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Evaluation of *PPAR-α*, *PPAR-γ*, *TLR2*, *TLR4* Gene Expression In Patients with Coronary Artery Disease (CAD): An Experimental Study

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Abstract

Background: Coronary artery disease (CAD) is one of the heart diseases that causes the death of many patients in the world. Many genes and molecular pathways are involved in the regulation of inflammation. However, some genes have a regulatory role and control immune responses. In recent studies, few studies have been done regarding the role of TLRs and PPARs in CAD. Hence, the present study aimed to determine and compare the mRNA expression of *PPAR-α* and *PPAR-γ* genes and genes of the innate immune system messenger pathway, including TLR2 and TLR4, in CAD patients in comparison to normal individuals.

Methods: This study (case-control) was conducted on 12 patients with coronary arteries and 10 healthy individuals as healthy controls. RNA extraction was performed, cDNA was produced, and then the mRNA expression levels of *TLR2*, *TLR4*, *PPAR*-α, and *PPAR*-γ genes were examined using Syber green Real-Time PCR. The t-test sample and the related non-parametric tests were used to investigate the relationship between the quantitative variables. The significance level in all tests was considered as less than 0.05.

Results: The results of data analysis showed that the expression level of *TLR2* and *TLR4* genes was significantly increased in the patient group compared to the controls (P=0.001). However, although *PPAR*-α and *PPAR*-γ genes were up-regulated in patients' samples, the comparison of gene expression levels did not significantly differ between the case and control groups.

Conclusion: we found meaningful results to the significant role of 2 and TLR4 in the pathogenesis of CAD and emphasize the hypothesis that TLR2 and TLR4 can be considered therapeutic options.

Keywords: cardiovascular disease; inflammation; Coronary Artery Disease, Toll Like Receptor, Peroxisome proliferator-activated receptorx

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Introduction

Cardiovascular diseases were described as the most prevalent disorders and the leading causes of death in recent decades worldwide (1-3). In that classification, coronary artery disease (CAD) and atherosclerosis, which account for a huge number of morbidity and mortality in most countries, are multifactorial ailments (4-6). The mortality rate related to CAD varies in different parts of the world. However, it has been shown that 160 CAD patients die per 100,000 people per year (7). In this regard, inflammation and its related pathways are the main factors that contribute to most atherogenesis procedures. Indeed, it is proposed that inflammation has a driving role in disease pathogenies that initiate plaque formation and subsequent events (8). However, CAD and atherosclerosis are considered multifactorial and polygenetic diseases, in which lots of human genes are involved in disease initiation and progression (9).

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↑What is "already known" in this topic:
In recent studies, few studies have been done regarding the role of TLRs and PPARs in CAD. Hence, the present study aimed to determine and compare the mRNA expression of *PPAR-α* and *PPAR-γ* genes and genes of the innate immune system messenger pathway, including TLR2 and TLR4, in CAD patients in comparison to normal individuals.

→What this article adds:
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Numerous studies have been performed in different populations to study genes affecting atherosclerosis and revealed that some Peroxisome proliferator-activated receptor (PPAR) PPAR and toll-like receptor (TLR) genes are the most important contributing agents (10).

PPAR genes are a group of ligand-activated transcription factors involved in regulating lipid and glucose homeostasis. In addition, they play a critical role in regulating the immune and inflammatory responses in the human body (11). Three groups of PPAR have been identified including PPAR-α, PPAR-β / δ and PPAR-γ; the expression level of each group is different in various cells (12). PPARs are expressed in endothelial cells, vascular smooth muscle cells, T lymphocytes, macrophages, and monocytes, which regulate the expression of key genes contributing to vascular biology. On the other hand, it is suggested that PPAR activation may reduce inflammation and reduce atherosclerosis (13, 14).

The TLR-like receptors are another group of genes involved in atherosclerosis development. They are a family of pattern recognition receptors that play a key role in innate immune responses against microbial agents (15). Recent studies have declared that some of these receptors may be up-regulated in atherosclerotic conditions and help disease progression (16). So, applying and designing new drugs based on their expression changes in CAD and atherosclerosis may provide new insights into patients' treatment processes and outcomes.

In these circumstances, the expression detection of disease-causing genes or those that contributed to its pathogenesis is effective in cardiovascular patients to imagine changes and modifications in their expression and benefit from disease-targeted therapy (17). Moreover, by modulation of these genes' expression with specific agonists and antagonists, an effective step would be taken in managing, following up, and treating these diseases.

Inflammation is one of the factors in the pathogenesis of CAD. Many genes and molecular pathways are involved in the regulation of inflammation. However, some genes have a regulatory role and control immune responses. In recent studies, few studies have been done regarding the role of TLRs and PPARs in CAD. Hence, the present study aimed to determine and compare the mRNA expression of PPAR-α and PPAR-γ genes and genes of the innate immune system messenger pathway, including TLR2 and TLR4, in CAD patients in comparison to normal individuals.

Methods

Study design and participants

This case-control study was conducted on 12 patients referred to clinics (cardiovascular disease of Rasoul Akram hospital) for angioplasty due to atherosclerotic plaque and 10 healthy individuals as the control group. The study was approved by the institutional ethics committee of Urmia University of Medical Science, and written informed consent was obtained from all subjects before inclusion.

Inclusion criteria included age ≥50 and coronary artery stenosis due to atherosclerotic plaque approved by a specialist. Exclusion criteria included patients with comorbidity of hepatic enzyme disorders or thyroid and kidney disease; patients consuming any specific medication, smoking, or alcohol abuse. Patients' demographic information was collected through interviews and their medical records. Patients with a discharge fraction (EF) of less than fifty percent were included.

The patient's peripheral blood was poured into sterile CBC tubes containing EDTA, and peripheral blood mononuclear cells (PBMCs) were isolated using density-gradient (Ficoll) according to its standard protocol.

RNA extraction and cDNA production

RNA extraction from mononuclear peripheral blood cells was performed using Gene All Trizol LS. The purity was examined by the Nanodrop (OD 260 / 280 nm ratio > 1.8) (Thermo Scientific, USA), and its quality was checked by observing ribosomal RNAs 18 s and 28 s bands on gel electrophoresis. The cDNA production (using Yekta Tajhiz Azma, Iran) was performed by PCR in conditions: 30 sec at 95°C, then 40 cycles at 95°C for 5 s denaturation, 60°C for 15 s annealing and 72°C for 32 s extension.

Reverse transcription-polymerase chain reaction

SYBR™ Green Real-Time PCR assay was carried out using qRT-PCR (Rotor-Gene 6000, Qiagen). The total reaction in the Real-Time PCR assay was 20 µL, including 10 µL of Eva Green Real-Time PCR Master Mixes (Amplicon, Denmark), 0.2 µL of both forward and reverse primers, 8.6 µL distilled water, and 1 µL of template cDNA. The expression level of target genes was analyzed by the ΔΔCT method. Primer sequences were shown in Table 1, and the GAPDH was used as a housekeeping gene. The Real-time PCR amplification efficiency was calculated using the following formula: Efficiency of PCR = $[10^{(-1 / Slope)}]-1$

Table 1. Primer sequences used in this study

Gene	Forward	Product size
PPAR-α	F: ATGGCATCCAGAACAAGGAG R: GGCGAATATGGCCTCATAAA	176 bp
PPAR-γ	F: AGAAATGAATCAAAGGCAGCCG R: CAGCAGCAGCAGCAACAAG	169 bp
TLR2	F: ATACTCCAATCAGGCTTCTCT R: ACACCTCTGTAGGTCACTGTTG	163 bp
TLR4	F: ATATTGACAGGAAACCCCATCCA R: AGAGAGATTGAGTAGGGGCATTT	300 bp
GAPDH	F: GAGCCACATCGCTCAGACAC R: CATGTAGTTGAGGTCAATGAAGG	150 bp

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Statistical analysis

The assessment of gene expression level was performed using $2^{-\Delta\Delta Ct}$. Data were analyzed by SPSS software (version 20). The normal distribution of data was evaluated by the Shapiro ilk and Kolmogorov Smirnov tests. Quantitative data were reported as mean ± standard deviation. The t-test sample and the related non-parametric tests were used to investigate the relationship between the quantitative variables. The significance level in all tests was considered as less than 0.05.

Results

The present study was carried out on 12 patients with a mean age of 55.2 ± 8 years and 10 healthy control groups with a mean age of 54.17 ± 7.3 years. The study included 5 males and 5 females in the healthy control group and 7 males and 5 females in the patients group.

Data analysis using t-test and comparison of mean ΔCT showed that the expression levels of TLR2 and TLR4 were increased in cases compared to the control group (P=0.001). Moreover, high expression levels were detected for PPARα and PPARγ in patients compared to healthy participants (P=0.234). The relative expression is shown in Figure 1.

Discussion

Atherosclerosis is a chronic inflammatory disease involving innate and acquired immune systems. Although the disease's main mechanisms are controversial, studies in animals and human populations suggest that immune and inflammation regulatory cells directly contribute to the development of atherosclerotic plaques (18). However, studies regarding aberrant gene expression in this disorder continue to find more evidence about its exact mechanism. Thus, we performed the present study and found that the expression of TLR-2 and TLR-4 genes in the patient groups significantly increased compared to the control group. Since TLRs activate macrophages through binding to specific ligands such as lipopolysaccharide and oxidized LDL; macrophages subsequently increase the production of inflammatory agents, we supposed that TLR upregulation enhances an accelerated atherosclerosis plaque formation and subsequently disease presentation. Inline, several studies have proved that the expression of TLR2 and TLR4 was increased in atherosclerotic plaques and blood samples that represented the association of these receptors with disease severity (16, 18).

For instance, in accordance with our findings, Meng Liu et al. revealed that TLR2 and TLR4 were significantly upregulated in CAD patients (19). Lukas Andreas Heger et al. also exhibited the high expression of TLR2 mRNA in acute

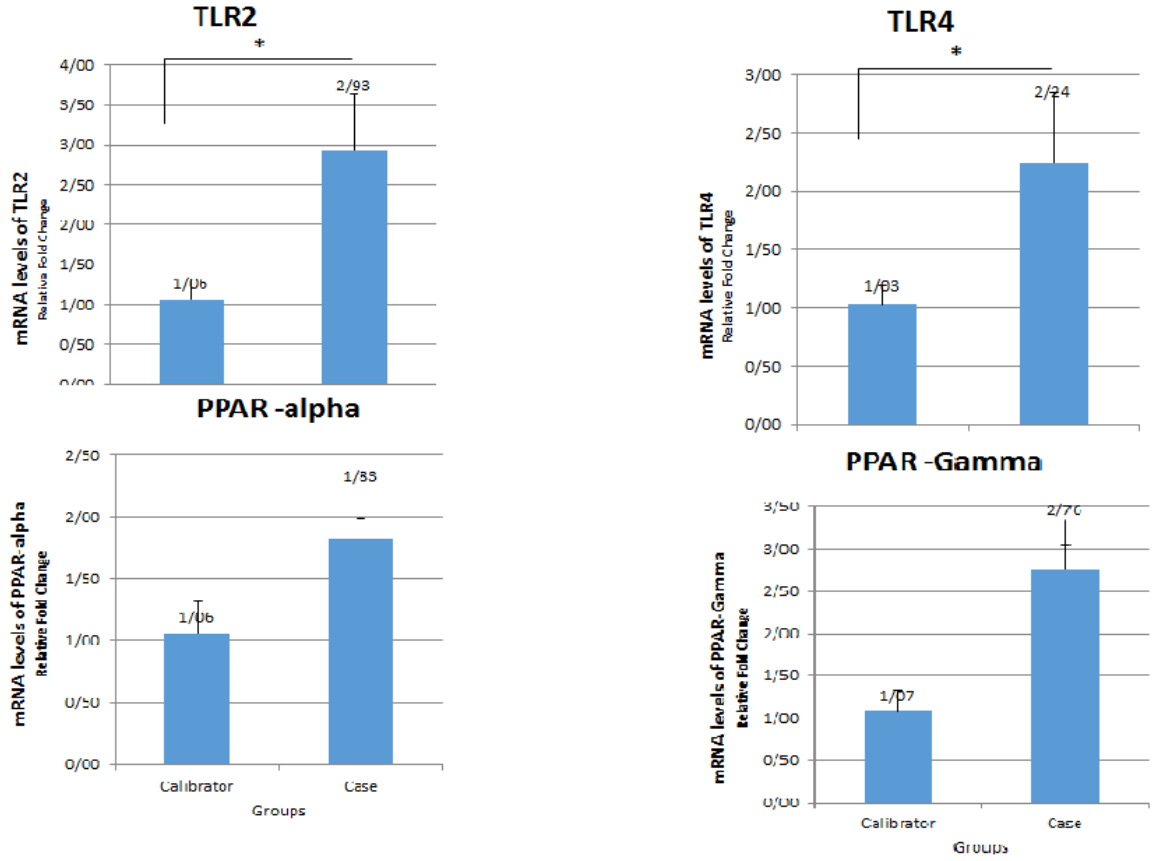

Figure 1. The comparison of relative expression of *TLR2*, *TLR4*, *PPAR-α*, and *PPAR-γ* genes between case and control groups. Error bars represent the standard deviation.

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coronary syndrome (ACS), which is a marker of inflammation's relationship with ACS and atherosclerosis severity (20). Similarly, in a study by Liang Shao et al., it was indicated that TLR-4 mRNA expression was significantly elevated in coronary artery stenosis (21). Since TLR-4 expression was raised by oxLDL, a macrophage stimulator in foam cells, it is thought that TLR-4 may have a critical function in plaque formation (22). Additionally, TLR-2 and TLR-4 may exert a role in atherosclerosis progression through the regulation of the NF-κB signaling pathway after activation by fatty acids (23, 24). Accordingly, it seems that TLR2 and 4 higher levels could be considered target genes and that their inhibition or reduced expression could be effective in preventive measures or disease management.

The present study results also showed that the expression of PPAR-α and PPAR-γ genes increased in the patient group compared to the control group, but this increase was not statistically significant. In this regard, it is demonstrated that the underlying mechanism of the PPARs effect goes back to the fact that PPAR-α and PPAR-γ activation can reduce disease-causing inflammation by increasing the adiponectin performance and obstructing macrophage activation. Unfortunately, studies researching the expression of these two genes were not enough to compare, but similar to our findings, Wen Gao et al. also reported an increase in PPAR-α expression in atherosclerotic and they assumed that it played an inhibitory role (23). Furthermore, in the study conducted by Sueyoshi et al., a PPAR gamma upregulation was obtained in studied plaques, and they proposed that PPAR gamma higher expression may be due to the local macrophages in early plaques, which was also observed in the present study. In contrast, a study by Constantinos Giaginis et al. who have been investigated the PPAR gamma expression in 134 atherosclerosis plaques and demonstrated that in most samples, PPAR expression was reduced in smooth muscle cells and macrophages, which was contrary to the results of the present study (25). Hua et al. showed that the increased expression of PPAR-γ in CAD patients can cause the production of reactive oxygen species (ROS) and ultimately aggravate the disease (26).

However, given the numerous and widespread applications of the PPAR family in lipid homeostasis, it would not be surprising to play an important role in atherosclerosis (11). Since the best, PPAR-α endogenous ligands are PUFA fatty acids and the most cells that express PPAR-γ are those involved in atherosclerosis, such as primary macrophages and vascular smooth muscle cells, PPAR-α and PPAR-γ activation probably reduces the risk of cardiovascular disease (27, 28). Therefore, activating the PPAR gene group at the earlier phases of plaque formation is probably one of the most important causes of cardiovascular disease risk reduction (29, 30). In agreement, multiple studies claimed that the PPAR-α and PPAR-γ genes could limit and reduce pro-inflammatory responses and inflammation events resulting in atherosclerosis inhibition (30).

Although the results of some studies contradict our findings, it can be explained by the fact that in the early plaques, the increase in PPAR expression is a disease-fighting mode, but with the progression of the disease, this effect decreases, and no increase in expression is observed. Probably the reason for this similarity was that plaques had just formed in the patients studied. However, their exact role and mechanism of action remain to be controversial.

In general, from the above topics, it can be claimed that the activation of PPARα and PPAR-γ genes may lead to the limitation and reduction of inflammation and, thus, the reduction of atherosclerosis at early stages of plaque formation. On the other hand, activating or increasing the expression of TLR-2 and TLR-4 genes causes the progression and spread of plaques and thus atherosclerosis. Therefore, more studies of the expression of these genes are of great importance. Finally, assessing the preventive and therapeutic potential of using PPAR and TLR gene ligands in many cardiovascular diseases, including atherosclerosis, requires a better understanding of the molecular mechanisms involved in the disease pathogenesis.

This study had a series of limitations. In this study, only the genes related to inflammation were evaluated, and their relationship with the treatment process of the patients was not investigated. Also, signaling pathways related to genes were not evaluated.

Conclusion

The present study's results demonstrated that TLR receptors increase in atherosclerosis at the lesion site and that their expression is associated with an increased risk of disease. On the other hand, it can be said that the increased expression of TLR4 activates endothelial cells and macrophages, and the presence of this receptor itself causes the differentiation of cells into antigen-presenting cells, and TLR2 leads to the resulting reaction. Perhaps this finding can be used to find new treatments based on inhibition of gene expression.

Authors' Contributions

Mahboubeh Pazoki and Mahbobe Abbasluo write the manuscript. Mahya Bakhshi Ardakani was done the experimental procedure. Negar Jafari has conducted the data collection.

Ethical Considerations

The authors declare no conflict of interest. All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or a comparable ethical strand (IR.UMSU.REC.1403.061).

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Conflict of Interests

The authors declare that they have no competing interests.

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