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Decreased Expression of Cytotoxic T Lymphocyte-associated Protein 4: A Risk Factor of Myocardial Infarction

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ABSTRACT

Cardiovascular diseases are the most prevalent disease worldwide and pose a considerable threat to human health. Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) plays a crucial role in the maintenance of immune diseases; however, its role in the progression of cardiovascular disorders is still unknown. The present study aimed to evaluate the relationship between CTLA-4 and myocardial infarction (MI).

The Kyoto encyclopedia of genes and genomes database and the pathway studio database has revealed the role of CTLA4 as an inhibitory receptor on activated T cells. The relative expression of the *CTLA-4* gene was assessed on the peripheral blood cells in 80 MI patients and 80 healthy individuals using quantitative real-time polymerase chain reaction (qRT-PCR) and also receiver operating characteristic (ROC) analysis was performed to evaluate the sensitivity and specificity of CTLA-4.

We noticed the decreased expression of CTLA-4 levels in patients, compared to the control group (2.73±1.55 vs. 5.36±1.34). The study revealed the sensitivity of 0.89, specificity of 0.83, the accuracy of 0.9, and the area under the ROC curve (AUC) of 0.901 (95% CI: 0.727-0.776) for CTLA-4.

The results highlighted the critical role of CTLA-4 as an inhibitory receptor in the maintenance of cardiovascular risks.

Keywords: Cardiovascular diseases; CTLA-4 protein; Myocardial infarction

INTRODUCTION

Cardiovascular diseases (CVDs) are the most frequent disorders of the heart, blood vessels, or coronary heart vessels. Heart attacks account for more than 80% of all CVD deaths or prolonged life-threatening disabilities.[1]

Despite many improvements in cardiovascular disease over years, early diagnosis is still the key to the successful treatment of heart diseases.[2] One of the main issues in researching cardiovascular diseases is to find out suitable and unique markers with clinical applications to decrease the mortality rate of CVDs.[3]

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One of the major factors limiting the survival of some affected patients might be the late diagnosis. Clinical and molecular predictive biomarkers have been developed for the screening purpose to provide the early detection of the disease.[4] Accordingly, it is necessary to detect new sensitive biomarkers that can be applied to detect early noninvasive cardiovascular disorders and the progression of myocardial infarction (MI),[5,6] impaired cardiovascular, caused by genetic and environmental factors such as infectious or inflammatory agents through the biomolecules are considered as the main mechanism in prompting arteriosclerotic plaques and providing inflammation conditions in one or more coronary vessels.[7]

The cytotoxic T lymphocyte-associated protein 4 (CTLA-4) gene is located on chromosome 2q33 and consists of 4 exons and 3 introns. This protein is identified to be involved in some inflammatory diseases such as autoimmune diseases, diabetes, and multiple sclerosis.[8] CTLA-4 mutations are attributed to hereditary diabetes, systemic lupus erythematosus, and Hashimoto's thyroiditis. Furthermore, CTLA-4, as an immune checkpoint inhibitor, is the most important factor on the activated T-cells as it inhibits their activation.[9] The question is whether the expression of *CTLA-4* genes is associated with cardiovascular risk. The expressions of mRNA would be highly valuable as a potential marker for the susceptibility of cardiovascular risk. Moreover, there was no study addressing this issue. Accordingly, the present study investigated the expression of *CTLA-4* in a patient with CVD and healthy samples using Real-time PCR and examined their relationship with CVD among the Iranian population. In this regard, CTLA-4 is appropriate for the elevated risk factors associated with autoimmune diseases, susceptibility to some diseases, and therapeutic target for its.[10] The administration of CTLA-4 is the main concern in future medicine, and the relevant knowledge can improve the management of cardiovascular diseases using a new target therapy. Such studies are of great importance in examining a variety of immune diseases in both healthy individuals and patients.

MATERIALS AND METHODS

Target Prediction and Pathway Analysis

Based on the Kyoto encyclopedia of genes and genomes (KEGG) database (https://www.kegg.jp/kegg/pathway) and the pathway studio database (https: //www. pathwaystudio.com), we identified a set of genes and activated T lymphocytes, significantly important in the response of the immune system. Recent studies have focused on the role of inhibitory receptors such as CTLA-4, PD-1, and CD45 on activated T cells.

Sample Collection

This research study complied with the Helsinki Declaration and obtained the approval of the Ethics Committee of Urmia University of Medical Sciences (IR. UMSU.REC.1398. 169).

Blood samples were obtained from Iranian individuals who were admitted to the Seydoshohada Hospital, Urmia, Iran. They were diagnosed by standard criteria. The participants provided written informed consent forms. The peripheral blood samples of 80 patients and 80 healthy individuals, with no family history of CAD, were included in the study. Demographic and clinical information about gender, age over 40 years, angiography results, family history of CVD, dyslipidemia, systolic blood pressure of ≥130 mm Hg, diastolic blood pressure of ≥85 mm Hg, smoking habits, and fasting blood glucose ≤110 mg/dL was collected from each participant. The patients were selected according to the angiography results of more than 50% vessel narrowing (stenosis) in at least one segment of a main coronary artery. Patients with a history of malignant diseases, congenital disease, diabetes mellitus, and infectious diseases were excluded from the study.

RNA Isolation and Quantification of *CTLA-4* Gene

Total RNA was extracted using QIAzol Lysis Reagent (Qiagen, Germany), and cDNA was synthesized using a Reverse Transcription kit (Qiagen, Germany) following the manufacturers' manual. Moreover, the *beta-actin* gene was considered the housekeeper gene in normalization. Real-time RT-PCR was performed using SYBR Green PCR kit (Qiagen, Germany) in an ABI Real-time PCR System (USA). CTLA-4 mRNA was amplified using primer pairs: forward 5'-CCCAACAGAGCCAGAATGTG-3', reverse 5'-ACACGTAATTTGGGTTCCGC-3', *beta-actin* gene forward: 5'-ACTCTTCCAGCCTTCCTTCC-3', and reveres: 5'-CGTACAGGTCTTTGCGGATG-3'. In this study, all primers were checked by Primer-BLAST and Oligoanalyzer software. The thermal cycling conditions

were as follows: 94°C for 10 minutes, 40 cycles at 94°C for 10 seconds, 55°C for 60 seconds, final extension at 72°C for 7 minutes and the cycle threshold (CT) was evaluated, followed by a melting curve analysis. The relative mRNA expression was determined using the delta-delta CT method, and $2^{-\Delta\Delta Ct}$ was calculated to determine the fold change of the *CTLA-4* gene.[11] All the experiments were performed at least twice.

Statistical Analysis

In this study, the mean and standard deviation (mean±SEM) are presented as descriptive statistics using SPSS 16.0 software (SPSS, Inc., USA). The two groups were compared using Student's t-test, and the relationship among *CTLA-4* gene expressions was evaluated; using the chi-square test. The fold changes in the gene expression were calculated with LinReg PCR Software. In this study, $p<0.05$ and 95% confidence interval were defined to be statistically significant.

RESULTS

Bioinformatics Prediction

Utilizing gene prediction pathways on KEGG and the pathway studio database, we identified several important genes involved in the T lymphocytes pathway, such as *CTLA-4*, *PD-1*, and *CD45*. CTLA-4 and inhibitory pathways have fundamental roles in MI risk, inflammation, and autoimmunity. Furthermore, altered expression levels and regulatory effects of predicted *CTLA-4* gene are very important in the MI patients compere with healthy controls.

Table 1. Laboratory results and comparison of quantitative and qualitative variables of enrolled people

Variables	Patients (n=80) Mean±SD	Healthy controls (n=80) Mean±SD	*p*
Age (years)	57.41±11.44	55.36±8.30	>0.05
Gender, male/Female	40/40	40/40	-
Weight (kg)	89.09±11.32	72.12±8.27	**0.01**
Systolic Pressure (mmHg)	146.79±3.6	123.22±1.5	**0.01**
Diastolic Pressure (mmHg)	82.00±1.1	79.36±0.9	**0.01**
Cigarette smoking (Positive)	23(46%)	15(37.5%)	-
FBS (mg/dL)	101.95±24.62	96.23±6.2	**0.01**
Blood Sugar (mg/dL)	101.30± 21.03	96.23±6.2	**0.01**
Serum Na (mEq/L)	140.54±0.21	140.98± 3.21	>0.05
Serum K (mEq/L)	4.22± 0.42	3.92 ±0.31	>0.05
Serum Mg (mg/dL)	2.41± 0.28	2.13±0.17	>0.05
Cholesterol (mg/dL)	173.43±56.22	125.26±21.66	**0.025**
Triglyceride (mg/dL)	153.55±36.32	125.17±21.57	**0.001**
HDL (mg/dL)	43.82±10.28	59.19±11.18	**0.001**
LDL (mg/dL)	111.45±39.96	65.44±17.45	**0.001**
Fold change $2^{-\Delta\Delta CT}$	2.73 ±1.55	5.36 ±1.34	**<0.05**

Molecular Analysis

We studied 80 MI patients (40 males and 40 females) as well as 80 healthy individuals (40 males and 40 females). The patients and the healthy individuals had an average age of 57.41±11.44 years and 55.36±8.30 years, respectively. All the laboratory data for the patients and healthy control group are presented in Table l. The circulating levels of CTLA-4 and *beta-actin* gene were analyzed using the fold change values for the patients and healthy individuals. Melting curve analysis confirmed the specificity of primers and PCR products in the present study.

The level of *CTLA-4* expression was lower in the patients, compared to the control group (p<0.05) (Figure 1A). This result suggested the altered *CTLA-4* expression in patients with CVD. To evaluate the sensitivity and specificity of circulating CTLA-4, receiver operating characteristic (ROC) analysis was performed for patients. The results revealed the sensitivity of 0.89, specificity of 0.83, the accuracy of 0.9, and the area under the ROC curve (AUC) of 0.901 (95% CI: 0.727-0.776) for CTLA-4 (Figure 1B).

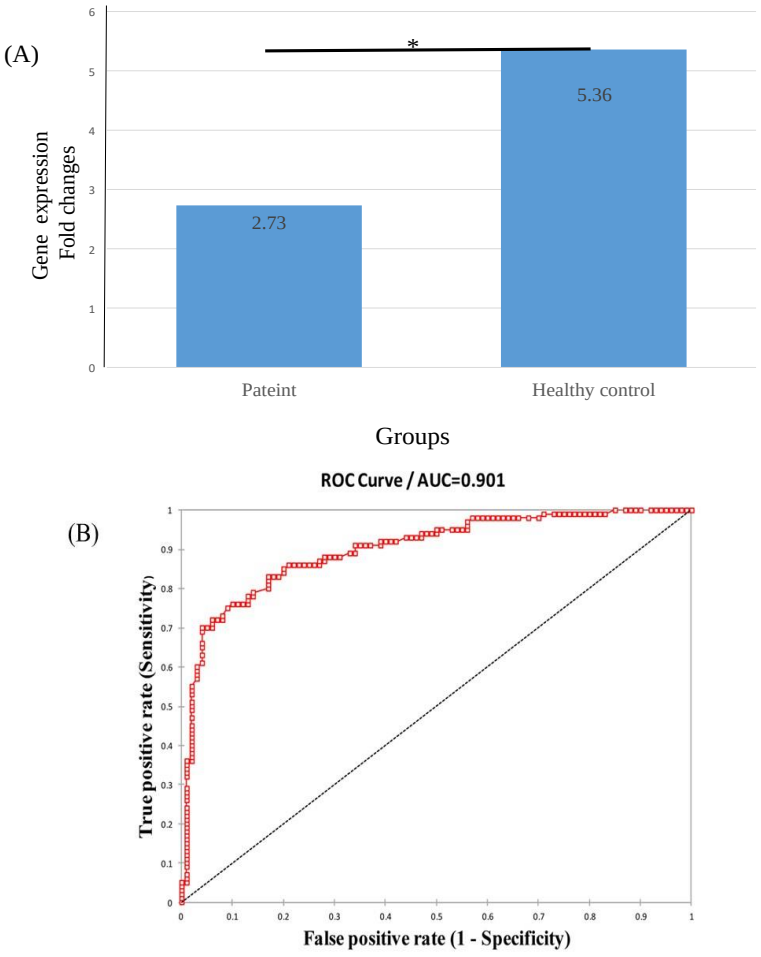

Figure1. The level of Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) expression by real-time PCR in study groups. (A) The fold changes of CTLA-4 expression in the myocardial infarction (MI) patients (n=80) and healthy groups (n=80). p<0.05 was considered statistically significant. $^{*}p$<0.05. (B) The ROC analysis was used to investigate the CTLA-4 expression levels in patients with the sensitivity of 0.89, specificity of 0.83, and AUC (0.901).

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DISCUSSION

The increasing prevalence of cardiovascular diseases is now considered as a major cause of cardiac and vascular dysfunction in some patients undergoing cardiovascular treatment.[12] Both environmental and genetic factors play roles in the pathogenesis of cardiovascular complications.[13,14] Cardiovascular diseases are complicated, and the impact of genetic factors in the pathogenesis of cardiovascular risk is of great importance.[15] The changes in some genes provide real-time information about their function and structure, inflammation, apoptosis, and oxidative stress in human diseases and many other heart cells and cancer cells.[16] Furthermore, previous studies have documented that immune cells are the major leading of inflammatory nature of cardiovascular risk.[17] CTLA-4 and CD28 play a fundamental role in T-cell activation. They are co-stimulatory receptors located on the surface of activated T-cells during antigen presentation. We assumed that the expression of *CTLA-4* genes was associated with cardiovascular risk and that this association was useful as biomarkers for the early diagnosis, monitoring, prediction of cardiovascular and therapeutic responses in patients at risk. Unfortunately, an ideal marker, in this case, is still unknown. Few studies have examined the role of *CTLA-4* gene expression in cardiovascular diseases. In the present study, the expression of CTLA-4 mRNA was detected using qRT-PCR in patients with vessels stenosis (greater than 50%) and healthy individuals. The analysis of our data showed that there were significant differences in fold changes. In this study, it was revealed that the relative expression of CTLA-4 was significantly down-regulated in patients when compared with that of healthy individuals. In this regard, the CTLA-4 can be a predictor for the pathogenesis of heart diseases. CTLA-4 seems to have an impact on the blood cells via T-cells and plays a pivotal role in the progression of atherosclerosis in the endothelial vessels and heart cells. This causes the accumulation and release of cytokine/T cells in the endothelial vessels and the activation of signaling pathways for plaque formation and rigidity of vessels. These signaling pathways have a great effect on the cardiac myocytes and cardiovascular systems. Consequently, dysregulation of CTLA-4, as a key genetic element in cellular immunity, can develop a particular pattern to establish peripheral tolerance and control the hemostasis, proliferation, and regulation of T cells.[18,19] The effects of blood cells and cytokines increase the stenosis in vessels and promote the development of MI risk. The CTLA-4 changes at different levels have been identified the risk of type 1 diabetes in children.[20] The antitumor activities of *CTLA-4* gene in mouse models were described by Zhuo et al, and Lewis et al.[10,21]

According to our observations, significant dysregulation of *CTLA-4* may be one of the important genes responsible for the pathogenesis of cardiovascular and individuals with this decrease in CTLA-4 could have a risk factor for susceptibility to MI risk. An assessment of CTLA-4 at baseline could be useful as immunotherapy to offer personalized monitoring for patients at high risk of CVD. The current study was evaluated the circulating CTLA-4 at the time of cardiac infarction on a limited number of patients and controls. It remains possible that later time points may provide additional information in patients with MI. Further studies are recommended to determine the predictive markers in patients with cardiovascular risks. Moreover, further studies with a larger sample size from multicenter are required to validate the CTLA-4 changes in cardiovascular diseases.

In summary, this study suggests that *CTLA-4* is expressed in blood patients and that *CTLA-4* expression is associated with cardiovascular diseases and MI risk; therefore, the CTLA-4 can act as a novel prognostic and therapeutic target for cardiovascular diseases. Future experiments are needed to demonstrate the potentials of human *CTLA-4* in regulating some genes.

CONFLICT OF INTEREST

The author declares no conflicts of interest.

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This study was approved by the Committee for Ethical Consent of Urmia University of medical sciences under the number IR. UMSU.REC.1398.169.

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