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Association between *NOS3* genetic variants and coronary artery disease in the Han population

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ABSTRACT. The enzyme endothelial nitric oxide synthase (NOS3) is an important mediator of atherosclerotic disease and is associated with coronary artery disease (CAD). There is growing evidence that polymorphisms in NOS3 influence the progression of CAD; however, there is also a controversy regarding the association of polymorphisms in the gene encoding *NOS3* and CAD. To determine if the *NOS3* genetic variants are associated with CAD in the Han Chinese, we examined the potential association between CAD and eight single nucleotide polymorphisms (rs1799983, rs2070744, rs11771443, rs3918188, rs2853796, rs7830, rs1541861, and rs2853792) of the *NOS3* using the MassARRAY system. The allelic and genotypic frequencies of the rs1799983 (promoter regions) and rs2070744 (intron 1) polymorphisms in patients with CAD were significantly different from those in healthy controls. These patients had significantly higher frequencies of the rs1799983 T allele (χ^2 = 7.717, P = 0.007, OR = 1.649, 95%CI = 1.41-2.382) and the rs2070744 G allele (χ^2 = 4.548, P = 0.033, OR = 1.490,

95%CI = 1.031-2.153). Strong linkage disequilibrium was observed in three blocks (D' > 0.9). In block 1, significantly more T-T-C haplotypes (χ^2 = 5.537, P = 0.019, OR = 0.632, 95%CI = 0.430-0.927) were found in controls. These findings point to a role for *NOS3* polymorphisms in CAD in the Chinese Han population, and may be useful for future investigations on the pathogenesis of CAD.

Key words: Coronary artery disease; Endothelial nitric oxide synthase; Single nucleotide polymorphisms

INTRODUCTION

Cardiovascular diseases, particularly coronary artery disease (CAD), are the leading cause of mortality in developed countries (Gaunt and Davey Smith, 2015). The etiology of CAD is multifactorial, and various well-known risk factors have been described in previous decades (Jayashree et al., 2015). As with other types of complex diseases, genetic predisposition has been shown to be a potential risk factor for CAD (Won et al., 2015). Genetic studies have consistently demonstrated a substantial genetic influence on the development of CAD, with inherited risk estimates in the range 20-60% (Kraus, 2000; Won et al., 2015; Yamada et al., 2015). Other studies have suggested that polymorphisms in the enzyme endothelial nitric oxide synthase (*NOS3*) may relate to CAD (Ben Ali et al., 2015; Gaunt and Davey Smith, 2015; Shim et al., 2015).

Nitric oxide (NO) is an important atheroprotective mediator that helps control endothelium-dependent vasodilatation. Abnormalities in NO generation could play an important role in the pathophysiology of CAD (Kugiyama et al., 1996). NO is synthesized from L-arginine by the action of nitric oxide synthase (NOS). There are at least three isoenzymes of NOS: inducible NOS, neuronal NOS, and endothelial NOS (NOS3) (Nathan and Xie, 1994). *NOS3* is encoded by a gene with the chromosomal locus 7q35-q36 (Marsden et al., 1993). Some single nucleotide polymorphisms (SNPs), including T786C, c.894G>T (Glu298Asp), and a variable-number tandem repeat (VNTR) in intron 4 (intron 4 a/b VNTR polymorphism), have been identified in the *NOS3* (Wang et al., 1996; Hingorani et al., 1999; Nakayama et al., 2000). NO production can be influenced by polymorphisms of the *NOS3*. It has been shown that certain *NOS3* polymorphisms that reduce NOS3 activity might be risk factors for atherosclerotic heart disease (Hibi et al., 1998; Nakayama et al., 2000).

Several polymorphisms have been identified in the *NOS3*, one of which is located in exon 7 and modifies the coding sequence (Glu298Asp). The Glu298Asp polymorphism (rs1799983) causes a structural change in the NOS3 protein and reduces NOS3 activity (Hingorani, 2003). In the present study, we investigated the association between eight SNPs to verify the putative association between *NOS3* SNPs and the risk of CAD in a Chinese Han population. These SNPs were rs1799983 (exon 7), rs2070744 (promoter), rs11771443 (promoter), rs3918188 (intron 12), rs2853796 (intron 13), rs7830 (intron 22), rs1541861 (intron 8), and rs2853792 (intron 12).

MATERIAL AND METHODS

Subjects

All participants were from a Chinese Han population in Shandong Province (China).

The study was performed according to the Guidelines of the Medical Ethics Committee of the People's Hospital of Jining City (Jining, China). Written informed consent was obtained from all the study participants. A total of 208 unrelated patients with CAD (mean age ± SD: 61.4 ± 6.2 years) were recruited from our hospital. The patients underwent the following examinations: coronary angiography, electrocardiogram, blood test, and/or stress test. The patients with CAD were also interviewed and their responses were recorded. The study exclusion criteria were: non-cardiac diseases including acute or chronic infections, malignancies, autoimmune diseases, hyperthyroidism, and medication with immunosuppressive agents. The control group consisted of 217 unrelated healthy subjects (mean age ± SD: 63.2 ± 5.8 years) who underwent the above-mentioned health examinations at the Medical Examination Center of the People's Hospital of Jining City (Jining, China).

Genotyping

Genomic DNA was extracted from blood leukocytes using an EZNA™ Blood DNA Midi Kit (Omega Bio-Tek, Norcross, GA, USA), according to the manufacturer instructions. Genotyping for the eight SNPs was performed using the MassARRAY platform (Sequenom, San Diego, CA, USA) by means of matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry, according to the manufacturer instructions. Primers were designed using the Sequenom software (Table 1), and the extension reaction produced allele-specific products with masses differing by 30 Da, or approximately one single nucleotide. Primer extension and polymerase chain reaction were performed according to the manufacturer instructions using an iPLEX enzyme (Sequenom) and HotStarTaq DNA polymerase (Qiagen, Hilden, Germany). Genotypes were automatically identified by the SpectroTYPER software (Sequenom), and only conservative and moderate calls, as defined by the software, were accepted for this study.

Statistical analysis

Each SNP was tested for deviation from Hardy-Weinberg equilibrium (HWE) using the Pearson chi-squared (χ^2) test or the Fisher exact test. The association between genetic polymorphisms was examined using the χ^2 test. The odds ratios (ORs) and their 95% confidence intervals (95%CIs) were calculated to estimate the strength of association by unconditional logistic regression analysis. The common genotype/allele served as a reference for calculating the OR. Pairwise linkage disequilibrium (LD) statistics (D' and r^2) and haplotype frequencies were computed using Haploview 4.0 to construct haplotype blocks. Haplotypes with a frequency of less than 5% were excluded. All the statistical tests mentioned above were conducted in SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

The distribution frequencies of the eight genotyped SNPs were in agreement with HWE. LD analyses of the patient and control data revealed that three SNPs (rs11771443, rs2070744, and rs1799983), two SNPs (rs1541861 and rs2853792), and two SNPs (rs3918188 and rs2853796) were located in haplotype block 1, block 2, and block 3, respectively (D' > 0.9; Figure 1). The genotype distributions, allelic frequencies, and haplotypes in the control and patient groups, together with the results of the statistical analysis, are listed in Tables 1-4.

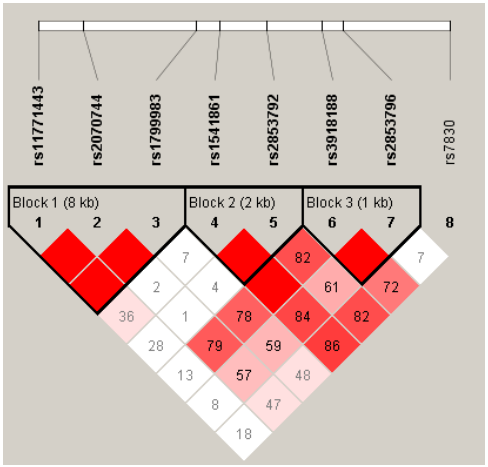

Figure 1. Linkage disequilibrium plot of the single nucleotide polymorphisms in the eNOS gene in controls.

Table 1. Genotypic and allelic frequencies of eNOS polymorphisms in controls and patients with coronary artery disease (CAD).

Variable	MAF	CAD (N = 208)		Controls (N = 217)		P value[a]	OR (95%CI)
		No.	%	No.	%		
rs1799983	0.131					0.000089	
CC		127	61.1	167	77.0	0.000435	0.469 (0.308-0.715)
CT		79	38.0	43	19.8	0.000045	2.478 (1.603-3.831)
TT		2	1.0	7	3.2	0.127	0.291 (0.060-1.419)
C allele		333	80.0	377	86.9	0.007	0.607 (0.420-0.876)
T allele		83	20.0	57	13.1		
rs2070744	0.136					0.062	
TT		138	66.3	166	76.5	0.021	0.606 (0.396-0.927)
GT		61	29.3	43	19.8	0.023	1.679 (1.073-2.628)
GG		9	4.3	8	3.7	0.737	1.182 (0.447-3.123)
T allele		337	81.0	375	86.4	0.033	0.671 (0.465-0.970)
G allele		79	19.0	59	13.6		
rs11771443	0.408					0.054	
TT		62	29.8	85	39.2	0.043	0.659 (0.441-0.987)
GT		85	40.9	87	40.1	0.871	1.033 (0.701-1.521)
GG		61	29.3	45	20.7	0.042	1.586 (1.018-2.472)
T allele		209	50.2	257	59.2	0.009	0.695 (0.530-0.912)
G allele		207	49.8	177	40.8		
rs3918188	0.435					0.236	
GG		67	32.2	74	34.1	0.679	0.918 (0.613-1.376)
GA		108	51.9	97	44.7	0.137	1.336 (0.912-1.957)
AA		33	15.9	46	21.2	0.159	0.701 (0.428-1.149)
G allele		242	58.2	245	56.5	0.612	1.073 (0.817-1.408)
A allele		174	41.8	189	43.5		
rs2853796	0.325					0.498	
TT		92	44.2	107	49.3	0.295	0.815 (0.557-1.194)
CT		87	41.8	79	36.4	0.252	1.256 (0.850-1.856)
CC		29	13.9	31	14.3	0.919	0.972 (0.563-1.679)
T allele		271	65.1	293	67.5	0.465	0.899 (0.677-1.196)
C allele		145	34.9	141	32.5		
rs7830	0.30					0.562	
GG		90	43.3	105	48.4	0.290	0.814 (0.555-1.192)
GA		98	47.1	94	43.3	0.432	1.166 (0.795-1.709)
AA		20	9.6	18	8.3	0.634	1.176 (0.603-2.292)
G allele		278	66.8	304	70.0	0.313	0.861 (0.645-1.151)
A allele		138	33.2	130	30.0		
rs1541861	0.258					0.856	
AA		122	58.7	127	58.5	0.979	1.005 (0.683-1.479)
GA		68	32.7	68	31.3	0.765	1.064 (0.708-1.600)
GG		18	8.7	22	10.1	0.601	0.840 (0.437-1.615)
A allele		312	75.0	322	74.2	0.787	1.043 (0.766-1.421)
G allele		104	25.0	112	25.8		
rs2853792	0.325					0.752	
AA		95	45.7	101	46.5	0.857	0.966 (0.659-1.414)
CA		93	44.7	91	41.9	0.564	1.120 (0.763-1.644)
CC		20	9.6	25	11.5	0.524	0.817 (0.439-1.521)
A allele		283	68.0	293	67.5	0.872	1.024 (0.768-1.365)
C allele		133	32.0	141	32.5		

[a]P value was calculated by 2 x 3 and 2 x 2 chi-squared tests based on codominant, dominant for the rare allele, heterosis and recessive for the rare allele models of inheritance.

Table 2. Frequencies of NOS3 haplotype in block 1 and the association with risk of coronary artery disease (CAD).

Haplotype	Cases [N (%)]	Controls [N (%)]	Statistics			
			χ^2	P	OR	95%CI
T-T-C	99 (47.596)	128 (58.986)	5.537	0.019	0.632	0.430-0.927
G-T-C	63 (30.288)	59 (27.189)	0.499	0.480	1.164	0.764-1.772
G-G-T	34 (16.346)	28 (12.903)	1.010	0.315	1.319	0.768-2.265

Table 3. Frequencies of NOS3 haplotype in block 2 and the association with risk of coronary artery disease (CAD).

Haplotype	Cases [N (%)]	Controls [N (%)]	Statistics			
			χ^2	P	OR	95%CI
A-A	141 (67.788)	146 (67.281)	0.012	0.911	1.023	0.682-1.536
G-C	52 (25.000)	56 (25.806)	0.036	0.849	0.958	0.619-1.484
A-C	15 (7.212)	15 (6.912)	0.014	0.904	1.047	0.498-2.199

Table 4. Frequencies of NOS3 haplotype in block 3 and the association with risk of coronary artery disease (CAD).

Haplotype	Cases [N (%)]	Controls [N (%)]	Statistics			
			χ^2	P	OR	95%CI
A-T	87 (41.827)	94 (43.318)	0.097	0.756	0.941	0.640,1.382
G-C	73 (35.096)	71 (32.719)	0.268	0.605	1.112	0.744,1.662
G-T	48 (23.077)	52 (23.963)	0.046	0.830	0.952	0.608,1.491

The difference in the distribution of genotype frequencies of rs1799983 between CAD subjects and healthy controls was significant (P = 0.000089). Patients with CAD had a significantly lower frequency of the G allele (χ^2 = 7.717, P = 0.007, OR = 1.649, 95%CI = 1.41-2.382). There was a significant between-group difference in the TT genotype distribution of rs2070744 (P = 0.021). Patients with CAD had a significantly higher frequency of the G allele of rs2070744 (χ^2 = 4.548, P = 0.033, OR = 1.490, 95%CI = 1.031-2.153) (Table 1).

Strong LD was observed in three blocks (D' > 0.9) (Tables 2-4). In block 1, significantly more T-T-C haplotypes (χ^2 = 5.537, P = 0.019, OR = 0.632, 95%CI = 0.430-0.927) were found in the controls.

DISCUSSION

It is well accepted that endothelial dysfunction occurs in response to cardiovascular risk factors and precedes the development of atherosclerosis (Ross, 1999). The constitutive NOS3 is expressed in the endothelium. A substitution from G894T in exon 7 of the *NOS3* that modifies its coding sequence (Glu298Asp) has been linked in some studies to the risk for coronary spasm, CAD, and diabetic nephropathy (Hibi et al., 1998; Hingorani et al., 1999; Colombo et al., 2002).

A total of 332 patients with CAD and 368 controls were included in a previous study. *NOS3* rs1799983 was significantly associated with CAD under the additive, dominant, but not the recessive models. This remained significant after adjustment for age, gender, diabetes, smoking, and hypertension. These findings suggest that the G894T (rs1799983) polymorphism of the *NOS3* was associated with CAD in Tunisian patients (Ben Ali et al., 2015). In our study, the difference in the distribution of genotype frequencies of rs1799983 between CAD subjects and healthy controls was significant. Patients with CAD had a significantly lower frequency

of the G allele. In contrast, the authors of a number of studies from different countries have reported that the *NOS3* 894G>T (Glu298Asp) polymorphism is not associated with an increased risk for CAD (Granath et al., 2001; Wang et al., 2001; Aras et al., 2002). In our study, subjects and controls were matched for ethnicity and drawn from a homogeneous population. Furthermore, the large sample size in our study provided sufficient statistical power to discern the epidemiologically relevant impact of *NOS3* polymorphisms.

The T786C (rs2070744) polymorphism, located in the promoter region of *NOS3*, may influence NOS3 expression levels; lower NOS3 mRNA expression and serum nitrite/nitrate levels have been found in individuals with the rs2070744 C allele (Miyamoto et al., 2000). Several studies have focused on the association between the *NOS3* rs2070744 polymorphism and susceptibility to CAD. However, the results were inconsistent (Miyamoto et al., 2000). In our study, patients with CAD had a significantly higher frequency of the G allele of rs2070744. In a meta-analysis, the overall analysis results showed that significant associations between the *NOS3* rs2070744 polymorphism and CAD risk were found in all six genetic models, confirming that the C allele of the *NOS3* rs2070744 polymorphism was associated with a statistically significant increase in CAD risk. Similarly, subgroup analysis based on the ethnicity of study populations showed that significant associations were found in Asians (Miyamoto et al., 2000).

In our study, haplotype analysis revealed a significantly greater frequency of the T-T-C haplotype (block 1) in the controls than in the CAD patients. Moreover, the point-wise associations of these variants with CAD were significant. These results indicate that people with the T-T-C haplotype of the *NOS3* are less susceptible to CAD. To some extent, this finding further supports a role of *NOS3* polymorphisms in CAD, while ethnic group differences may exist.

In conclusion, our study suggests a potential role of the *NOS3* SNPs (rs1799983 and rs2070744) in CAD. A broader examination of the genetic variation in *NOS3* in the Han Chinese may reveal other variants associated with disease risk.

Conflicts of interest

The authors declare no conflict of interest.

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