

Endothelial nitric oxide gene polymorphisms and their association with coronary artery disease in Tunisian population

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ABSTRACT

Objective: By releasing mediators, like nitric oxide (NO), vascular endothelium is considered so significant in the process of atherosclerotic. In fact, the major functions of NO consist in inhibiting the activation of platelet, relaxing the muscles (vascular and smooth ones), and modulating the growth and the migration of cells (vascular and smooth ones). Therefore, this process makes the endothelial nitric oxide synthase (NOS3) considerably important because it possesses atheroprotective activity. Polymorphisms, rs1808593 (10G/T) as well as rs891512 (G24943A) within NOS3 gene, play major role in the coronary artery disease (CAD) development. The aim of the study is to evaluate the relationship between the 10G/T and G24943A polymorphisms and the CAD among Tunisian individuals.

Methods: We included, in this survey, a set of 274 patients suffering from CAD together with 162 normotensive subjects. The PCR-RFLP was applied to analyze the polymorphism of intron 23 (10G/T) gene, while the ASA-PCR was used to analyze the intronic G24943A gene polymorphism. Overall and subgroup analyses were performed. Odds ratio (OR) and 95% confidence interval (CI) were used to evaluate the association between NOS3 10G/T and G24943A polymorphisms as well as CAD risk. Statistical analysis was performed with SPSS V.10.

Results: The genotype frequencies for G24943A polymorphism differed significantly between the CAD patients and the controls. The former had a frequency of 11.4% for the AA genotype, 34.7% for the GA genotype and 53.9% for the GG genotype. The latter had a frequency of only 2.5% for the AA genotype, 29.7% for the GA genotype and 67.7% for the GG genotype ($\chi^2=7.62$; OR=1.79; $p=0.006$). The CAD patient group showed a significantly-higher frequency of the A allele compared to the controls (0.28 vs. 0.16; $\chi^2=15.20$; $p<0.001$). The odds ratio of CAD for A vs. G allele frequency was statistically significant 1.99 (1.4–2.82) at 95% CI. The genotype distribution for the 3 investigated variants of 10G/T were not significantly different between CAD and control subjects ($\chi^2=1.46$; OR=1.72; $p=0.22$). Whereas, 10G/T has revealed barely allelic ($\chi^2=4.45$; OR=2.3; $p=0.034$) correlation with coronary artery disease

Conclusion: The present study was designed so that there would be an association between the CAD and NOS3 polymorphism (G24943A). However, these results have proven that the polymorphism of 10G/T is not associated with CAD in the Tunisian population.

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Keywords: intron 23; risk factors, CAD; NOS3, Gene polymorphism

Introduction

Coronary artery disease (CAD) is one of the main causes resulting in mortality. It leads to almost 30% of the total number of deaths all over the world (1, 2). Obviously, this disease can be caused by some environmental factors as well as genetic ones (3). Actually, the role that the abnormalities within nitric oxide availability play in the pathophysiology of the CAD has been experimentally and clinically proven. This is in accordance with the assumption that nitric oxide (NO) is so crucial in maintaining the endothelial function, causing vasodilatation, preventing the migration and proliferation of vascular smooth muscle cell (4) as well as atherogenic low-density lipoprotein oxidation (5),

and inhibiting the adhesion of platelet and leucocyte. Our growing interest about the NO clinical relevance and its biology has resulted in the appearance of some surveys that tried to find the association between the CAD and polymorphisms located in the gene containing the enzyme responsible for synthesizing NO in the endothelial cells (eNOS). These studies have also dealt with the effect of this enzyme on the availability of the NO (6).

The gene of eNOS is involved in only one copy of the gene on chromosome 7q35-36. It contains 26 exons spanning 21kb as well as 23 introns. NOS gene codes 4052 nucleotides related to an mRNA (7). It has been noticed that various polymorphisms exist within the gene of NOS3. Some surveys have addressed the potential correlations with the CAD (8), stroke, myocardial in-

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farction and hypertension (9-11), recurrent early pregnancy loss (12) and intracranial aneurysm (13).

The afore-mentioned polymorphisms can be found within T786C, A922G and T1468 A, in the flanking region and exon7 (894 G/T), intron 4b/4a, 11(-30 A/G), 18 (27 A/C), 23 (10G/T) in the coding region (14).

Nevertheless, we have not widely studied polymorphisms G24943A and 10G/T within intron 23. They have been related to endothelial dysfunction. Thus, in some populations, they can cause CAD (15, 16). However, it was not the case in some other studies (17,18).

Considering the contradictory results obtained by previous surveys, the aim of the present study is to investigate the association between 10G/T and G24943A polymorphisms in intron 23 of the NOS3 gene and the risk of CAD severity among the Tunisian population.

Methods

Study design

The present survey was a case-control study in which the studied population consisted of 274 patients suffering from CAD, and 162 healthy subjects. The mean age of CAD group was 64.6 years (SD,10.6). Patients, belonging to this group, were enrolled in the Department of Cardiology, Farhat Hached University Hospital of Sousse. We perform the coronary angiography by using standard technique. Besides, coronary tree images were provided through routine and standardized projections. In fact, we noticed the importance of coronary artery stenosis while we have a luminal diameter shrinkage of $50\geq\%$ of left anterior descending, right coronary, circumflex artery or their primary branches. The number of the significantly stenosed coronary arteries (one, two, or three vessels) determined the CAD severity. Actually, diagnosis of myocardial infarction (MI) was confirmed according to the European Society of Cardiology criteria; a typical rise and fall of serum creatine kinase-MB isoenzyme (CK-MB) is explained by at least one of the following criteria: ischemic symptoms, development of pathologic Q waves on the ECG, ECG changes indicative of ischemia (ST segment elevation or depression).

For this study, we recruited 162 control subjects. Their mean age was 53.7 (SD, 7.4). Furthermore, the used questionnaire was structured to determine the controls that did not suffer from vascular disease, and exclude those who were estimated to be potential patients. In this way, we asked the controls if they have CAD family history, personal antecedents [diabetes, hypertension, dyslipidemia, CAD, angioplasty, stent, stroke, arthrits of the lower limbs, ongoing treatment, lifestyle (smoking/ex-smoking/no smoking, physical inactivity)]. We also eliminate controls having CAD familial history. In addition, to be qualified as a control, subjects had to be free from any clinical vascular event including reduced ankle-brachial index which indicates arterial stiffness reflecting atherosclerosis by measuring the skin wave propagation velocity. Resting ECG had to be without signs of possible CAD or left ventricular hypertrophy.

Actually, in each subject, we evaluated demographic data as well as history of diabetes mellitus, hypercholesterolemia, cigarette smoking and hypertension. The latter was considered as elevated systolic >140 mm Hg and/or diastolic >90 mm Hg blood pressure, and/or being administered antihypertensive medication.

The index of body mass was computed as weight in kilograms divided blood samples for biochemical and genotype analysis.

The study protocol was approved by the Ethics Committee, Farhat Hached University Hospital of Sousse (Approval No: 35220228) and written informed consents were taken from all patients and healthy individuals.

Laboratory analysis

By using the standard methods applied in the hospital clinical laboratory, we measured the serum concentrations related to the total cholesterol, HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), and triglyceride (TG).

We used the standard technique (promega kit, USA) to extract Genomic DNA from the peripheral leukocytes of the blood. Then, we stored the Genomic DNA at 4°C till we need it in the analysis.

We utilized the PCR-RFLP to analyze G24943A genotype and NOS3 10G/T by ASA-PCR and HinII, respectively.

Genetic analysis

As far as the polymorphism of 10G/T is concerned, we amplified the DNA through these primers: 5' - AGC TCT GGC ACA GTC AAG AAT-3' (reverse) and 5'-CCC CTG AGT CAT CTA AGT ATT-3' (forward). The amplification was done for a set of 30 cycles. Each one involved annealing at 56°C , denaturation at 94°C , and extension at 72°C . All these stages took 1 min separately, while the ultimate extension lasted for 10 min, and the initial denaturation lasted 5 min. By using the restriction enzyme HinII at a temperature equal to 37°C for 16h, the fragments of 676 bp were digested. The wild allele, with a site of only one HinII cleavage, digested to 99 bp and 577 fragments. However, 577 bp fragments, in the mutant allele were attached to 203 bp as well as to 374 fragments. Actually, we separated the afore-mentioned fragments using electrophoresis on 2% agarose gel. They were then visualized with ethidium-bromide staining.

Genotypes for the G24943A polymorphism of NOS3 gene were determined by ASA-PCR amplification using the primers 5'-TGC TTT AAG ACC CAG CTC CT-3' (Forward 1), 5'- GAG GAA CAC AGA AGC TTT CAG A-3' (Reverse 2), 5'-GGG GGT AAG TGA GAT GGA G-3' (Forward 2) and 5'-CCT GGG CAG CTC CCC ACC AAG TCT-3' (Reverse1). Initial denaturation was done at 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 1 min, and the final extension of 10 min at 72°C .

The amplified fragments were separated on 2% agarose gel with ethidium-bromide staining.

Fragments 612 bp PCR (F1, R1), 279 bp PCR (F2, R2) and 849 bp PCR (F1, R2) represented respectively the wild-type, the mutant allele and the common allele.

Statistical analysis

Statistical analyses were performed with using the statistical Package for Social Sciences (SPSS V.10.0 for Windows; SPSS Inc., Chicago, IL, USA).

Numeric data were shown as mean±SD. We either used an independent student t-test or χ^2 test, computed the discrepancy between the studied groups. The choice of test type depended on the case in which we calculated this difference. To compare the three groups, we accomplished the ANOVA. Besides, the method of gene counting was applied to assess the allele frequencies. The latter and the genotypes were compared among control groups and patients. We evaluated 95% confidence interval (CI) and the odd ratio (OR). Actually, we used the χ^2 test to analyze the genotype distribution deviation from the point of Hardy-Weinberg equilibrium. A binary logistic regression analysis was performed for the determination of the independent predictors for CAD. A p value <0.05 was considered to be significant.

Results

Clinical variables

We present in Table 1 the studied subjects clinical features. It is obvious that the triglycerides, HDL-C and CPK mean serum concentrations did not differ considerably among control and CAD groups. In fact, frequencies of age, diabetes, gender, smo-

Table 1. Demographic and clinical characteristics of coronary artery disease patients and controls

Characteristic	Patients n=274	Controls n=162	P*
Men/Women, n	166/108	68/94	<0.001
Age, years	64.65±10.69	53.76±7.44	<0.001
BMI, kg/m ²	28.79±4.47	26.61±3.58	<0.001
Hypertension, n, %	161 (58.7%)	10 (6.17%)	<0.001
Diabetes, n, %	164 (59.8%)	32 (17.7%)	<0.001
Smoking, n, %	94 (34.3%)	29 (17.9%)	<0.001
Number of diseased vessels			
Single vessel, n, %	165 (60.22%)	–	–
Double vessels, n, %	69 (25.18%)	–	–
Triple vessels, n, %	40 (14.60%)	–	–
Total-C, mmol/L	4.91±1.01	4.65±1.19	0.039
LDL-C, mmol/L	2.78±0.99	2.18±1.08	<0.001
HDL-C, mmol/L	1.18±0.46	1.28±0.35	0.102
TG, mmol/L	2.45±7.278	1.63±1.20	0.212
Homocysteine, μ mol/L	28.37±56.26	21.39±25.89	0.334
Creatinine, μ mol/L	100.13±58.04	82.08±63.83	0.009
CPK, U/L	271.31±432.93	82.21±65.17	0.059

Data are expressed as means±SD and as percent in other variable. *P values from Student's t-test for continuous variables, Pearson χ^2 test for categorical variables. BMI - body mass index; CPK - creatine phosphokinase; HDL-C - high-density lipoprotein cholesterol; LDL-C - low-density lipoprotein cholesterol; n - number; TG - triglycerides; Total-C - total cholesterol

king and hypertension in the controls were considerably lower than in patients ($p<0.001$). We also notice that the baseline serum concentrations of the total cholesterol, LDL-C and creatinine amount were more elevated in patients if compared to the controls ($p<0.05$). Similarly, serum concentrations of homocysteine and CPK were higher in patients compared to the controls, but it is not significant ($p>0.05$).

Allele frequencies

In this study, we examined the G24943A polymorphisms and The NOS3 gene 10G/T. In Table 2 and Table 3, we show their distributions as well as their allele frequencies in controls and patients. These investigated elements in control subjects and CAD patients were compatible using Hardy-Weinberg equilibrium.

The TT genotype frequency of the polymorphism 10G/T reached 0.7% in control subjects and 4.2% in the patients. Nevertheless, the distribution of the genotype did not differ considerably between the control groups ($p=0.226$, Table 2) and the patients. Besides, the CAD odd ratio within T allele carriers reached 2.30 (95% CI:1.04–5.11); $p=0.034$). Equally, AA genotype frequency for the polymorphism of G24943A had an average of 2.5% in the group of control, while it reached 11.4% among patients. We noticed that the distribution of the genotype and the allele frequencies are clearly dissimilar among the control groups and the

Table 2. Genotype and allele frequencies of 10 G/T polymorphism in intron 23 of the NOS3 gene in CAD patients and controls

	Patients n=274	Controls n=162	χ^2	P	OR, 95% CI
GG, n, %	217 (92%)	138 (95.2%)	4.03	0.079	1
GT, n, %	9 (3.8%)	6 (4.1%)			
TT, n, %	10 (4.2%)	1 (0.7%)			
GG, n, %	217 (92%)	138 (95.2%)	1.46	0.226	1.72 (0.7–4.21)
GT + TT, n, %	19 (8%)	7 (4.8%)			
G, n, %	443 (93.86%)	282 (97.24%)	4.45	0.034	2.30 (1.04–5.11)
T, n, %	29 (6.14%)	8 (2.76%)			

Distributions of the 10G/T genotypes in both patients and controls were in Hardy-Weinberg equilibrium, calculated by Pearson χ^2 test

Table 3. Genotype and allele frequencies of G24943A polymorphisms in intron 23 of the NOS3 gene in CAD patients and controls

	Patients n=274	Controls n=162	χ^2	P	OR, 95% CI
GG, n, %	132 (53.9%)	107 (67.8%)	13.39	0.002	1
GA, n, %	85 (34.7%)	47 (29.7%)			
AA, n, %	28 (11.4%)	4 (2.5%)	7.62	0.017	0.258 (0.085–0.78)
GG	132 (53.9%)				
GA + AA, n, %	113 (46.1%)	51 (32.3%)	15.20	<0.001	1.79 (1.18–2.72)
G, n, %	349 (71.22%)	271 (83.13%)			
A, n, %	141 (28.78%)	55 (16.87%)			1.99 (1.4–2.82)

Distributions of the G24943A genotypes in both patients and controls were in Hardy-Weinberg equilibrium, calculated by Pearson χ^2 test

Table 4. Binary logistic regression modeling using CAD as dependent variable (n=436)

Variables	Beta-coefficient	SE	P	OR (95% CI)
Age	0.109	0.18	<0.001	1.11 (1.07–1.15)
Gender	0.968	0.333	0.004	1.37 (1.37–5.05)
Hypertension	-2.702	0.419	<0.001	0.067 (0.029–0.15)
Diabetes	-1.197	0.321	<0.001	0.30 (0.16–0.56)
Smoking	-0.588	0.236	0.013	0.55 (0.35–0.88)
10G/T polymorphism				
GT	-0.374	1.105	0.73	0.68 (0.079–5.99)
TT	-0.322	1.349	0.81	0.72 (0.052–10.19)
Constant	0.541	1.827	0.76	–

CAD - coronary artery disease; CI - confidence interval; GT - heterozygotes; OR - odds ratio; SE - standard error; TT - homozygotes for the T allele

Table 5. Binary logistic regression modeling using CAD as dependent variable (n=436)

Variables	Beta-coefficient	SE	P	OR (95% CI)
Age	0.101	0.17	<0.001	1.10 (1.07–1.14)
Gender	0.682	0.336	0.042	1.97 (1.024–3.81)
Hypertension	-2.707	0.416	<0.001	0.067 (0.030–0.15)
Diabetes	-1.600	0.321	<0.001	0.20 (0.10–0.37)
Smoking	-0.535	0.235	0.023	0.58 (0.37–0.92)
G24943A polymorphism				
GA	-2.109	0.699	0.003	0.121 (0.031–0.47)
AA	-1.580	0.714	0.027	0.72 (0.051–0.83)
Constant	3.360	1.588	0.034	–

AA - homozygotes for the T allele; CAD - coronary artery disease; CI - confidence interval; GA - heterozygotes; OR - odds ratio; SE - standard error

Table 6. Clinical characteristics and 10G/T genotypes in patients with CAD

Variable	Genotype			P*
	GG (n=217)	GT (n=9)	TT (n=10)	
Hypertension with/without	132/82	7/2	5/5	0.45
Diabetes mellitus, with/without	135/79	5/4	7/3	0.80
Total-C, mmol/L	4.56±1.16	5.46±1.14	4.89±1.29	0.075
HDL-C, mmol/L	1.20±0.47	0.96±0.25	1.1±0.43	0.30
LDL-C, mmol/L	2.07±1.02	1.21±0.0	1.87±0.0	0.68

*P values from Pearson χ^2 test, ANOVA test. CAD - coronary artery disease; GG - homozygotes for the G allele; GT - heterozygotes; HDL-C - high-density lipoprotein cholesterol; LDL-C - low-density lipoprotein cholesterol; n - number; Total-C - total cholesterol; TT - homozygotes for the T allele

CAD patients ($p < 0.05$, Table 3). The odd ratio related to the CAD patients having A allele reached 1.99 (95% CI: 1.4–2.82); $p < 0.001$).

We used binary logistic regression model to test the independent correlates of the CAD presence. In this model, we included age, gender, hypertension, diabetes, smoking, and 10G/T poly-

Table 7. Clinical characteristics and G24943A genotypes in patients with CAD

Variable	Genotype			P*
	GG (n=132)	GA (n=85)	AA (n=28)	
Hypertension, with/without	87/41	45/38	17/11	0.12
Diabetes mellitus, with/without	87/41	54/30	16/12	0.53
Total-C, mmol/L	4.63±1.30	4.65±1.10	4.56±1.22	0.95
HDL-C, mmol/L	1.18±0.42	1.17±0.54	1.20±0.39	0.96
LDL-C, mmol/L	2.18±1.02	1.9±0.84	2.34±1.48	0.39

*P values from χ^2 test, ANOVA test. AA - homozygotes for the A allele; CAD - coronary artery disease; GA - heterozygotes; GG - homozygotes for the G allele; HDL-C - high-density lipoprotein cholesterol; LDL-C - low-density lipoprotein cholesterol; n - number; Total-C - total cholesterol

Table 8. Genotype frequencies of 10G/T and G24943A polymorphisms of the NOS3 gene and CAD severity

	CAD group (n=274)			P*
	G1, n=165	G2, n=69	G3, n=40	
10 G/T polymorphism, n, %				
GG	100 (92.6%)	59 (93.6%)	32 (88.8%)	0.72
GT	3 (2.8%)	3 (4.8%)	2 (5.6%)	
TT	5 (4.6%)	1 (1.6%)	2 (5.6%)	
G24943A polymorphism, n (%)				
GG	66 (61.1%)	31 (47.7%)	21 (52.5%)	0.28
GA	29 (26.9%)	28 (43.1%)	14 (35.0%)	
AA	13 (12.0%)	6 (9.2%)	5 (12.5%)	

*P values from Pearson χ^2 test. AA - homozygotes for the A allele; CAD - coronary artery disease; G1 - single vessel; G2 - double vessels; G3 - triple vessels; GA - heterozygotes; GG - homozygotes for the G allele; GT - heterozygotes; n - number; TT - homozygotes for the T allele

morphism. Age ($p < 0.001$) and gender ($p = 0.004$) were independent correlates of the presence of CAD. Whereas, 10G/T polymorphism, hypertension, diabetes and smoking were not CAD independent risk factors (Table 4). Likewise, the logistic regression analysis has shown that G24943A polymorphism, hypertension, diabetes, and smoking were not significant predictors of CAD. However, age ($p < 0.001$) and gender ($p = 0.042$) were found to be independent predictors of CAD in this case (Table 5).

Relationship between clinical characteristics and 10G/T and G24943A genotypes in CAD patients

The clinical and laboratory values were compared among genotypes of 10G/T and G24943A in the CAD patients. No significant difference was noted for the two polymorphisms (Table 6, 7).

The correlation between the 10G/T and G24943A polymorphisms of the NOS3 gene and CAD severity

We sub-classified the CAD patients into three distinct groups depending on the affected coronary arteries number (Table 8). Statistically, the polymorphism of 10G/T was not correlated to

the CAD extent ($p=0.72$). The NOS3 gene's G24943A polymorphism did not result in angiographically-defined CAD severity that was about 0.28 (Table 8).

Discussion

In this study, we have dealt with the correlations between the polymorphisms: G24943A and 10G/T at the NOS3 gene. We have also shown that the CAD presence can be angiographically proven among the Tunisian population. The obtained results demonstrate that 10G/T polymorphism genotype frequencies in intron 23 were not considerably different among control subjects and CAD patients.

Nevertheless, frequency of 10G/T polymorphism T allele was remarkably lower in controls than in patients. Such finding is the same as that obtained by some other researchers belonging to various ethnic groups (17,18). For example, Akhter et al. (19) have proven that, in Asian Indian ethnic group, deep vein thrombosis (DVT) cannot be caused by 10G/T polymorphism.

In addition, for the latter, no discrepancies at the level of allele frequencies or the distribution of the genotype were observed between control subjects and CAD patients within Iranian people (20). However, many studies have proposed that NOS3 gene's 10G/T polymorphism contribute to CAD. For instance, it was observed by Yoon et al. (21) that 10G/T polymorphism's G allele frequency was noticeably lower in the control subjects than in the CAD patients. In our study, the T allele frequencies in control groups and in CAD patients were 0.063 and 0.009, respectively (21).

Furthermore, Wang et al. (22) concluded that the rs4496877, rs1808593 and rs3918186 polymorphisms of NOS3 contributed to the genetic susceptibility of hypertension, and that rs3918186 was associated with spontaneous bacterial peritonitis (SBP) in the Han Chinese population. As far as the CAD and the G24943A polymorphism are concerned, the obtained results proved that G24943A polymorphism A allele frequency varied significantly from CAD patients to the control subjects (0.28 vs. 0.16, $p<0.001$). To our best knowledge, we carried the first survey that deals with the correlation between NOS3 polymorphisms and CAD within Tunisian individuals.

Seelenfreund et al. (18) obtained similar results within the Chilean ethnic group. These researchers concluded that NOS3 polymorphism rs891512 (G24943A) is associated with hypertension ($p<0.05$). In the same way, the association of the G24943A and hypertension has been reported in the Caucasian population (23-24).

Also, in our study, the NOS3 gene's 10G/T and G24943A polymorphisms did not result in a severe CAD that is angiographically-defined. As for the 2 NOS3 polymorphisms, the obtained results are similar to those provided by some studies, while they are contradictory with other surveys. This difference is due to the discrepancies between the ethnic groups and the sample sizes. It can also be explained by two other factors: first, the criteria of classifying the studied subject as control or patients;

second the effects of the environment interaction and the importance of gene-gene and gene-environment interactions in circulatory disorders.

Study limitations

Here, we will mention some of our study weaknesses. First, this work is based on a limited number of patients and controls. Second, we did not apply the coronary angiograph on the control groups that had no signs and no history of vascular events. Finally, we did not measure eNOS activity. Besides, to prove the correlation between plasma NOx concentrations and polymorphisms, a wide sample of studied subjects should be considered.

Conclusion

This work has shown that the polymorphism of G24943A can cause Coronary artery disease among Tunisian people. However, we have proven that the 10G/T is not related to the CAD risk in the studied population. Furthermore, 10G/T and G24943A polymorphisms of the NOS3 gene were not associated with the severity of angiographically-defined CAD.

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