Lack of association between endothelial nitric oxide synthase gene polymorphisms and risk of premature coronary artery disease in the Greek population

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Objective — Genetic polymorphisms in the gene for endothelial nitric oxide synthase have been considered as potential risk factors for the development of coronary artery disease in some populations.

Methods — We studied two polymorphisms of the NOS3 gene, the VNTR in intron 4 (4VNTR) and the Glu298Asp polymorphism in exon 7, in relation to the existence of premature coronary artery disease and the occurrence of myocardial infarction. A total number of 370 individuals of the Greek population was examined by PCR-RFLP method. The patient group consisted of 209 subjects, aged less than 58 years presenting symptomatic coronary artery disease, documented by coronary angiography.

Results — The frequencies for bb, ab and aa genotypes of 4VNTR polymorphism were 0.67, 0.29, 0.04, respectively, for the patient group and 0.73, 0.24, 0.03 for the control group. The frequencies for GG (Glu/Glu), GT (Glu/Asp), TT (Asp/Asp) of the Glu298Asp polymorphism were 0.52, 0.41, 0.07, respectively, in patients compared to 0.47, 0.46, 0.07, in control subjects. Statistical analysis indicated that there are no significant differences in the frequencies of the genotypes between patients and control subjects for both polymorphisms. The combined analysis of the two polymorphisms indicated no synergistic effect of the a and T alleles on coronary artery disease.

Conclusions — We have found no evidence for association between the a allele of the 4VNTR polymorphism, or the T allele of Glu298Asp polymorphism and the risk for premature coronary artery disease or occurrence of myocardial infarction. Furthermore, no synergistic contribution of these polymorphisms to the development of premature coronary artery disease has been observed.

Keywords: *coronary artery disease – endothelial nitric oxide – NOS3 gene polymorphism – risk factor.*

Introduction

Nitric oxide (NO) produced by endothelial cells is a key molecule in maintaining normal vascular integrity and function[1] while it has several potentially anti-atherosclerotic effects[2]. It mediates vasodilation and suppresses platelet aggregation and vascular smooth muscle cell proliferation[3]. In endothelium NO is produced from L-arginine by the action of homodimeric enzyme nitric oxide synthase (eNOS). The enzyme is encoded by the NOS3 gene, located on chromosome 7q35-36[4,5]. eNOS catalyses NO production in the vascular wall[6]. Different studies have shown that genetic polymorphisms in the NOS3 gene are influencing local haemodynamics and at least two of them, VNTR in intron 4 and Glu298Asp are considered as potential risk factors for the development of premature coronary artery disease (CAD) in some populations. VNTR (variable number tandem repeats) in intron 4 has been associated with lower concentrations of NO metabolites[7,8]. Two alleles have been identified in intron 4 of NOS3 gene, the larger of which has five tandem 27-bp repeats (allele b) and the smaller has four repeats (allele a). The larger is a major allele in the human

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population, while the smaller, although its frequency varies among different populations, is a minor allele. The exonic polymorphism Glu298Asp is the result of a G > T substitution at nucleotide position 894 of the gene and is associated with breakdown of the enzyme[9]. Contradictory results have been published for both polymorphisms, VNTR in intron 4 and Glu298Asp, and the risk of CAD among different populations. Since genetic and environmental background differ among populations, ethnic differences in the allelic frequencies of NOS3 polymorphisms and the genetic associations with disease may exist. In order to further elucidate the question whether these two NOS3 polymorphisms are associated with CAD, we investigated the potential association of these gene polymorphisms in a CAD patient group from a Greek population and compared the genotype distribution with a sample of healthy subjects from the general population. We also investigated the occurrence of myocardial infarction (MI) in CAD patients.

Material and methods

Our study population consisted of 370 unrelated individuals, 209 CAD patients and 161 healthy control subjects. All individuals were Greeks, from Greek parents. The study was approved by the local Ethics Committee and informed consent from all participants was obtained. The patient group consisted of subjects aged less than 58 years presenting symptomatic CAD, documented by coronary angiography, at the "Laiko" hospital of Athens. The control group consisted of healthy individuals. None of the control subjects had evidence of cardiovascular disease, such as coronary artery disease or stroke, and they all had normal electrocardiograms. Individuals were defined as hypertensive if their blood pressure was > 140/90 mm Hg or if they were receiving any antihypertensive treatment. In addition, they were considered as diabetic when they had fasting glucose > 126 mg/dl or if they were receiving any antidiabetic medication. Hypercholesterolaemia was defined as total cholesterol > 220 mg/dl. Family history was considered positive for CAD if at least one first-degree relative was diagnosed with CAD or MI by the age of 65 years. Subjects were defined as smokers if they were current or past smokers. Genomic DNA was extracted from peripheral blood leukocytes by a standard salting out method[10]. The genotyping of the 4VNTR polymorphism was carried out using PCR-based method. The genotyping of the Glu298Asp polymorphism was carried out using the PCR-RFLP method. For the first polymorphism the DNA samples were subjected to amplification using primers flanking the polymorphic region at intron 4 of the NOS3 gene. The PCR oligonucleotide primers were as follows: forward primer: 5'- AGGCCCTATGGTAGTGCCTTT-3', reverse primer: 5'- TCTCTTAGTGCTGTGGTCAC-3'[10,20]. Each reaction mixture was heated to 95°C for 5 min (initial denaturation) followed by 35 cycles of 95°C for 50 sec, 54°C for 50 sec and 68°C for 45 sec, and a final extension at 68°C for 5 min. Following amplification, 8 µl of each PCR product was analysed by electrophoresis on a 2% agarose gel and fragments were visualized by ethidium bromide staining and ultraviolet transillumination. For genotyping the Glu298Asp polymorphism of the NOS3 gene, the PCR amplification primers were: forward: 5'–CATGAGGCTCAGCCCCAGAAC-3', reverse: 5'-AGTCAATCCCTTTGGTGCTCAC-3'. DNA was amplified for 35 cycles, each cycle comprising denaturation at 95°C for 45 sec, annealing at 58°C for 50 sec, extension at 72°C for 45 sec with a final extension time for 5 min at 72°C. PCR products were digested with the restriction enzyme MboI, at 37°C, for 16 h. The 206–bp PCR product in the presence of T at nucleotide 894 of the coding sequence is cleaved into two fragments of 119 and 87 bp. The PCR products were separated on agarose gel, as previously described for 4VNTR polymorphism.

Statistical analysis

Statistical analysis was carried out using the SPSS v10 for Windows statistical package (SPSS, Chicago, IL, USA). Data are presented as a total number, percent or mean. To test for independent relationships between categorical variables, such as genotype distribution, the x^2 test was performed. A P value of less than 0.05 is considered as statistically significant. The odds ratios as estimators of relative risk together with their 95% confidence interval were calculated for the interaction between 4VNTR and Glu298Asp genotypes on CAD risk.

Results

CAD

The clinical characteristics of the CAD patients and the control group, used in this study, are shown in table 1. As seen, the patient group had a significantly higher prevalence of risk factors such as hypertension, diabetes, family history of CAD, cholesterol, compared to the control subjects. The genotype distribution for 4VNTR and Glu298Asp polymorphisms of NOS3 gene are shown in table 2. For 4VNTR polymorphism, the frequencies of bb, ab, aa, in the CAD patient group were 0.67, 0.29, 0.04, respectively, and 0.73, 0.24, 0.03, respectively, for the control group. Allele frequencies for the 4VNTR polymorphism among patients and control subjects were 0.81 and 0.85, respectively, for

Table 1. – *Baseline characteristics of CAD patients and control subjects*

	Patients	Control subjects	*P*
	n = 209	n = 161	
Mean age	52.3 (35-57)	65.6 (52-72)	< 0.01
Male sex, n (%)	173 (82.7)	82 (50.9)	< 0.01
Hypertension, n (%)	98 (46.8)	29 (18.0)	< 0.01
Diabetes, n (%)	52 (24.8)	8 (4.9)	< 0.01
Hypercholesterolaemia, n (%)	117 (55.9)	31 (19.2)	< 0.01
Smoking, n (%)	111 (53.1)	61 (37.9)	< 0.01
Family history, n (%)	67 (32.0)	27 (16.8)	< 0.01

the most frequent allele b and 0.19 and 0.15, respectively, for the allele a (table 2). The data of the two groups were analysed by chi-square test. We compared individuals who were either homozygous aa or heterozygous ab with individuals who were homozygous bb because the frequency of aa genotype was very low. As can be seen in table 2 no significant differences have been observed in the genotype and allele frequencies between the patient and control group ($P = 0.484$). Furthermore, for Glu298Asp polymorphism, the observed frequencies for GG, GT, TT genotypes were 0.52, 0.41, 0.07, respectively, for the patient group and 0.47, 0.46, 0.07 for the control group. Allele frequencies for the G allele of Glu298Asp polymorphism were 0.72 and 0.70 for patients and control subjects, respectively, and for the mutant allele T were 0.28 and 0.30, respectively, for the patient and the control group (table 2). Chi-square analysis of these results indicated that there is no significant difference between the patient and the control group ($P = 0.669$).

Previous myocardial infarction

Within the CAD group, we examined the 4VNTR and Glu298Asp polymorphisms in a subgroup of 50 patients who previously suffered an MI. The genotype distribution of the two polymorphisms among MI patients is shown in table 3. As can be seen, comparison of the genotype distribution between MI and control groups has shown that there were no significant differences between the two groups for both the studied polymorphisms ($P = 0.205$ for 4VNTR and $P = 0.281$ for Glu298Asp). Comparison of the genotype distribution among MI patients and CAD patients indicated similar results ($P = 0.240$ for 4VNTR and $P = 0.512$ for Glu298Asp).

Combined analysis of 4VNTR and Glu298Asp

We also investigated a possible synergistic effect between the 4VNTR and Glu298Asp polymorphisms of NOS3 gene. We found that individuals-carriers of one or two a alleles which are either homozygous or heterozygous for T allele (genotypes: aa-GT, ab-TT, ab-GT. There were no aa-TT genotypes) do not show a statistically significant association with an increased risk for premature CAD (OR = 0.970, $P = 0.963$) (table 4).

Table 2. – *Genotype distribution and allele frequencies of the 4VNTR and Glu298Asp polymorphisms in CAD patients and control subjects. P value determined by X^2 analysis*

Genotype 4VNTR	Patients n = 209	Control subjects n = 161	
bb	140 (0.67)	117 (0.73)	$X^2 = 1.453$
ab	60 (0.29)	39 (0.24)	$P = 0.484$
aa	9 (0.04)	5 (0.03)	
Allele frequencies a/b	0.19/0.81	0.15/0.85	
Glu298Asp			
GG	109 (0.52)	76 (0.47)	$X^2 = 0.803$
GT	85 (0.41)	74 (0.46)	$P = 0.669$
TT	15 (0.07)	11 (0.07)	
Allele frequencies T/G	0.28/0.72	0.3/0.7	

Data are expressed as number (percentages) of each genotype and gene frequency.

Discussion

Cardiovascular diseases are a major cause of mortality in the human population all over the world and coronary artery disease is well known as a multifactorial disease resulting from the interaction of genetic predisposition and environmental risk factors. NO produced by the endothelial cells is known to play a major role in maintaining normal vascular function and the NOS3 gene is considered to be a candidate gene for the mediation of initial endothelial cell damage in atherosclerosis. Several research groups have studied genetic polymorphisms in the NOS3 gene and showed associations with CAD, MI, or hypertension. Among the reported polymorphisms, two of the most studied are the VNTR in intron 4 (4 or 5 repeats) and the Glu298Asp, in exon 7, with contradictory results for different populations. The different genetic background of the populations studied has to be considered as a putative explanation of these contradictory results. However, both these polymorphisms have been identified as potential risk factors for the development of premature CAD in some populations. In the present study, we investigated the association of the VNTR polymorphism in intron 4 and the Glu298Asp polymorphism in exon 7 of the NOS3 gene with the risk of coronary artery disease and MI in a south-east European population, the Greek population. Our results for 4VNTR polymorphism showed that there is no significant difference in the frequency of aa and ab genotypes between CAD patients and healthy control subjects, in this Caucasian

Table 3. – *Genotype distribution of the 4VNTR and Glu298Asp polymorphisms in the subgroup of MI patients.*

4VNTR n = 50		Glu298Asp n = 49	
Genotype frequency			
bb	33 (0.66)	GG	30 (0.61)
ba	17 (0.34)	GT	16 (0.33)
aa	0 (0)	TT	3 (0.06)
X^2= 3.166	*P* = 0.205 *	X^2 = 2.539	*P* = 0.281*
X^2= 2.857	*P* = 0.240**	X^2 = 1.339	*P* = 0.512**

* Test between MI patients and control subjects.
** Test between MI patients and total of CAD patients.

population. Thus, we cannot confirm the findings of those groups who reported the ecNOS4aa genotype to be associated with CAD[11-15]. The a allele could not be associated neither with the risk for CAD, nor with the risk for MI in our study group. However, our results are in agreement with previous results for some other Caucasian populations (Germans, Australian Caucasians, Brazilian Caucasians) that have shown no association of this polymorphism and the risk for CAD[16-19]. Furthermore, our study indicated no association between the T allele and TT or GT genotype of the Glu298Asp polymorphism and the risk for premature CAD or occurrence of MI. Previous studies have shown that the T allele of the polymorphism Glu298Asp was significantly higher in MI patients or CAD patients in different Caucasian populations. Association between the NOS3 Glu298Asp polymorphism and CAD has been reported for the British population[20,21], for Germans[22], Italians[23] and Dutchmen[24]. In a recent study for the Greek population Antoniadis et al.[25] reported that the genotype TT of Glu298Asp polymorphism leads to an increased risk for premature MI. Similar results were also reported for non-Caucasian populations, i.e. a Japanese[26,27], a Turkish[28] and an Egyptian population[29]. In contrast to these results, other studies do not support the finding that the Glu298Asp is a risk factor for cardiovascular alterations. Granath et al.[17] in a large case-control study found no evidence for association between several NOS3 gene polymorphisms, including Glu298Asp, and premature CAD in an Australian Caucasian population. No association between the Glu298Asp and premature CAD has been reported for the same population[30] as well as for patients from the UK[31], French patients[32], Italians[33] and in CAD patients from two Austrian populations[31]. In addition, the Glu298Asp polymorphism was not associated with CAD in several non-Caucasian populations such as Taiwanese[34], Korean[35] and Turkish[36,37]. It is clear that different studies for the same populations indicated contradictory results for the Glu298Asp polymorphism and risk of premature CAD, i.e. for British, Italian and Turkish populations. Our findings for the Greek population are in contrast with those of Antoniades et al.[25] and do not support an association between the Glu298Asp polymorphism and risk of occurrence of MI. We do not have a convincing explanation for the contradictory results of Antoniades et al.[25] and our results reported here. However, their results and ours are similar for the control groups. The observed frequencies of the TT genotype (candidate genotype) in the control groups were 0.7 (in our study) and 0.9 in the study by Antoniades et al.[25]. Perhaps a larger sample of cohorts should be studied in order to reach a more definite conclusion as regards the role of this polymorphism of NOS3 gene in CAD and MI in the Greek population.

Table 4. – *Combined analysis of 4VNTR and Glu298Asp genotypes for CAD and control subjects*

Genotypes	OR (CI 95%)	*P*
aa-GT+ab-TT+ab-GT *	0.970 (0.268-3.512)	0.963
aa-GG	1.273 (0.237-6.821)	0.778
ab-GG	1.594 (0.491-5.175)	0.437
bb-GG	1.257 (0.415-3.809)	0.686
bbGT	0.922 (0.308-2.757)	0.884

* No individuals of genotype aa-TT have been observed.

Conclusions

In contrast to some earlier findings our results do not support an association between the a allele of the 4VNTR polymorphism, or the T allele of Glu298Asp polymorphism of the ecNOS gene and the risk for premature CAD or occurrence of MI. Furthermore, no synergistic contribution of these polymorphisms to the development of premature CAD has been observed. We may conclude that these two polymorphisms of the ecNOS gene could not serve as risk factors for premature CAD in this population.

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Conflict of interest: none declared.

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