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Expression Levels of miR-27a, miR-329, *ABCA1* and *ABCG1* Genes in Peripheral Blood Mononuclear Cells and Their Correlation with Serum Levels of Oxidative Stress and hs-CRP in the Patients with Coronary Artery Disease

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Abbreviations:

ABC, ATP-binding cassette
ABCA1, ATP-binding cassette transporter A1
ABCG1, ATP-binding cassette sub-family G member 1
AHA, American heart association
BG, blood glucose
BMI, body mass index
BUN, blood urea nitrogen
CAD, coronary artery disease
CPK-MB, creatine phosphokinase myocardial band
CRP, C-reactive protein
CVD, Cardiovascular disease
DBP, diastolic blood pressure
FRAP, ferric-reducing antioxidant power
HDL, high-density lipoprotein
HDL-C, high-density lipoprotein-cholesterol
hs-CRP, high-sensitivity C-reactive protein
LDL, low-density lipoprotein
LDL-C, low-density lipoprotein-cholesterol
MDA, malondialdehyde
miRs, microRNAs
ox-LDL, oxidized LDL
PBMCs, peripheral blood mononuclear cells
RCT, Reverse cholesterol transport
SBP, systolic blood pressure
TC, total cholesterol
TG, triglyceride

Abstract

Atherosclerosis is a chronic inflammatory disease with high mortality worldwide. The reverse cholesterol transport (RCT) pathway in macrophage plays an important role in the pathogenesis of coronary artery disease (CAD) and is strongly controlled by regulatory factors. The microRNAs (miRs) can promote or prevent the formation of atherosclerotic lesions by post-transcriptional regulation of vital genes in this pathway. Therefore, this study was conducted to investigate the relationship between the expression levels of miR-27a, miR-329, *ABCA1*, and *ABCG1* genes and serum levels of hs-CRP, ox-LDL, and indices of oxidative stress in the patients with established CAD and controls. A total of 84 subjects (42 patients with CAD and 42 controls) were included in this study. Expression levels of miR-27a-3p, miR-329-3p, *ABCA1*, and *ABCG1* genes in the peripheral blood mononuclear cells (PBMCs) and serum concentration of hs-CRP and ox-LDL were measured by real time-PCR and ELISA, respectively. Also, oxidative stress parameters in the serum were evaluated by ferric reducing antioxidant power (FRAP) and malondialdehyde (MDA) assays. *ABCA1* and *ABCG1* gene expression in PBMC and serum concentration of FRAP were significantly lower in the CAD group compared to the control group. Expression levels of miR-27a and miR-329 and serum levels of hs-CRP, ox-LDL, and MDA were significantly higher in the CAD group compared to the control group. Serum levels of hs-CRP, ox-LDL, and expression level of miR-27a have inversely related to *ABCA1* and *ABCG1* gene expression in all the subjects. An increase in the expression levels of miR-27a and miR-329 may lead to the progression of atherosclerosis plaque by downregulating the expression of *ABCA1* and *ABCG1* genes.

Keyword: Atherosclerosis; microRNA; *ABCA1*; *ABCG1*; Oxidative stress; hs-CRP

INTRODUCTION

Cardiovascular disease (CVD) is among the leading causes of death globally [1]. Atherosclerosis, is the major cause of CVD including coronary artery disease (CAD), which is a chronic inflammatory disease characterized by the presence of immune cells in vascular lesions . The accumulation of lipids in the wall of arteries is the main feature of this disease due to formation of foam cells and these contribute to arterial stenosis [2]. Risk factors for atherosclerosis include age, obesity, diabetes mellitus, hypertension, smoking and inactive lifestyle [3]. Oxidative stress is a condition characterized by the elevated production of free oxygen radicals is closely associated with endothelial dysfunction and pathogenesis processes of atherosclerosis [4]. Low-density lipoprotein (LDL) enters arterial wall and converts into the oxidized LDL (ox-LDL) through exposure to oxidant agents [5]. Furthermore, ox-LDL attracts innate immune cells, such as monocytes and lymphocytes [6].

Reverse cholesterol transport (RCT) is a pathway for preventing atherosclerosis by which the excess cellular cholesterol is transported to the liver for excretion. Ox-LDL accumulated in the vessels wall is ingested by macrophages through endocytosis [7]. Cholesterol efflux from the macrophage is an important process in the RCT pathway that performed by the ATP-binding cassette (ABC) family transporters. The most important members of this family are ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette sub-family G member 1 (ABCG1) [8]. ABCA1 is a critical regulator of high-density lipoprotein (HDL) synthesis in the liver [9]. Dysfunction of ABCA1 and ABCG1 leads to accumulation of cholesterol within macrophages and their transformation into foam cells [10, 11].

C-reactive protein (CRP) is another factor that may be involved in the pathogenesis of atherosclerosis. The American heart association (AHA) has approved serum levels of high-sensitivity C-reactive protein (hs-CRP) as the biomarker predicting CVD [12]. CRP deposits are likely to form in atherosclerotic lesions before entry of monocytes into the tissue and this is one of the factors for monocyte recruitment to atherosclerotic plaque [13]. Therefore, CRP may be more directly involved in atherogenesis.

The microRNAs (miRNAs) are small noncoding (including 20-24 nucleotides) and single-stranded RNA molecules bind to mRNA of target gene and regulate their expression [14]. Circulating miRs can act as diagnostic biomarkers in many diseases, including cardiovascular

disease [15]. Recently, it has been indicated that levels of miR-27a and miR-329 are increased in atherosclerotic plaques in mice [16-18].

Since, the exact association of miR-27a-3p and miR-329-3p with *ABCA1* and *ABCG1* genes in peripheral blood mononuclear cells (PBMCs) of humans have not been determined yet. Therefore, the present study was conducted to assess expression levels of miR-27a, miR-329, *ABCA1*, and *ABCG1* genes in the PBMC of the patients with CAD compared with control subjects and investigate their correlation with serum levels of hs-CRP, ox-LDL, MDA and FRAP.

MATERIALS AND METHODS

Study Subjects and Collection of Anthropometric Data

The study population included 84 Iranian patients who underwent coronary angiography in Hajar Hospital in Shahrekord city, Chaharmahal and Bakhtiari province, Iran. Ethical approval was issued by the Research Ethics Committee of Shahrekord University of Medical Sciences (IR.SKUMS.REC.1398.129). The blood samples were collected between October 2019 and December 2019. In addition, the study was done in accordance with the declaration of Helsinki and an informed written consent was obtained from all the subjects. A definitive diagnosis of CAD was made by a cardiologist using angiography. Patients with arterial stenosis above 50% in at least one of the major coronary arteries were assigned to the CAD group. Patients without arterial stenosis or a stenosis of <20% were assigned to the control group [19]. Patients with liver disease, cancer, diabetes, chronic infections, and kidney disease were excluded from the study. In the control group, patients with any history of atherosclerotic plug and angina were excluded from the study. Demographic information of the patients, such as drug treatment, smoking habit, family history, height, weight, body mass index (BMI), systolic blood pressure (SBP) and diastolic blood pressure (DBP) was also recorded by the researcher. SBP and DBP were recorded at the beginning of patients' examination and after 15 minutes of sitting according to the standard sphygmomanometer.

Bioinformatics Analysis

Using http://www.targetscan.org/vert_72/, TarBase v.8, <http://mirdb.org/index.html>, <http://mirwalk.umm.uni-heidelberg.de/> databases, it was found that miR-27a-3p and miR-329

could potentially reduce the expression of *ABCA1* and *ABCG1* genes by targeting 3-UTR region of their mRNA.

Collection of Venous Blood Samples and Separation of Serum

Ten mL of venous blood was taken from each patient, 5 mL was used in the tube containing ethylenediaminetetraacetic acid (EDTA) for separation of PBMC, and 5 mL was used in the tube containing clotting agent for serum separation. Serum samples were stored at -80 °C until subsequent analyses.

Measurement of Biochemical Parameters

After separation of serum, levels of biochemical parameters, such as blood glucose (BG), blood urea nitrogen (BUN), creatinine, sodium, potassium, creatine phosphokinase myocardial band (CPK-MB), troponin I, total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), and triglyceride (TG) were measured based on enzymatic and spectrophotometric standards method using the Pars Azmoun Company's kit.

Isolation of PBMCs and RNA Extraction

Separation of PBMCs was performed under sterile conditions. After dilution of blood with phosphate buffer solution (PBS) (1:1), it was centrifuged using Lympholyte (Cedarlane, Ontario, Canada) and then, PBMCs were isolated by density-gradient centrifugation according to the manufacturer's instructions. The PBMCs were washed with PBS (2×) and were stored at - 80°C prior to RNA extraction step [20]. Extraction of total RNA was carried out by RNX-Plus Solution (SinaClon, Iran) according to the manufacturer's protocol. Concentration of RNA samples was measured by Thermo Scientific™ NanoDrop 2000 (Thermo Scientific, UAS). 260/280 and 260/230 ratios were determined for each sample to evaluate purity and concentration of RNA.

First-Strand cDNA Synthesis and Quantification of *ABCA1* and *ABCG1* mRNA

cDNA was synthesized by Revert Aid first-strand cDNA synthesis kit (Thermo Scientific, K1622) using 2000 ng of RNA sample in 20 µL of reaction volume. Quantitative real-time PCR was performed using a Rotor-Gene RG-3000 (Corbett Research, Sydney, Australia) by the SYBR Green qPCR master mix 2x (Yektatajhz, Iran). Expression level of *ABCA1* and *ABCG1*

mRNA was assessed with respect to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA using the $2^{-\Delta\Delta C_t}$ method. Specific primers were designed using the following website: <https://primer3plus.com>. Table 1 shows the list of primer sequences used for RT-PCR analysis in this study.

First-Strand cDNA Synthesis of miRNAs and Quantification of miR-27a-3p and miR-329-3p

First-strand cDNA was synthesized using BONmiR High-Sensitivity MicroRNA 1st-Strand cDNA Synthesis Kit (Bonbiotech, Iran). Quantitative real-time PCR was performed using the BON MicroRNA QPCR Master Mix Kit and specific primers for miR-27a, miR-329 and SNORD-47 (Bonbiotech, Iran). PCR amplification was carried out using the Rotor-Gene 3000 device (Corbett Research, Australia). Expression level of miR-27a and miR-329 was determined with respect to SNORD-47 using the $2^{-\Delta\Delta C_t}$ method.

Measurement of Serum Concentration of hs-CRP and ox-LDL by ELISA

Serum level of hs-CRP was measured using an ELISA kit (LDN; Germany) with intra-assay coefficient of variation (CV) < 15% and inter-assay CV < 10% and a sensitivity of 10 ng/ml. Serum level of ox-LDL was determined by an ELISA kit (Mercodia, Sweden) with intra- and inter-assay CV of 8.3% and 7.3% and detection limit of 0.6 mU/L, respectively. Serum levels of hs-CRP and ox-LDL were also measured using standard samples containing known concentrations of hs-CRP and ox-LDL provided by the manufacturers.

Measurement of Oxidative Stress Parameters in the Serum

Serum lipid oxidation was evaluated by MDA assay. In this method, serum MDA reacts with thiobarbituric acid (TBA) in the presence of trichloroacetic acid (TCA) and hydrochloric acid (HCl). After boiling for 15 min, flocculent precipitates were separated by cooling and centrifugation and then, its absorbance was measured by a spectrophotometer at 535 nm of wavelength compared to a blank. The broken product of 1,1,3,3-tetraethoxypropane was used as the standard [21].

Serum antioxidant capacity was measured by a colorimetric method called as FRAP. In this method, ferric-tripyridyltriazine compound (FeIII – TPTZ) is converted into the form of ferrous

in the presence of serum antioxidants. This product has a blue color and its absorbance was read at 593 nm of wavelength. The difference in the absorbance is positively related to electron-donating potential of serum antioxidants [22].

Statistical Analysis

Laboratory evaluation was investigated by the Independent-Samples t-test. Normal distribution of data was checked by the Kolmogorov-Smirnov and Shapiro-Wilk normality test. Quantitative data were assessed by the Mann-Whitney U test and Kruskal-Wallis H test depending on non-normality of data distribution. Quantitative data were shown as median with 95% confidence interval (CI) by GraphPad Prism software version 8.4.3 (GraphPad Software, La Jolla, CA, USA). Spearman correlation analysis was used for non-parametric data to investigate the correlation between parameters. A *P*-value less than 0.05 (typically ≤ 0.05) was considered as statistically significant.

RESULTS

Basic Characteristics of the Patients and Laboratory Evaluation

Table 2 shows clinical and anthropometric characteristics of the studied subjects. There was no significant difference in the gender, age, BMI, TG, HDL-C, BUN, creatinine, sodium, potassium, drug use, SBP, and DBP between the subjects. In contrast, Troponin I, TC, LDL-C, CPK-MB, and BG levels were considerably higher in the CAD group compared to the control group. This difference in Troponin I level between the two groups was very much (Mean of control group = 93.38 ng/ml, Mean of CAD group = 11753 ng/ml). Besides, participants in the CAD group smoked more than the control group.

Expression Levels of *ABCA1* and *ABCG1* Genes as Well as miR-27a-3p and miR-329-3p in PBMCs of the Subjects

As shown in Fig. 1 (a) and 1 (b), the expression levels of *ABCA1* and *ABCG1* genes were significantly lower (1.65 and 2.18 fold) in the CAD group compared to the control group (*ABCA1*; *P*= 0.009 and *ABCG1*; *P*< 0.0001), respectively. As shown in Fig. 1 (c) and 1 (d), expression levels of miR-27a and miR-329 were significantly higher (2.01 and 2.07 fold) in the CAD group compared to the control group (miR-27a; *P*= 0.006 and miR-329; *P*= 0.004), respectively.

Expression Levels of *ABCA1*, *ABCG1*, miR-27a, and miR-329 in PBMCs of the Subjects and Their Relation with the Number of Clogged Arteries

CAD-positive subjects were classified into three groups based on the cardiologists' medical report: patients with cardiac arterial stenosis in one main vessel (CS1), patients with cardiac arterial stenosis in two main vessels (CS2), and patients with cardiac arterial stenosis in three main vessels (CS3). Dunn's multiple comparison analysis showed that expression level of *ABCA1* gene was significantly decreased by 2.6 and 3.3 folds in the CS3 group compared to the control and CS1 groups, respectively. Also *ABCA1* gene expression was significantly decreased by 2.3 fold in CS2 group compared to CS1 group (Fig. 2 (a): CS3 vs. control; $P= 0.0003$, CS3 vs. CS1; $P= 0.0003$ and CS2 vs. CS1; $P= 0.046$). There was no significant difference in *ABCA1* expression between CS1 and control groups as well as CS3 and CS2 groups. Expression levels of the *ABCG1* gene was significantly lower (3.8 and 3.2 fold respectively) in the CS2 and CS3 groups compared to the control group, respectively. In addition, *ABCG1* gene expression was significantly reduced by 3.9 and 3.2 folds in CS2 and CS3 groups compared to CS1 group, respectively. (Fig. 2 (b): CS2 vs. control; $P= 0.0003$, CS3 vs. control; $P= 0.0004$, CS2 vs. CS1; $P= 0.021$, CS3 vs. CS1; $P= 0.023$). As shown in Fig. 2 (c), expression level of miR-27a was significantly higher (3.17 and 2.64 fold respectively) in the CS3 group compared to the control and CS1 groups, respectively. (CS3 vs. control; $P= 0.001$ and CS3 vs. CS1; $P= 0.027$). miR-27a expression was not significantly different between CS2 and other groups. Also, expression level of miR-329 was increased significantly by 2.4 fold in the CS3 group compared to the control group (Fig. 2 (d); $P= 0.03$). miR-329 expression was not significantly different between CS1, and other groups as well as CS2 and other groups.

Correlation between Expression Levels of *ABCA1*, *ABCG1*, miR-27a, and miR-329 in PBMCs of All the Subjects

The expression level of the *ABCA1* gene was positively correlated with expression level of *ABCG1* gene in PBMCs of all the subjects (Fig. 3 (a): $r = 0.623$, $P < 0.0001$). Also, the expression of miR-27a was positively correlated with expression level of miR-329 in PBMCs of all the subjects (Fig. 3 (b): $r = 0.368$, $P= 0.0006$). The expression of miR-27a was inversely correlated with the expression of *ABCA1* and *ABCG1* genes in PBMCs for all the subjects (Fig. 3 (c): *ABCA1*; $r = -0.332$, $P= 0.002$ and *ABCG1*; $r = -0.296$, $P= 0.006$). Furthermore, the

expression of miR-329 was negatively correlated with expression level of *ABCG1* genes in PBMCs of all the subjects but was not correlated with *ABCA1* gene (Fig. 3 (d): *ABCA1*; $r = -0.2$, $P = 0.067$ and *ABCG1*; $r = -0.227$, $P = 0.037$).

Serum Concentration of hs-CRP, ox-LDL, FRAP, and MDA in the Subjects

Serum concentrations of hs-CRP, ox-LDL, and MDA were significantly higher (3.41, 1.32, and 1.21 fold) in the CAD group compared to the control group, respectively (Figs. 4(a), (b), and (d): hs-CRP; $P < 0.0001$, ox-LDL; $P = 0.0009$ and MDA; $P = 0.0004$). Also, serum level of FRAP was significantly lower by 1.17 folds in the CAD group than the control group (Fig. 4 (c): $P = 0.005$).

Serum Concentration of hs-CRP, ox-LDL, FRAP, and MDA and Their Relation with the Number of Clogged Arteries

Serum level hs-CRP was significantly higher in the CS1, CS2, and CS3 groups by 3.32, 4.7, and 4.34 fold compared to the control group, respectively (Fig. 5 (a): CS1 vs. control; $P = 0.047$, CS2 vs. control; $P = 0.005$ and CS3 vs. control; $P = 0.001$). hs-CRP level was not significantly different between CS1, CS2 and CS3 in CAD groups. Serum level of ox-LDL was significantly higher in the CS3 group by 1.45 fold compared to the control group, (Fig. 5 (b): $P = 0.001$). Serum FRAP was significantly lower by 1.21 fold in the CS3 group compared to the control group, (Fig. 5 (c), $P = 0.018$). Serum level of MDA was increased significantly by 1.31 fold in the CS3 group compared to the control group, (Fig. 5 (d), $P = 0.003$). Serum levels of ox-LDL, FRAP and MDA were not significantly different between CS1 and other groups as well as CS2 and other groups.

Correlation between Serum Levels of hs-CRP, ox-LDL, FRAP, and MDA and Expression Levels of *ABCA1* and *ABCG1* Genes in PBMC of All the Subjects

Serum hs-CRP was positively correlated with serum level of ox-LDL in all the subjects (Fig. 6 (a): $r = 0.442$, $P < 0.0001$). Additionally, there was an inverse correlation between serum levels of FRAP and MDA in all the subjects (Fig. 6 (b): $r = -0.698$, $P < 0.0001$). Serum level of hs-CRP was negatively correlated with expression levels of *ABCG1* and *ABCA1* genes in PBMCs of all the subjects (Fig. 6 (c): *ABCG1*; $r = -0.546$, $P < 0.0001$ and *ABCA1*; $r = -0.478$, $P < 0.0001$). Furthermore, serum level of ox-LDL was negatively correlated with expression level of *ABCG1* and *ABCA1* genes in all the subjects (Fig. 6 (d): *ABCG1*; $r = -0.305$, $P = 0.005$ and *ABCA1*; $r = -$

0.609, $P < 0.0001$). Moreover, serum level of FRAP was positively correlated with expression levels of *ABCG1* and *ABCA1* genes in all the subjects (Fig. 6 (e): *ABCG1*; $r = 0.268$, $P = 0.0137$ and *ABCA1*; $r = 0.392$, $P = 0.0002$). Serum level of MDA was negatively correlated with expression level of *ABCG1* and *ABCA1* genes in all the subjects (Fig. 6 (f): *ABCG1*; $r = 0.331$, $P = 0.002$ and *ABCA1*; $r = 0.287$, $P = 0.008$).

Correlation between the Studied Parameters in the CAD Group

As shown in Table 3, expression level of *ABCA1* gene was positively correlated with expression level of *ABCG1* gene in the CAD group ($r = 0.639$, $P < 0.0001$). Also, expression level of *ABCA1* gene was negatively correlated with expression level of miR-27a and serum levels of hs-CRP and ox-LDL in the CAD group, respectively (miR-27a; $r = -0.340$, $P = 0.027$ and hs-CRP; $r = -0.333$, $P = 0.035$ and ox-LDL; $r = -0.469$, $P = 0.002$). *ABCA1* gene expression was not correlated with level of miR-329, FRAP and MDA in the CAD group. Expression level of *ABCG1* gene was only negatively correlated with serum level of hs-CRP in the CAD group but was not correlated with other parameters ($r = -0.385$, $P = 0.014$). There was a positive correlation between expression levels of miR-27a and serum level of ox-LDL in the CAD group ($r = 0.330$, $P = 0.034$). miR-329 expression was not significantly correlated with other factors in CAD group. Furthermore, serum levels of hs-CRP and ox-LDL were positively correlated with each other in the CAD group ($r = 0.350$, $P = 0.028$). In contrast hs-CRP was not correlated with oxidative stress parameters in CAD group. Serum level of ox-LDL was positively correlated with MDA level ($r = 0.324$, $P = 0.038$) and also was negatively correlated with FRAP level ($r = -0.452$, $P = 0.003$) in the CAD group.

DISCUSSION

Atherosclerosis is a complex disease involving the immune system, dyslipidemia, and vascular involvement [23]. Immune system cells play a vital role in initiating the process of atherogenesis. A balance between immune system and cholesterol homeostasis system is essential to prevent onset of atherosclerosis because monocytes/macrophages act as the main link between inflammation and atherogenesis by regulating RCT pathway [24].

Since, *ABCA1* and *ABCG1* genes are important genes involved in the cholesterol efflux, herein, expression level of *ABCA1* and *ABCG1* genes was evaluated in PBMCs of the subjects. The

results of our study showed that expression level of *ABCA1* and *ABCG1* genes was significantly decreased in the CAD group compared to the control group. Also, higher grade of arterial stenosis showed a lower expression level of *ABCA1* and *ABCG1* genes in the CAD group. Previous studies found that Kuwanon G (a flavonoid extracted from the root bark of *Morus alba* L) and baicalin exerts anti-atherosclerosis effects possibly through LXR α -peroxisome proliferator-activated receptor gamma (PPAR γ) and nuclear factor kappa B (NF- κ B) pathways and upregulating the expression of *ABCA1* and *ABCG1* genes [25, 26]. Zhao *et al.*, found that 70 kilodalton heat shock protein (Hsp70) suppresses the expression of *ABCA1* and *ABCG1* genes through c-Jun N-terminal kinase (JNK)/ETS like-1 protein (ELK)-1 pathway and promotes progression of atherosclerosis [27]. van Eck *et al.*, demonstrated that *ABCA1* regulate recruitment of inflammatory cells and mice that were selectively deficient in leukocytes *ABCA1* genes (*ABCA1*^{-/-}) have a more advanced atherosclerotic lesions [28].

In agreement with other studies, our results suggested that expression levels of *ABCA1* and *ABCG1* genes was decreased in PBMC before migration of monocytes and lymphocyte into endothelial tissue. Generally, it can be said that decreased expression of these genes in PBMCs plays an important role in progression of atherosclerosis. Studying various factors are involved in their expression regulation, can effectively help to mitigate development of atherosclerosis.

Our results showed that serum level of hs-CRP was increased in the CAD group compared to the control group. Also, this increase was positively correlated with grade of arterial stenosis. Moreover, serum hs-CRP was negatively correlated with expression levels of *ABCG1* and *ABCA1* genes in PBMCs of all the subjects particularly in the CAD group.

In this regard, Hashimoto *et al.*, found that CRP concentration in the early stages of atherosclerotic plaque formation is the best marker for evaluation of atherogenic activity [29]. Mangge *et al.*, observed that children with type 1 diabetes had low levels of inflammation in their body representing onset of atherosclerotic [30]. Regarding the potential role of CRP in atherogenesis, Wang *et al.*, demonstrated that CRP reduced expression of *ABCA1* and *ABCG1* genes by activating extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) pathway [31]. Paul *et al.*, confirmed that ApoE^{-/-} mice with human transgene CRP expression showed accelerated aortic atherosclerosis by an increase in the C3 deposition, vascular cell adhesion molecule-1 (VCAM-1) [32].

In line with the previous studies, our results showed that hs-CRP may involved in atherosclerosis progression. The inverse relationship between serum level of hs-CRP and expression levels of *ABCA1* and *ABCG1* in PBMCs probably indicates the role of hs-CRP in atherogenesis. Of course, further studies are needed to confirm this relationship.

Dysregulation of miRNAs has been shown to be related to cardiovascular diseases including CAD, and they are important and powerful as a diagnostic biomarker [33]. This study showed that expression levels of miR-27a and miR-329 were significantly higher in the CAD group compared to the control group and positively correlated with grade of arterial stenosis. Expression levels of miR-27a was negatively correlated with expression levels of *ABCA1* and *ABCG1* genes in PBMCs of all the subjects. In line with this finding, Yao *et al.*, found that glucagon-like peptide-1 (GLP-1) enhanced expression level of *ABCA1* gene by decreasing miR-27a expression, thereby reducing lipid accumulation in pancreatic β cells [34]. In another study, inhibition of miR-27a expression enhanced expression levels of *ABCA1* and *NF-kB* genes and played a protective role in heart damage by reducing apoptosis [35]. A study by Ingen *et al.*, in LDL receptor-deficient(*LDLr^{-/-}*) mice demonstrated that inhibition of miR-329 expression controls development of atherosclerotic plaque and further stabilizes the advanced lesions [36]. Also, silencing of myocyte-specific enhancer factor 2A(MEF2A) attenuates expression of miR-329 and increasing neovascularization in the patients with the peripheral arterial disease [37]. In accordance with the previous studies, our results demonstrated the relationship between miR-27a and miR-329 and cholesterol efflux genes. Therefore confirmed the possible role of miR-27a and miR-329 in suppressing expression of *ABCA1* and *ABCG1* genes and their potential role in progression of atherosclerotic plaque.

Our results confirmed that the increase in the oxidative stress correlated with increase of arterial stenosis. It was found that serum levels of ox-LDL and MDA were significantly increased and serum level of FRAP was decreased in the CAD group compared to the control group. This difference was particularly significant between CS3 and control groups. Furthermore, serum level of ox-LDL was positively correlated with hs-CRP level and was negatively correlated with expression level of *ABCA1* gene in the CAD group. In line with this finding, Mutlu-Turkoglu *et al.*, demonstrated that MDA level was elevated and FRAP level was reduced in the patients with CAD compared to the control group [38]. As mentioned in previous

studies increase in the levels of hs-CRP or ox-LDL predicted the risk of CVD occurrence and future myocardial infarction (MI) [39, 40]. In addition, our results showed a significant negative correlation between serum levels of ox-LDL and expression levels of *ABCA1* and *ABCG1* genes in PBMC of all the subjects. Our results suggested that expression of *ABCA1* and *ABCG1* genes could be possibly altered by oxidative stress and the increase in the LDL oxidation.

CONCLUSION

In conclusion, our results demonstrate that increased expression of miR-27a and miR-329 in PBMCs of the patients with CAD may lead to progression of atherosclerosis plaque by downregulating expression of *ABCA1* and *ABCG1* genes. In addition, other factors such as, oxidative stress, ox-LDL and hs-CRP may involve in disease progression, regulation of miRs and genes function.

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CONFLICT OF INTEREST

This manuscript was approved by all authors. No competing interests declared.

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Figure 1. Column bar graph for the relative expression levels of ABCA1 and ABCG1 genes as well as miR-27a-3p and miR-329-3p in PBMCs of the Subjects. A and B) Expression levels of ABCA1 and ABCG1 genes were significantly decreased by 1.65 and 2.18 folds in the CAD group compared to the control group, respectively. C and D) Expression levels of miR-27a and miR-329 were significantly increased by 2.01 and 2.07 folds in the CAD group compared to the control group, respectively. P -value ≤ 0.05 were considered as significant using nonparametric Mann-Whitney test. Results are expressed as the median with 95% CI.

Figure 2. Column bar graph for the relative expression levels of *ABCA1* and *ABCG1* mRNA as well as miR-27a-3p and miR-329 in PBMC of the subjects and their relation with the number of clogged arteries. A) Expression level of *ABCA1* gene was significantly decreased by 2.6 and 3.3 folds in CS3 group compared to control and CS1 groups, respectively. Also expression level of *ABCA1* gene was significantly decreased by 2.3 fold in CS2 group compared to CS1 group. B) Expression level of *ABCG1* gene was significantly decreased by 3.8 and 3.2 folds in CS2 and CS3 groups compared to the control group, respectively. Also, expression level of *ABCG1* gene was significantly decreased by 3.9 and 3.2 folds in CS2 and CS3 groups compared to CS1 group, respectively. C) Expression level of miR-27a was significantly increased by 3.17 and 2.64 folds in the CS3 group compared to the Control and CS1 groups, respectively. D) Expression level of miR-329 was significantly increased by 2.4 fold in the CS3 group compared to the control group. $P\text{-value} \leq 0.05$ were considered as significant using Dunn's multiple comparison analysis. Results are expressed as the median with 95% CI.

Figure 3. Correlation between expression levels of *ABCA1*, *ABCG1*, miR-27a and miR-329 in PBMCs of all the subjects. A) Expression level of *ABCA1* gene was positively correlated with expression level of *ABCG1* gene. B) Expression level of miR-27a was positively correlated with expression level of miR-329. C) Expression level of miR-27a negatively correlated with expression level of *ABCA1* and *ABCG1* genes. D) The expression level of miR-329 negatively correlated with expression level of *ABCG1* gene but was not correlated with *ABCA1* gene. $P\text{-value} \leq 0.05$ were considered as significant using Spearman rank correlation.

Figure 4. The column bar graph for Serum concentration of hs-CRP, ox-LDL, FRAP, and MDA in the CAD group compared to the control group. A and B) Serum concentration of hs-CRP and ox-LDL were significantly elevated by 3.41 and 1.32 folds in the CAD group compared to the control group. C) Serum concentration of FRAP was significantly reduced by 1.17 fold in the CAD group compared to the control group. D) Serum concentration of MDA was significantly increased by 1.21 fold in the CAD group compared to the control group. $P\text{-value} \leq 0.05$ was considered as significant using a nonparametric Mann-Whitney test. Results are expressed as the median with 95% CI.

Figure 5. Column bar graph for the serum levels of hs-CRP, ox-LDL, FRAP, and MDA in the CAD patients with different stages of stenosis. A) Serum level of hs-CRP was significantly elevated by 3.32, 4.7 and 4.34 folds in the CS1, CS2, and CS3 group compared to the control group, respectively. B). Serum level of ox-LDL was significantly increased by 1.45 fold in the CS3 group compared to the control group. C). Serum level of FRAP was significantly decreased by 1.21 fold in the CS3 group compared to the control group. D). Serum level of MDA was significantly increased by 1.31 fold in the CS3 group compared to the control group. $P\text{-value} \leq 0.05$ was considered as significant using Dunn's multiple comparison analysis. Results are expressed as the median with 95% CI.

Figure 6. Correlation between the serum levels of hs-CRP, ox-LDL, FRAP and MDA in all the subjects. A) Serum level of hs-CRP positively correlated with the serum level of ox-LDL. B) Serum level of FRAP negatively correlated with the serum levels of MDA. C) Serum levels of hs-CRP negatively correlated with the expression levels of *ABCG1* and *ABCA1* genes. D) Serum levels of ox-LDL negatively correlated with the expression levels of *ABCG1* and *ABCA1* genes. E) Serum levels of FRAP positively correlated with the expression levels of *ABCG1* and *ABCA1* genes. F) Serum levels of MDA negatively correlated with the expression levels of *ABCG1* and *ABCA1* genes. $P\text{-value} \leq 0.05$ were considered as significant using Spearman rank correlation.

Tables

Table 1. Primers used for real time-PCR analysis

Gene	Forward Primer	Reverse Primer	Product size
ABCA1	5'-TGCAAGGCTACCAGTTACATT-3'	5'-TTAGTGTTCTCAGGATTGGCT-3'	180 bp
ABCG1	5'-AAGGTGTCCTGCTACATCAT-3'	5'-CAGTATCTCCTTGACCATTTC-3'	135 bp
GAPDH	5'-ACACCCACTCCTCCACCTTTG-3'	5'-GTCCACCACCCTGTTGCTGTA-3'	113 bp

Table 2. Anthropometric characteristics and biochemical parameters of the study participants.

	Group	Number	Mean	Std. Error Mean (SEM)	P-value
Gender (male/female)	control	42	-----	-----	1.000
	case	42	-----	-----	
Age (year)	control	42	59.76	1.884	0.065
	case	42	64.69	1.841	
BMI (kg/m ²)	control	42	25.58	0.573	0.981
	case	42	25.56	0.711	
Troponin I (ng/ml)	control	42	93.38	70.994	<0.0001
	case	42	11753	2627.034	
TC (mg/dl)	control	42	139.88	5.461	0.02
	case	42	168.17	6.872	
TG (mg/dl)	control	42	145.48	12.317	0.613
	case	42	153.43	9.640	
HDL-C (mg/dl)	control	42	57.19	1.957	0.678
	case	42	55.95	2.242	
LDL-C (mg/dl)	control	42	56.02	5.223	0.003
	case	42	81.53	6.621	
CPK-MB (mg/dl)	control	42	9.74	0.618	<0.0001
	case	42	17.64	1.520	
BS (mg/dl)	control	42	107.71	4.288	0.004
	case	42	125.60	4.260	
BUN (mg/dl)	control	42	18.31	0.778	0.131
	case	42	20.17	0.936	
creatinine (mg/dl)	control	42	0.898	0.028	0.08
	case	42	1.029	0.038	
Sodium (mEq/L)	control	42	139.95	0.588	0.212
	case	42	140.95	0.534	
Potassium (mEq/L)	control	42	4.014	0.063	0.178
	case	42	4.145	0.072	
Drug use	control	42	0.33	0.074	0.49
	case	42	0.55	0.078	
Family history	control	42	0.50	0.092	0.388
	case	42	0.38	0.102	
Smoking	control	42	0.17	0.058	0.04
	case	42	0.45	0.078	
SBP (mmHg)	control	42	122.62	2.157	0.231
	case	42	126.74	2.641	
DB (mmHg)	control	42	75.3571	2.523	0.579
	case	42	77.0238	1.608	

Table 3. Spearman' correlation between the levels of the different parameters in the study subjects.

		ABCG1	miR-27a	miR-329	hs-CRP	ox-LDL	FRAP	MDA
ABCA1	Total	0.623****	-0.332**	-0.200	-0.478****	-0.609****	0.392***	-0.287**
	CAD	0.639****	-0.340*	-0.041	-0.333*	-0.469**	0.265	-0.231
	Control	0.641****	-0.244	-0.162	-0.511***	-0.600****	0.372*	-0.215
ABCG1	Total		-0.296**	-0.227*	-0.546****	-0.305**	0.268*	-0.331**
	CAD		-0.157	-0.090	-0.385*	-0.303	0.064	-0.090
	Control		-0.172	-0.147	-0.481**	-0.113	0.271	-0.284
miR-27a	Total			0.386***	0.328**	0.330**	-0.262*	0.138
	CAD			0.253	0.164	0.330*	-0.165	0.148
	Control			0.275	0.342*	0.209	-0.179	-0.083
miR-329	Total				0.366***	0.342**	-0.049	0.046
	CAD				0.182	0.172	-0.104	0.109
	Control				0.375*	0.278	0.197	-0.168
hs-CRP	Total					0.442****	-0.252*	0.247*
	CAD					0.350*	-0.287	0.082
	Control					0.341*	-0.050	0.060
ox-LDL	Total						-0.381***	0.242*
	CAD						-0.452**	0.324*
	Control						-0.200	-0.064
FRAP	Total							-0.698****
	CAD							-0.650****
	Control							-0.626****

* P -value ≤ 0.05

** P -value ≤ 0.01

*** P -value ≤ 0.001

**** P -value ≤ 0.0001

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6