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The Synergistic Effect of Fluvastatin and IFN-λ on Peripheral Blood Mononuclear Cells of Chronic Hepatitis C Virus (HCV) Patients with IL-28B rs12979860 CC Genotype

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ABSTRACT

There is a relationship between the life cycle of the hepatitis C virus (HCV) and the synthesis and hemostasis of lipids as well as lipid metabolism and interferon (IFN) regulatory system. This study aimed to examine the effect of fluvastatin and IFN-λ in the expression of mediators involved in lipid metabolism and HCV proliferation in patients with rs12979860 CC polymorphism.

Thirteen patients with HCV and five controls with rs1297986CC polymorphism were included in this study. Peripheral blood mononuclear cells (PBMCs) of patients and controls were treated by fluvastatin, IFN-λ or fluvastatin+IFN-λ. Assessment of IL-28B polymorphism, RNA extraction, and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed. The mRNA expression of sterol regulatory element-binding protein 1 c (SREBP1c), ATP-binding cassette transporter A1 (ABCA1), diacylglycerol acyltransferase 1 (DGAT1), and HCV core as well as measurement of ABCA1 protein level were evaluated before and after treatment.

The results indicated that IFN-λ +fluvastatin acted as an inhibitor in mRNA expression of SREBP1c; while acting as an inducer in the expression of ABCA-1. The results of ABCA1 assay showed a significant increase of this protein after treatment with fluvastatin and IFN-λ compared with untreated cells (p=0.02). Moreover, the mRNA expression of HCV core was suppressed in all experimental groups treated with fluvastatin, IFN-λ or their combination which was more significant after treatment with fluvastatin+IFN-λ (p<0.001).

The results of this study demonstrated the significant effect of treatment with fluvastatin+IFN-λ in PBMCs of HCV patients with rs12979860 CC polymorphism. According to the drug resistance of viruses and prevention of virus-induced steatosis in patients with HCV, using regulatory agents of lipid mediators in parallel with current medications could be considered as an effective therapeutic strategy.

Keywords*:* ATP-binding cassette transporter A1 (ABCA1); Diacylglycerol acyltransferase 1 (DGAT1); Fluvastatin; Hepatitis C virus; Interferon lambda; IL-28B; Sterol regulatory element-binding protein 1c (SREBP1c)

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INTRODUCTION

Hepatitis C Virus (HCV) as a hepatophilic virus with positive single-stranded RNA develops chronic liver diseases such as cirrhosis and hepatocellular carcinoma.[1,2] This virus has affected about 3% of the world population.[3] A high risk of developing HCV infection was found in patients with human immunodeficiency virus (HIV), drug users, and homosexual men.[4] Moreover, lipid metabolism could change as a result of HCV and HIV infections with different alternations.[5] The close relationship of the viral life cycle (HCV assembly, secretion, and pathogenesis) with lipid and lipoprotein metabolism has been documented.[6,7] Furthermore, lipoproteins facilitate the HCV entry to hepatocytes. The literature review shows that the metabolism of fatty acids and phospholipids is essential for HCV proliferation. Moreover, the synthesis of fatty acids increases during virus replication.[6]

Statins such as fluvastatin act as an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) which play a vital role in cholesterol biosynthesis and are extensively used in the treatment of patients with hypercholesterolemia. Recently, considerable attention has been paid to the effects of statins in the life cycle and the proliferation of HCV viruses.[8] In the last years, there has been a growing interest in the application of fluvastatin to treat HCV because of its higher efficacy compared to other statins.[9] On the other hand, there is a negative regulation between lipid biosynthesis and Interferon (IFN) type I signaling. IFNs have also shown to increase cell import of cholesterol and fatty acids.[10] The previous study has demonstrated synergistic effects of fluvastatin and Interferon-α to inhibit HCV proliferation.[9]

Moreover, in CC genotype of IL-28 B as one of the most important host factors, decreased steatosis and increased response to Interferon have been documented.[11] IFN- λ as the newest characterized IFN, consist of IL-29 (IFN-lambda1), IL-28A (IFN-lambda2), and IL-28B (IFN-lambda3).[12] It has been suggested that there is a significant interaction between IFNλ family and HCV response to IFN. Moreover, a higher sustained viral response (SVR) was revealed in HCV patients with IL-28B rs12979860CC polymorphism treated with peginterferon compared with other polymorphism (CT and TT).[13] This class of IFNs performs important functions to overcome HCV.[12] To the best of our knowledge, no previous research has investigated the synergic effect of fluvastatin and Interferon-λ on regulated factors of fatty acids and lipids in peripheral blood monuclear cells (PBMCs) of patients with chronic HCV infection.

Because of the close relationship between viral life cycle with lipids and fatty acids, using regulatory factors of fatty acids and lipids including ATP-binding cassette transporter A1 (ABCA-1), diacylglycerol acyltransferase 1 (DGAT1), and sterol regulatory element-binding protein 1 (SREBP-1), could be helpful as indirect therapeutic agents. There is a question of whether factors involved in the synthesis and metabolism of lipids could be used to control the infection and virus replication. Although several studies have been performed regarding the expression of fatty acids regulators and lipids metabolisms in different alleles of IL-28B,[14,15] little attention has been given to the synergic effect of fluvastatin and IFN-λ on fatty acids regulators and lipids metabolisms in patients with rs12979860 CC. Hence, the aim of this study was to investigate the effects of fluvastatin and IFN-λ on lipid metabolism in PBMCs of HCV patients with IL-28B polymorphismsrs12979860 CC and healthy controls as well as their effect on HCV proliferation in PBMCs.

MATERIALS AND METHODS

Patients

This interventional and in vitro study has been conducted on PBMCs of patients with HCV referred to Hepatitis and Liver clinic of Imam Khomeini Hospital from June 2017 to August 2017. This study was approved by the Ethics committee of Tarbiat Modares University (N. IR.TMU.REC.1394.263). Informed consent was obtained from all patients and controls.

Inclusion criteria for HCV group included treatment-naïve patients with chronic HCV, IL-28 polymorphisms rs12979860 CC, not receiving any kind of medications, and lack of fatty liver. Patients with metabolic diseases, those individuals who received metabolic, anti-diabetic and anti-lipid and anti-inflammatory drugs and those with other viral diseases (HIV, HBV) were excluded from both patient and control groups. A total of thirteen blood samples of chronic HCV infected patients and five blood samples from healthy controls without a history of infectious diseases in the last three months were taken. Moreover,

the HCV genotype of the patients was obtained from their clinical records.

Biochemical Parameters and Lipid Profile

Biochemical parameters and lipid profile [including serum total cholesterol (Chol), triglycerides (TGs), high-density lipoprotein Cholesterol (HDL-C) and low-density lipoprotein Cholesterol (LDL-C)] of patients were assessed using Cobas Integra 400Plus system (Roche Diagnostic).

Peripheral Blood Mononuclear Cells (PBMC), Cell Culture and Viability

Five milliliters (mL) whole-blood samples with EDTA (EthyleneDiamine Tetraacetic Acid) were taken from patients. To isolate peripheral blood mononuclear cells (PBMC), Ficoll (GE HealthCare) was used. PBMCs were used to evaluate IL-28B polymorphion, RNA extraction and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) for baseline expression of genes as well as cell culture for treatment.

The 10^5cell /well of PBMCs were cultured in a 96-well plate. The cells were treated with different dilutions of fluvastatin (10, 20, 30 µmol /mL) and IFN-ƛ (50 and 100 ng/mL) in time intervals of 48 and 72 hours. Untreated cells with RPMI1640 and Dimethyl sulfoxide (DMSO) 0.5% were used as control. Accordingly, MTT (soluble tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide) solution (Dacell) was added to the wells and incubated at 37°C for 3 hours. To dissolve the dark blue crystals, Dimethyl sulfoxide (DMSO) was added to the plate. Finally, the optical absorbance was measured using a microplate reader at 570 nm and cell viability of treated PBMCs was determined.

Primer Design

Primer design was performed for IL-28B polymorphism, ABCA-1, DGAT-1 and SREBP-1c as key factors in lipid metabolism, HCV core and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as an internal control using AllelID software version 6 (Biosoft International, Palo Alto, CA, USA). Table 1 lists designed and used primers to do experiments.

DNA Extraction and IL-28B Polymorphism

DNA was extracted from PBMCs; using Bio Basic Inc. kit (Canada). The DNA concentration was assessed; using NanoDrop 2000 (Thermo Scientific, Wilmington, USA) at 260 and 280 nm.

The genotyping of IL28B rs12979860 was carried out using polymerase chain reaction (PCR). The designed forward and reverse primers have been presented in Table 1. PCR was done in 40 cycles as follows: annealing step for 20 S at 62 °C , followed by an extension at 72° for 35 S. The PCR product was sequenced using the Sanger method[16].

Treatment of PBMCs with Fluvastatin and IFN-ƛ

The number of 10^5 PBMCs/ well were treated with 20 µM fluvastatin (Cayman Chemical Company, USA) or/and 50 ng IFN-ƛ (Sigma Company) in RPMI 1640 medium, fetal calf serum, FBS 10%, penicillin 100U/ML, and streptomycin 100 µg /mL in a 96-well plate with 5% CO_2 condition for 48 hours.

RNA Extraction, cDNA synthesis, and Real Time PCR

Following treatment of PBMC with fluvastatin and IFN-ƛ, in order to determine the expression of SREBP1C, DGAT1, and ABCA1 and CORE HCV virus mRNAs, RNA extraction was performed by QIAzole (Qiagen, Germany) RNA method according to the manufacture's structure.

The extracted RNA was immediately collected in RNAase Free tubes. After measuring the RNA concentration, cDNA was synthesized using the RT BIOFACT kit (Korea) protocol.

The mRNA expression for SREBP1c, DGAT1, ABCA1, and HCV Core was measured using Relative Real Time PCR method. In this technique, HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Korea) is applied accompanying with primers (Table 1) using ABI StepOnePlus Real-Time PCR instrument. The final volume of each reaction was 20 µL containing 0.3 µL forward and 0.3 µL reverse primers (10 ng), 1 µL DNA, 4 µL of master mixes and 14.4 µL nuclease-free water. PCR protocol was as follows: holding phase at 95°C for 15 minutes, 40 cycles in 95°C for 15 S on denaturation steps, 58°C for 20 S on annealing steps and 72°C for 30 S on extension steps. Results were analyzed using the Livak method[17]and the prism software.

Table 1. Designed Primers for evaluating the regulators of lipid metabolism, HCV core, IL-28B, in addition to GAPDH as a housekeeping gene

Primer	Forward	Reverse	Location of the Primers on the Genomes
IL28B	5'-CCACAATTCCCACCACGAGAC-3'	5'-GGACGAGAGGGCGTTAGAGC-3'	Forward: 39248304 TO 39248284 Reverse: 39247858 TO 39247877
ABCA1	5'-GGCATCGTGTATGAGAAGGAG-3'	5'-AGGAATGAGGCTACTAATGAACC-3'	Forward: 2062 TO 2082 Reverse: 2166 TO 2144
DGAT1	5'-ATCCTTGAGATGCTGTTC-3'	5'-GATGATGCGTGAGTAGTC-3'	Forward: 849 TO 866 Reverse: 965 TO 948
SREBP1C	5'-GTCGTAGATGCGGAGAAG-3'	5'-TTGATGGAGGAGCGGTAG -3'	Forward: 364 TO 381 Reverse: 494 TO 477
HCV-Core	5'-CGYAAYTTGGGTAARGTCATCG-3'	5'-AGRAAGATAGARAARGAGCAACC-3'	Forward: 349 TO 370 Reverse: 533 TO 511
GAPDH	5-'AAGCCTGCCGGTGACTAAC-3'	5'-CGCCCAATACGACCAAATCAGA-3'	Forward: 121 TO 139 Reverse: 269 TO 248

HCV-core (Hepatitis C virus –core), ABCA1 (ATP-binding cassette transporter A1); DGAT1 (Diacylglycerol acyltransferase 1); SREBP1C (Sterol regulatory element-binding protein 1C), GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)

ABCA1 Assay

The measurement of ABCA1 protein was performed; using ELISA kit (Cat No. ZB-11483C-H9648; ZellBio GmbH, Germany). This is based on biotin double antibody sandwich technology. The wells precoated with an anti-ABCA1 monoclonal antibody. The cell lysate was added to each well. Following adding anti-ABCA1 antibodies labeled with biotin and streptavidin-HRP for 60 minutes at 37C, Chromogen was added. After pouring the stop solution, they were read at 450 nm. The concentration of ABCA1 protein was assessed before and after treatment. The sensitivity of the assay was 0.04 ng/mL.

Statistical Analysis

To determine the normality of variables, the Shapiro-Wilk test was applied. The mean, standard deviation (SD), and standard error of mean (SEM) were used to describe variables. To compare two quantitative variables with normal and non-normal distribution in two groups, independent t-test or Mann-Whitney was used, respectively. Paired t-test was used to compare the concentration of ABCA1 before and after treatment. One Way ANOVA was used to compare a quantitative variables in more than two groups. Statistical analysis was performed by using the IBM SPSS software version 20 (IBM Corp., Armonk, N.Y., USA) applying a significance level of 0.05. To draw graphs, Prism 5 (Graphpad Software Inc., La Jolla, CA, USA) software was applied.

RESULTS

Thirteen HCV patients and five controls with CC genotype of rs1297986polymorphism were included in the study. The Mean±SD (range) age of patients and controls were 45.53±10.21 (30-62) and 39.9±10.88 (26-51) years, respectively. The HCV genotypes of subjects were as follows; 3a (n=6), 1a (n=5), 2a (n=1), 1b (n=1). The demographic and biochemical profile of patients and controls are presented in Table 2. The result of evaluating the biochemical profile of patients and

controls revealed that the concentration of liver enzymes (ALT, AST) and lipid profile including cholesterol, triglyceride, and LDL was higher in HCV patients with CC genotype of IL-28B than the control group.

According to the results of MTT assay, the percentage of viable cells treated with fluvastatin and IFN-λ was evaluated after 48 and 72 hours. The dilution of 20 µmol fluvastatin and 50 ng IFN-λ were selected for this study (Figure 1). The effect of concentrations of 10 and 20 µmol Fluvastatin was also evaluated on the expression of ABCA1. According to the results, the best results were obtained from 20 µmol fluvastatin. The qualitative PCR product (447bp) for IL-28B and ladder 100 bp were visualized on 3% agarose gel. The result of PCR product for three patients are shown in Figure 2.

Experimental results carried out showed that fluvastatin acted as an inhibitor on the expression level of SREBP1c in patients with HCV and controls (Figure 3). However, no statistically significant difference was found between case and controls.

On the other hand, in spite of the more inductive effect of IFN-λ on mRNA expression of SREBP1c in HCV-infected cells compared with normal cells, we found no significant difference between the two groups (Figure 3). Moreover, the inhibitory effect of fluvastatin and IFN-λ combination was observed and the difference between the two groups was significant (p=0.03).

According to the results of the current study, mRNA expression of ABCA1 was induced as the result of treatment with fluvastatin, IFN-λ, and their combination. A significant statistical difference was found between two groups after the combinational treatment of fluvastatin and IFN-λ (p=0.02).

Our results revealed a significant statistical difference in mRNA expression of DGAT1; following treatment with fluvastatin between two groups (p=0.03). This statistical difference was not found after treating with IFN-λ or combinational treatment.

As shown in Figure 3, the expression of HCV core mRNA was inhibited as the result of treatment with fluvastatin, IFN-λ and their combination. This effect was statistically significant following a combination of two treatments (p<0.001).

The concentration of ABCA1 protein was measured before and after treatment with fluvastatin and IFN-λ. The results showed a significant increase in ABCA1 protein after treatment rather than before treatment (Figure 4).

Table 2. The demographic data and biochemical profile of HCV patients and healthy controls

Variables	Patients Mean± SD	Controls Mean± SD
Weight (Kg)	71.84±11.92	77.60±6.10
Height (Cm)	176.84±7.65	177.80±5.06
BMI	22.92±3.16	24.62±2.70
ALT(IU/L)	56.00±28.08	31.80±8.55
AST(U/L)	57.23±43.57	28.20±6.14
ALP	214.45±61.98	101.60± 13.27
Albumin (g/dl)	4.36±0.47	-
Cholesterol (mg/dl)	151.00±20.33	109.60±8.73
Triglyceride (mg/dl)	108.91±49.65	72.40±23.74
Bilirubin (mg/dl)	0.44±0.43	-
Total Bilirubin (mg/dl)	1.31±0.81	-
Creatinine (mg/dL)	0.98±0.24	-
HDL-C (mg/dl)	47.81±6.61	60.20±3.96
LDL-C (mg/dl)	64.54±11.05	57.40±8.84
Hemoglobin	14.96±1.55	-
Platelet	201600±59240	-
Viral load(Iu/mL)	1184130±1862409	-

HCV= Hepatitis C virus; BMI=Body Mass Index; ALT=Alanine Aminotransferase; AST=Aspartate Aminotransferase; ALP=Alkaline phosphatase; HDL-C=high-density lipoprotein Cholesterol; LDL-C=low-density lipoprotein Cholesterol

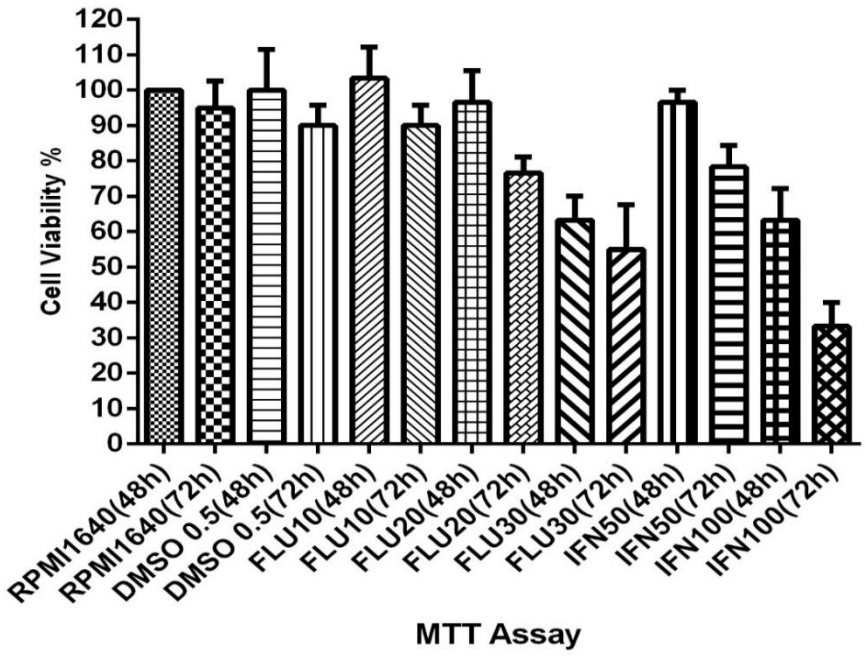

Figure 1. The percentage of cell viability following treatment with different dilutions of fluvastatin (FLU) (µM), IFN-λ (ng). The cells were treated with different dilutions of fluvastatin (10, 20, 30 µM) and IFN-ƛ (50 and 100 ng) in time intervals of 48 and 72 hours. Untreated cells with RPMI1640 and Dimethyl sulfoxide (DMSO) 0.5% were used as control. The treatment of cells for each dilution was performed as triplicate.

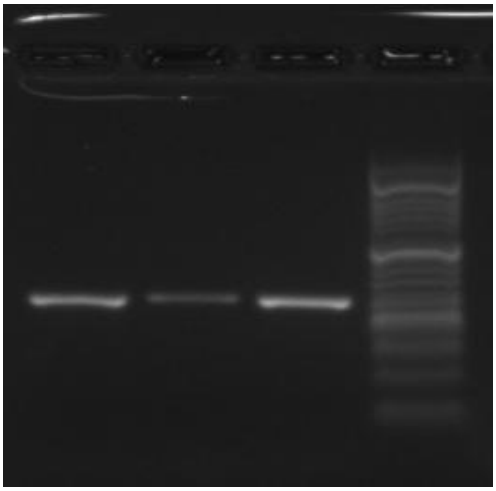

Figure 2. Polymerase chain reaction (PCR) product (447bp) for IL-28B and ladder 100 bp on 3% agarose gel

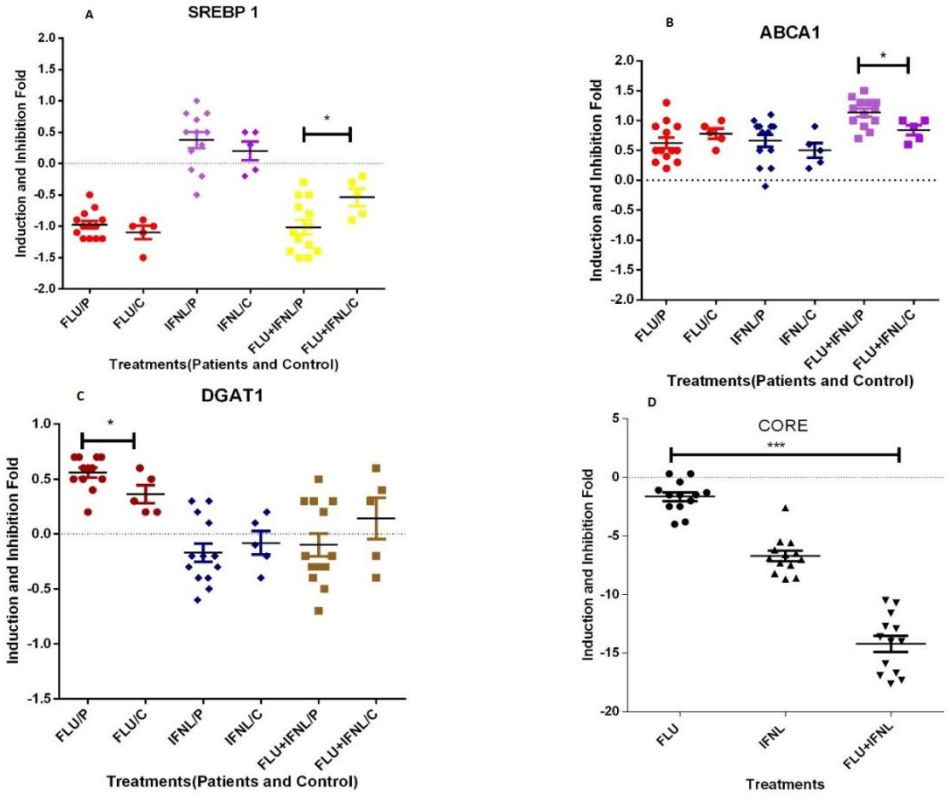

Figure 3. The mRNA expression of sterol regulatory element-binding protein 1 (SREBP1) (A), ATP-binding cassette transporter A1 (ABCA1) (B), Diacylglycerol acyltransferase 1 (DGAT1) (C) and HCV core (D) following treatment with fluvastatin (FLU), Interferon (IFN)-ƛ and their combination. P (Patients); C (control). FLU (Fluvastatin); IFNL (Interferon-Lambda). The Relative Real Time PCR was used to determine the mRNA expression for SREBP1c, DGAT1, ABCA1, and HCV core. The induction and inhibition fold (fold change) was reported as mean±SEM. Independent t-test or Mann-Whitney was used to compare fold changes of mRNA expression between two groups. $^{*}p<0.05$, $^{***}p<0.001$

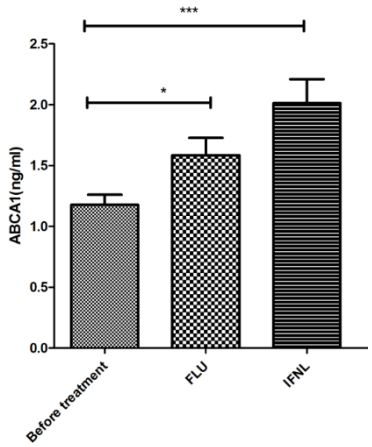

Figure 4. The concentration (Mean±SEM) of ATP-binding cassette transporter A1 (ABCA1) protein in peripheral blood mononuclear cells (PBMCs) of patients before and after treatment with fluvastatin (FLU) or Interferon-Lambda (IFNL). The concentration of ABCA1 protein was measured using sandwich enzyme linked immunosorbent assay (ELISA). Paired t-test was used to compare the concentration of ABCA1 before and after treatment. $^{*}p<0.05$, $^{***}p<0.001$

DISCUSSION

The results of the present study provided evidence regarding the significant effects of combination therapy with fluvastatin and IFN-λ in PBMCs of HCV patients with rs12979860 CC polymorphism. As the finding revealed, the statistically significant difference in inhibition of SREBP1c and induction of ABCA-1 was obtained as the result of combination therapy with IFN-λ and fluvastatin.

According to the study of Rojas et al, a higher concentration of LDL was found in patients with CC genotype of IL28B polymorphism and genotype 1 of HCV. This result suggested the presence of an association between IL28B polymorphism (especially CC) and lipid profile.[14] It is worth mentioning that rs12979860 polymorphism is located between IFN-λ and IL-28 A/B genes.[12]

Considering previous studies, hepatic steatosis and exacerbation have been developed in 40-86% of patients with chronic HCV.[11,18] SREBP1c is one of the mediators involved in lipogenesis as well as hepatic steatosis in patients with HCV infection. Hepatic lipids are accumulated as the result of SREBP1c up-regulation; resulting from the inductive effect of HCV core protein.[19] Moreover, an important enzyme called liver microsomal triglyceride transfer protein (MTP) which participates in the assemblage of VLDL could contribute to HCV-related steatosis.[18] In this study, the combination of respective inhibitory and inductive effects of fluvastatin and IFN-λ led to inhibition of SREBP1c expression as well as HCV core. The difference of the inhibitory effect of FLV/ IFN-λ combination was statistically significant between patients and controls. Ikeda et al found similar results to our findings by a combination of fluvastatin and IFN-α.[9] Various researchers have suggested the use of fluvastatin as supplementary medication to enhance the response to treatment with PEG-IFN and ribavirin[20] and decrease viral relapse.[21]

DGAT-1 protein as a mediator in triglyceride synthesis recruits viral core protein from the endoplasmic reticulum to cytosolic lipid droplets and subsequently, virion assembly is induced.[18,22] As literature review showed DGAT-1 inhibitors cause a defect in viral particle production.[23,24] The results of this study showed that fluvastatin has an inducer role in the expression of DGAT-1 in PBMCs of patients with HCV and controls while IFN-λ and combination of fluvastatin and IFN-λ suppressed the expression of DGAT-1. According to the study of Rojas et al the expression of lipid-related genes in cultured cells depends on HCV genotype and IL-28B polymorphism. For instance, the expression of DGAT-1 was increased in patients, a responder to therapy rather than non-responders.[14]

One of the important proteins expressed in liver and tissue macrophages is ABCA1. This large protein performs essential functions to produce the plasma HDL and cholesterol homeostasis.[25] Furthermore, the cholesterol efflux from liver cells to extracellular apo A-I is implemented through ABCA1 mediation and HDL is formed accordingly. Overexpression of ABCA1 and cholesterol efflux leads to impairment in HCV proliferation.[6] Our results demonstrated that ABCA-1 mRNA was overexpressed in PBMCs of patients with HCV and control group after treatment with fluvastatin, IFN-λ and their combination. Additionally, the results of ABCA-1 protein assay revealed its increase in treated cells compared to untreated cells. In line with our study, Bocchetta et al demonstrated that up-regulation of ABCA-1 resulting from medications, could suppress virus–cell fusion and entrance of HCV to cell through dysfunction of cholesterol homeostasis in the cell membrane. Accordingly, ABCA-1 could be considered for targeted therapy in patients with HCV.[26]

As the results of the present study revealed, combinational therapy with fluvastatin and IFN-λ significantly suppressed the mRNA expression of HCV core protein. Similar finding was found by Kurincic T et al, suggesting a significant increase in sustained virus response in patients with CHC (HCV genotype 1) treated with fluvastatin and PegIFN-ribavirin.[27]

There are also several limitations to this study. The first limitation was the sample size of patients and controls. The second limitation was the assessment of one protein (ABCA1) by ELISA technique. Moreover, the HCV genotype of the patients was different but regarding the variety of genotypes and a limited number of patients, statistical comparison of the expression of measured factors with viral genotype and polymorphism was not valuable. Because of lacking results related to the side effects of treatment on the liver of HCV patients, further investigations be needed.

In conclusion, our results demonstrated a significant effect of combination therapy with fluvastatin and IFN-λ in the SREBP1c inhibition and also the ABCA-1

induction as vital mediators in synthesis and hemostasis of lipids as well as steatosis in PBMCs of HCV patients with rs12979860 CC polymorphism. Regarding the drug resistance of viruses and prevention of virus-induced steatosis in PBMCs of patients with HCV, using regulatory agents of lipid mediators as therapeutic targets and combination with IFN- λ could be considered in parallel with current medications.

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