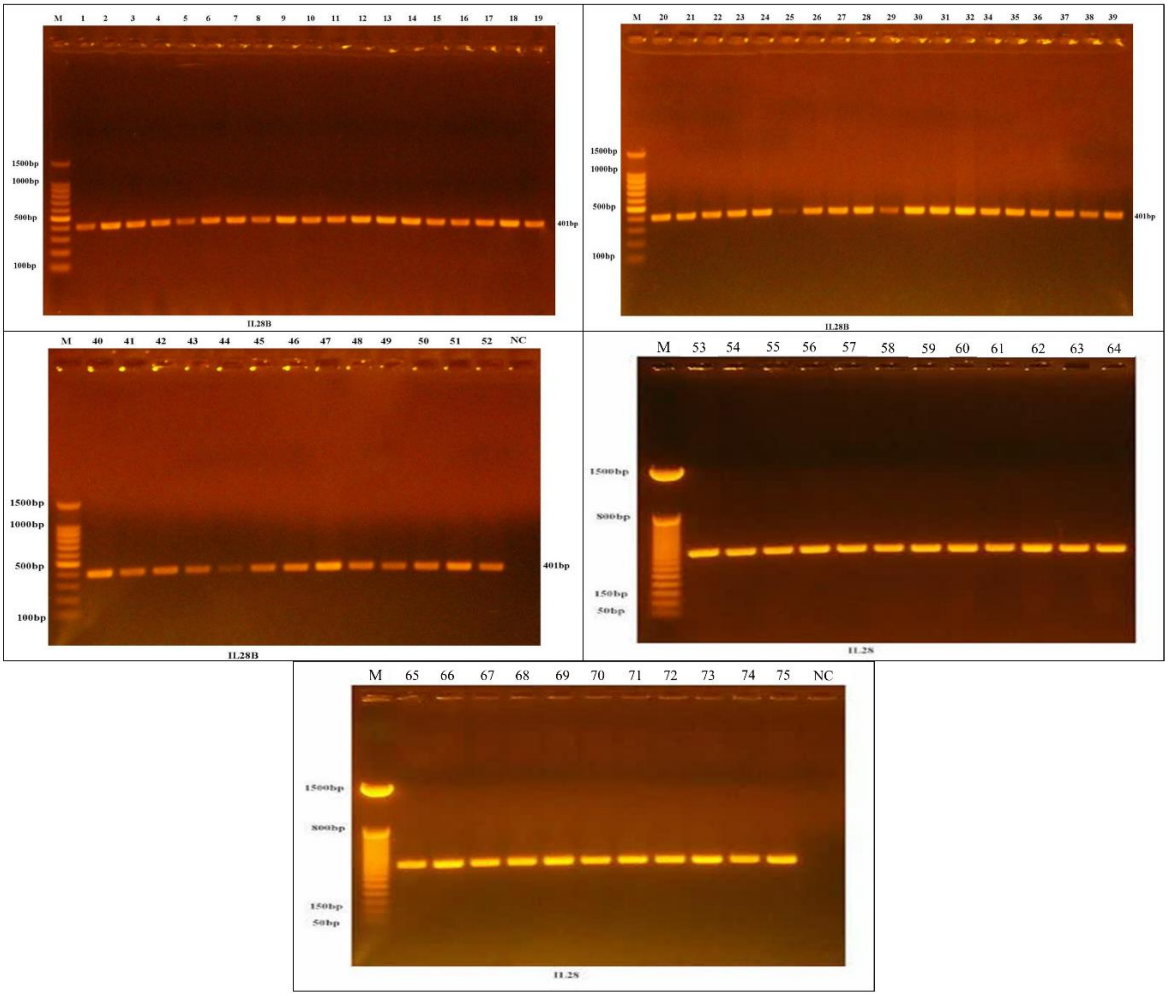

Figure 2: Agarose gel electrophoresis of the PCR products (400 bp) of the IFNL3 gene from HCV-infected thalassemia patients. M: 100 bp ladder marker (ExcelBand 100 bp DNA Ladder; Cosmo Bio, Tokyo, Japan).

Table 4. Allele frequencies of SNPs detected in HCV-infected thalassemia patients treated with ledipasvir/sofosbuvir and their association with SVR

Gene	SNP	Allele (F-%)	Male (F-%)	Female (F-%)	SVR (%)	NSVR (%)
MTTP	rs1800591	GT (24-30.37)	9-11.39	15-19	0	100
		TT (15-18.98)	4-5.06	11-13.92	1.26	98.74
		GG (40-50.63)	18-22.78	22-27.84	2.53	97.46
IFNL3	rs 8099917	TG (26-32.9)	11-23.92	15-19	1.26	98.74
		TT (49-62)	20-25.31	29-36.7	2.53	97.46
		GG (4-5)	0-0	4-5.06	0	100
	rs8113007	AA (30-18.74)	12-15.2	18-22.78	1.26	98.74
		AT (39-49.36)	15-19	24-30.4	2.53	97.46
		TT (10-12.6)	4-5.06	6-7.6	0	100

F: frequency; SVR: sustained virological response; NSV: non-sustained virological response

Table 5. Comparison of liver enzyme levels pre- and post-treatment

Enzyme	Gender	UL-1 (Mean ± SD)		P-value
		Before treatment	After treatment	
ALP	Male	136.22±6.92	93.58±2.66	P<0.001
	Female	133.13±6.92	89.16±2.66	P<0.001
	Total	140.9±6.92	88.77±2.66	P<0.001
AST	Male	71.68±4.87	49/77±3.22	P<0.001
	Female	74.68±4.87	53/32±3.22	P<0.001
	Total	76.26±4.87	54/32±3.22	P<0.001
ALT	Male	73.35±4.9	54/74±3.31	P<0.001
	Female	81.77±4.9	54/22±3.24	P<0.001
	Total	84.1±4.9	56/71±3.33	P<0.001

ALT: alanine transaminase, AST: aspartate transaminase, ALP: alkaline phosphatase

The frequency of different alleles across various SNPs of the 31 cases with a decrease in liver enzymes to normal (≤ 45 UL^{-1} for ALT and AST and 35-85 UL^{-1} for ALP) after treatment with ledipasvir/sofosbuvir is shown in Table 6. Considering the MTTP gene, in the case of ALT, the frequency of **the** GG allele was significantly higher than that of the GT allele. In the case of AST, the frequency of the GG allele was significantly higher than **that of the TT and GT alleles**. For ALP, the frequency of the GG allele was significantly higher than that of the TT and GT alleles. For IFNL3 (rs8099917), regarding ALT, significant differences were observed among TT, TG, and GG. Furthermore, the difference between TT and TG was significant. A similar pattern was observed for AST. For ALP, the frequency of TT was significantly higher than that of GG. The same was true for TG compared with GG. For IFNL3 (rs8113007), **regarding ALT**, significant differences were observed only between AA and TT alleles. For AST, significant differences were observed between the frequency of AA and TT, as well as AT and TT. For ALP, significant differences were observed between AA and TT and also AT and TT. Based on the results, the GG allele of the MTTP gene was the most effective allele associated with a reduction ALT, AST, and ALP levels. For IFNL3 (rs8099917) and IFNL3 (rs811307), the TT and AT alleles were the most effective alleles for the ALP enzyme, respectively.

Table 6. Frequency of alleles in SNPs of the 31 cases with normalized liver enzymes (≤ 45 for ALT and AST, and 35-85 UL^{-1} for ALP) after ledipasvir/ sofosbuvir treatment

Enzyme	MTTP (rs1800591)			IFNL3 (rs 8099917)			IFNL3 (rs8113007)		
	Allele	F	P-value	Allele	F	P-value	Allele	F	P-value
ALT	GG	16	a*	TT	20	a*, b**	AA	14	a*
	TT	9		TG	10	a*, c**	AT	12	
	GT	6	a*	GG	1	b**, c**	TT	5	a*
AST	GG	17	b*, c**	TT	20	a*, b**	AA	13	b*
	TT	8	b*	TG	10	a*, c**	AT	13	c*
	GT	6	c**	GG	1	b**, c**	TT	5	b*, c*
ALP	GG	18	d**, e**	TT	19	d****	AA	13	d*
	TT	7	d**	TG	13	e****	AT	16	e***
	GT	7	e**	GG	0	d****, e****	TT	3	d*, e***

F: frequency, ALT: alanine transaminase, AST: aspartate transaminase, ALP: alkaline phosphatase, In each column, data with the same letters have significant differences, *= P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001

4. Discussion and conclusion

Beta-thalassemia is an inherited blood disorder characterized by reduced or absent production of beta-globin chains, leading to anemia and potential complications. Regular blood transfusions in beta-thalassemia patients pose a risk for viral infections, including HCV (18). While no direct causal relationship exists between beta-thalassemia and HCV, the overall health of individuals with beta-thalassemia is often further compromised by co-infection (19). Antiviral medications like ledipasvir/sofosbuvir (Harvoni; Gilead Sciences) have been used to clear HCV in beta-thalassemia patients (20). This study investigated the impact of three SNPs- 493G/T, T/T and G/G (rs1800591) in the MTTP gene fragment, and A/T, A/A and T/T (rs8113007) and G/T, T/T and G/G (rs8099917) in the IFNL3 gene on viral clearance and their relationship with ledipasvir/sofosbuvir treatment in beta-thalassemia patients co-infected with HCV. Studies have revealed that carriers of this polymorphism exhibit elevated levels of steatosis, increased quantities of HCV RNA, and more advanced fibrosis in their liver (21). The possibility of applying rs1800591 polymorphism as a biomarker for early detection of non-alcoholic fatty liver disease (NAFLD) has been previously investigated (22). Tan *et al*. (2020) (23) also concluded that the G allele of rs1800591 was more likely to be associated with NASH susceptibility in their systematic review of a total of 10 case-control studies. According to gene expression analyses, the -493T allele is associated with elevated MTTP expression in healthy individuals, while the -493G allele is linked with reduced MTTP transcription (24). The association between genetic polymorphisms in the MTTP gene and HCV-related hepatic steatosis appears to be complex and dependent on population characteristics. While the -493G/T polymorphism of the MTTP gene shows no significant association with HCV genotype 1-related hepatic steatosis in the Turkish population (24), other studies have reported links between different MTTP gene polymorphisms and non-alcoholic fatty liver disease (NAFLD) susceptibility in various populations (22). These conflicting findings highlight the need for further research to clarify the role of MTTP gene polymorphisms in HCV-related hepatic steatosis. However, MTTP may serve as a potential biomarker of SVR in antiviral therapy of patients with HCV genotypes 1, 3, and 4 infections (21). In addition, the MTTP -493 G/T polymorphism has been linked to insulin resistance in chronic hepatitis C infection. Still, some studies have indicated that it is not universally applicable (24). In this study, the polymorphism of rs1800591 (-493G/T allele) was observed in 30.37% of the patients, while the T/T allele was present in 18.98% and the G/G allele in 50.63%. However, it should be noted that the presence of T/T or G/G alleles was associated with SVR rates of only 1.23% and 2.56%, respectively. Therefore, these rates of clearance are not sufficiently reliable predictors of antiviral treatment outcomes. The association between the influence of MTTP polymorphism in rs1800591 and treatment with ledipasvir-sofosbuvir has not been previously investigated. Nevertheless, based on our results, it appears that this treatment may not be effective for patients with polymorphisms in rs1800591 (-493GT, TT, and GG alleles) across all patient groups. Patients with TT or GT allele polymorphisms showed only a 1.26% and 2.56% SVR, respectively, indicating that the effectiveness of ledipasvir-sofosbuvir treatment in patients with these polymorphisms is limited. On the other hand, the weak results obtained in this study may be corelated to thalassemia, which needs more sophisticated studies.

The causal role of nucleotide variations of the IFNL3 gene in HCV therapy success and spontaneous viral clearance has been reported by some researchers (25, 26). However, the exact mechanism of this association is not fully understood. In a study on a population of 368 cases with beta thalassemia and anti-HCV antibodies in Italy, the total frequencies of the genotypes TT, GT, and GG of rs8099917 were 40.5%, 47%, and 12.5%, respectively (27). In addition, the obtained data revealed no correlation between IFNL3 polymorphisms and liver fibrosis stage. In another report, by contrast, spontaneous viral clearance was found to be more frequent among patients with the T/T genotype of rs8099917 or C/C genotype of rs12979860 than other genotypes variants in HCV-infected thalassemia major patients. Also, G/T or G/G genotypes of rs8099917 and C/Tor T/T

genotypes of rs12979860 were associated with severe liver fibrosis (28). In addition, the IFNL3 gene polymorphisms have been linked to inflammation activity and fibrosis of the liver (29, 30). Nevertheless, according to genomic analyses, rs8099917 can be used as a valuable candidate SNP in clinical algorithms to predict SVR to IFN-based therapies (31). IFNL3 polymorphisms have been consistently associated with treatment outcomes in HCV infection, even with the introduction of modern highly effective direct-acting antivirals (32). Unfavorable IFNL3 SNPs are associated with high baseline expression of interferon-stimulated genes (ISGs) and insufficient induction of ISGs by exogenous interferon, resulting in poor treatment outcomes with interferon-based therapy (33, 34). These genetic markers can aid in individualizing treatment strategies, maximizing therapeutic efficiency, and identifying patients at risk of being refractory to treatment due to multidrug-resistant HCV (35). IFNL3 polymorphisms, specifically rs12979860 and rs8099917, have been identified as predictors of treatment response in pediatric patients infected with HCV genotypes 1 or 4 (36). The CC genotype of rs12979860 and the TT genotype of rs8099917 were associated with higher rates of SVR in treatment with pegylated interferon alpha and RBV (pegIFNα/RBV). Notably, the rs12979860 genotype was found to be a better predictor of treatment response among HCV/HIV-1 coinfected patients compared to rs8099917 (31, 37). These findings emphasize the importance of IFNL3 genotyping in predicting treatment outcomes, especially in specific patient groups. The T/T genotype of rs8099917 and the C/C genotype of rs12979860, along with age, female gender, and specific HCV genotypes, were independently associated with SVR in patients treated with interferon-alpha (28). These findings suggest that genetic factors, along with demographic characteristics and viral factors, contribute to treatment response. Patients with favorable IFNL3 genotypes may benefit from abbreviated treatment courses, which could potentially improve adherence and reduce the burden of therapy without sacrificing efficacy. However, caution is advised when considering this approach for patients with a less favorable genotype, as it may result in considerably lower SVR rates (38). In the ION-3 study, 423 previously untreated patients infected with HCV genotype 1 and without cirrhosis received ledipasvir/sofosbuvir for 8 weeks, and their outcome data were analyzed. Upon reevaluating the published ION-3 data, the researchers observed that SVR rates varied significantly based on gender and rs12979860 genotype. Notably, SVR rates exceeded 98% in women and individuals with the rs12979860-CC genotype (16). A study has been performed on 75 patients with genotype 3a HCV to evaluate the effect of allelic associations of 50 SNPs in the interferon-λ gene in response to interferon-α and RBV therapy in Pakistan. Data indicated the significant association of rs8109886, rs8113007, and rs12979860 among the 13 most effective SNPs in HCV clearance (39). Also, in a cohort study in China, the allele polymorphisms in rs8099917 and rs8113007 for IFNL3 were not associated with HCV infection susceptibility, and these polymorphisms showed similar frequencies in healthy and infected individuals (40). However, our study's evidence suggests that polymorphisms in IFNL3 rs8099917 (TG, TT, and GG) and rs8113007 (AA, AT, and TT alleles) do not significantly influence response to ledipasvir-sofosbuvir therapy. Based on the information provided, it appears that Harvoni (Harvoni®; Gilead Sciences, Inc., Foster City, CA, USA) has demonstrated higher success rates in treating patients with HCV genotype 1, compared to other viral genotypes. Additionally, for patients with genotype 1 or 4 of HCV, with or without cirrhosis, the use of Harvoni in combination with RBV is recommended for optimal viral clearance. In the Iraqi population, the most dominant HCV genotypes are genotypes 4 and 1 (41). Among thalassemic HCV patients, the rate of genotype 4 is even higher, reaching 94%, and in patients with chronic liver disorder due to hepatitis C, genotype 4 accounts for 85% of cases (42). Considering the high prevalence of genotype 4 in the Iraqi population and especially among thalassemic HCV patients, Harvoni® monotherapy may be insufficient for effective treatment in these patients. Instead, combination therapy with Harvoni® and RBV may be necessary to optimize treatment efficacy (43). As shown in Table 5, the liver enzyme levels decreased following treatment

compared to baseline in the studied subjects. Similar results have been reported in previous studies. For example, Noureddin *et al* (2018) reported that ALT decreased from 63.1 ± 62.6 UL^{-1} to 17.8 ± 12.3 UL^{-1} and AST from 51.8 ± 41.1 UL^{-1} to 21.5 ± 8.0 UL^{-1} after achieving SVR compared with baseline values in chronic hepatitis C patients (44). However, other risk factors for elevated liver enzymes should be interpreted considering additional risk factors. For example, Chadha *et al*. (2023) have reported abnormal ALT at SVR-12 (12 weeks after SVR) in 9.6% of those with steatosis and 6.7% of those without and identified that abnormal ALT was related to increased body mass index but not ongoing alcohol use among HCV- infected patients (45). Although, a high rate of SVR was not observed in the patients, significant reduction of the liver enzymes was observed. Considering that the patients were thalassemic, it can be considered a positive outcome of the treatment. The periodic blood transfusions of patients with β-thalassemia major result in the accumulation of iron and this overload affects the liver along with other organs. Affected or damaged liver and also some treatments lead to significant increases of ALT, AST, and ALP enzymes (46). Co-infection with HCV is significantly correlated with the increased iron overload (47). Acute or chronic injuries to the liver increase the concentrations of AST and ALT (48). The high level of liver enzymes before treatment could be correlated with this. On the other hand, it has been reported that an increased risk of hepatocellular carcinoma and death is associated with elevated liver enzymes after SVR-24 (49). The decreased levels of liver enzymes indicate that the treatment at least can decrease these outcomes.

In this study, the high levels of liver enzymes significantly decreased after treatment with ledipasvir-sofosbuvir. The GG allele of the MTTP gene was the most effective allele involved in decreasing ALT, AST, and ALP enzymes. For IFNL3 (rs8099917) and IFNL3 (rs8113007), the TT and AT alleles were the most effective alleles for the ALP enzyme, respectively. Therefore, it seems there is a correlation between the frequent alleles detected and liver enzymes and in designing treatment strategies, these polymorphisms should be taken into consideration.

In conclusion, in this study, we conducted a thorough evaluation of the polymorphisms in the PCR-synthesized regions of IFNL3 and MTTP genes using 79 blood DNA samples obtained from HCV-infected thalassemia patients in Wasit province, Iraq. The analysis of genotypic alleles revealed a high prevalence of the G allele of rs1800591, the T allele of rs8099917, and the A allele of rs8113007 among the subjects under investigation. The analysis also indicated that these allelic polymorphisms in the MTTP and IFNL3 genes do not exhibit any meaningful correlation with the response to 12-week ledipasvir-sofosbuvir treatment in β-thalassemia patients with HCV infection. The results also show that other treatment strategies may be more successful. Given that the drug manufacturer's prescription indicates a higher efficacy of Harvoni® in treating HCV viral genotypes 1 and 4, it is advisable to avoid using this drug as a standalone treatment in countries like Iraq, where genotype 4 is the prevailing strain. In regions with predominant genotype 4 HCV prevalence, using Harvoni® alone may not yield optimal results. Instead, a more effective treatment approach may involve combining Harvoni® with RBV or other supplementary medications to enhance treatment outcomes.

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