Human Immunology 76 (2015) 13–18

Contents lists available at ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

TNF-alpha-308G>A polymorphism and the risk of familial CAD in a Pakistani population

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ARTICLE INFO

Article history:
Received 13 December 2013
Accepted 3 December 2014
Available online 11 December 2014

Keywords:
TNF-alpha
Polymorphism
TDT
Association
Familial CAD

ABSTRACT

A case–control and trio-families study was performed to establish a potential association between TNF-alpha gene promoter SNPs at -308 and -238, and occurrence of CAD in a Pakistani population. In the first phase, 150 patients and 150 controls were enrolled in the case–control association study. In the second phase, heritability of susceptible alleles was investigated from 88 trio-families with CAD affected offspring. Biochemical analysis of lipids and hs-CRP was carried out spectrophotometrically, while serum TNF-alpha concentrations were determined by enzyme-linked immunosorbent assay. Genotyping of the TNF-alpha SNPs were determined by PCR-RFLP method. Elevated serum TNF-alpha and hs-CRP were observed from CAD vs. controls ($P < 0.0001$; for both). The evaluation of TNF-alpha-308G>A polymorphism in case–control study revealed that the said SNP was significantly associated with the increased risk of CAD. The findings demonstrated a significant link between the TNF-alpha variant allele A at -308 and CAD ($P = 0.0035$), whereas the -238 SNP was not associated with the disease. Haplotype A–G of the TNF-alpha gene at -308G>A and -238G>A showed higher frequency in the patient group compared with controls ($P < 0.05$). Moreover, data showed preferential transmission of the disease susceptible allele A at TNF-alpha-308 from parent to affected offspring in a trio-family study ($P < 0.0001$). The current research leads to conclusion that the TNF-alpha-308G>A polymorphism is associated with CAD in the study population. Furthermore, for the first time, we showed that the TNF-alpha-308A allele was significantly associated with the familial CAD in our high risk population.

1. Introduction

Coronary artery disease (CAD) is a complex disorder resulting from interactions between environmental and genetic factors and has been accepted as the leading cause of death around the world. Mortality and morbidity can be reduced by at least 40% by treating the known risk factors [1]. Familial genetic studies suggest that genetic propensity accounts for about 50% of susceptibility to CAD [2]. Despite significant efforts and the wide range of strategies employed, genetic variants of CAD still remain elusive. The use of candidate genes in case–control study designs to investigate complex disorders has become an increasingly preferred method owing to its cost effectiveness and reasonably high success rates. However, case–control association studies have some intrinsic limitations: the population stratification, it is important to use a family based approach to purge population stratification [3]. A transmission disequilibrium test (TDT) has been used to demonstrate the association between susceptible allele and disease in parents-offspring trios [4,5]. The TDT is a useful statistical analysis in familial genetic studies and it is protected against the population stratification [6].

Inflammatory cytokines have been shown to be significant predictors in the development of CAD. In terms of association studies, TNF-alpha is a well studied risk marker for CAD [7]. Several single nucleotide polymorphisms (SNPs) have been identified in the TNF-alpha gene [8], while functional SNPs in the regulatory region of TNF-alpha gene have an important role in regulation of TNF-alpha production [9]. Several studies have reported the link of TNF-alpha-308G>A and -238G>A polymorphisms with cardiovascular disease, however, the results are inconclusive [10]. Some case–control studies support the association between TNF-alpha polymorphisms and CAD [11–13]; while others failed to demonstrate a significant link with the disease [7,14,15]. Since,

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http://dx.doi.org/10.1016/j.humimm.2014.12.010
0198-8859/

controversy exists regarding the role of TNF-alpha gene polymorphisms along with the circulating levels in the development of cardiovascular complications. This inconsistency requires further investigations to clarify the issue of TNF-alpha gene polymorphisms and its link with the disease, as there is no clear evidence available to demonstrate the association between TNF-alpha circulating levels, polymorphism and familial CAD. Therefore, we hypothesized that TNF-alpha polymorphism at -308 and -238 might be a genetic risk factor for CAD in our high risk population. We investigate the distribution of both SNPs of TNF-alpha gene in sporadic CAD patients and controls. After the significant association between TNF-Alpha-308G>A polymorphism and CAD, we further examine the transmission of polymorphic alleles at -308 and -238 from familial history parents to affected offspring using TDT method. To our knowledge, this is the first and comprehensive case–control and family trios study to assess the association between TNF-alpha polymorphisms and CAD in a Pakistani population. The relationship of TNF-alpha genotypes with circulating levels of TNF-alpha has not been reported yet in patients with CAD. Therefore, the present trio family study is novel which underscoring the genetic insights of CAD in such a high risk population.

2. Materials and methods

2.1. Study population

The work presented here includes biochemical and genetic analyses of the study samples comprising of patients with CAD and their respective controls. The present study protocol is adherent to the Helsinki Declaration of 1975 as revised in 1997 and was approved from the Institutional Review Board (IRB), Quaid-i-Azam University, Islamabad. All participants signed informed consents for the genetic and biochemical analyses, including demographic observations. In this study, 150 patients (mean age 48.9 ± 10.2) with documented CAD and 150 healthy control subjects (mean age 50.2 ± 10.5) were enrolled for the case–control association of TNF-alpha gene polymorphisms. We ascertained those patients whose were not in blood relation with controls. The inclusion criterion was applied for both, the patients and the controls in this study. Study subjects were evaluated by physical and clinical history, ECG, and coronary angiography. The patients were confirmed on the basis of angiographic criteria established by Ledru et al. [16]. Control subjects were from the same ethnic region and their clinical histories were reviewed by a cardiologist being unaware of the objectives of study. The control subjects lacked any history of cardiac disease or any symptoms of other type of CVD were included. The subjects for the case–control analysis and the TDT were not overlapping. Eighty-eight (88) trio-families were investigated for the transmission of the risk allele to affected offspring using TDT. For TDT analysis, 72 and 52 trios were informative for TNF-alpha-308G>A and -238G>A allele transmission, respectively.

2.2. Sample collection and processing

Standard protocol was adopted for serum and DNA isolation from whole blood samples as described earlier [4]. Biochemical analysis of TC, TG, LDL and HDL were carried out by using AMP Diagnostic kits (Austria). Assays were performed according to manufacturing recommendation by using Vitalab Selectra E chemistry analyzer (Netherlands). High-sensitive C-reactive protein (hs-CRP) and TNF-alpha concentrations were determined by using Roche/Hitachi-904 chemistry analyzer, Roche Diagnostics (Indianapolis, USA), and AMP Platos R 496 Micro-plate reader (AMP Diagnostics, Austria), respectively. The TNF-alpha gene -308G>A polymorphism was investigated by the polymerase chain reaction (PCR) using forward primer F-5′-AGG CAA TAG GTT TTG AGG GCC AT-3′and reverse primer R-5′-CAT CAA GGA TAC CCC TCA CAC TC-3′. For the -238G>A polymorphism of the cytokine gene, forward primer F-5′-AGA AGA CCC CCC TCG GAA CC-3′ and reverse primer R-5′-ATC TGG AGG AAG CGG TAG TG-3′ were used for amplification. PCR amplification was carried out in 0.2 mL tubes (Axygen, CA, USA) in a total volume of 50 μL. The reaction mixture contained 3 μL of genomic DNA, 2.5 μL of each forward and reverse primer (20 μM stock), 5 μL of 10× PCR buffer (200 mM of $(NH4)2SO_4$, 750 mM of Tris–HCl (pH 8.8), and 0.1% Tween 20), 4 μL of 25 mM $MgCl_2$ (MBI-Fermentas, England), 1 μL of 10 mM dNTPs (MBI-Fermentas, England), and 0.5 μL (5 U/μL) of *Taq* DNA polymerase (MBI Fermentas, England) in 31.5 μL of PCR water. PCR reactions were performed by means of GeneAmp PCR System 9700 (Applied Biosystems Inc, Foster City, CA, USA). PCR was carried out with the following thermal cycling conditions: an initial denaturation step at 94 °C for 12 min, followed by amplification for 35 cycles at 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min, followed by a final extension step at 72 °C for 2 min. The PCR products were analyzed by 2% agarose gel electrophoresis.

N*co*I (MBI-Fermentas, England) restriction endonuclease was used for the detection of the TNF-alpha gene -308GA polymorphism. RFLP was performed in 0.2 mL tubes (Axygen, CA, USA) in a total volume of 20 μL containing 12 μL of amplified products, 2 μL of 10× G buffer (10 mM Tris–HCl (pH 7.5), 10 mM MgCl2, 50 mM NaCl, and 0.1 mg/mL BSA), 0.3 μL of N*co*I enzyme, and 5.7 μL of PCR water. Digested products were analyzed by 3% agarose gel electrophoresis and visualized under ultraviolet light. Similarly, M*sp*I (MBIFermantas, England) enzyme was used to investigate the TNF-alpha gene -238G>A polymorphism using 10× R buffer (10 mM Tris HCl (pH 8.5), 10 mM $MgCl_2$, 100mMKCl, and 0.1 mg/mL BSA).The digested products were subjected to 3% agarose gel electrophoresis and visualized under ultraviolet light.

2.3. Statistical analysis

Basic and biochemical variables are mentioned as mean ± SD. Comparison of these variables between patients and control was carried out by Chi-Square test and independent samples *t*-test. Allele and genotype frequencies were calculated by direct counting. Hardy–Weinberg equilibrium (HWE) was calculated using the software Arlequin V3.0. Fisher's exact test, used for comparison between the patient and controls groups, odds ratio (OR) and 95% confidence intervals were calculated using the statistical software GraphPad Instat 3.05 for 2 way contingency table analysis (GraphPad Software Inc., San Diego, Calif.). The TDT was used for the association of TNF-alpha polymorphisms at -308 and -238 with familial CAD. The allele transmitted from parent to an affected offspring and the allele not transmitted by the same parent to the same offspring is treated as a case–control pair, and the analysis was carried out by using the McNemar test. An excess of a particular allele transmitted from heterozygous parents to affected offspring is taken as evidence of an association between disease and that allele. Our analyses were restricted to the trios with both parents typed. In trios where both parents and offspring were heterozygous considered as uninformative and excluded from the transmission disequilibrium analysis. *P* values <0.05 were considered as statistically significant.

3. Results

3.1. Baseline characteristics and clinical parameters of the study subjects

Clinical and biochemical characteristics of patients with CAD and those of healthy controls are summarized in Table 1. Patients