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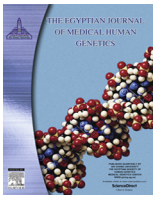

Original article

Association assessment of *Interleukine-10* gene polymorphism and its expression status with susceptibility to coronary artery disease in Iran

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ABSTRACT

Background: The cytokines are potent inflammatory factors that regulate each stage of atherosclerosis leading to the disease development. Interleukin-10 (IL-10) as an anti-inflammatory cytokine can develop atherosclerosis by inhibiting the synthesis of metalloproteinase. Moreover, IL-10 promotes the plaque stability by preserving the extracellular matrix and fibrous cap.

Aim: We evaluated the association of the two *IL-10* promoter gene polymorphisms with susceptibility to coronary artery disease (CAD) in Iranian population.

Subjects and methods: We used the Sequence Specific Primer-Polymerase Chain Reaction method to determine genotypes. We also studied mRNA expression of the *IL-10* gene in Iranian CAD patients using quantitative real-time PCR.

Results: There was a significant association between *IL-10*(−819) T allele and *IL-10*(−819) T/T genotype, and CAD ($p = 0.041$ and $p = 0.042$ respectively). There also was a significant association between *IL-10* (−1082) G allele and *IL-10*(−1082) G/G genotype, and CAD ($p = 0.017$ and $p = 0.020$ respectively). Genotype T/T of *IL-10*(−819) polymorphism significantly associated with the two vessel disease type ($p = 0.017$). In addition, there was a significant association between *IL-10*(−1082) G/G genotype and the three vessel disease type ($p = 0.009$). IL-10 mRNA expression was significantly decreased 3.36-fold in samples with *IL-10*(−819) polymorphism and 1.98-fold in individuals with *IL-10*(−1082) polymorphism.

Conclusions: Our results suggest that *IL-10* gene promoter polymorphisms may influence both coronary artery disease risks and severity in Iranian patients.

1. Introduction

Growing evidence confirms the role of inflammatory responses in the pathophysiology of atherosclerotic events such as coronary artery disease (CAD). CAD develops by plaque formation and deposition in the arteries walls, causing disruption of blood flow, and is called atherosclerosis [1]. Atherosclerosis can be stimulated by multiple molecular pathways and genetic variants, which play a crucial role in the determination of susceptibility to CAD [2]. Several factors increase the risk of CAD such as age, gender, heredity, smoking, high blood cholesterol, high blood pressure, physical inactivity, and obesity. These factors make people prone to the coronary heart disease [3]. The cytokines are potent inflammatory factors that regulate each stage of atherosclerosis and leading to the development of diseases such as CAD [4]. Interleukin-10 (IL-10), as one of the most important anti-inflammatory cytokines, serves as an anti-inflammatory agent by down-regulation of the Th1 and suppression of pro-inflammatory cytokines such as IL1, IL6, IFN-γ and TNF-α [5]. It also develops atherosclerosis by inhibiting the synthesis of metalloproteinase. Moreover, IL-10 promotes the stability of plaque by preserving the extracellular matrix and fibrous cap [2]. The *IL-10* gene has variable sites (polymorphisms and microsatellites) which influence the level of *IL-10* expression. Therefore, it may be associated directly and indirectly with the development of the CAD. In the previous study, we evaluated the association of *platelet-derived growth factor b* polymorphisms with CAD [6]. In this study, we investigated the

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association of two single nucleotide polymorphisms (SNP) in *IL-10* gene promoter and its mRNA expression level with CAD.

2. Subjects and methods

2.1. Study subjects

This case-control study with randomized sampling was performed to evaluate the genetic association of the two *IL-10* polymorphisms [−1082G/A (rs1800870) and −819C/T (rs1800871)] with CAD. We enrolled 303 CAD patients with evidence of atherosclerosis, with known number of the vessel involved, who scheduled to undergo diagnostic coronary angiography, from March 2013 to March 2014, in Kouwsar heart center of Golestan University of Medical Sciences, Kordkuy, Iran. Inclusion criteria for patients group were the presence of a stenosis less than 50% in at least one major coronary artery. The controls group involved 343 individuals with normal coronary artery angiograms. Inclusion criteria for the control group also were the presence of normal electrocardiograms at rest, without symptoms of myocardial ischemia during exercise. In agreement with the Helsinki Declaration, all participants were aware of the study details and signed the relevant written informed consent. The Ethics Committee of Golestan University of Medical Sciences approved this study.

2.2. DNA extraction and amplification

Genomic DNA was extracted by the phenol–chloroform method following standard protocol with some modifications as described previously [7]. The *IL-10*(−819)C/T and (−1082)G/A polymorphisms were investigated by Sequences Specific Primer-polymerase chain reaction (SSP-PCR) using two primer sets (Table 1). PCR reaction included 2 μl of DNA, 13 μl master mix containing 1.5 μl dNTP, 1.5 μl 10x buffer, 0.9 μl MgCl2, 2.2 μl 12% Sucrose and 0.2 unit Taq Polymerase (Qiagen, Hilden, Germany), 0.5 μl human growth hormone (hGH) primer (MWG, Ebersberg, Germany) as internal control (Table 1), 5.2 μl dH2o and 1 μl of each specific primer (MWG, Ebersberg, Germany). The PCR reaction was performed in a Thermal Cycler (Techne, Staffordshire, UK), with the following program: 1 min at 95 °C followed by 10 cycles of 15 s at 95 °C, 50seconds at 62 °C, 40 s at 72 °C, followed by 20 cycles of 30 s at 95 °C, 30seconds at 57 °C and 30 s at 72 °C with 5 min at 72 °C as final extension. Then, PCR products were transferred on 1.5% agarose gel containing DNA safe stain and the bands were visualized on a gel documentation system (UviTec, Warwickshire, UK).

2.3. Quantitative Reverse Transcriptase PCR (RT-qPCR)

Total RNA was isolated from peripheral blood mononuclear cells (PBMCs) by the Trizol reagent (Thermo Fisher Scientific, Massachusetts, USA). In this stage, samples were selected from 80 patients and 80 normal individuals. RNA concentration was measured using a NanoDrop ND 1000 (Thermo Fisher Scientific, Massachusetts, USA). All RNA samples had a 260 nm OD equivalent to 0.4–0.9 nanograms per microliter, and a 260/280 ratio of 1.8–2.2. Total RNA was reversely transcribed to cDNA by the QuantiTect Reverse Transcriptase kit (Qiagen, Hilden, Germany). Quantitative real-time PCR was performed using a Corbett Rotor-Gene 6000 (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The information of primers used to quantify IL-10 mRNA expression are shown in Table 1. The phosphoglycerate kinase 1 (PGK1) gene was amplified as the normalized control. Real-time PCR was performed in triplicate using optimal conditions including 3 min at 95 °C as a PCR initial activation step followed by two-step cycling at 95 °C for3 s and 60 °C for 30 s, repeated for 40 cycles. The relative expression was determined by threshold cycle (Ct) values and ΔΔCt method as described previously [10].

2.4. Statistical analysis

All data were analyzed by GraphPad software (version 6; San Diego, United States). Deviation from the Hardy–Weinberg assumption was checked for each polymorphism. Comparisons of the frequencies of the IL-10 alleles and genotypes between the patient and control groups were carried out using a chi-square test with Yates' correction. Data are presented as the mean ± standard deviation (SD) for parametric variables and as percentages for non-parametric values. Allele and genotype frequencies were calculated and compared between groups by non-parametric tests, and followed by Fisher's exact test. A p value of less than 0.05 was considered statistically significant.

3. Results

This study consisted of 646 subjects including 303 CAD patients and 343 normal subjects as the control group. The mean age of cases and controls were 59.04 ± 10.61 and 52.43 ± 10.89 years respectively. The male/female ratio was 1.50 in patients and 0.59 in controls. Deviation from the Hardy-Weinberg equation was not observed in any of the groups ($\chi^2 < 3.84$, df = 1, $p < 0.05$). The allele and genotype distribution of *IL-10* polymorphisms in CAD patients and matched controls are shown in Table 3. Results of this table show that there is a significant association between *IL-10* (−819) T allele, and *IL-10*(−819) T/T genotype and CAD (p = 0.041 and p = 0.042 respectively). In addition, there is a significant asso-

Table 1
Information of primers used in this study.

Primer name [reference]	Direction	Sequence (5′ – 3′)	Size (bp)	Gene accession number
IL-10 (−819) C/T [8]	Forward C	CCCTTGTACAGGTGATGTAAC	234	NG_012088
	Forward T	ACCCTTGTACAGGTGATGTAAT		
	Reverse	AGGATGTGTTCCAGGCTCCT		
IL-10 (−1082) G/A [8]	Forward G	CTACTAAGGCTTCTTTGGGAG	258	
	Forward A	ACTAGTAAGGCTTCTTTGGGAA		
	Reverse	CAGTGCGAACTGAGAATTTGG		
hGH [6]	Forward	GCCTTCCCAACCATTCCCTTA	430	NG_011676.1
	Reverse	TCACGGATTTCTGTTGTGTTTC		
IL-10 cDNA [9]	Forward	GTGATGCCCCAAGCTGAGA	138	NM_000572
	Reverse	CACGGCCTTGCTCTTGTTTT		
PGK1 cDNA [6]	Forward	GCAGATTGTGTGGAATGGTC	101	NM_000291.3
	Reverse	CCCTAGAAGTGGCTTTCACC		