−308G>A and −1031T>C tumor necrosis factor gene polymorphisms in Tunisian patients with coronary artery disease

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Abstract

Background: Recent research has shown that inflammation plays a key role in coronary artery disease (CAD) and other manifestations of atherosclerosis. Several lines of evidence support a key role for tumor necrosis factor-α (TNF-α), a potent immunomodulator and pro-inflammatory cytokine, in the development of atherosclerosis and in complications of CAD.
Methods: We investigated the possible association between CAD and the *TNF* gene promoter polymorphisms −308G>A and −1031T>C in a Tunisian population. We compared the distribution of these polymorphisms between 418 patients with CAD and 406 healthy controls using polymerase chain reaction restriction fragment length-polymorphism analysis.
Results: The frequency of the TNF-α −308A allele in the control group was similar to that observed in CAD patients [p=0.78; odds ratio (OR)=1.15; 95% confidence interval (CI)=0.86–1.55], but higher than those described in other Europeans, such as in the French, Finnish and Spanish. Concerning the TNF-α −1031T/C polymorphism, the same distribution was observed between patients with CAD and controls (p=0.12; OR=1.27; 95% CI=0.94–1.72). In addition, the genotype and allele frequencies of control individuals were comparable to those previously reported in healthy Tunisian controls and other ethnic groups. Haplotype analysis (TNF-α −308G>A and −1031T>C) demonstrated no significant association between *TNF* haplotypes and CAD.

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Conclusions: We conclude that *TNF* promoter gene polymorphisms at position −308G>A and −1031T>C do not play a major role in the pathogenesis of CAD in the Tunisian population.
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Introduction

Cardiovascular disease, including coronary artery disease (CAD), is the leading cause of death in most industrialized nations and will soon be the leading cause of death in the developing world (1, 2). CAD is a chronic inflammatory process in which pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukine-6 (IL-6) may play a crucial role in its pathogenesis (3, 4). In recent years, markers of subclinical inflammation and subclinical atherosclerosis have been studied in order to try to understand how they predict adverse outcomes in patients with CAD (5). Several lines of evidence support a key role for TNF-α in the development of atherosclerosis and its complications (6). High concentrations of plasma-soluble TNF and its soluble receptors are considered to play a central part in the inflammatory cascade that is a key feature in cardiovascular diseases, such as atherosclerosis (7, 8), CAD (9) and acute myocardial infarction (MI) (10, 11). Prospective studies suggest that elevated concentrations of TNF-α are independent predictors of first-time cardiovascular disease (12) and that it is also a marker for recurrent coronary events following a previous MI (7). Circulating concentrations of TNF are increased in patients with ischemic artery disease (13), and TNF-α has been shown to reduce the bioavailability of nitric oxide (NO), a potent endothelium-derived vasodilator, in cultured endothelial cells (14). Furthermore, TNF-α concentrations correlate with progression of early carotid atherosclerosis (15). It also stimulates endothelial expression of adhesion molecules for mononuclear cells and induces endothelial apoptosis (16). These processes are highly relevant to the initiation and progression of atherosclerosis. While many factors can affect TNF-α production, genetic regulation also plays a significant role. Among the many DNA variants in the TNF-α gene, a G to A transition at the −308 bp position (*Nco*I polymorphism) was shown to be associated with increased promoter activity (17), and increased plasma TNF-α concentrations (18) and is considered to play an important pathogenic role

(19). We also chose to genotype TNF-α T-1031C on the basis of its association with increased TNF-α secretion, and its implication in susceptibility to several autoimmune diseases, such as systemic lupus erythematosus, insulin-dependent diabetes, and inflammatory bowel disease (20, 21).

The goal of the present study was to investigate the potential role played by two *TNF* promoter single nucleotide polymorphisms (SNPs) and the subsequent risk of CAD in a population sample of 406 controls and 418 patients with CAD from whom information regarding most known risk factors for CAD had been obtained.

Subjects and methods

Patient and control groups

All participants were genetically unrelated Tunisian subjects. The study population included 418 patients with CAD (331 males and 87 females, mean age 58.1 $\pm$ 12.0 years) who were seen at the Farhat Hached Hospital in Sousse, Tunisia. Patients enrolled in our study had evidence of CAD documented by coronary angiography (presence of one or more coronary arteries with $\geq$ 50% stenosis), prior cardiac bypass surgery or documented acute coronary syndrome. Diagnosis of MI was confirmed following review of the patients' records using the World Health Organization (WHO) (22) criteria based on the diagnosis of chest pain and clinical symptoms, increases in cardiac markers or electrocardiographic changes. None of the study patients had evidence of significant atherosclerotic vascular diseases, auto immune disease, cancer, renal or hepatic disease. The control group comprised 406 apparently healthy subjects (299 males and 107 females, mean age 56.7 $\pm$ 14.1 years), undergoing pre-employment examination. Written informed consent was obtained from all participants after explanation of the goals and details of the study. The study was approved by the Ethics Committee of Sousse, and all institutional ethnic requirements were met.

During the interview, a standard questionnaire was used to carefully ascertain information on smoking habits, history of medication for hypertension, diabetes mellitus and hypercholesterolemia. Serum lipids [triglycerides, total cholesterol, low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol] were measured. For coronary risk factors, the following definitions were used: individuals were defined as hypertensive if their systolic blood pressure was $\geq$ 140 mm Hg and/or diastolic blood pressure was $\geq$ 90 mm Hg on more than one occasion, or taking antihypertensive medication. Blood pressure (right arm) was measured twice, using mercury sphygmomanometer with participants in the sitting position following a 5-min rest; the mean of two readings measured 1 min apart was used. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2). Individuals with a history of diabetes mellitus or those receiving medication for diabetes were considered to be diabetic. Smoking history was coded as 'never' or 'current smoker'.

Gene analysis

Peripheral blood was collected, separated within 1 h and the samples maintained at –80°C until analysis. Genomic DNA was extracted from blood leukocytes using the proteinase K/salting-out method. TNF-α gene polymorphisms –308G>A and –1031T>C were determined using polymerase chain reaction-restriction fragment-length polymorphism analysis. The DNA was amplified using the following primers: for TNF-α –308G>A: sense 5'-GAG GCA ATA GGT TTT GAG GGC CAT-3', antisense 5'-GGG ACA CAC AAG CAT CAA G-3'; for TNF-α –1031T>C: sense 5'-TATGTGATGGACTCAC-CAGGT-3', anti-sense 5'-CCTCTACATGGCCCTGTCTT-3'; Genotype determination was made after restriction enzyme digestion (*Nco*l for TNF-α –308G>A and *Bbs*l for TNF-α –1031T>C). Digested fragments were separated by electrophoresis on 3% ethidium bromide-staining agarose gels, and were visualized by UV transillumination.

Statistical analysis

Statistical analysis was performed with SPSS version 13.0 statistics software (SPSS, Chicago, IL, USA). Comparisons of categorical variables were performed with the Pearson χ^2-test. Continuous variables were compared with Student's t-test. Data were expressed as mean $\pm$ standard deviation (SD) (continuous variables) or as percentages of the total (categoric variables). The overall power (69.2%) was calculated as the average power over the two TNF-α SNPs that were genotyped (Genetic Power Calculator; SGDP Statistical Genetics Group). Allele frequencies were calculated using the gene-counting method, and both polymorphisms were tested for Hardy-Weinberg equilibrium using the χ^2 goodness-of-fit-test with HPlus 2.5 software. Comparisons of the genotype and allele frequencies of the gene between cases and controls were performed using the Pearson χ^2-test and Fisher's exact tests. The degree of linkage disequilibrium (LD) between polymorphisms was assessed using the Thesias software (http://genecanvas.ecgene.net) (23). For all analyses, odds ratios (OR) and their 95% confidence intervals (CI) were calculated assuming an additive effect of alleles. The significance level was p<0.05.

Results

Study subjects

Table 1 shows the demographics and clinical biochemistry parameters of individuals in the CAD and control groups. CAD cases and controls were matched for age and gender, but typical differences for several conventional risk factors for CAD including habitual smoking, hypertension, diabetes mellitus, and BMI were observed (all p<0.001). The CAD group showed higher concentrations of triglyceride, total cholesterol, LDL-cholesterol, and lower concentrations of HDL-cholesterol compared with the control group. We also found a statistically significant difference between cases and controls with respect to systolic and diastolic blood pressure, uric acid, urea and creatinine.

TNF-α genotyping

The association of G-308A and T-1031C *TNF* polymorphisms with CAD risk was examined. There was no significant departure from Hardy-Weinberg equilibrium among participants in the genotype frequency distributions of the two polymorphisms. TNF-α –308A allele frequency was 19.6 in CAD patients and 19.0 in healthy controls (p=0.78; OR=1.15; 95% CI=0.86–

Table 1 Clinical characteristics of patients with coronary artery disease and controls.

Characteristics	Controls (n = 406)	Patients (n = 418)	p-Value
Females, n (%)	107 (26.4)	87 (20.8)	0.071
Mean age, years	56.7 ± 14.1	58.1 ± 12.0	0.092
Mean BMI, kg/m^2	25.22 ± 2.35	27.08 ± 4.20	< 0.001
Hypertension, n (%)	54 (13.3)	134 (32.1)	< 0.001
Systolic BP, mm Hg	120.0 ± 13.7	133.8 ± 26.4	< 0.001
Diastolic BP, mm Hg	73.7 ± 8.9	76.2 ± 13.7	0.006
Fasting glucose, mmol/L	5.7 ± 1.7	9.5 ± 5.3	< 0.001
Diabetes, n (%)	45 (11.1)	198 (47.4)	< 0.001
Smoking, n (%)	104 (27.4)	234 (56.8)	< 0.001
Urea, mmol/L	4.64 ± 1.86	6.77 ± 4.31	< 0.001
Uric acid, mmol/L	301 ± 106	292 ± 119	0.59
Creatinine, mmol/L	75 ± 16	104 ± 72	< 0.001
Total cholesterol, mmol/L	3.98 ± 1.24	4.78 ± 1.22	< 0.001
Triglycerides, mmol/L	1.35 ± 0.86	1.76 ± 0.95	< 0.001
HDL-cholesterol, mmol/L	1.30 ± 0.44	1.07 ± 0.45	< 0.001
LDL-cholesterol, mmol/L	2.27 ± 1.19	3.00 ± 1.09	< 0.001
LDL/HDL ratio	2.00 ± 1.39	3.50 ± 2.76	< 0.001

For continuous variables, mean and SD are given. BP, blood pressure.

Table 2 TNF-α G-308A and T-1031C genotypic and allelic distributions.

SNP	Genotype frequency, %			p[a]	Allele frequency, %		p[a]
	G/G	G/A	A/A		G	A	
G-308A				0.43			0.78
Controls[b]	267 (65.8)[b]	124 (30.5)	15 (3.7)		81.0	19.0	
Patients[b]	265 (63.4)	142 (34.0)	11 (2.6)		80.4	19.6	
	T/T	T/C	C/C		T	C	
T-1031C				0.25			0.12
Controls[b]	284 (70.0)	111 (27.3)	11 (2.7)		83.6	16.4	
Patients[b]	270 (64.6)	134 (32.1)	14 (3.3)		80.6	19.4	

[a]Pearson's χ^2-test; [b]number (% of total). SNP, single nucleotide polymorphism.

1.55). Similarly, TNF-α –1031C allele frequency was 19.4 in CAD patients and 16.4 in healthy controls (p = 0.12; OR = 1.27; 95% CI = 0.94–1.72) (Table 2). Based on the calculated OR, the calculated power of –308G/A (66.49%) and –1031T/C (71.79%) was obtained, which translated to an overall power of 69.14%. In this study, the frequency of *TNF* G-308A (p = 0.43) and *TNF* T-1031C (p = 0.25) genotypes were not significantly different between patients with CAD and healthy controls (Table 2).

Haplotype distribution

Table 3 shows the two-locus *TNF* haplotype analysis stratified by study subjects. Of the four possible *TNF* haplotypes, none was associated with CAD. The 'double-mutant' haplotype (–308A/–1031C) was uncommon, and was present at very low frequencies in both controls and patients. Using the –308G/–1031T haplotype as reference, no significant association between the two remaining common haplotypes and CAD was observed.

Discussion

Previous cardiovascular studies have investigated the association between several SNPs located in the promoter region of the TNF-α gene and CAD or atherosclerosis (24–27), but conclusive data regarding the role of TNF-α genotypes in CAD pathogenesis is need-

Table 3 TNF-α G-308A and T-1031C haplotype distribution.

Haplotype	Controls	Patients	OR (95% CI)	
Common haplotypes				
–308G/–1031T	0.66	0.62	1[a]	
–308A/–1031T	0.17	0.18	1.09 (0.82–1.45)	p = 0.54
–308G/–1031C	0.14	0.17	1.26 (0.95–1.67)	p = 0.1
Uncommon haplotypes				
–308A/–1031C	0.01	0.01	1.01 (0.24–4.15)	p = 0.98

[a]Haplotype frequency determined by the maximum likelihood method; df = 3; χ^2 = 2.05; p = 0.56. OR, odds ratio; CI, confidence interval.

ed. The present study investigated the association between two biallelic polymorphisms of the TNF-α gene and their relation to CAD in an Arab African population. The polymorphisms studied were two common substitutions in the 5'-flanking region.

No differences were found in the distribution of allelic or genotypic frequencies of TNF-α G-308A and TNF-α T-1031C polymorphisms between patients with CAD and healthy controls. In our hands, the frequency of the TNF-α –308A allele in the control group was similar to that observed in CAD patients. The lack of association between –308 TNF-α polymorphism and CAD is consistent with other studies (24, 28), and confirm the results of Francis et al. (29) who found no association between TNF-α –308A allele and clinically or angiographically-documented coronary stenosis. Our data confirm some previous studies that investigated Caucasian populations and failed to demonstrate that this locus could be a marker for greater risk of CAD. The same studies affirm that the allelic distribution is different according to the geographical origin of the study group (ranging from 24% to 12% for the rare allele) (25, 30). However, in contrast to other published data (26, 31), this may be due to different genetic and environmental risk factors or to different selection criteria of CAD patients.

Concerning the TNF-α –1031T/C polymorphism, the same distribution was observed between CAD patients and controls. The frequency of the –1031C allele was similar to that reported in a case control study on 1213 post-MI patients and 1561 healthy controls (32). In addition, the genotype and allele frequencies of control individuals were comparable to those previously reported for healthy Tunisian controls (33), which were similar to frequencies established for other ethnic groups, including healthy Swedish (32) and pan-Brazilian (34) individuals. The association between the two TNF-α loci and CAD was also studied by haplotype analysis. Of the possible four haplotypes, the ''doublemutant'' –308A/–1031C haplotype was under-represented in both groups, and no significant preferential association with incident CAD was seen.

In conclusion, our study demonstrates that neither –308G>A nor –1031T>C TNF-α gene polymorphisms are significantly associated with CAD among Tunisians, even before controlling for potential confounders. However, our study has some limitations. First, we were not able to measure plasma TNF-α concentrations in patients and controls. It was also underpowered; a total of 636 (–308G/A) or 517 (–1031T/C) patients (compared to the current 418 patients) would have been needed for detection with 80% power. In addition, our study was limited to a specific ethnic group (North African Tunisian Arabs), thereby necessitating follow-up studies in patients from other ethnic groups. Another limitation includes the selection of the control group; while deemed healthy based on the routine pre-employment health examination, the possibility that some of the controls that were included had asymptomatic stenosis needs follow-up. Also, the potential linkage of the TNF-α promoter polymor-

phisms studied with other TNF-α gene variants, or possibly polymorphisms in nearby genes (HLA region) cannot be excluded.

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