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eNOS 4a/b polymorphism and its interaction with eNOS G894T variants in type 2 diabetes mellitus: Modifying the risk of diabetic nephropathy

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Abstract. To investigate the possible association between eNOS 4a/b polymorphism and the risk of developing type 2 diabetes mellitus (T2DM) and diabetic nephropathy (DN) 173 T2DM patients with and without DN and 101 healthy subjects with ethnic background of Kurds were examined for the frequency of eNOS variants using PCR-RFLP method. The frequency of eNOS 4a/b genotypes between T2DM and controls was not significantly difference. Studying eNOS 4a/b variants alone indicated that the presence of *eNOS* 4a allele was not associated with the risk of developing DN. However, considering both polymorphisms of eNOS 4a/b and G894T indicated that the risk of macroalbuminuria significantly increased in the presence of either *eNOS* 4a or 894T allele by 2.45 times ($p = 0.014$) and 3.7-fold ($p = 0.016$), respectively. However, the concomitant presence of both alleles was not associated with the risk of macroalbuminuria. In microalbuminuric patients, in the presence of each allele, the risk of microalbuminuria increased 2.2 times ($p = 0.028$) and 2.72-fold ($p = 0.057$) for *eNOS* 4a and 894T alleles, respectively. However, the combined presence of both *eNOS* 894T and 4a alleles was not associated with the risk of microalbuminuria. The present study indicates the absence of association between eNOS 4a/b variants and the risk of developing T2DM and DN. Also, we demonstrated that *eNOS* 4a or 894T allele alone increased the risk of developing DN but this effect was modified by the concomitant presence of both alleles.

Keywords: T2DM, eNOS variants, diabetic nephropathy, Western Iran

1. Introduction

Familial aggregation of diabetic nephropathy (DN) and interethnic variation in developing DN among type 2 diabetes mellitus patients (T2DM) are indicative of the complexity of the disease and suggest a role for genetic susceptibility in developing DN among diabetic patients [1].

Nitric oxide (NO), a vasodilator molecule, is produced from L-arginine by endothelial nitric oxide synthase (eNOS). NO regulates endothelial function and is an important factor in the maintenance of homeostasis. The presence of eNOS variants might further complicate endothelial dysfunction and nephropathy through reduced production of NO [2,3]. Two polymorphisms of eNOS 4a/b and G894T are associated with a decreased eNOS activity and a reduced plasma level of NO [4,5].

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A variable number of tandem repeats (VNTR) in intron 4 of eNOS (NOS3) have been reported in association with cardiovascular and renal diseases [2]. This polymorphism comprises the two alleles of *eNOS* 4a with 4 tandem 27-repeats and *eNOS* 4b with 5 repeats [2].

The G894T polymorphism of eNOS results in a substitution of aspartate for glutamate at amino acid position 298 of the NOS3 protein [6].

There are conflicting reports related to the role of eNOS 4a/b variants on the risk of developing diabetes and its renal complications (DN) in various populations [2–10]. Due to differences in the distributions of eNOS variants among different ethnic groups, association of the variants of this gene with T2DM is ethnically dependent [5].

We have recently reported a non significant association between eNOS G894T polymorphism and DN among Kurdish population of Western Iran [11].

In the present study we intend to investigate the possible association between eNOS 4a/b variants and the risk of T2DM and DN. In addition, we want to find out the possible synergistic effects between eNOS 4a/b and G894T polymorphisms on the risk of DN in T2DM patients from Western Iran.

2. Materials and methods

2.1. Subjects

We have studied 274 individuals including 173 with T2DM (63 with microalbuminuria, 58 with macroalbuminuria, 121 DN, and 52 with normoalbuminuria) and 101 healthy subjects without history of diabetes according to fasting blood sugar. The controls were selected from medical staff and students. The power of test for studying eNOS polymorphisms among diabetic patients and controls based on our previous work [12] was 90% with confidence level of 95% using the following formula:

$$n = \frac{(z_{1-\alpha/2} + z_{1-\beta})^2 (p_1 q_1 + p_2 q_2)}{(P_1 - P_2)^2} = 163$$

$$p_1 = 11.9 \quad p_2 = 25.7$$

$$q_1 = 88.1 \quad q_2 = 74.3$$

$$z_{1-\alpha/2} = 1.96 \; z_{1-\beta} = 1.28$$

All subjects were from Kermanshah Province of Iran with Kurdish ethnic backgrounds who were admitted to Taleghani Diabetes Research Center of Kermanshah University of Medical Sciences. Type 2 diabetes mellitus was diagnosed according to WHO criteria [13].

Microalbuminuria and macroalbuminuria were defined as albumin to creatinine ratio, (ACR) of 30–299 mg/g and $\geqslant$ 300 mg/g, respectively in a random spot collection of urine in three specimens collected within a 3–6 month period. The ACR was measured in 24 h urine collection in those samples with ACR higher than 30 mg/g to confirm the presence of micro- or macroalbuminuria. Diabetic patients with ACR $<$ 30 mg/g made up the normoalbuminuric patients [11].

Detailed medical history of each patient including the presence of nephropathy, retinopathy, neuropathy, coronary artery disease (CAD) and hypertension was obtained. Informed written consent was obtained from each individual before participation. The study was approved by the Ethics Committee of Kermanshah University of Medical Sciences and was in accordance with the principles of the Declaration of Helsinki II.

DNA was extracted from the leukocyte fraction of the EDTA-treated whole blood using the phenol-chloroform method [14].

2.2. Genotype analysis

eNOS 4a/b polymorphism was detected using the forward primer of 5′ AGG CCC TAT GGT AGT GCC TTT 3′ and the reverse primer of 5′ TCT CTT AGT GCT GTG GTC AC 3′ flanking the 27-bp repeat in intron 4 of the gene. The PCR products were electrophoresed on a 3% agarose gel. In the presence of *eNOS* a allele a fragment with 393 bp is produced while the *eNOS* b allele produced a fragment with 420 bp [3].

For detection of eNOS G894T polymorphism, a region in exon 7 of eNOS gene containing this polymorphism was amplified using forward primer of 5′-AAG GCA GGA GAC AGT GGA TGG A-3′ and the reverse primer of 5′- CCC AGT CAA TCC CTT TGG TGC TCA-3′ [15]. After amplification, PCR products were digested overnight by 5 units of MboI restriction endonuclease at 37°C. The genotyping of the eNOS gene was determined by fragment separation on a 3.0% agarose gel. The MboI restricted products of eNOS G894T polymorphism including GG, GT, and TT genotypes had band sizes of 248 bp, 248 bp/158 bp/90 bp, and 158 bp/90 bp, respectively.

Table 1
The demographic and biochemical characteristics of diabetic patients

Variables	Normoalbuminric patients ($n = 52$)	Microalbuminuric patients ($n = 63$)	Macroalbuminuric patients ($n = 58$)
Age (years)	54.5 ± 8.8	55.7 ± 8.7 $p = 0.45$	57.8 ± 8.4 $p = 0.04$
BMI (Kg/m^2)	27.3 ± 4.8	27.9 ± 4.1 $p = 0.47$	26.8 ± 4.5 $p = 0.56$
HbA_{1C} (%)	7.8 ± 1.57	8.04 ± 1.58 $p = 0.48$	8.04 ± 1.58 $p = 0.48$
Diabetes duration (years)	7.8 ± 5.6	8.4 ± 5.3 $p = 0.57$	11.3 ± 6.6 $p = 0.04$
Systolic blood pressure (mmHg)	130.5 ± 20.9	129.9 ± 21.3 $p = 0.88$	141.8 ± 20.7 $p = 0.005$
Diastolic blood pressure (mmHg)	81.3 ± 9.2	80.4 ± 10.7 $p = 0.65$	85.5 ± 9.3 $p = 0.017$

*Comparison has been made with normoalbuminuric patients.

2.3. Statistical analysis

The allelic frequencies were calculated by the chromosome counting method. The observed genotype frequencies for eNOS polymorphism among patients and controls were tested for Hardy–Weinberg equilibrium using the χ^2 method [16]. The degrees of significance of differences in genotype and allele frequencies of eNOS between patients and controls were calculated using χ^2 test. Odds ratios (OR) were calculated as estimates of relative risk for the disease and 95% confidence intervals (CI) were obtained by SPSS logistic regression. The interaction between the two polymorphisms of eNOS 4a/b and eNOS G894T was determined using logistic regression model. The correlation values of biochemical and clinical data with the eNOS polymorphism between studied groups were calculated using linear regression and an unpaired t test. Two-tailed Student's t-test and ANOVA analysis were also used to compare quantitative data. The categorical variables among groups were compared using χ^2 test. Statistical significance was assumed at the $p < 0.05$ level. The SPSS statistical software package version 16.0 was used for the statistical analysis.

3. Results

Among T2DM patients with microalbuminuria there were 23 males and 40 females with a mean age of 55.9 ± 8.8 years. Patients with macroalbuminuria consisted of 26 males and 32 females with a mean age of 57.4 ± 8.5 years and normoalbuminuric T2DM patients were 15 males and 37 females with a mean age of 54.5 ± 8.9 years. Controls consisted of 31 males and 70 females with a mean age of 37.6 ± 13.3 years.

Table 2
Comparison of the frequency of eNOS genotypes and alleles between diabetic patients and controls

	Diabetic patients ($n = 173$)	Healthy subjects ($n = 101$)
eNOS genotypes		
aa	3 (1.7%)	0 (0%)
ab	46 (26.6%)	28 (27.7%)
bb	124 (71.7%)	73 (72.3%)
ab + bb	49 (28.3%)	28 (27.7%)
eNOS alleles		
a	52 (15%)	28 (13.9%)
b	294 (85%)	174 (86.1%)
	($\chi^2 = 0.13$, $p = 0.71$)	
	OR = 1.1 (0.66–1.79, $p = 0.71$)	

The characteristics of patients are depicted in Table 1. As demonstrated in Table 1 macroalbuminuric patients were older than normoalbuminuric patients ($p = 0.04$) and also had higher duration of diabetes ($p = 0.04$) and higher systolic and diastolic blood pressure ($p = 0.005$ and $p = 0.017$, respectively) than normoalbuminuric patients. Neuropathy, and history of CAD were detected in 67.6 and 33.8% of macroalbuminuric patients, respectively compared to the respective values of 58.3 ($p = 0.25$), and 22.2% ($p = 0.12$) in microalbuminuric and 52.8 ($p = 0.07$), and 19.4% ($p = 0.054$) in normoalbuminuric patients.

The frequencies of eNOS 4a/b genotypes and alleles were in Hardy-Weinberg equilibrium in all diabetic, normoalbuminuric, microalbuminuric and macroalbuminuric patients ($\chi^2 = 0.29$, $\chi^2 = 1.26$, $\chi^2 = 1.78$, $\chi^2 = 1.96$, $p > 0.1$) and in controls ($\chi^2 = 2.62$, $p > 0.1$).

In macroalbuminuric patients with eNOS aa + ab genotype, the risk of neuropathy was 77.8% compared with the value of 62.5% ($p = 0.25$) in the eNOS 4bb

Table 3
The distribution of eNOS genotypes and alleles in diabetic patients with and without nephropathy

	T2DM with Microalbuminuria ($n = 63$)	T2DM with Macroalbuminuria ($n = 58$)	All diabetic nephropathy ($n = 121$)	Normoalbuminuric T2DM ($n = 52$)
eNOS genotypes				
aa	3 (4.8%)	0 (0)	3 (2.5%)	0 (0%)
ab	14 (22.2%)	18 (31%)	32 (26.4%)	14 (26.9%)
bb	46 (73%)	40 (69%)	86 (71.1%)	38 (73.1%)
	($\chi^2 = 2.74$, df $= 2$, $p = 0.25$)	($\chi^2 = 0.23$, df $= 1$, $p = 0.63$)	($\chi^2 = 1.31$, df $= 2$, $p = 0.51$)	
OR (95%CI, p)	1.2 (0.58–2.49, $p = 0.61$)	1.22 (0.53–2.79, $p = 0.63$)	1.21 (0.61–2.37, $p = 0.58$)	
aa + ab	17 (27%)	18 (31%)	35 (28.9%)	14 (26.9%)
eNOS alleles				
a	20 (15.9%)	18 (15.5%)	38 (15.7%)	14 (13.5%)
b	106 (84.1%)	98 (84.5%)	204 (84.3%)	90 (86.5%)
	($\chi^2 = 0.26$, df $= 1$, $p = 0.60$)	($\chi^2 = 0.19$, df $= 1$, $p = 0.66$)	($\chi^2 = 0.29$, df $= 1$, $p = 0.59$)	
OR (95%CI, p)	1.21 (0.58–2.54, $p = 0.60$)	1.09 (0.74–1.58, $p = 0.66$)	1.2 (0.61–2.31, $p = 0.59$)	

*The distribution and comparisons of alleles and genotypes frequencies of eNOS in diabetic patients with nephropathy compared with diabetic patients without nephropathy were made using χ^2 test.

genotype. In microalbuminuric and macroalbuminuric patients carrying aa + ab genotype, the frequencies of hypertension were 76.5 and 72.2%, respectively compared to the microalbuminuric (56.5%, $p = 0.12$) and macroalbuminuric (67.5%, $p = 0.48$) patients with the eNOS 4bb genotypes.

Distribution of eNOS 4a/b genotypes between diabetic patients and healthy individuals showed 46 subjects (26.6%) with eNOS ab and 3 subjects (1.7%) with eNOS 4aa genotype in T2DM patients compared to 28 subjects (27.7%) with eNOS 4ab genotype in the controls ($p = 0.41$). The frequency of a allele was 15% in all diabetic patients compared to 13.9% ($p = 0.71$) in healthy controls (Table 2).

The frequency of eNOS 4a/b genotypes and alleles in diabetic patients are shown in Table 3. The frequency of eNOS 4ab genotype was 31% ($p = 0.63$) in macroalbuminuric patients, 27% ($p = 0.99$) in microalbuminuric and 26.9% in normoalbuminuric ones. Considering each gender there was no significant difference in the distribution of eNOS 4a/b genotypes between patients and controls. The eNOS 4aa genotype was only observed in microalbuminuric patients with a frequency of 4.8%. The frequency of eNOS 4a allele was 15.5% ($p = 0.66$) in macroalbuminuric, 15.9% ($p = 0.60$) in microalbuminuric and 13.5% in normoalbuminuric group. The presence of *eNOS* 4a allele was not associated with the risk of diabetic nephropathy [OR = 1.2, 95%CI 0.61–2.31, $p = 0.59$] (Table 3).

We had previously, reported [11] that the presence of GT genotype of eNOS G894T polymorphism increased the risk of developing macroalbuminuria and microalbuminuria by 1.86-($p = 0.11$) and 1.21-($p = 0.63$) fold, respectively in T2DM patients. In the present study we investigated the effects of the interaction between *eNOS* 4a and 894T alleles on the risk of DN. Combined analysis of both *eNOS* 4a and 894T alleles indicated that the presence of either *eNOS* 4a or 894T allele significantly increased the risk of macroalbuminuria by 2.45-($p = 0.014$) or 3.7-fold ($p = 0.016$), respectively. However, the concomitant presence of both alleles was not associated with the risk of macroalbuminuria. In microalbuminuric patients, almost the same values were obtained for the increased risk of developing microalbuminuria in the presence of each allele (2.2-($p = 0.028$) and 2.72-fold ($p = 0.057$) for *eNOS* 4a and 894T allele, respectively). In contrast, the concomitant presence of both alleles of *eNOS* 894T and 4a was not associated with a reduced risk of developing microalbuminuria [OR = 0.77, 95%CI 0.47–1.25, $p = 0.28$] (Table 4).

4. Discussion

Susceptibility to nephropathy is different among diabetic patients and could be attributed to genetic backgrounds. DN starts with various renal functional changes including glomerular hyperfilteration and hyperperfusion, and increased glomerular filteration rate (GFR) [17]. Endothelial dysfunction has been involved in the pathogenesis of DN. eNOS has an essential role in the regulation of endothelial function through production of NO [4]. eNOS polymorphism might be involved in the pathogenesis of hypertension, atherosclerosis and renovascular injury in diabetic patients through the production of an abnormal level of eNOS [9].

Table 4
Carrier odds ratios interaction between eNOS a with eNOS T alleles in T2DM patients with macroalbuminuria compared to normoalbuminuric and microalbuminuric patients

eNOS a	eNOS T	Macroalbuminuric patients n (%) [OR (95% CI, p)]	Microalbuminuric patients n (%) [OR (95% CI, p)]	Normoalbuminuric patients (control group) n (%)
−	−	14 (28.6%) Reference group	19 (36.5%) Reference group	23 (54.8%) Reference group
+	−	11 (22.4%) [2.45 (1.19–5.04, $p = 0.014$)] ($\chi^2 = 6.74$, df = 1, $p = 0.009$)	12 (23.1%) [2.2 (1.09–4.43, $p = 0.028$)] ($\chi^2 = 5.38$, df = 1, $p = 0.02$)	3 (7.1%)
−	+	18 (36.7%) [3.7 (1.27–10.72, $p = 0.016$)] ($\chi^2 = 6.02$, df = 1, $p = 0.014$)	18 (34.6%) [2.72 (0.97–7.63, $p = 0.057$)] ($\chi^2 = 3.72$, df = 1, $p = 0.052$)	8 (19%)
+	+	6 (12.2%) [1.07 (0.7–1.62, $p = 0.74$)] ($\chi^2 = 0.1$, df = 1, $p = 0.74$)	3 (5.8%) [0.77 (0.47–1.25, $p = 0.28$)] ($\chi^2 = 1.16$, df = 1, $p = 0.28$)	8 (19%)

In the present study, the frequency of *eNOS* 4a allele tended to be higher in DN patients than in the normoalbuminuric ones. However, an association between eNOS 4a/b variants and the risk of developing DN and T2DM was not found.

Several reports have examined the role of eNOS 4a/b polymorphism in the susceptibility to T2DM and DN with inconsistencies. Ezzidi et al. [7] indicated an association between eNOS 4a/b and T2DM among North African Tunisian (OR $= 1.46$, $p < 0.001$), but they did not find any association with DN. Also, an association between *eNOS* 4a allele and aa + ab genotype and an increased risk of developing T2DM by about 2-fold has been reported among a group of Iranians with Fars ethnic background [8]. In contrast, the study of Thameem et al. [5] indicated an absence of association between this polymorphism and the risk of developing T2DM among Mexican Americans. Neugebauer et al. [9] observed a statistically significant difference in the genotype distribution and the frequency of *eNOS*a allele of eNOS 4a/b polymorphism between T2DM patients with and without nephropathy with a 2.87-fold increased risk of progression to advanced diabetic nephropathy among Japanese patients. They suggested that the association between DN and eNOS 4a/b polymorphism is not through an elevation of hypertension. Furthermore, in the study of Ahluwalia et al. [3] the presence of eNOS 4a/b was associated with a 6.03-fold ($p < 0.0001$) increased risk of developing DN among Indians. Bellini et al. [2] demonstrated a strong association between *eNOS* 4a allele and end stage renal disease (ESRD) among a multiethnic group from Brazil. They suggested that this polymorphism might be a genetic marker for susceptibility to ESRD even in multiethnic populations. However, eNOS 4a/b variants were not significantly associated with the risk of developing DN in T2DM patients from Iran [8]. Association of eNOS 4a/b polymorphism with the risk of developing T2DM and not with its complications such as DN might be attributed to the diverse effects of eNOS 4a/b variants on diabetes and the microvascular complications of diabetes [8]. In a recent meta-analysis by He et al. [4] eNOS 4a/b polymorphism (*eNOS* 4a versus 4b allele) were significantly associated with developing DN in an East-Asian population but not in the Caucasians. In contrast to all of the above studies, Caucasian-Brazilian VNTR intron 4a/b was neither associated with T2DM nor with the renal complications of the disease [10].

There are ethnic differences in the endothelial function and the development of DN leading to a higher development of DN and ESRD among T2DM patients from South Asia compared to those from Europe [3]. The controversial reports related to the role of eNOS variants on the risk of developing T2DM and DN could be attributed to ethnic differences (even within the same country), the sample size, the multifactorial nature of the DN and the gene-environment interactions [10].

The role of eNOS G894T polymorphism upon the risk of develop and progression of DN has been investigated in several reports [3,17–19]. While most studies observed an association between the variants of eNOS G894T and the risk of DN [3,7,18] Zanchi et al. [19] failed to demonstrate an association.

In the present study, analyzing both *eNOS* 4a and 894T alleles revealed in the presence of either *eNOS* 4a or 894T allele, the risk of developing DN increased significantly. However, the concomitant presence of both alleles was not associated with the increased risk of macroalbuminuria or even with the reduced risk of microalbuminuria. Among Indians, the presence of either

eNOS T786C or G894T polymorphism alone significantly elevated the risk of developing DN [3]. However, in their study the concomitant presence of both *eNOS* 894T and 786 C alleles were associated with a non significant increase in the risk of developing DN. There may be several explanations for the absence of significant positive influence of the two alleles of *eNOS* 4a or 894T on the risk of developing nephropathy in spite of the significant effect of each allele on the risk of developing DN including the modulation of the risk of developing DN in the presence of both polymorphisms combined [3], reduction of sample size in the presence of both alleles and unidentified mechanisms underlying decreased risk of DN in the presence of both mutant alleles.

5. Conclusion

The present study indicates the absence of association between eNOS 4a/b variants and the risk of developing T2DM and DN and suggests a role for ethnicity and genetic background for susceptibility to diabetes and its complications even within the same country. It also demonstrates that *eNOS* 4a or 894T allele alone increases the risk of developing DN but the effect is modified by the concomitant presence of both alleles.

6. Limitations of the study

One of the limitations of this study is the higher mean age of the diabetic patients compared with controls. The presence of a younger cohort of controls could also bias the results by including cases that have not yet presented themselves. The lower number of patients available for the analysis of the concomitant presence of both polymorphisms may also be considered as another limitation.

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