Original Article
Association between genetic polymorphisms of ACE, eNOS, DDAH-2 and ADMA, SDMA and NOx with coronary artery disease in Tunisian population

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Abstract: Background: While a number of genetic and environmental risk factors for coronary heart diseases have been identified. This study was designed to determine the association between NOS, ACE and DDAH-2 gene polymorphism and CAD with regard to the plasma levels of ADMA, SDMA and NOx in Tunisian population. Methods: Relevant clinical parameters were measured and peripheral whole blood obtained for genetic analysis on 193 patients with stable coronary artery disease (CAD) and 104 CAD free control subjects. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) was used to determine genotype distributions for three polymorphic DDAH-2 rs3131383, ACE rs4291 and NOS rs2070744 and evaluated the association with plasma NOx, ADMA and SDMA concentration, them a potential predictor of CAD in a Tunisian population. Results: Significant differences in the distribution of the ACE and DDAH-2 polymorphisms were observed between CAD and controls but no difference was detected in eNOS gene T-786C polymorphism. Conclusion: ACE and DDAH-2 polymorphism were associated with an increased risk of CAD in Tunisian population.

Keywords: Coronary artery disease, DDAH2, ADMA

Introduction

Coronary artery disease (CAD) is a leading cause of mortality worldwide and accounts for 30% of death [1, 2]. The pathophysiology of CAD remains unclear but may involve interactions between genetic and environmental factors. Many epidemiological studies have highlighted numerous risk factors for CAD. Those include hypertension, diabetes, dyslipidemia, obesity, smoking habit and history of CAD. However, predictive factor, such as genetic association may also be helpful stratifying patients at risk of CAD [3-6]. One of the hallmark of CAD is an alteration in nitric oxide (NO°) metabolism. NO° is a major vasodilator factor that is produced by the endothelium, which exhibit anti-thrombotic, anti-inflammatory, and anti-proliferative actions [7]. Patients with CAD display reduced NO° production, which is believed to participate in the establishment and progression of CAD. Endothelium-derived NO° is produced from L-arginine by the endothelial NO synthase (NOS3, eNOS). Several allelic variants of the *NOS3* gene have been shown to influence basal production of NO° [8]. The T-786C polymorphism (rs2070744) is one of several promoter SNPs of eNOS that have been associated with the reduced eNOS expression and have been linked to CAD risk [9].

Factors that influence the production of NO° are numerous and include asymmetric dimethyl-arginine (ADMA), which inhibits NO production [10]. ADMA is metabolized into inactive compound by the dimethylargininase (DDAH). DDAH exists under two isoforms, of which DDAH-2 share similar endothelial expression pattern as NOS3 [11].

Table 1. Demographic and clinical characteristics of the study population

	CAD (n = 193)	Controls (n = 104)	P
Age	62 [55, 70]	50 [41-57]	< 0.0001
Male, n (%)	50 (66.3%)	45 (43.3%)	0.0002
BMI	27.7 [24.8-31.2]	26.8 [24.7-30.5]	0.44
Hypertension, n (%)	104 (53.9%)	1 (0.9%)	< 0.0001
Dyslipidemia, n (%)	43 (22.3%)	0 (0%)	< 0.0001
Diabetes, n (%)	115 (59.6%)	3 (2.9%)	< 0.0001
Smoking, n (%)	107 (55.4%)	6 (5.8%)	< 0.0001

CAD: coronary artery disease, BMI: body mass index.

The *DDAH-2* expression is related to the renin-angiotensin-aldosterone system since ADMA infusion promotes the expression of the angiotensin converting enzyme (ACE), which is the major angiotensin II-producing enzyme [11]. Renin angiotensin system (RAS), known for having a helpful role in physiological functions of cardiovascular system and also CAD, this association has been frequently reported by many studies [12, 13].

From a genetic standpoint, polymorphism of the *NOS3*, *DDAH-2* and *ACE* genes have been implicated as risk factors of CAD. We therefore studied the association of the SNPs *DDAH-2* rs3131383, *ACE* rs4291 and *NOS3* rs2070744 with regard to the plasma levels of ADMA, SDMA and NOx and evaluated them as potential predictor of CAD in a Tunisian population.

Methods

This study was approved by local ethic committees and written consent was obtained from all patients and controls, whose are Tunisian origins. This study using human samples was performed according to the current revision of the Helsinki Declaration.

Study population

The studied population (N = 297) consisted of 193 patients with stable coronary artery disease (CAD) and 104 CAD-free control subjects recruited from the health professional department while undergoing routine check-up. The patients with CAD were included when presenting at least 50% stenosis in at least one of the major coronary arteries. Patients who suffered from inflammatory disease, heart failure, renal disease, rheumatoid arthritis or cancer were excluded. Patients with fasting plasma glucose ≥ 7.0 mmol/L, or who were under anti-diabetic treatment were considered as diabetic [14]. Patients with cholesterol levels > 5.17 mmol/L, triglycerides levels > 1.70 mmol/ L, both, or who were under hypo-cholesterolemic drugs were considered dyslipidemic.

Plasma samples were assayed for NOx, the stable end product of NO, by using a commercially available kit (Cayman Chemical, Ann Arbor, MI). Briefly, the plasma and urine samples were first filtered through a 0.45-μm Minisart column (Amersham, U.K.) [15].

Plasma ADMA and SDMA were measured by HPLC-fluorescence after derivatization with naphthalene-2,3-dicarboxaldehyde (NDA) according to Marra et al [16].

DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes using salting out method [17]. Extracted DNA were dissolved in sterile distilled water and stored at 4°C for further PCR analysis. The *NOS3*-786C>T (rs2070744), the *ACE*-240A>T (rs4291), and the *DDAH-2*-871T>G (rs3131383) were analyzed by restriction fragment length polymorphism (RFLP). The oligonucleotides used for PCR were: 5'-TGGAG-AGTGCTGGTGACCCCA-3' and 5'-GCCTCCACCC-CCACCCTGTC-3' for rs2070744, 5'-TCGGGCT-GGGAAGATCGAGC-3' and 5'-GAGAAAGGGCCT-CCTCTCTCT-3' for rs4291, and 5'-AGCACAAC-ACAAGCTCAATCAG-3' and 5'-AGTCCCCTCCC-CAGGTTC-3' for rs3131383. PCR products were digested with BsiSI (rs2070744, Thermo scientific), XbaI (rs4291, Thermo scientific), or SmaI (rs3131383, Thermo scientific). Restriction fragments were analyzed on 2% agarose gel stained with ethidium bromide. For technical reasons, genetic analysis was performed on a subset of 94 controls and 99 CAD patients.

Statistical analysis

All statistical analyses were performed using the R-statistical software (http://www.r-project.org/). Continuous variables are expressed as median and interquartile range and were ana-
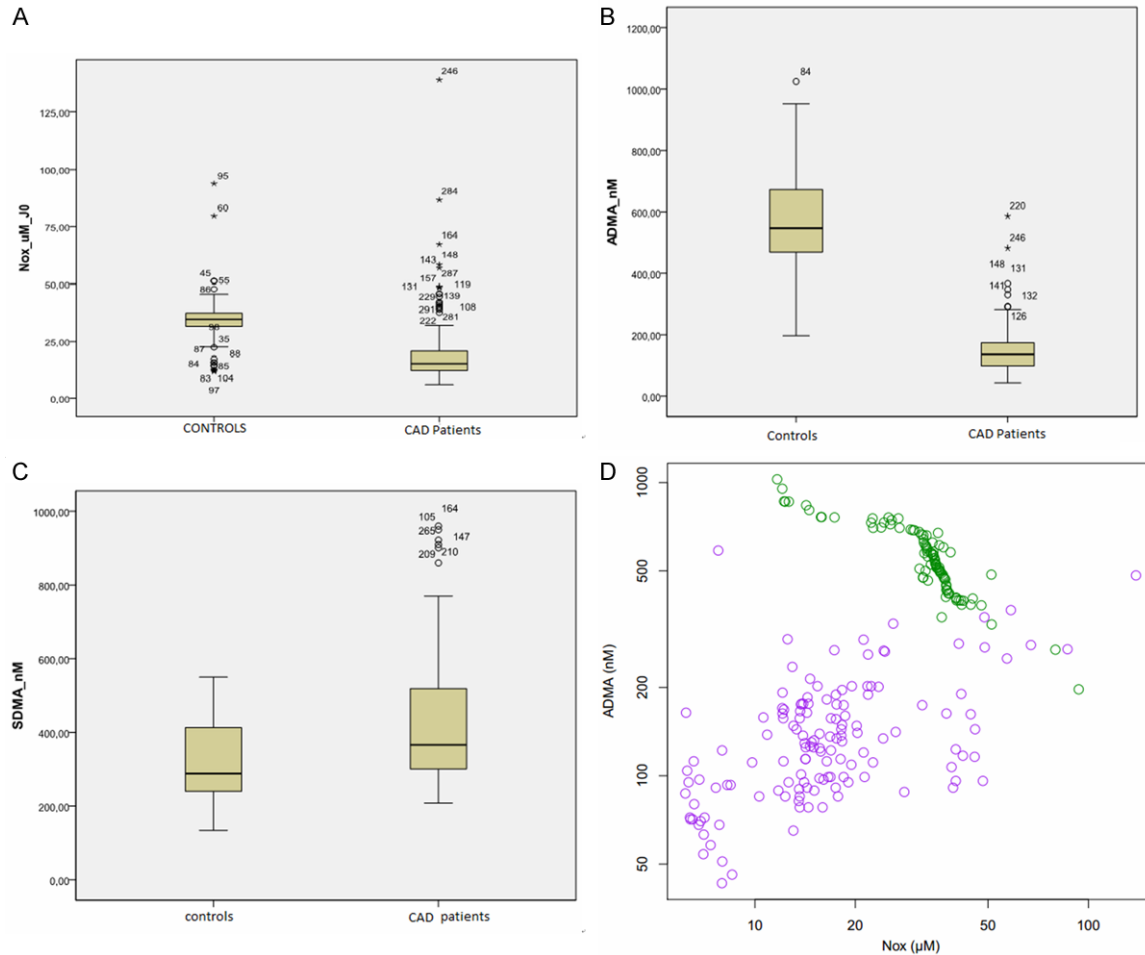
Figure 1. A. Plasma levels of NOx in controls and CAD patients. B. Plasma levels of ADMA in controls and CAD patients. C. Plasma levels of SDMA in controls and CAD patients. D. Correlation between NOx and ADMA levels.

Table 2. Genetic characteristics of the population for the three polymorphisms tested

Gene	Genotype	CAD	Controls	P
NOS3	-786TT	57	63	0.21
	-786CT	32	27	
	-786CC	10	4	
ACE	-240AA	38	54	0.01
	-240AT	48	36	
	-240TT	13	4	
DDAH-2	-871TT	46	41	0.02
	-871TG	27	40	
	-871GG	26	12	

CAD: coronary artery diseases.

lyzed using Wilcoxon rank-sum test. Categorical data were analyzed using Pearson's χ^2 test. Genetic association analyses were performed using the SNPassoc package from R. A p value < 0.05 was considered significant.

Results

Baseline characteristics of control and CAD groups

We investigated 193 patients with stable coronary artery diseases and 104 apparently healthy subjects which demographic and clinical characteristics are reported in **Table 1**. CAD patients had higher risk factors of cardiovascular diseases (*e.g.* hypertension, diabetes) compared to the control group.

Plasma levels of NOx, ADMA and SDMA

Plasma levels of NOx were lower in CAD patients (15.3 μM [12.2-20.5]) compared to controls (34.5 μM [31.5-37.3], P < 0.0001, **Figure 1A**).

Table 3. Odd ratio associated with the ACE-240T and DDAH-2-867G alleles according to the genetic model

	Dominant model		Recessive model		Additive model	
	OR [95% CI]	P	OR [95% CI]	P	OR [95% CI]	P
Univariate analysis						
ACE-240T	2.17 [1.22-3.85]	0.007	3.40 [1.07-10.84]	0.03	2.02 [1.27-3.22]	0.002
DDAH-2-867G	2.62 [1.42-4.83]	0.002	3.23 [1.71-6.09]	< 0.001	2.00 [1.40-2.85]	< 0.001
Multivariate analysis[b]						
ACE-240T	0.56 [0.15-2.12]	0.38	3.28 [0.21-2.12]	0.38	0.83 [0.30-2.32]	0.72
DDAH-2-867G	20.94 [2.93-149.85]	< 0.001	3.24 [0.90-11.61]	0.06	3.49 [1.42-8.57]	0.002

[b]Multivariate analysis adjusted for age, sex, hypertension, diabetes, smoking habit, and dyslipidemia.

Similarly, ADMA levels were lower in CAD (136 nM [98-174]) compared to controls (547 [470-671], P < 0.0001, **Figure 1B**). Conversely, SDMA levels were higher in CAD patients (366 nM [301-519]) than in controls (288 [241-407], P < 0.0001, **Figure 1C**). There was a strong negative correlation between NOx and SDMA (ρ = -0.90, P < 0.0001). In contrast, there was a moderate positive relationship between NOx and ADMA in CAD patients (ρ = 0.49, P < 0.0001, **Figure 1D**).

Association of ACE, eNOS and DDAH-2 gene polymorphism with CAD

No deviation from the Hardy-Weinberg equilibrium was observed for *NOS3* rs2070744, *ACE* rs4291 and *DDAH-2* rs3131383 (P > 0.05) in the control population. Significant differences in distribution of the *ACE* and *DDAH-2* polymorphisms were observed (**Table 2**) between CAD and controls. No difference was observed for *NOS3* between the two groups (**Table 2**).

Univariate analysis of *ACE* and *DDAH-2* polymorphism showed that the *ACE*-240T was associated with an increased risk of CAD regardless the genetic model used; dominant or recessive (**Table 3**). In contrast, the *DDAH-2-*871G allele was also associated with increased risk of CAD only when a recessive model was considered (**Table 3**). In a multivariate model that was adjusted with common CAD risk factor, *i.e.* age, gender, hypertension, diabetes, smoking habit, and dyslipidemia, none of the polymorphism was associated with risk of CAD (**Table 3**). Of note, there was no association between the plasma levels of NOx, ADMA or SDMA and the polymorphisms tested.

Discussion

Three gene polymorphisms *NOS3-*786C>T, *ACE*-240A>T, and DDAH-2-871T>G, in this present study, were determined in CAD patients and healthy individuals.

NOS3-786C>T

In the present study, the *NOS3* SNP T-786C was investigated in Tunisian population and the distribution of genotypes was not different between case and control groups significantly.

Similarly to many previous report, we found that genetic polymorphism in the -786T>C not associated to the risk of development of coronary heart disease [18-20].

In contrast, many studies have both shown a significant association between -786T>C gene polymorphism and CAD risk [21-24].

ACE-240A>T and DDAH-2-871G

Results showed that the *ACE*-240T and *DDAH-2*-871G alleles were strongly associated with CAD and that *DDAH-2*-871G was strongly associated with CAD, even after adjustment for risk factors in a dominant model.

We found the *DDAH-2*-871G allele was an independent predictor of CAD in the study population, regardless the recessive or dominant genetic model. In type 2 diabetic patients, this allele has been associated with ADMA serum level [25]. In our population, the presence of *DDAH-2*-871G allele is therefore anticipated to dictate ADMA plasma levels. Further studies are required to test this hypothesis but these results suggest *DDAH-2* as a significant CAD

predictor in the Tunisian population, possibly through alteration of the nitric oxide metabolism. Interestingly, the genotype distribution of the *DDAH-2*-871T>G was in the Hardy-Weinberg equilibrium, with a lower representation of heterozygous.

The DDAH-2 gene, rs3131383 are significantly associated with ADMA levels in individuals with type 2 diabetes [22].

The *ACE*-240T allele was also a strong predictor of CAD but failed once adjusted to common CAD risk factors. These data are no exception to the body of literature which found no association between this polymorphism and cardiovascular diseases. It is worth mentioning that some association was found with early-onset Alzheimer's disease [26], suggesting a potential specific effect of this polymorphism. However, since *ACE*-240T was associated with higher risk of CAD in univariate analysis; these data suggest that the *ACE*-240T allele is most likely associated with a risk factor of CAD rather than CAD itself.

Finally, associations between *NOS3*-786C>T polymorphism and cardiovascular diseases have been extensively studied in various populations, albeit conflicting results. *NOS3*-786C allele was found an independent predictor of internal carotid artery stenosis [21] and MI in young subjects [24]. In contrast, we and others failed to find any association between this polymorphism and the risk of cardiovascular diseases [18]. These data suggest that while alterations of nitric oxide metabolism can be a risk factor for cardiovascular disease, it appears to be context [27] and possibly population-dependent.

In conclusion, this study identified the *DDAH-2* G allele as a potential genetic predictor for CAD in the Tunisian population. Moreover, *ACE*-240T is most likely a predictor of a CAD risk factor than a CAD predictor itself.

Disclosure of conflict of interest

None.

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