



Association of the endothelial nitric oxide synthase gene G894T polymorphism with the risk of diabetic nephropathy in Qassim region, Saudi Arabia—A pilot study



Amal Mohammed Husein Mackawy^{a,*}, Amjad Ali Khan^b,
Mohammed El-Sayed Badawy^c

^a Medical Biochemistry and Molecular Biology, Faculty of Medicine, Zagazig University and Medical Laboratory department, Applied Medical Science, Qassim University, Saudi Arabia

^b Applied Medical Science College, Qassim University, Saudi Arabia

^c King Fahd Specialist Hospital, Saudi Arabia

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ABSTRACT

Background: Diabetic nephropathy (DN) is a chronic microangiopathic complication of type 2 diabetes mellitus (DM). Vascular endothelial dysfunction resulting from impaired nitric oxide synthase (NOS) activity in the vascular endothelial cells has been suggested as playing an important role in the pathogenesis of diabetic nephropathy (DN). Endothelial nitric oxide synthase (E-NOS) gene G894T polymorphism has been reported to be associated with endothelial dysfunction leading to DN.

Our objective was to evaluate the association of G894T polymorphism of eNOS gene with the risk of DN among type 2 diabetic Saudi patients. **Methods:** One hundred and twenty subjects were included in this study. They were divided into three groups. Group I, 40 controls. Group II, 40 type 2 diabetic patients without nephropathy. Group III, 40 type 2 diabetic patients with nephropathy. Endothelial nitric oxide synthase (eNOS) G894T polymorphism was detected by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). Plasma nitric oxide (NO) levels were estimated.

Results: E-NOS genotype frequency showed non-significant differences among the all studied groups ($p > 0.05$). Both diabetic groups had significantly higher plasma nitrate levels than in controls with a significant increase in group III than in group II patients (all $p < 0.0001$).

* Corresponding author. Tel.: +20 966508129407 (mobile).

E-mail address: amalmackawy@yahoo.com (A.M.H. Mackawy).

E NOS 894TT genotype was associated with higher plasma nitrate levels in all groups.

Conclusion: E-NOS gene SNP is not considered as genetic risk factor for DN among type 2 diabetic Saudi patients. The higher plasma levels of nitrates as a marker of oxidative stress in diabetic patients with nephropathy suggest the possible role of oxidative stress but not e-NOS gene SNP in pathogenesis of the DN.

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Introduction

In the Eastern Mediterranean Region more than 16 million people are living with diabetes, a staggering number which is expected to rise to almost 43 million by the year 2025 (Stakeholder, 2008). Diabetes has reached near-epidemic proportions in the United Arab of Emirates (UAE) and Saudi Arabia, with nearly one out of every five individuals suffering from diabetes in the UAE. In Saudi Arabia, the prevalence is expected to rise to between 40 and 50% by 2020 (Stakeholder, 2008).

Vascular endothelium modulates blood vessel wall homeostasis through the production of factors regulating vessel tone coagulation state, cell growth, cell death and leukocyte trafficking (Mohsen et al., 2006). One of the most important endothelial cell products is nitric oxide (NO), which is synthesized from L-arginine by the enzyme endothelial nitric oxide synthase (eNOS). Endothelial nitric oxide synthase (eNOS; NOS3) produces nitric oxide (NO) from L-arginine. NO has diverse physiologic regulatory functions and is involved in smooth muscle relaxation, inhibition of platelet aggregation, immune regulation, neurotransmission and blood pressure regulation (Rabbani et al., 2011). An increased production of reactive oxygen species (ROS), including superoxide anion (O_2^-) may contribute to diabetic complications (McLennan et al., 2000). O_2^- reacts with NO with great affinity to produce peroxynitrite ($ONOO^-$) (Gryglewski et al., 1986) which is a weak agonist for activation of cyclic guanosine monophosphate (cGMP) (Villa et al., 1994). Therefore, O_2^- effectively inactivates nitric oxide (NO). The activation of NAD (P)H oxidase in diabetes mellitus (DM) is postulated to suppress the action of NO (Ohishi and Carmines, 1995) to increase the expression of the mRNA for transforming growth factor- β 1 (TGF- β 1) and fibronectin in the glomerulus, to decrease the expression of matrix metalloproteinases, and to increase the expression of the tissue inhibitor of metalloproteinases in the kidney (Chiarelli et al., 2009). These diverse effects could contribute to the pathophysiology of diabetic nephropathy (DN) (Schnackenberg and Wilcox, 2001).

DN is a chronic microangiopathic complication of both type 1 and type 2 diabetes mellitus and is the primary cause of end-stage renal disease (Gross et al., 2008).

It has been shown that eNOS inhibition accelerates atherosclerosis in animal models, and that abnormalities of the endothelial NO pathway are present in humans with atherosclerosis (Salimi et al., 2010). This evidence suggests that NO may inhibit several key steps in the atherosclerotic process and that an alteration of NO production within the vascular endothelium could contribute to the pathogenesis of atherosclerosis. Thus eNOS could be a candidate gene for atherosclerosis (Manjula, 2011). A single base exchange (G894 \rightarrow T) in exon 7 of the human endothelial nitric oxide synthase (eNOS) gene results in a Glu \rightarrow Asp substitution at residue 298 of the eNOS gene.

The functional significance of this single nucleotide polymorphism (SNP) remains an issue of controversy since homozygosity for the Asp298 variant has been related to reduced enzyme activity (Manolio et al., 2008). Two meta-analyses have discussed the association of eNOS gene polymorphisms with the risk of DN, the first one supported lack of association between them (Zintzaras et al., 2009). Although the second meta-analysis supports the effects of the three polymorphisms (894G $>$ T, 4b/a, and T-786C) in the eNOS gene on the risk of DN, it reported that none of them has been convincingly proved as determinants responsible for the development of DN, and referred that association to the linkage disequilibrium of these polymorphisms with other unidentified functional causative mutations that exist in the eNOS gene and affect the susceptibility to DN (Zeng et al., 2010). Associations between this variant and coronary spasm, coronary artery disease and acute myocardial infarction have been reported, but data on its relation with disease severity are lacking (Manjula et al., 2011).

Until now there are not enough researches on the effect of the eNOS G894T SNP and the incidence of diabetic nephropathy in type 2 DM in Saudi Arabia.

Our objective in this study was to evaluate the association of G894T polymorphism of eNOS gene with the risk of DN among type 2 diabetic Saudi patients.

Subjects and methods

This case-control study was started in June 2012 to October 2013. It was carried out at Medical Laboratory Department, Applied Medical Science, Qassim University. One hundred and twenty subjects were included in this study. The subjects were recruited from Endocrinology and Nephrology outpatient clinics of the Internal Medicine Department of the public and private hospitals in Qassim region and they belonged to the same ethnic group: mixed.

- Diagnosis of type 2 DM based on the criteria established by the American Diabetes Association Expert Committee [confirmed fasting blood glucose >126 mg/dl (7 mmol/l) and/or 2-h postprandial glucose level > 200 mg/dl (11.1 mmol/l) on more than one occasion] ([World Health Organization, 2006](#)) and history of hypoglycemic treatment. Type 2 DM subjects were selected on the basis of at least 5 years from diagnosis without insulin treatment and absence of concomitant autoimmune disease. Patients who did not meet these criteria as under treatment but who gave a history of T2DM were also included in the study. DN was defined as urinary albumin excretion of >30 mg in 24 h urine collections ([Lamb et al., 2009](#)). Patients with collagen vascular diseases, chronic infections, liver diseases as well as those with kidney diseases other than DN were excluded from the study.

All included subjects were divided into three main groups:

Group I: 40 non diabetic subjects with no family history of diabetes or any chronic disease may interpret our results. Exclusion criteria for control subjects were: the presence of type 2 DM or of a first-degree relative with type 2 DM, the presence of collagen vascular diseases, chronic infections and presence of chronic liver and kidney diseases.

- Diabetic patients were further classified according to the progress of the disease into:

II-Group II 40 patients suffered from type 2 DM without nephropathy who had T2DM for at least 10 years or more after diagnosis and urinary albumin excretion of <30 mg in 24 h urine collections ([Lamb et al., 2009](#)).

III-Group III 40 patients suffered from type 2 DM with DN who had persistent proteinuria. DN was defined as urinary albumin excretion of >30 mg in 24 h urine collections ([Lamb et al., 2009](#)). Diabetic subjects without proteinuria but on antihypertensive drug treatment were excluded from the study group in order to avoid misclassification of phenotype.

For all subjects, a complete medical history was obtained by questionnaire. History taking included questions about smoking habits, history of hypertension and type 2 diabetes, and current medication used. Local Ethics Committee approval and a written informed consent were obtained from all patients.

For all the subjects the following were done

I- Full history and clinical examination.

II- Research investigations:

- 1- Estimation of fasting blood glucose (FBG) level by enzymatic method. ([Trinder, 1969](#)).
- 2- Determination of glycosylated hemoglobin (HbA1c) in blood ([Abraham et al., 1978](#)).
- 3- Total lipid profile levels:
 - Triglyceride was determined enzymatically ([Bucolo and David, 1973](#)).
 - Total cholesterol was estimated by enzymatic method ([Assmann et al., 1983](#)).
 - Lipoprotein cholesterol (HDL-C) ([Assmann et al., 1983](#)).
 - Low density lipoprotein cholesterol (LDL-C) (LDL-C concentration was calculated by the Friedewald formula).
 - Estimation of high density ed by the Friedewald formula.
- 4- Measurement of plasma creatinine ([Murray and Kaplan, 1989](#)).
- 5- 24 h urine samples were collected for protein analysis ([Orsonneau et al., 1989](#)).

- 6- Determination of plasma nitric oxide (NO) levels (Green et al., 1982).
- 7- Determination of endothelial nitric oxide synthase (eNOS) G894T polymorphism by PCR-based RFLP (Nishevitha et al., 2009).

Sampling

Under complete aseptic conditions, 5 ml of venous blood was collected after 12 hour fasting in sterile EDTA containing tubes and divided into two tubes:

One tube of whole blood was collected for DNA extraction and eNOS gene G894T SNP detection and kept immediately at -20°C . Plasma specimen of the other tube was obtained by centrifugation at low speed centrifugation at 2500 \times g; 15 min for estimating the plasma nitrate levels and other research investigations.

DNA analysis

DNA extraction and DNA analysis for eNOS G894T polymorphism were achieved by PCR-based RFLP. (Nishevitha et al., 2009). Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit purchased from Promega. The average DNA concentration was determined from absorbance at 260 nm. All DNA samples were examined at a 260/280 nm absorbance ratio. The integrity of the DNA was checked by electrophoresis on 1.5% agarose gel with an ethidium bromide. Isolated DNA was used for determination of the eNOS G894T SNP. Genomic DNA (~ 50 ng) was incubated in a total reaction volume of 50 μl containing equal concentration of the forward primer 5' TCC CTG AGG GCA TGA GGC T-3' (70 picomoles) and reverse primer 5' TGA GGG TCA CAC AGG TTC CT-3', 200 μM d NTP, 10X PCR buffer pH=8.3 containing 15 mM MgCl_2 and 1.5 units of Taq DNA polymerase. DNA was initially denatured at 95°C for 5 min prior to amplification. PCR amplification was accomplished using 30 cycles, consisting of 2 min denaturation at 95°C , 45 sec annealing at 62°C , and 1 min extension at 72°C and the final extension included a 1 min extension at 72°C . Restriction digestion was performed in a total volume of 10 μl amplicons, 1 μl NE buffer (50 mM potassium acetate, 20 mM tris-acetate, 10 Mm magnesium acetate, 1 mM dithio-threitol pH=7.9 at 25°C) and 8 units of *Ban II* enzyme. Samples then were incubated for 5 h at 37°C and the digested PCR products were separated by 2.5% agarose gel electrophoresis stained with ethidium bromide and visualized on a UV transilluminator with 100 base pair ladder and photographed.

Statistical analysis

Statistical power for linkage or association analysis is computed by specifying genetic model parameters such as the disease allele frequency and the conditional probabilities. The appropriate sample size and power of the study were calculated using PAWE-3D. PAWE-3D calculations showed that the sample size, together with the specified study design, allele frequencies, the incidence of nephropathy among T2DM patients, and allowable error rates, can give as high as 90% power and can detect variant allele frequency of at least 0.05.

The results for continuous variables are expressed as means and standard deviation (SD). The differences between mean values for each element were tested by one way analysis of variance test (Anova "F" test) and Post Hoc Tukey HSD (high significance difference). Tukey's test calculates a new critical value that can be used to evaluate whether differences between any two pairs of means are significant. The statistical significance of differences in frequencies of variants between the groups was tested using the chi-square (χ^2) test. In addition, the odds ratios (ORs) and 95% confidence intervals (CIs) were calculated as a measure of the association of the e-NOS alleles with groups. *P*-values were considered significant when $p < 0.05$. All data analysis was using SPSS version 16.0.

Results

Demographic, clinical, and laboratory characteristics with ANOVA (F-test) and Post Hoc Tukey HSD values of the studied groups are shown in Tables 1 and 2.

The mean age–SD of diabetic patients without nephropathy (group II) was 51.67–6 years, for diabetic patients with nephropathy (group III) it was 50.45–8 years and that of the controls 51.67–6.01 years.

Plasma nitrate, FBG and HbA1c levels showed statistical significant difference among all studied groups. Plasma nitrate levels were significantly increased in diabetic patients more than controls with a more significant increase in group III patients comparing to patients of group II (Table 2). Plasma levels of TG, total cholesterol, LDL-C and FBG were significantly higher in groups II and III patients as compared to controls (Table 2), but no significant differences were found between group II and III diabetic patients regarding LDL-C levels (Table 2). HDL-C were significantly decreased in diabetic patient groups when compared to controls with no significant differences between group II and III patients (Table 2). No significant differences were found between both groups of diabetic patients (group II and III) as regarding to HbA1c (Table 2). ANOVA (F) test values are shown in Table 1.

The genotype and allele frequencies with chi square (χ^2) value for the eNOS G894T polymorphism in all studied groups are illustrated in Fig. 1 and Table 3.

The results showed non-significant differences between controls and patients with DN and patients without DN regarding the genotype and allele distributions of the G894T SNP ($\chi^2 = 0.621, P = 0.733, \chi^2 = 2.17, P = 0.33$) ($\chi^2 = 0.681, P = 0.409, \chi^2 = 2.146, P = 0.14$), respectively. Moreover, no significant differences of G894T genotypes could be detected between group II and group III ($\chi^2 = 0.687, P = 0.71$) ($\chi^2 = 0.41, P = 0.521$), respectively.

Effect of +894 G/T SNP on plasma nitrate levels (ANOVA F-test, Post Hoc Tukey HSD test and P values) (Table 4).

Carriers of the T allele and TT subjects exhibited higher levels of plasma nitrates compared with GT and GG carriers in each group ($p < 0.05$ for each).

Discussion

NO plays a fundamental role in the regulation of endothelial function and vascular tone in many organs, including the kidney (Drexler and Horning, 2009). NO inhibits platelet aggregation and leukocyte adhesion to vascular endothelium with inhibition of LDL oxidation (Casas et al., 2006).

Alterations of endothelial function play a pivotal role in the development of atherosclerosis and predict the occurrence of atherosclerotic complications (Suwaidi et al., 2000; Schachinger et al., 2000). Oxidative stress has played a critical role in the pathogenesis of both micro- and macrovascular complications of diabetes, and increased intracellular glucose leads to electron transport chain and increased formation of secondary reactive oxygen species (ROS) (Forbes et al., 2008; Brownlee, 2005). A unifying hypothesis has been proposed whereby mitochondrial production of ROS in response to chronic hyperglycemia may be the key initiator for these pathogenic pathways. This increases the importance of mitochondrial

Table 1
Basic characteristics of all participant groups.

Parameters	Group I (n = 40)	Group II (n = 40)	Group III (n = 40)	Chi (χ^2) square, ANOVA (F) value with P values
Age (years)	51.67 ± 6.01	53.40 ± 7.96	50.45 ± 8.03	F = 1.851, P = 0.160
Sex	21 F (52.5%) 19 M (47.5%)	22 F (55%) 18 M (45%)	17 F (42.5%) 23 M (57.5%)	$\chi^2 = 0.05, P^* = 0.823$ (NS) $\chi^2 = 0.110, P^{**} = 0.740$ (NS) $\chi^2 = 1.018, P^{***} = 0.313$ (NS) F = 218.658, P = 0.000
Plasma nitrate (µmol/ml)	11.38 ± 2.17	18.40 ± 1.81	20.42 ± 2.08	F = 24.63, P = 0.000
TC (mg/dl)	197.98 ± 22.3	229.1 ± 41.16	271.51 ± 68.73	F = 34.315, P = 0.000
TG (mg/dl)	145.62 ± 18.31	167.08 ± 24.97	184.70 ± 31.05	F = 43.165, P = 0.000
LDL-C (mg/dl)	93.6 ± 12.3	106.52 ± 8.23	109.32 ± 8.60	F = 22.921, P = 0.000
HDL-C (mg/dl)	54.25 ± 6.65	47.50 ± 6.68	48.93 ± 6.44	F = 195.483, P = 0.000
FBG (mg/dl)	103.1 ± 11.07	136.21 ± 8.87	138.83 ± 8.09	F = 83.919, P = 0.000
HbA1c	4.73 ± 0.98	7.34 ± 0.98	7.67 ± 1.38	F = 83.919, P = 0.000

Table 2

Post Hoc Tukey test with high significance difference (HSD) of biochemical parameters in all studied groups.

Dependent variable	(I) VAR00001	(J) VAR00001	Mean difference (I – J)	Std. error	Sig.	95% confidence interval	
						Lower bound	Upper bound
TC (mg/dl)	1	2	-32.34750*	10.64771	.008	-57.6242-	-7.0708-
		3	-74.51000*	10.64771	.000	-99.7867-	-49.2333-
	2	1	32.34750*	10.64771	.008	7.0708	57.6242
		3	-42.16250*	10.64771	.000	-67.4392-	-16.8858-
	3	1	74.51000*	10.64771	.000	49.2333	99.7867
		2	42.16250*	10.64771	.000	16.8858	67.4392
TG (mg/dl)	1	2	-21.05000*	5.30369	.000	-33.6405-	-8.4595-
		3	-43.92500*	5.30369	.000	-56.5155-	-31.3345-
	2	1	21.05000*	5.30369	.000	8.4595	33.6405
		3	-22.87500*	5.30369	.000	-35.4655-	-10.2845-
	3	1	43.92500*	5.30369	.000	31.3345	56.5155
		2	22.87500*	5.30369	.000	10.2845	35.4655
LDL (mg/dl)	1	2	-13.32500*	1.89700	.000	-17.8283-	-8.8217-
		3	-16.67500*	1.89700	.000	-21.1783-	-12.1717-
	2	1	13.32500*	1.89700	.000	8.8217	17.8283
		3	-3.35000-	1.89700	.186	-7.8533-	1.1533
	3	1	16.67500*	1.89700	.000	12.1717	21.1783
		2	3.35000	1.89700	.186	-1.1533-	7.8533
HDL (mg/dl)	1	2	6.52425*	1.13781	.000	3.8232	9.2253
		3	6.80975*	1.13781	.000	4.1087	9.5108
	2	1	-6.52425*	1.13781	.000	-9.2253-	-3.8232-
		3	.28550	1.13781	.966	-2.4156-	2.9866
	3	1	-6.80975*	1.13781	.000	-9.5108-	-4.1087-
		2	-.28550-	1.13781	.966	-2.9866-	2.4156
FBG (mg/dl)	1	2	-34.12500*	2.07374	.000	-39.0479-	-29.2021-
		3	-36.75000*	2.07374	.000	-41.6729-	-31.8271-
	2	1	34.12500*	2.07374	.000	29.2021	39.0479
		3	-2.62500-	2.07374	.417	-7.5479-	2.2979
	3	1	36.75000*	2.07374	.000	31.8271	41.6729
		2	2.62500	2.07374	.417	-2.2979-	7.5479
HbA %	1	2	-2.53725*	.24701	.000	-3.1236-	-1.9509-
		3	-2.95750*	.24701	.000	-3.5439-	-2.3711-
	2	1	2.53725*	.24701	.000	1.9509	3.1236
		3	-.42025-	.24701	.209	-1.0066-	.1661
	3	1	2.95750*	.24701	.000	2.3711	3.5439
		2	.42025	.24701	.209	-1.661-	1.0066
Plasma nitrate (µmol/ml)	1	2	-7.22625*	.48603	.000	-8.3801-	-6.0724-
		3	-9.21900*	.48603	.000	-10.3728-	-8.0652-
	2	1	7.22625*	.48603	.000	6.0724	8.3801
		3	-1.99275*	.48603	.000	-3.1466-	-.8389-
	3	1	9.21900*	.48603	.000	8.0652	10.3728
		2	1.99275*	.48603	.000	.8389	3.1466

* The mean difference is significant at the 0.05 level.//([Badawy et al., 2011](#)) for Group I ([Abraham et al., 1978](#)) Group II and ([Ahluwalia et al., 2008](#)) for Group III.

dysfunction in the progression and development of diabetic complications including nephropathy ([Busik et al., 2008](#); [Evans et al., 2002](#)).

Accumulated evidence strongly suggests that eNOS gene polymorphisms are associated with the bioavailability of eNOS and endothelial function and have been implicated with DN ([Tanus-Santos et al., 2001](#); [Rossi et al., 2003](#)). However, differences in biochemical phenotypes and clinical findings among the polymorphisms have not yet been fully elucidated.

The mechanism responsible for this potential association is not known yet. However, variants of the eNOS gene may cause defective NO synthesis and decreased NO levels, enhancing the susceptibility to glomerular disease and deteriorating the renal function ([Shin et al., 2004](#); [Ahluwalia et al., 2008](#)). Therefore, this metabolic pathway of diabetes may be involved in renal complications of diabetes.

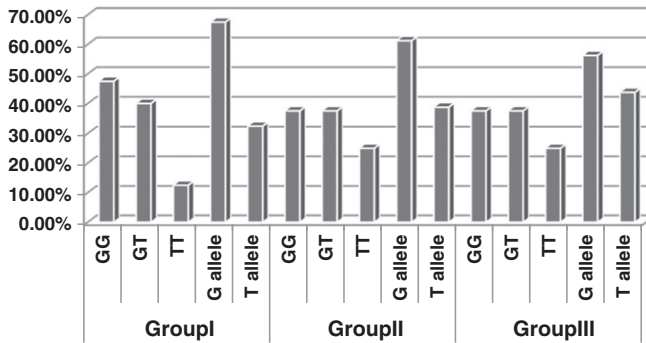


Fig. 1. Allele and genotype frequencies for eNOS gene polymorphism at +894 G/T polymorphism in all studied groups.

The present study investigated the impact of eNOS +894G/T SNP on plasma nitrate levels and tried to clarify the effects of this SNP on the rapid progression of DN in type2 diabetic Saudi patients in Qassim region. Our results revealed non-significant differences in genotype and allele frequencies of G894T polymorphism between the diabetic patients with and without nephropathy and the controls.

These results coincided with [Badawy et al. \(2011\)](#) and [Zanchi et al. \(2000\)](#), who revealed non significant differences in the frequencies of genotypes and alleles between the studied diabetic patients with and without DN and the control group.

However, the results of the present study disagreed with those of [Ahluwalia et al. \(2008\)](#), [Ezzidi et al. \(2008\)](#), [Sun et al. \(2004\)](#) and [Noiri et al. \(2002\)](#) who recorded a significant increase of homozygous mutated TT genotype, and mutant T allele of G894T polymorphism among cases of types 1 and 2 diabetes with nephropathy compared to control and concluded that TT genotype and T allele may be considered genetic risk factors for DN.

In contrary to our results [Ezzidi et al. \(2008\)](#) found increased TT genotype in DN compared to diabetics without nephropathy in Tunisian patients.

A recent meta-analysis assessed the association between the alleles of the eNOS gene 894G/T polymorphism and DN and a subgroup analysis was performed based on ethnicity. The evidence accumulated suggested that

Table 3

Genotype and allele frequency for e NOS gene polymorphism at +894 G/T in all studied groups.

+894 G/T			
Genotype	Groups		
	Group I (N = 40) N (%)	Group II (N = 40) N (%)	Group III (N = 40) N (%)
GG	19 (47.5%)	16 (40%)	15 (37.5%)
GT	16 (40%)	17 (42.5%)	15 (37.5%)
TT	5 (12.5%)	7 (17.5%)	10 (25%)
χ^2	0.621 ^a	P value	0.733 ^a (NS)
	2.17 ^b		0.33 ^b (NS)
	0.687 ^c		0.71 ^c (NS)
G allele	54 (67.5%)	n (%)	n (%)
	T allele	26 (32.5%)	49 (61.25%)
χ^2	0.681 ^a	38 (38.75%)	35 (43.75%)
	0.409 ^a	2.146 ^b	0.41 ^c
P	0.409 ^a	0.14 ^b	0.521 ^c
	OR 95%CI	1.314 (0.687–2.51)	1.615 (0.849–3.074)

Comparison of Group I and Group II ^a, Group I and Group III ^b, and Group II and Group III ^c.

Table 4

Plasma nitrate parameters and different e NOS +894G/T genotypes in all studied groups (ANOVA *F*-test, Post Hoc Tukey's HSD test and *P* values).

Parameters	GG	GT	TT	ANOVA (<i>F</i>), Tukey HSD value and <i>P</i> values
Plasma nitrate ($\mu\text{mol/ml}$) Mean–SD				
Group I (<i>n</i> = 40)	9.92–1.65	12.11–1.04	14.55–2.18	<i>F</i> = 21.635, <i>P</i> = 0.000 HSD* = –2.89, <i>P</i> = 0.000 HSD** = –4.84, <i>P</i> = 0.000 HSD*** = –1.94, <i>P</i> = 0.028
Group II (<i>n</i> = 40)	16.86–1.69	18.98–0.76	20.51–0.74	<i>F</i> = 22.332, <i>P</i> = 0.000 HSD* = –1.95, <i>P</i> = 0.023 HSD** = –4.44, <i>P</i> = 0.000 HSD*** = –2.49, <i>P</i> = 0.024
Group III (<i>n</i> = 40)	18.65–2.01	20.82–1.05	22.47–0.79	<i>F</i> = 21.658, 0.000 HSD* = –1.78, <i>P</i> = 0.000 HSD** = –3.45, <i>P</i> = 0.000 HSD*** = –1.67, <i>P</i> = 0.000

*compares GG to GT/**compares GG to TT/P***compares GT to TT.

G894T polymorphism in the eNOS gene was associated with susceptibility to DN in Asian populations but not in Caucasian populations (He et al., 2011).

Shoukry et al. (2012) examined the three eNOS polymorphisms (894G > T, –786 T > C and 27-bp-VNTR) and reported that two SNPs (894G > T and –786 T > C) were associated with increased risk of DN among type 2 diabetic subjects, whereas no association was found between the 27-bp-VNTR polymorphism and the risk of DN in Egyptian patients.

The discrepancy of the results between the different studies can be attributed to the variability of the number of the patients studied or to the ethnic differences regarding the distribution of this pattern of polymorphism.

In the present study, plasma nitrate levels exhibited significant increased levels in diabetics with nephropathy as compared to diabetics without nephropathy and controls. In a trial to examine the functional aspects of the 894G > T SNP with plasma NO levels, we compared the nitrate levels according to 894G > T genotypes in all studied groups. The results provided evidence of an association between this variant and plasma NO levels. T allele and TT genotypes carriers exhibit higher plasma nitrate levels than G allele and GG genotypes carriers in all studied groups.

Our findings were supported by a study of Shoukry et al. (2012) and Badawy et al. (2011) who showed that serum NO levels were significantly decreased in the 894(G > T) GT and TT genotypes in relative to the GG genotype in diabetic patients with and without nephropathy.

However, in contrast to our results, Ritt et al. (2008) stated that 894G > T had no impact on basal NO activity in the renal circulation of patients with or without DM, and Moon et al. (2002) revealed that there was no substantial effect of 894G > T polymorphism on the variance of plasma NO levels in a healthy Korean population.

Increased amounts of eNOS have been detected in pre-glomerular blood vessels in diabetic rat and NOS inhibitors have been shown to decrease the glomerular filtration rate (Veelken et al., 2000). These findings suggest that NO and the eNOS can contribute to the glomerular damage resulting in nephropathy in diabetic patients (Mollsten et al., 2009).

There are a number of studies that have previously investigated the functional aspects of the eNOS gene polymorphisms. The protein product of eNOS 894 T is more susceptible to selective proteolytic cleavage in endothelial cells and vascular tissues (Tesauro et al., 2000), which could lead to reduced vascular NO generation. However, other expression studies have demonstrated no difference in NO generation between G894 (298Glu) and 894 T (298Asp) (Bank and Aynedjian, 1993; Fairchild et al., 2001).

Jeerooburkhan et al. (2001) investigated whether the intron 4 variable number tandem repeat (VNTR), –922A/G, –786 T/C and G894T polymorphisms in the eNOS gene could influence NO production; they recorded that none of the studied polymorphisms had influenced the serum NO even if corrected for the

serum creatinine level. Another study demonstrated that the median serum NO level in G/T and T/T genotype of the G894T polymorphism was significantly higher than that of G/G genotype in the healthy population but not in the CAD patients (Yoon et al., 2000). Decreased renal NO accelerates the progression of DN in the rat model, presumably through mechanisms, such as increased renal vascular tone and potentiation of angiotensin II effects (Zanchi et al., 2000). Thus, reduced eNOS activity associated with –786 T > C and 894G > T polymorphism could play a role in down regulation of eNOS expression and endothelial dysfunction (Shoukry et al., 2012).

There are some limitations of our study. We need a larger sample size to identify significant associations of genetic variants with complex qualitative trait, such as DN. Moreover we have to examine the association of other SNPs of eNOS gene with increased risk of DN among type 2 diabetic subjects.

Conclusion

The present study revealed non significant differences in the frequencies of G894T eNOS gene genotypes and alleles between the studied diabetic patients and the controls. Our findings indicated that eNOS gene SNP may not be considered as genetic risk factors for DN among type 2 diabetic Saudi patients. The higher plasma levels of nitrates as a marker of oxidative stress in diabetic patients with nephropathy suggest the possible role of oxidative stress but not endothelial nitric oxide synthase gene SNP in pathogenesis of the disease. Further studies with larger sample size and on a wider scale of type 2 diabetic patients are needed to confirm this conclusion. Our data may enable type II diabetic patients who are at high risk of nephropathy and renal failure to be identified and, as such, benefit from more targeted therapy and prevention which could save their life.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.mgene.2014.04.009>.

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