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NQO1 C609T Polymorphism is Associated with Coronary Artery Disease in a Gender-Dependent Manner

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Abstract Findings on the association of NQO1 C609T polymorphism in the NQO1 gene and cardiovascular disease susceptibility are controversial. The objective of the current study was to examine the relationship between this polymorphism and the presence and severity of angiographically determined coronary artery disease (CAD). One-hundred and forty-five patients with newly diagnosed angiographically documented CAD (≥50 % luminal stenosis of any coronary vessel) as case group were compared to 139 controls (subjects with no luminal stenosis at coronary arteries). The presence of C609T polymorphism was analyzed using polymerase chain reaction-based restriction fragment length polymorphism. Among total population, those with combined CT/TT (T allele carrier) genotype showed a trend toward lower odds of CAD compared to those with CC (wild type) genotype, but it did not reach a statistically significant level ($p = 0.061$). When data were analyzed separately for men or women, CT + TT group as compared to CC genotype was associated with decreased odds of CAD in women (adjusted OR 0.4, 95 % CI 0.2–0.9; $p = 0.043$), but not in men (adjusted OR 0.8, 95 % CI 0.3–1.9; $p = 0.612$). The C609T polymorphism within NQO1 is independently associated with CAD in women, but no association was observed in whole study population or in men.

Keywords Coronary artery disease · NAD(P)H: quinone oxidoreductase 1 · NQO1 C609T polymorphism · Gender

Introduction

Coronary artery disease (CAD), the leading cause of death in most parts of the world [1], is a multifactorial disease involving many genetic and environmental factors [2, 3]. Although the main risk factors for CAD, such as tobacco use, hypertension, diabetes, and dyslipidemia, have been identified, the underlying causes of the disease remain largely unclear [1].

A distorted balance between reactive oxygen species (ROS) and antioxidant defense causes oxidative stress which may play a major role in the beginning of atherosclerotic cardiovascular disease [4]. ROS, chemically reactive molecules containing oxygen, are formed as a natural by-product of the normal metabolism and have roles in cell signaling and homeostasis; however, enhanced generation of ROS may occur in environmental stress such as UV or heat exposure, toxicant exposure, radiation damage, and disease, leading to local oxidative stress [5]. NAD(P)H: quinone oxidoreductase 1 (NQO1) is an important detoxifying cytosolic flavoenzyme widely

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distributed on the surface of endothelial and epithelial cells [6]. NQO1 is considered an antioxidant enzyme by catalyzing a two-electron reduction of quinone compounds and detoxification of the electrophilic compounds [7], but in certain conditions NQO1 can act as a pro-oxidant enzyme contributing in the formation of ROS [8]. The gene coding for NQO1 is therefore being investigated in relation to progression of CAD.

The human NQO1 gene is located on chromosome 16q22.16; it is approximately 17 kb long and contains six exons. There are more than 93 gene single nucleotide polymorphisms (SNPs) related to NQO1 gene, but the most widely studied SNP is dbSNP: rs1800566, a cytosine (C) to thymine (T) change at nucleotide position 609 in exon six which results in a proline-to-serine amino acid change at codon 187 of the amino acid sequence of the protein [9]. In comparative studies with wild-type protein, the mutant NQO1 protein showed a dramatically reduced stability due to ubiquitination and proteasomal degradation [10]. The heterozygote variant genotype (CT) showed a threefold reduction in enzyme activity level, while homozygous variant (TT) had merely 2–4 % of the quinone reductase activity, as compared to wild-type genotype (CC) [11]. Although the association of NQO1 C609T polymorphism and cancer susceptibility has been extensively studied and can be considered conclusive [12, 13], findings on the relationship between this SNP and cardiovascular disease are still conflicting [7, 14–16], and there is no study that we are aware of that has tested this association in Iranian population. Therefore, the aim of this study was to examine whether there is an association between C609T SNP in the NQO1 gene with the presence and severity of angiographically determined CAD in an Iranian population.

Materials and Methods

Study Population

Between September 2013 and January 2014, we recruited 145 patients with CAD from patients attended to in our clinics at Tehran Heart Center. All cases had clinical manifestations of CAD and consequently showed angiographically documented CAD. The control group ($n = 139$) was selected from those attended to in our Valvular Heart Disease clinics during the same period of time in whom the results of coronary angiography were normal. Exclusion criteria were previous history of acute myocardial infarction (MI), stent implantation, cardiopulmonary resuscitation (CPR), and coronary artery bypass graft surgery. All angiograms were performed at the catheterization laboratory of our center. All participants signed written informed consent wherein explicitly provided permission for gathering the relevant clinical data and for DNA analyses. This study was conducted in agreement with the Declaration of Helsinki for research involving human subjects and was approved by the local ethical committee of our center.

Coronary Angiograms

Using standard techniques, coronary angiographies were performed by the percutaneous femoral approach. The presence of CAD was determined by the evidence of atherosclerosis, i.e., ≥50 % luminal stenosis in at least one coronary artery or major branch segment in their epicardial coronary tree. The degree of stenosis was defined as the visually determined greatest percentage of reduction in luminal diameter in any view compared to the nearest normal segment. Based on the number of vessels involved, scores ranged from one to three. Left main artery stenosis was scored as one-vessel disease. Controls had no luminal stenosis at coronary angiogram. The severity of CAD was determined by vessel score as well as a semi-quantitative scoring system (Gensini score) which has been previously described [17]. Briefly, the coronary arterial tree was divided into segments with multiplying factors according to geographic functional importance of any given segment (five points for the left main stem to 0.5 for the most distal segments), and the percent reduction in lumen diameter of each narrowing was assigned a score (0, 1, 2, 4, 8, 16, or 32, according to the degree of stenosis). The sum of the scores of all segments results in the Gensini score, which put emphasis on the severity of the disease.

Definitions of CAD Risk Factors

The following risk factors were assessed: body mass index (BMI), current smoking status, hypertension, diabetes mellitus, and dyslipidemia. Height and weight were measured to the nearest 0.1 cm and 0.1 kg, respectively, by qualified trained staff. BMI was calculated as weight (kg) divided by height squared (m^2). Patients were considered current smokers if they currently smoked any kind of tobacco or had quit smoking for less than 1 month. Patients were considered to have hypertension if they had received such a diagnosis with arterial pressure more than 140/90 mmHg or were being treated with antihypertensive drugs. Patients were considered to have diabetes providing that they were taking insulin or oral hypoglycemic agents. Patients with lack of awareness of their past history of diabetes were defined as a fasting blood glucose >110 mg/dl. Dyslipidemia was defined as plasma total cholesterol level ≥200 mg/dl, low-density lipoprotein cholesterol (LDL cholesterol) level ≥130 mg/dl, triglyceride level

≥150 mg/dl, and HDL cholesterol level ≤40 mg/dl or being on lipid-lowering drugs at the time of study.

Laboratory Measurements

Peripheral blood samples were obtained by vein puncture after a 10-h overnight fast from an antecubital vein. About 5 mL blood was collected in plain tubes and used for biochemical assays immediately; the rest of the sample (5 ml) placed in ethylenediamine tetraacetic acid (EDTA)-containing tubes and stored deep-frozen (−70 °C) until later use. Biochemical measurements such as total cholesterol, HDL cholesterol, triglycerides, and fasting blood sugar (FBS) levels were carried out by an auto analyzer (Roche/Hitachi 902; Roche Diagnostics GmbH, Manheim, Germany) using standard methods and commercial kits. LDL cholesterol was estimated based on Friedewald formula [18]. LDL cholesterol was not calculated if the serum triglyceride level was more than 400 mg/dl. The uric acid concentration was measured in milligrams per deciliter by an enzymatic colorimetric method using uricase.

DNA Extraction and NQO1 C609T Genotyping

Genomic DNA was extracted from leukocytes using the buffy coat of the stored EDTA whole blood samples. DNA extraction was carried out using 'salting-out' method. DNA quantity was evaluated by calculating absorbance at $\lambda = 260$ nm, and the quality was assessed by a ratio of $\lambda = 260/280$ nm being close to 1.8. The purified DNA was stored in Tris–EDTA buffer (pH 8.0) at −70 °C until the analysis of genotypes. The polymorphism of NQO1 C609T (rs1800566) was determined using PCR-restriction fragment length polymorphism (PCR-RFLP) as previously described by Naoe et al. [19]. Briefly, a 273-bp fragment of NQO1 gene was amplified using the following primers: The forward primer was 5′-AGTGGCATTCTGCATTT CTGTG-3′, and the reverse was 5′-GATGGACTTGCCC AAGTGATG-3′. The amplicon was then digested with using 1 unit of the HinfI (Takara, Japan) restriction enzyme and separated on a 3 % agarose gel. The genotypes were determined by the pattern on the digested bands: 188 and 85 bp bands as the Pro/Pro (wild type: CC) genotype, 151 and 85 bp as the Ser/Ser (homozygote: TT) genotype, and all three bands of 181, 151, and 85 bp as the Pro/Ser (heterozygote: CT) genotype.

Statistical Analysis

Continuous variables were expressed as mean ± standard deviation (SD) and categorical variables as frequency (%). Variables were tested for normality by visually inspecting box-plots and by using Kolmogorov–Smirnov normality test. Gensini score, which was skewed, presented as median and inter-quartile range (25th to 75th percentiles). The case and control groups were compared using the independent two-sample Student's t test (or Mann–Whitney U test if required) for the continuous variables and the Chi-square test (or the Fisher's exact test, as appropriate) for the categorical variables. Due to the highly skewed distribution of Gensini scores, to compare Gensini score among C609T genotypes (CC vs. CT + TT), the Mann–Whitney U test was chosen and subjects with normal coronary arteries (Gensini score = 0, $n = 168$) were excluded to ensure that any observed effect on graded severity was not being driven by those without any CAD in whom risk allele frequency is expected to be significantly lower. The logistic regression model was used to determine the association between the NQO1 C609T genotypes and CAD in a univariate model and in a multivariate regression in the presence of potential confounders. Odds ratio (OR) and 95 % confidence intervals (CI) were calculated in all patients and in males and females separately. A p value ≤0.05 was considered statistically significant. All statistical analyses were carried out by PASW Statistics for Windows, version 18.0 (Chicago: SPSS Inc.).

Results

The mean age of the studied subjects was 58 ± 12 years and 50.6 % were women. Clinical and demographic characteristics of the study sample stratified by sex are listed in Table 1. The women were younger and had a greater BMI as compared to men, whereas the prevalence of dyslipidemia and cigarette smoking was much more frequent in men. There was no significant difference in the prevalence of family history of CAD, hypertension, and diabetes between men and women. The distribution of genotype for the examined polymorphism in controls was as expected from the Hardy–Weinberg equilibrium (HWE) in both men and women subgroups, but in entire study population it was not consistent with that predicted by HWE ($\chi^2 = 5.43$, $p = 0.019$). This may be due to rarity of TT genotype. Allele and genotype frequencies for the NQO1 (rs1800566) polymorphism in the case and control groups, separated by sex, are presented in Table 2. Due to the rarity of the homozygous mutant genotype, the clumped TT + CT genotypes as carriers of T allele were compared with CC genotype. There was a statistically significant difference in the distribution of alleles and genotypes of this variant among women subgroup, but not among whole population or men.

Table 3 shows that after applying a binary logistic regression model with adjusting for age, sex, dyslipidemia,

Table 1 Baseline characteristics of the study population stratified by sex

	All ($n = 318$)	Men ($n = 157$)	Women ($n = 161$)	p value*
Age (years)	57.9 ± 12.0	59.5 ± 12.0	56.2 ± 11.8	0.014
Body mass index (kg/m^2)	26.9 ± 4.0	26.3 ± 3.8	27.4 ± 4.2	0.020
Cigarette smoking				<0.001**
Current smoker	30 (9.4)	27 (17.2)	3 (1.9)	
Ex-smoker	44 (13.8)	40 (25.5)	4 (2.5)	
Non-smoker	244 (76.7)	90 (57.3)	154 (95.7)	
Hypertension	146 (45.9)	74 (47.1)	72 (44.7)	0.666
Diabetes	54 (17.0)	28 (17.8)	26 (16.1)	0.766
Dyslipidemia	146 (45.9)	87 (55.4)	59 (36.6)	0.001
Family history of CAD	80 (25.2)	33 (21.0)	47 (29.2)	0.093
Triglyceride (mg/dl)	138.8 ± 68.6	133.4 ± 66.6	144.4 ± 70.6	0.234
HDL cholesterol (mg/dl)	41.3 ± 10.9	38.9 ± 9.8	43.9 ± 11.4	0.001
LDL cholesterol (mg/dl)	109.2 ± 37.4	102.9 ± 34.7	115.8 ± 39.1	0.010
Total cholesterol (mg/dl)	174.8 ± 49.1	164.2 ± 41.9	185.8 ± 53.7	0.001

Data are presented as mean ± SD or n (%)

CAD coronary artery disease, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein

* p values for men versus women

** Current smokers and ex-smoker were compared to non-smoker

Table 2 Genotype and allele frequencies of rs1800566 polymorphism in the NQO1 gene according to CAD status in whole study group and in subgroups separated by sex

	Non-CAD patients			CAD patients			p value*		
	All ($n = 175$)	Male ($n = 66$)	Female ($n = 109$)	All ($n = 143$)	Male ($n = 91$)	Female ($n = 52$)	All	Male	Female
rs1800566 (C > T)							0.190	0.962	0.033
CC	114 (65.1)	44 (66.7)	70 (64.2)	103 (72.0)	61 (67.0)	42 (80.8)			
CT + TT	61 (34.9)	22 (33.3)	39 (35.8)	40 (28.0)	30 (33.0)	10 (19.2)			
Alleles							0.250	0.932	0.043
C	288 (82.3)	110 (83.3)	178 (81.7)	245 (85.7)	151 (83.0)	94 (90.4)			
T	62 (17.7)	22 (16.7)	40 (18.3)	41 (14.3)	31 (17.0)	10 (9.6)			

Data are presented as n (%)

CAD coronary artery disease, *NQO1* NAD(P)H: quinone oxidoreductase 1

* p values for non-CAD versus CAD

diabetes mellitus, cigarette smoking, hypertension, and family history of CAD, rs1800566 polymorphism was found to be associated with the presence of CAD in women, but not in total population and in men. Among total population, those with combined CT/TT genotype showed a trend toward lower odds of CAD compared to those with CC genotype, but it did not reach a statistically significant level ($p = 0.061$). When data were analyzed separately for men or women, CT + TT group as compared to CC genotype was associated with decreased odds of CAD in women (adjusted OR 0.4, 95 % CI 0.2–0.9; $p = 0.043$), but not in men (adjusted OR 0.8, 95 % CI 0.3–1.9; $p = 0.612$).

Table 4 shows the association between the studied polymorphism and the severity of CAD with respect to Gensini score. The median and inter-quartile range for Gensini score was not significantly different between the CC genotype (25, 7–54) and CT + TT group (26, 6–54) with adjusted $p = 0.823$. There was also no statistically significant association between the examined polymorphism and the severity of CAD within men or women subgroups.

Table 3 Unadjusted and adjusted effect of rs1800566 genotypes on CAD in whole and in men and women separately

rs1800566 (C > T)	Unadjusted		Adjusted[a]	
	Odds ratio (95 % CI)	*p* value	Odds ratio (95 % CI)	*p* value
All participants				
CC	1.0 (Ref.)	–	1.0 (Ref.)	–
CT + TT	0.7 (0.5–1.2)	0.190	0.6 (0.3–1.1)	0.061
Male sex				
CC	1.0 (Ref.)	–	1.0 (Ref.)	–
CT + TT	1.0 (0.5–1.9)	0.962	0.8 (0.3–1.9)	0.612
Female sex				
CC	1.0 (Ref.)	–	1.0 (Ref.)	–
CT + TT	0.4 (0.2–0.9)	0.036	0.4 (0.2–0.9)	0.043

CI confidence interval, *Ref.* the reference category

[a] Adjusted for age, sex, dyslipidemia, diabetes mellitus, cigarette smoking, hypertension, and family history of coronary artery disease in all participants, and for all aforementioned covariates except for sex in male and female subgroups

Table 4 Genotypes of rs1800566 SNP in association with CAD severity measured by Gensini score

rs1800566 (C > T)	Median (25th to 75th percentiles), *n*[a]	*p* value*
All participants		0.823
CC	24.5 (7.250–54.125), 108	
CT + TT	26.0 (6.375–53.875), 42	
Male sex		0.909
CC	28.0 (10.000–63.000), 63	
CT + TT	29.5 (7.250–58.375), 32	
Female sex		0.793
CC	10.0 (5.000–50.000), 45	
CT + TT	15.5 (4.125–50.750), 10	

[a] A total number of 150 patients after excluding 168 with normal coronary arteries (Gensini score = 0)

* *p* values for CC versus CT + TT

Discussion

NQO1, a cytosolic enzyme with a molecular mass of 31 kDa, was first identified in rat liver and has been widely studied since then; it is broadly distributed in mammalian tissues, but the highest levels are generally expressed in liver and cardiovascular tissues [7]. NQO1 has a role in detoxification of numerous compounds primarily by catalyzing the two-electron reduction of the quinones to hydroquinones. The NQO1 variant C609T, which leads to an amino acid change from proline to serine at the site of codon 187 (nucleotide 609 position), is of essential importance because it directly affects the catalytic potential of the enzyme. This SNP makes the enzyme vulnerable to proteasomal degradation; the heterozygote genotype has a 60 % less catalytic efficiency, while a negligible amount of enzyme is found in homozygous mutant variant [10, 11]. A lack of activity in the detoxification enzyme NQO1 associated with the C609T variant may result in a susceptibility to oxidative stress [20] and could consequently play a role in cardiovascular disease [21]. In the present study, we evaluated the effects of C609T variant within NQO1 gene on CAD presence and severity in an Iranian sample. We found, contrary to our expectations, that this polymorphism has a protective effect on CAD in the studied women, as there was a lower frequency of T allele in the female patients with CAD than in corresponding controls, indicating a detrimental role of NQO1 in the development and progression of CAD.

The frequency of NQO1 C609T allele varies widely in different ethnic populations ranging from 0.22 to 0.45; the TT genotype is as rare as 2–5 % in Caucasians and Blacks, but as frequent as 20 % in Asians [22]. Among our study population, the variant allele of SNP rs1800566 (T allele) frequency was 0.162 in total, 0.143 in case group, and 0.177 in reference group. The T allele frequency of near 18 % in control group is consistent with the frequency reported for other Iranian healthy populations [23] and with that in general Caucasian population [24]. The allele

frequency of the NQO1 C609T polymorphism determined in a study of 245 Iranian healthy individuals in eight regions within the country was 0.149 for the T allele in total, ranging from 0.080 to 0.280 in various regions [23]. The T allele frequency of the examined polymorphism in a study of 2248 Chinese was reported to be 0.457 among 1142 CAD patients and 0.465 in 1106 controls, which is higher than that obtained in the present study [25].

Several experimental models have demonstrated that NQO1 might exert a protective effect on cardiovascular disease and related conditions [7]. For example, in an animal model of balloon injury of carotid artery, the activation of NQO1 prevented arterial restenosis by suppressing vascular smooth muscle cell proliferation [26]. Previous studies of the influence of NQO1 C609T polymorphism on cardiovascular disease in human subjects have, however, produced conflicting results [14–16, 25, 27, 28]. In 2008, for the first time, Martin et al. [15] reported the role of NQO1 polymorphism in CAD and biomarkers of oxidative stress. The investigators found higher frequencies of CT and combined CT + TT genotypes in controls compared to cases (OR 0.29 and 0.18 vs. control, respectively), suggesting a protective effect of the variant allele on CAD. On the other hand, they reported significantly higher levels of C-reactive protein, a biomarker for CAD, to occur among CAD patients with TT genotype as compared to CAD patients with CT or CC genotypes, but there was no significant difference between the CT and CC genotypes, which suggests a protective role of NQO1 in cardiovascular inflammation. As the author stated their findings should be interpreted with caution due to the low frequency of the TT genotype in the general population and the relatively small population sampled, only three of the cases had this genotype.

Ramprasath et al. [28] showed that NQO1 C609T variant might contribute to the development of CAD in people with type 2 diabetes mellitus of South Indian population. As compared to CC genotype, type 2 diabetes patients with TT genotype were found to have a 1.6-fold increased risk of developing CAD. This NQO1 polymorphism was also associated with carotid artery plaques in patients with type 2 diabetes [14]. In contrast, Shyu et al. [16] found a higher frequency of NQO1 C609T allele in the controls than in the patients with large-artery atherosclerotic stroke. These conflicting results may be attributed to different study design, differences in study population (e.g., age and gender of the studied subjects), small sample size, and variation in the definition of phenotype. The precise definition of phenotype in studies on CAD association is of essential importance because of the lack of proper control; for example, classification of subjects with significant CAD who are clinically silent or those with nonsignificant <50 % stenosis in coronary arteries as controls could lead to a higher likelihood of null results.

In a recent study on patients with angiographically documented CAD, Yu et al. [25] suggested that NQO1 C609T polymorphism was not associated with risk of CAD; there was no significant difference in the genotype and allele distribution between CAD patients and controls, which is consistent with our results in the entire population. However, in our study, when the analysis was stratified by sex, C609T SNP was associated with CAD in the female but not the male patients. Yu et al. [25] did not examine this association separately for men and women; thus, we were unable to compare our findings with those of these authors. The biological role of NQO1 in different physiological and pathophysiological processes, e.g., atherosclerosis, can be explained by its possible role in the regulation of translation through protection of eukaryotic translation initiation factor 4GI (eIF4GI) from proteasomal degradation [29]. Another notable issue is that significant sex-dependent association between this genetic variant and CAD may be justified by a likely interaction between NQO1 genotype and estrogen-dependent regulation of NQO1 expression. A higher expression of NQO1 in the biopsies of female than male patients with cutaneous melanomas has been shown [30]. In another study on the Sprague–Dawley strain of rats, significant higher mRNA, protein, and activity of NQO1 in female rats have been reported [31]. Bauer et al. [32] also demonstrated that benzene hematotoxicity was greater in $NQO1^{-/-}$ mice than in $NQO1^{+/+}$ mice in a gender-specific manner. Female $NQO1^{-/-}$ mice were sixfold more sensitive to benzene-induced genotoxicity than the female $NQO1^{+/+}$ mice, but this genotoxic response in male mice was similar between the two genotypes.

In conclusion, our findings suggest that the SNP C609T within NQO1 is independently associated with CAD in a sex-dependent manner. Using angiographic data and thus refining the phenotype, we found this association in women, but no association was observed in whole study population or in men. Further studies with larger sample size in subgroups of men and women are required to confirm such association in other racial or ethnic group. In addition, the exact mechanism and biological basis of possible role of this variant in pathogenesis of CAD need to be explained.

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