

RESEARCH ARTICLE

Functional interleukin-10 promoter variants in coronary artery disease patients in Tunisia

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ABSTRACT. *Objectives.* The contribution of interleukin (IL)-10 promoter variants -1082G/A, -819C/T, and -592C/A to the risk of coronary artery disease (CAD) was investigated in 291 CAD patients and 291 age- and gender-matched control subjects. *Methods and results.* IL-10 genotyping was performed using PCR-allele-specific amplification (PCR-ASA). Regression analysis was employed in assessing the contribution of the IL-10 variants to the overall CAD risk. A higher frequency of the -592A allele ($p = 0.004$), but not the -1082A ($p = 0.828$) or -819T ($p = 0.952$) alleles, was seen in CAD patients. A higher frequency of -592C/A ($p = 0.011$), and a lower frequency of -592C/C ($p = 0.015$) genotypes was noted in patients compared to healthy controls. Regression analysis demonstrated an association of -592C/A [OR (95% CI) = 1.82 (1.02-3.23)] and -592A/A [OR (95% CI) = 3.33 (1.27-9.09)] genotypes with 1-artery disease. Haplotype analysis revealed that none of the eight possible IL-10 haplotypes was associated with CAD or with the severity of CAD, and was confirmed by multivariate regression analysis, after adjusting for a number of confounders (smoking, systolic and diastolic blood pressure, hypertension, diabetes, glucose, cholesterol, and triglycerides). *Conclusions.* Our results suggest that the -592C/A, more so than the -1082G/A or the -819C/T IL-10 promoter variant alleles, may be considered to be a risk factor for CAD in Tunisians.

Keywords: interleukin-10, PCR, gene polymorphism, coronary artery disease

Acute coronary events are the consequence of the rupture of coronary atherosclerotic plaque and thrombus development [1, 2], and their pathogenesis is influenced by a fine balance between (pro-inflammatory) T helper (Th)1 and (anti-inflammatory) Th2 cytokines [3, 4]. In this regard, it was demonstrated that interleukin (IL)-10, a pleiotropic, anti-inflammatory cytokine, is expressed in coronary plaques, and contributes to reduced inflammation and coagulation during atherosclerotic and thrombotic processes [5]. Functionally, IL-10 acts by inhibiting Th1 cytokine production, induction of apoptosis and tissue factor (TF) expression by activated monocytes/macrophages [6-8], and downregulation of thrombin generation triggered by TF-expressing monocytes and TF-bound microparticles [9]. In addition, IL10 stimulates B-cell growth and lipid accumulation in oxidized, low-density lipoprotein (LDL)-stimulated macrophages [10].

IL-10 is produced by activated Th2 cells, B-cells, monocytes and macrophages, and its production is genetically controlled, with a reported two thirds of the variance in IL-10 production being genetically determined [11].

Several IL-10 gene variants, mainly in the promoter region, have been associated with altered IL-10 production [11, 12]. These include -1082A/G, -819T/C and -592C/A single nucleotide polymorphisms (SNP), which are associated with altered IL-10 secretion as demonstrated by lower transcriptional activity and IL-10 secretion linked with the ATA haplotype [11].

Coronary artery disease (CAD) is viewed as a state of low-grade, chronic inflammation [13], and a role for IL-10 in its pathogenesis has been proposed [14, 15]. This was demonstrated by studies involving IL-10-transgenic and IL-10-knockout mouse models, in which increased IL-10 expression reduced both early and advanced atherosclerotic lesions [10, 16]. In addition, serum IL-10 levels induced by specific IL-10 promoter polymorphisms, were associated with the development of coronary events and their outcome by some [13, 17, 18], but not all [19, 20] studies. Here we investigated the notion that the IL-10 promoter -1082G/A, -819C/T, and -592C/A variants, by regulating IL-10 production, influence the pathogenesis of CAD in Tunisian population.

DONORS AND METHODS

Study subjects

This was a retrospective, case-control study involving 291 consecutive CAD patients (220 males and 71 females), who were admitted for cardiac catheterization at Menzel Bourguiba Hospital Centre (Bizerte, Tunisia) and CHU Fattouma Bourguiba (Monastir, Tunisia), and 291 age- ($p = 0.69$) and gender-matched ($p = 1.00$) healthy controls from the same geographical area; all subjects were of Tunisian Arab origin (Berbers excluded) (table 1). The study was carried out in accordance with the Helsinki Declaration of 1975 guidelines, and was approved by the University of Monastir's institutional review board. Informed consent was obtained from all subjects.

The diagnosis of CAD was based on coronary angiography performed using the standard femoral or brachial approach (at least $\geq 50\%$ stenosis in one of the coronary arteries), prior cardiac bypass surgery or documented acute coronary syndrome (including elevation in cardiac enzymes or typical ECG changes). A complete clinical history, including cardiovascular risk factors, was taken from all participants. Diabetes mellitus was assessed according to elevated fasting blood glucose (WHO criteria: > 7.0 mmol/dL), and/or use of glucose-lowering drugs (including insulin). Obesity was defined as a body mass index (BMI) of 30 kg/m^2 or higher, and hypertension was defined according to seated blood pressure readings of $> 140/90$ mmHg on two separate occasions, and/or the use of anti-hypertensive therapy (ACE inhibitors, angiotensin receptor blockers, beta blockers, diuretics, and calcium antagonists). Hyperlipidemia was defined as fasting total cholesterol > 5.17 mmol/L (200 mg/dL), LDL-cholesterol > 3.36 mmol/L (130 mg/dL), fasting

triglycerides > 2.66 mmol/L (200 mg/dL), or treatment with lipid-lowering medication. The severity of CAD was defined according to the presence of a $\geq 50\%$ stenosis in one (*one-vessel*), and $\geq 50\%$ stenosis in two (*two-vessel*) or three (*three-vessel*) of the major coronary arteries [21]. The control subjects comprised 291 healthy subjects (220 males and 71 females), who were undergoing a routine checkup as part of pre-employment requirements, which included ECG and chest X-ray, and serum analysis (table 1). As their physical examination was unremarkable, coupled with the absence of a personal or family history of, and reasons to suspect CAD, they were classified as healthy. Demographic details for all participants were assessed by a standardized questionnaire, and included age, gender, BMI, age-at-onset and duration of disease, angiography, ECG results, associated co-morbidities, and CAD treatment. Fasting venous blood samples were collected from all participants into plain tubes [creatinine phosphokinase (CPK), glucose and serum lipids determination], and EDTA-treated vacuum tubes for DNA extraction; phlebotomy being performed on patients and controls at 8.00 am (± 30 min). While most of the requested information was provided for each study subject, the missing historical information was verified from the clinic records where available; any remaining unavailable information was entered into the SPSS file as "missing data".

Biochemical analysis

Venous blood samples were taken for measuring glucose following an overnight fast. Samples were collected in fluoride oxalate tubes using the hexokinase method (Cobas Integra 800; Roche, Mannheim, Germany). Serum lipids [total cholesterol, high-density lipoprotein

Table 1
Characteristics of study participants

	Cases (n = 291)	Controls (n = 291)	p ¹
Mean age $\pm$ SD (years)	56.7 $\pm$ 12.2	56.3 $\pm$ 12.5	0.692
Gender (M:F)	220:71	220:71	1.00
Smokers (%)	120 (44.3) ²	81 (30.3) ²	1.6×10^{-15}
Systolic BP (mmHg)	132.2 $\pm$ 21.0	119.8 $\pm$ 12.2	1.2×10^{-16}
Diastolic BP (mmHg)	75.0 $\pm$ 11.5	73.3 $\pm$ 8.6	0.046
Hypertension	118 (41.4) ²	39 (13.4) ²	5.8×10^{-14}
CAD severity:			
1-vessel	163 (56.0)	N/A	N/A
2-vessel	63 (21.6)	N/A	N/A
3-vessel	65 (22.3)	N/A	N/A
BMI	27.0 $\pm$ 4.1	24.8 $\pm$ 1.6	1.3×10^{-14}
Diabetes	126 (43.9) ²	35 (12.0) ²	1.1×10^{-14}
Glucose (mmol/L)	8.9 $\pm$ 4.5	5.5 $\pm$ 1.3	3.4×10^{-27}
Cholesterol (mmol/L)	4.9 $\pm$ 1.2	4.3 $\pm$ 1.2	2.7×10^{-8}
Triglycerides (mmol/L)	1.8 $\pm$ 1.2	1.6 $\pm$ 0.8	0.041
HDL (mmol/L)	1.1 $\pm$ 0.4	1.2 $\pm$ 0.4	0.084
CPK (U/L)	156.0 (19.0-3312.0) ³	133.0 (38.0-365.0)	0.297
LDH (U/L)	497.0 (472.0-993.0)	402.0 (370.0-415.0)	0.014
CRP (mg/L)	6.0 (2.0-93.0)	2.3 (1.0-5.1)	0.004
Fibrinogen (g/L)	4.6 (4.4-8.3)	3.5 (3.4-4.0)	0.014

¹ Student's *t* test (2-sided) for continuous variables, Pearson's chi square test for categorical variables.

² Number of subjects (percentage of total).

³ Median (range).

(HDL) and LDL, and triglycerides] were measured using an enzymatic colorimetric method (Integra 800; Roche); serum creatinine was assayed using the Jaffe reaction method (Integra 800). Additional testing for liver function tests, renal function tests, serum electrolytes, was performed using a Dade Boehringer instrument.

IL-10 genotyping

IL-10 genotyping was performed using PCR-allele-specific amplification (PCR-ASA). Quality control for DNA amplification was evaluated using the Factor IX gene. Positive controls were selected by amplifying and sequencing two regions of IL10 promoter; the first containing -592 and -819 SNPs (Control 1) for -592C/A and -819C/T, while the second contained -1082 SNP (Control 2). Primer sequences and amplification conditions are listed in *table 2*.

Statistical analysis

Statistical analysis was performed using SPSS v. 15.0 software (SPSS, Chicago, IL, USA). Data were expressed as mean $\pm$ SD (continuous variables), or as percentages of the total (categorical variables). The overall power was calculated as the average power over the genotyped SNPs (Genetic Power Calculator; SGDP Statistical Genetics Group). Based on the calculated OR, the calculated power of -1082G/A (76.22%), -819C/T (57.24%), and -592C/A (81.34%) was obtained, which translated to an overall study power of 71.6%. Pearson χ^2 or Fisher's exact test were used to assess inter-group significance, and Student's *t*-test was used to determine differences in means. Allele frequencies were calculated by the gene counting method, and each polymorphism was tested for Hardy-Weinberg equilibrium using χ^2 goodness-of-fit test (HPlus 2.5).

IL-10 haplotype estimation was performed using the expectation maximization method, where the sum of the probability estimates for all possible haplotypes equals 1.00. Where haplotype assignment was uncertain (heterozygous carriers), the haplotype assignment probability estimate was used to determine the individual contribution to that haplotype. IL-10 haplotypes were coded as per the allele

at each locus; the first letter refers to -1082 (G, A), the second to -819 (C, T), and the third to -592 (C, A) alleles. Multivariate regression analysis was performed using HPlus 2.5 haplotype analysis software; results being expressed as *P* value, odds ratio (OR) and 95% confidence intervals (CI).

RESULTS

Study subjects

The characteristics of the CAD patients and control subjects are shown in *table 1*. Patients were matched to controls with regards to gender ($p = 1.000$) and age ($p = 0.692$), with CAD patients having a higher BMI ($p < 0.001$), higher systolic ($p < 0.001$) and diastolic ($p = 0.046$) blood pressures, together with higher total cholesterol ($p < 0.001$), glucose ($p < 0.001$), and triglyceride levels ($p = 0.041$). The greater prevalence of hypertensive ($p < 0.001$), diabetic ($p < 0.001$) individuals, and cigarette smokers ($p < 0.001$) were seen in the patient group. In addition, increased lactate dehydrogenase (LDH; $p = 0.014$), C-reactive protein (CRP; $p = 0.004$), and fibrinogen ($p = 0.014$) levels were seen in cases than in control subjects.

Genotype analysis

Genotype frequency distributions of the three IL-10 variants did not deviate from Hardy-Weinberg equilibrium among participants. While -1082A ($p = 0.828$) and -819T ($p = 0.952$) allele frequencies were comparable between CAD patients and controls, a higher frequency of the -592A allele was seen in CAD patients ($p = 0.004$) (*table 3*). A varied distribution of the IL-10 genotypes was noted between CAD patients and controls; a higher frequency of -592C/A ($p = 0.011$), and a lower frequency of -592C/C ($p = 0.015$) genotypes being seen in patients compared to healthy controls. The distribution of IL-10 -1082G/A and -819C/T genotypes was comparable between patients and controls (*table 3*). The genotype distribution of the IL-10 variants studied according to CAD severity is

Table 2
Optimized PCR Conditions

SNP	Primer	Sequence (5'3')	Tm (°C)	Size (bp)
-592C/A	Common	GCTCACTATAAAAATAGAGACGG	53.07	223
	Forward C	CTGGCTTCCTACAGG	44.89	
	Forward A	GACTGGCTTCCTACAGT	45.71	
-819C/T	Common	CTTCTTCCACCCCATCT	51.30	192
	Forward C	GCTCACTATAAAA ATAGAGACGG	49.83	
	Forward T	AACTGAGGCACAGAGATA	46.59	
-1082G/A	Common	GTAAGCTTCTGTGGCTGGAGTC	57.59	161
	Forward G	AACACTACTAAGGCTTCTTTGGGTG	55.06	
	Forward A	AACACTACTAAGGCTTCTTTGGGT A	57.53	
Control 1	Forward	AATCCAGACAACACTACTAAGG	51.39	256
	Reverse	TTCCATTTTACTTTCCAGAG	50.67	
Control 2	Forward	TTTCCAGATATCTGAAGAAGTCCTG	58.80	313
	Reverse	GTAAGCTTCTGTGGCTGGAGTC	58.99	

Table 3
IL-10 allele and genotype frequency

SNP	Allele/genotype	Cases (291)	Controls (291)	p ¹	OR (95% CI)
-1082G/A	-1082A	260 (45.6) ²	204 (44.7)	0.828	1.04 (0.81-1.33)
	G/G	101 (35.4)	76 (33.3)	0.362	1.00 (Reference)
	G/A	108 (37.9)	100 (43.9)	0.313	0.81 (0.54-1.22)
	A/A	76 (26.7)	52 (22.8)	0.686	1.10 (0.69-1.75)
-819C/T	-819T	107 (19.2)	96 (19.2)	0.952	1.00 (0.74-1.36)
	C/C	191 (66.3)	162 (64.8)	0.898	1.00 (Reference)
	C/T	87 (30.2)	80 (32.0)	0.668	0.92 (0.64-1.33)
	T/T	10 (3.5)	8 (3.2)	0.904	1.06 (0.41-2.75)
-592C/A	-592A	159 (27.4)	115 (20.0)	0.004	1.51 (1.15-1.98)
	C/C	156 (53.8)	188 (65.5)	0.015	1.00 (Reference)
	C/A	109 (37.6)	83 (28.9)	0.011	1.58 (1.11-2.26)
	A/A	25 (8.6)	16 (5.6)	0.061	1.88 (0.97-3.65)

¹ Pearson's chi square test.

² Number (percentage of total).

presented in *table 4*. Using the all wild-type genotype as reference, univariate regression analysis showed an association of -592C/A [$p = 0.043$; OR (95% CI) = 1.82 (1.02-3.23)] and -592A/A [$p = 0.015$; OR (95% CI) = 3.33 (1.27-9.09)] genotypes with severity (1-vessel, 2-vessel, 3-vessel disease). There was no significant difference in the distribution of the -1082G/A and -819 C/T genotypes among CAD patients according to the severity of CAD.

Haplotype distribution

Haplotype analysis was performed to explore further the possible association of the IL-10 variants with CAD. None of the eight possible IL-10 haplotypes was asso-

ciated with CAD (*table 5*). Using the (all wild-type) GCC haplotype as reference (OR = 1.00), univariate and multivariate regression analysis confirmed the lack of association between any of the IL-10 haplotypes and CAD, or the severity of CAD, after adjusting for a number of confounders (smoking, systolic and diastolic blood pressure, hypertension, diabetes, glucose, cholesterol, and triglycerides) (*table 5*).

DISCUSSION

We report on a preliminary association between the functionally significant IL-10 promoter variants and the risk of CAD in Tunisians. Our working hypothesis is that CAD is a state of chronic inflammation [2, 22], in which an altered Th1-Th2 cytokine balance significantly influences its outcome [13, 15, 17, 18, 23]. This was based on the findings that the onset of CAD was linked with changes in the levels of inflammatory mediators [15, 24, 25], including pro-inflammatory cytokines (TNF α , IL-6, IL-1 β , and TGF β), which were reportedly elevated in CAD patients [4, 22]. The pathogenesis and progression of CAD is to be viewed as an orchestrated shift in the Th1-Th2 balance towards Th1 dominance, with pro-inflammatory markers and activated leukocytes contributing significantly to the development of CAD [23].

Table 4
Association of IL-10 variants with CAD severity

SNP	Genotype	p	OR (95% CI) ²
-1082G/A	G/G	0.190	1.00 (Reference)
	G/A	0.288	1.43 (0.74-2.74)
	A/A	0.431	0.75 (0.37-1.52)
-819C/T	C/C	0.808	1.00 (Reference)
	C/T	0.514	0.83 (0.46-1.47)
	T/T	0.703	1.26 (0.39-4.09)
-592C/A	C/C	0.019	1.00 (Reference)
	C/A	0.043	1.82 (1.02-3.23)
	A/A	0.015	3.33 (1.27-9.09)

Table 5
IL-10 haplotypes in patients and controls

Haplotype ¹	Patients (282)	Controls (211)	p ²	OR (95% CI)
G C C	0.299 $\pm$ 0.019	0.309 $\pm$ 0.028	0.830	0.96 (0.73-1.26)
A C C	0.314 $\pm$ 0.024	0.326 $\pm$ 0.021	0.832	0.96 (0.73-1.26)
G C A	0.114 $\pm$ 0.021	0.106 $\pm$ 0.020	0.813	1.07 (0.72-1.60)
G T C	0.078 $\pm$ 0.018	0.103 $\pm$ 0.019	0.188	0.73 (0.47-1.12)
A C A	0.085 $\pm$ 0.020	0.056 $\pm$ 0.029	0.118	1.54 (0.92-2.53)
G T A	0.057 $\pm$ 0.019	0.046 $\pm$ 0.016	0.499	1.28 (0.71-2.25)
A T C	0.031 $\pm$ 0.024	0.030 $\pm$ 0.030	0.899	0.98 (0.47-1.99)
A T A	0.022 $\pm$ 0.066	0.024 $\pm$ 0.076	0.971	0.90 (0.39-2.04)

¹ IL-10 haplotype (-1082GA/-819CT/-592CA) frequency determined by the maximum likelihood method.

² Fisher's exact test.

The proposed role for IL-10 in the pathogenesis of CAD was based on a number of observations. IL-10 was detected in human atherosclerotic plaques in association with reduced inducible nitric oxide synthase (iNOS) expression and low levels of cell death, indicating that IL-10 expression may attenuate the inflammatory response, hence promoting plaque healing [7]. IL-10 inhibited IL-12 release (and thus the activation of the Th1 program) by oxidized LDL-stimulated monocytes, and local production of IL-10 counter-regulated IL-12-induced atherosclerotic lesions, thus demonstrating a role for IL-10 in limiting the local immunity in atherosclerosis [26]. IL-10 also modulated coagulation and inflammatory activity in atherosclerosis by inhibiting phosphorylation of key intracellular molecules (ERK1/2, MEK-1/2, and Elk-1) linked to TF expression [6, 8]. Since IL-10 production is genetically controlled, as demonstrated by altered IL-10 secretion mediated by specific IL-10 promoter polymorphisms [11, 16], there have been only a few studies that have examined the possible association of IL-10 promoter gene variants with CAD [13, 17-20]. Our findings support the hypothesis that IL-10 (-592C/A) promoter polymorphisms influence the risk of CAD among Tunisian patients.

A limited number of studies have investigated the possible association between IL-10 promoter variants and CAD, but with inconsistent findings. In our hands, only the -592C/A IL-10 promoter variant was linked with CAD, as the frequencies of -592A allele and -592C/A genotype, more so than the homozygous -592A/A genotype, were higher in CAD patients. Univariate analysis confirmed the association of -592C/A and -592 A/A genotypes with the severity of CAD, suggesting a possible role for the -592C/A SNP as a disease-modifying polymorphism in determining CAD severity. However, we did not establish any significant association of the low IL-10-producing haplotype, ATA, with CAD in the Tunisian study group tested. While the -592A allele was strongly associated with CAD when examined at the single SNP level ($p = 0.004$), the lack of the association of the -592A allele-containing haplotypes with CAD is likely attributed to epistasis between this locus and nearby (-819, -1082), and possibly distant loci, as was demonstrated in studies on IL-10 variants associated with many other conditions [27, 28]. This provides evidence of a genetic basis for the clinical heterogeneity of CAD.

Our results are reminiscent of the findings of Trompet *et al.*, in which the -592A-containing IL-10 promoter haplotype was significantly associated with an increased risk for coronary events [29], but, are in apparent disagreement with a small Greek study which documented the association of the low IL-10-producing haplotype, ATA, with myocardial infarction [30], with the study of Bown *et al.* who reported that the -1082A allele was linked with an increased propensity for cardiovascular disease in Caucasian (UK) patients [31], and with the study of Bis *et al.* in which no IL-10 promoter SNP or haplotype was associated with CAD or myocardial infarction [20]. In addition, a recent study on 2260 young Finns showed that high IL-10 production (precipitated by the GCC haplotype) was devoid of anti-atheromatous capacity [19]. Also, two German studies

failed to demonstrate any association between the IL-10 promoter variants (-1082G/A, -819C/T, and -592C/A) and CAD pathogenesis, as highlighted by the comparable allele and genotype distribution of the three promoter SNPs between CAD and MI patients compared to healthy controls [32, 33].

These discrepancies may be attributable to differences in ethnicity [30-33], study design and selection of cases [19, 20, 31, 33], sample size [30], and in not testing for other IL-10 variants [31]. While not tested, our results suggest that the contribution of the IL-10 -592C/A variant to the pathogenesis of CAD may result from reduced IL-10 expression, highlighted by the reported association of the -592 C/A variant with lower IL-10 expression [34]. As for studies of a similar nature, caution is warranted in the extrapolation of the association of specific gene variants and haplotypes with disease states, given the multi-factorial nature of CAD [2, 4], and the likely participation of additional genetic and non-genetic factors in influencing overall risk of CAD.

The strengths of this study lie in it being the first to examine the association of IL-10 promoter variants with CAD in ethnically homogenous Tunisian Arabs, and in utilizing regression analysis to confirm the association of these variants with the severity of CAD. This study has some shortcomings however, namely that we did not correlate IL-10 genotypes with serum IL-10 levels; thus posing the question as to whether the selective IL-10 genotype association is functional, or alternatively, is a genetic marker for increased CAD risk. The potential association of the IL-10 variants investigated with other IL-10 variants, as well as nearby or distant functional gene variants remains to be seen [27, 28]. Another shortcoming lies in the selection of controls groups from blood donors and friends, which, in principle, may introduce bias, thereby necessitating the need for selection of control groups from the community at large. Despite these limitations, the association of IL-10 variants with susceptibility to CAD will strengthen our understanding of the link between altered Th1-Th2 balance and the pathogenesis of CAD.

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