The Role of Endothelial Nitric Oxide Synthase Gene 4a/b Polymorphism and its Interaction with eNOSG894T Variants in Egyptian Type 2 Diabetes Mellitus as a Risk Factor to Nephropathy


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Abstract

Background: Studies from different parts of the world have given controversial results regarding the association of eNOS gene variation with T2DM and DN.

Aim of the Work: The aim of this study is to evaluate the role of eNOS 4a/b polymorphism and its interaction with eNOS G894T variants in type 2 diabetes mellitus as a risk factor to nephropathy in patients attending the outpatient clinic of National Institute of Diabetes and Endocrinology (NIDE).

Subjects and Methods: This study was conducted on a total number of 120 subjects which were subdivided into three groups Group I: Included 40 type 2 diabetic patients without nephropathy. Group II: Included 40 type 2 diabetic subjects with nephropathy. All patients are selected from the outpatient clinic of National Institute of Diabetes and Endocrinology (NIDE) Group III: Included 40 age and sex matched normal healthy subjects (as controls). In addition to the routine investigation genotyping for the intron 4a/b polymorphisms for the eNOS gene was performed by PCR amplification of the target genes while genotyping of the G-894T polymorphism was performed by PCR amplification of the target genes followed by allele specific restriction enzyme digestion (PCR-RFLP).

Results: When we compared the different studied groups we found that there was no statistical significant association between them and mutant genotypes (p>0.05) nor mutant alleles (p>0.05). It was found that; individuals with eNOS-4 polymorphism were 0.9 times more likely to develop nephropathy (Group I and Group II), when compared to normal control subjects it were 1.49 times more likely to develop nephropathy. Individual eNOS (G-894T) mutation were 0.11 times more likely to develop nephropathy (Group I and Group II), when compared to normal control subjects it were 1.36 times more likely to develop nephropathy.

Conclusion: It can be concluded that there was absence of association between eNOS 4a/b variants and the risk of developing type-2DM and DN. It also demonstrates that eNOS 894T allele alone does not increase the risk of developing DN and the effect is not modified by the concomitant presence of both allele.

Key Words: T2DM – DN – eNOS – NIDE.

Introduction

DIABETES mellitus is a chronic illness that requires continuing medical care and ongoing patient self-management education and support to prevent acute complications and to reduce the risk of long-term complications [1].

Type-2 Diabetes Mellitus (T2DM) is characterized by a decrease production of insulin, together insulin resistance, multifactorial disease metabolic syndrome results from a complex interaction of genetic and environmental factors which influence beta-cell mass, insulin secretion, insulin action, fatdistribution, and obesity. With the increase related complications cardiovascular disease, retinopathy, neuropathy, and nephropathy [2]. Diabetic Nephropathy (DN) is the leading cause of chronic renal disease in patients starting renal replacement therapy. It is associated with increased cardiovascular mortality. Diabetic nephropathy has been classically defined as increased protein excretion in urine. Early stage is characterized by a small increase in Urinary Albumin Excretion (UAE), also called microalbuminuria or incipient DN. More advanced disease is defined by presence of macroalbuminuria or proteinuria. The latter is classically named overt DN. In most cases, proteinuria and decreased Glomerular Filtration Rate (GFR) occur in parallel. Traditionally, GFR has been expected to decrease when proteinuria is established, but not before.

The two main risk factors for DN are hyperglycemia and arterial hypertension. However, DN
Laboratory investigations:

Eight ml of venous blood were withdrawn from each subject in dry sterile vacutainers after fasting 6-8 hours. First two ml of collected blood was taken on EDTA tubes for DNA extraction and analysis of eNOS gene polymorphisms (4a/b, G894T) using Polymerase Chain Reaction (PCR) followed by restriction fragment length polymorphism, the sample was stored at –20ºC till time of assay.

Second two ml collected in EDTA vacutainer, this sample was freshly used for measurement of HBA1C level by HPLC technique according to manufacturer's instructions.

The last four ml was centrifuged at 3000rpm for 5min. After centrifugation serum was separated for the routine chemistry investigations (fasting blood glucose and serum creatinine) by using ARCHITECT 8000 chemistry analyzer (USA, supplied by Abbott, Al-Kamal Company Cairo, Egypt).

A random urine sample was collected in sterile cup for measurement of micro-albumin and creatinine in urine and calculation of micro-albumin/creatinine ratio by using ARCHITECT 8000 chemistry analyzer (USA, supplied by Abbott, Al-Kamal Company Cairo, Egypt).

Statistical analyses:

Statistical analysis was performed using the statistical package for social sciences (SPSS, USA). Data are expressed as means ± standard deviation, median and range, or frequencies (number of cases). Odd Ratio (OR) and its 95% confidence interval (95% CI) was calculated for mutation in relation to albuminuria groups statistical differences between groups were evaluated by using the student’s t-test. Comparison of numerical variables between more than two groups was done using (ANOVA) test. Correlations between various variables was done using Pearson moment correlation equation for linear relation in normally distributed variables and Spearman rank correlation equation for non-normal variables. p-values less than 0.05 was considered statistically significant.

Results

The study was conducted on 120 subjects, 80 of them recruited from the outpatient clinic in the national institute of diabetes and endocrinology and 40 age and sex matched normal subjects as control group, they were divided into three groups according to presence of DN in type-2 diabetes patients.
Group I: Included 40 type-2 diabetes patients without diabetic nephropathy (albuminuria).

Group II: Included 40 type-2 diabetes patients with diabetic nephropathy (albuminuria).

Group III: Included 40 age and sex matched normal subjects as control.

The studied groups were well matched in age and sex. There was no statistically significant difference regarding the BMI, family history of hypertension and family history of diabetes, this is shown in (Table 1). On comparing different laboratory investigation in the three studied groups there was statistically significant difference in FBG \( (p=0.000) \), HbA1c \( (p=0.000) \), eGFR \( (p=0.031) \). There was no significant difference in the other laboratory, (Table 2). Frequency distribution of eNOS intron-4 genotypes in the studied groups showed that there was no association between eNOS intron-4 polymorphism and DN \( (p=0.770) \), (Table 3). Frequency distribution of eNOS (G-894T) genotypes in the studied groups showed that there was no association between eNOS (G-894T) polymorphism and DN \( (p=0.911) \), (Table 4). Frequency distribution of wild (bb) versus mutant (ab+aa) in eNOS intron-4 genotypes among the studied groups showed that there was no association between eNOS (G-894T) polymorphism and DN \( (p=0.535) \), (Table 5). Frequency distribution of wild (GG) versus mutant (GT+TT) in eNOS (G-894T) genotypes in the studied groups showed that there was no association between eNOS (G-894T) polymorphism and DN \( (p=0.635) \), (Table 6). On performing Odds Ratio (OR) on the different study groups as an estimate of relative risk of nephropathy, it was found that; individuals with eNOS intron-4 mutation were 0.9 times more likely to develop nephropathy (type-2 DM without nephropathy and type-2 DM with nephropathy), when compared to normal control subjects, it were 1.49 times more likely to develop T2DM (Group 2 and 3). Also individuals with eNOS (G894T) mutation were 0.11 times more likely to develop nephropathy (type-2 DM without nephropathy and type-2 DM with nephropathy), when compared to normal control subjects it were 1.35 times more likely to develop T2DM (Group 1 and 3). The eNOS intron-4 a allele frequency and the eNOS-894 T allele frequency showed non significant difference in the three studied groups \( (p=0.462) \) and \( (p=0.627) \) respectively, (Tables 7,8).

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Diabetes mellitus as the most prevalent metabolic disease is a multifactorial disease which is influenced by environmental and genetic factors.

Diabetic nephropathy is the leading cause of chronic renal failure. Progression of Diabetic Nephropathy (DN) is commonly defined by an increase in albuminuria from normoalbuminuria to microalbuminuria and from microalbuminuria to macroalbuminuria. Accumulated evidences suggest that both environmental and genetic factors are related with the etiology of DN.

Among which eNOS may play a critical role because endothelial dysfunction is considered as an important pathophysiologic factor for DN. Genetic polymorphism of endothelial Nitric Oxide Synthase (eNOS) has been implicated in the risk of Diabetic Nephropathy (DN), but the published findings were inconsistent. Variants of the NOS gene have been shown to modify its expression or activity, thus leading to reduced or excessive NO production and consequently contributing to many pathological processes.

The aim of this study is to evaluate the role of eNOS 4a/b polymorphism and its interaction with eNOS G894T variants in type-2 diabetes mellitus, modifying the risk of diabetic nephropathy.

This study was conducted on 120 subjects, eighty of them recruited from diabetes outpatient clinic of national institute of diabetes and endocrinology...
nology and 40 normal subjects as control, they were divided into 3 groups (forty type-2 diabetic patients without nephropathy, forty type-2 diabetic patient with nephropathy and 40 age and sex matched normal subjects serve as control).

To achieve our aim, genotyping for the intron 4a/b polymorphism and for the G-894T polymorphism of the eNOS gene were performed.

Regarding gender distribution in the three studied groups, they were (62.5%), (55.0%), (37.5%) males and (37.5%), (45.0%), (62.5%) females in Group 1, 2 and 3 respectively which shows no statistically significant difference between groups in this study (p=0.072), this was consistent with [14] (p=0.5) and [7] in which they were (45.0%), (57.5%), (47.5%), (55.0%), (42.5%), (52.5%), females in Group 1, 2 and 3 respectively (p=0.313).

Regarding mean age distribution in the three studied groups it showed no statistically significant difference between all the three studied groups in our study (p=0.338), as the mean age was 52.7±7.2, 53.1±7.2 and 49.8±8 in Groups 1, 2 and 3 respectively and that was close to the frequency of [7] study in which the mean age was 51.6±6, 50.4±4 and 51.6±6 in Groups 1, 2 and 3 respectively (p=0.16), this was also consistent with the study done by [14] in which there were no statistically significant difference in the mean age distribution between the studied groups (p=0.21) and disagreed with a study done by [15] (p=0.001) in which the mean age was 40.1±9.6 and 49.6±10 in control and patient group respectively.

The mean BMI was 28.3±2.3, 27.8±2.2, 28.9±2.5 in Groups 1, 2 and 3 respectively which shows non statistically significant difference between groups (p=0.098), this was in agreement with the study done by [5] who reported that the mean BMI showed non statistically significant difference between groups (p=0.47) 27.3±4.8, 27.9±4.1, 26.8±4.5 in Groups 1, 2 and 3 respectively. Also [14] study was agreement (p=0.15).

Concerning the frequency of hypertension in the current study 15 (37.5%), 14 (35.0%), 18 (45.0%) were hypertensive in Groups 1, 2 and 3 respectively. It was found that there was no statistically significant difference in frequency of hypertension among the 3 studied groups (p=0.635), this was disagreed with a study done in Iran where there was statistically significant difference in hypertension frequency between patients with DN [5] (p=0.005).

Regarding laboratory data, the FBG was found to be statistically different between all the studied groups in our study (p=0.000) with medians 183, 190, 82 in Groups 1, 2 and 3 respectively. This finding was consistent with similar study made by [7] who found statistically significant difference in between cases and controls. (p=0.000) and also a study done by [15] (p<0.001) from India.

The mean value of HbA1c were 8.7±1.6, 9±2.6 and 5.6±0.3 in Groups 1, 2 and 3 respectively in the current study which showed statistically significant difference between type-2 diabetic patients with and without nephropathy and the control group (p=0.000), this was in agreement with the study done by [7] in Saudi Arabia who found statistically significant difference in between cases and controls (p=0.000), and in contrast with the studies done by [5] in which the mean value of HbA1c were 8.04±1.58, 8.04±1.58, 7.8±1.57 in Groups 1, 2 and 3 respectively, and [14] in which the mean value of HbA1c were 7.3±1.5, 7.5±1.4 in Groups 1, 2 respectively who found no statistically difference (p=0.48), (p=0.17) respectively.

Regarding the creatinine levels; it showed no statistically significant difference between groups (p=0.291) with medians 0.7, 0.7 and 0.7 in Groups 1, 2 and 3 respectively this is not agreed with the studies done by [16] (p<0.05), but the study [17] showed significant difference only between macroalbuminuria and normoalbuminuria groups (p<0.001).

In this study, on comparing endothelial nitric oxide synthase genotypes among the studied groups, it was found that the mutations was not associated with nephropathy (p=0.635) in eNOS (G-894T) polymorphism, (p=0.535) in eNOS 4a/b polymorphism. And also the mutant alleles were not associated with nephropathy (p=0.462) of a allele in eNOS4a/b polymorphism, (p=0.627) of T allele NOS (G894T) polymorphism among the studied groups.

Concerning the polymorphism 4a/b upon the risk of development and progression of DN.

Our study was in agreement with a study by [18] who could not observe a significant influence of this polymorphism on the course of the disease and the clinical data of the patients. Also our results goes in accordance with the study done by [19] who indicated an absence of association between this polymorphism and the risk of developing T2DM among Mexican Americans. A study done by [13], also goes in accordance with our results, that no association were observed between the
-786T>C, the VNTR intron 4a/b and the 894G>T (Glu 298Asp) polymorphisms in the eNOS gene and renal disease in type-2 diabetic Caucasian-Brazilians (p>0.05 for all comparisons).

This goes in accordance with [20] who could not observe a significant influence of this polymorphism on the clinical data of the patients, and also this was in agreement with a study done by [21] in which eNOS 4a/b variants were not significantly associated with the risk of developing DN in T2DM patients from Iran.

In another hand results of our study was in partial agreement with a study done by [22]. These results implied that 4a/b polymorphism of eNOS gene may be not associated with the occurrence of type-2 diabetes mellitus but in his study the presence of a allele may be related to the development of diabetic nephropathy (p=0.01).

In contrast to our results [23] observed a statistically significant difference in the genotype distribution and the frequency of 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.

Also in the study of [24] demonstrated a strong association between eNOS 4a/b 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 which is in contrast to results of our study.

Furthermore, in the study of [25] the presence of eNOS 4a/b was associated with a 6.03 fold (p<0.0001) increased risk of developing DN among Indians which is also in contrast to results of our study.

Also a meta-analysis study done by the [26] was in contrast to our results, showed that an evidence accumulated suggested that 4a/b polymorphism in the eNOS gene were associated with susceptibility to DN in Asian population, but not in Caucasian populations. Allele contrast (4a versus 4b) of 4a/b polymorphism produced significant results in the global population (random effects model (re) Odds Ratio (OR)=1.33; 95% Confidence Interval (CI)=1.10 versus 1.61, p=0.003) and East-Asian population (re) OR=1.68; 955 CI=1023 versus 2.30, p=0.001), but not in the Caucasian population.

Also in contrast to our results a study done by [20] showed that the frequency of allele a was significantly increases in patients compared to the controls (p=0.001, OR=2.8, 95%CI (1.4-5.9).

Also a study done in Egypt by [14], their results were in contrast to our results. The haplotypes CTA (with all the mutant alleles) and CTb were significantly more common in patients with DN (p=0.01) and 0.003, respectively). These results suggested that the eNOS polymorphisms might represent genetic determinants for developing DN in type-2 diabetic Egyptians.

Also in contrast to our results a meta-analysis done by [11] supported an association between the 4b/a polymorphism of eNOS gene and increased risk of DN in type-2diabetes. However, significant association only existed in Asian populations, especially in Chinese population. As for non-Asian populations, no significant association was found under all the genetic models for Asian population, the 4a allele was found contributing significantly to increased DN risk in all cohorts model (REM; OR=1.59, 955CI=1.22-2.09), additive model (FEM; OR=3.94, 95% CI=2.72-5.71), dominant model (REM; OR=1.48, 95% CI=1.12-1.95), and recessive model (FEM; OR=4.01, 95% CI=2.78-5.80), respectively. No significant association was found in any of the above mentioned models for non-Asian population.

A meta-analysis study done by [12] concerning eNos-4b/4a allele was found contributing significantly to increased DN risk in the global population. Suggests that three polymorphisms of eNOS may be increased the risk factors of DN development, especially in Asian population and T2DM group which is in contrast to results of our study.

Also in contrast to our results study done by [10] revealed significant association between DN and eNOS 4b/a a allele (p=0.01) and also positive significant association between 4a or 894T allele carriers and DN (p=0.02).

Concerning the role of eNOS G894T polymorphism upon the risk of development and progression of DN.

Results of our study was in agreement with a study done [27] who found no statistically significant association between mutant genotypes and the disease (p=0.471). [28] also found no statistically significant difference between patients and healthy control (p=0.09). In contrast to our results a study done by the [26] showed that an evidence accumulated suggested that G894T polymorphisms in the eNOS gene were associated with susceptibility to DN in Asian populations. In allele contrast of
G894T, an obvious significant results was observed in the East-Asian population [fixed effects model OR=1.69; 95% CI=1.37 versus 2.08 p<0.0001] but not in the Caucasian population due to ethnic variations as mentioned above.

Also in contrast to our results a study done in Egypt by Shoukry et al. [14] the T allele for 894G>T were significantly more frequent in diabetics with nephropathy than in diabetics without nephropathy [p<0.001; OR and 955 CI=1.7 (1.27-2.26)] for the T allele. These results suggest that the eNOS polymorphisms might represent genetic dominants for developing DN in type-2 diabetic Egyptians.

Also in contrast to our results a meta-analysis study done by Zhang et al. [12] concerning for eNOS-G894T, there was an association between T allele and DN risk in the global, Asian and African population in DN/T2DM group.

Furthermore, a study done by Khodaeian et al. [10] revealed significant association between DN and eNOS 894T allele carrier (p=0.02) which was also in contrast to results of our study.

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 gene-environment interactions.

**Conclusion:**

The present study indicates the absence of association between eNOS 4a/b variants and the risk of developing type-2 DM and DN. It also demonstrates that eNOS 894T allele does not increase the risk of developing DN and the effect is not modified by the concomitant presence of both alleles and from other research results we can suggest a role of ethnicity and genetic background for susceptibility to diabetes and its complications even within the same country. Our recommendation is performing the study on a large group to increase the precision of the statistical analysis and studying of the other polymorphisms affecting the eNOS gene as C786T to detect if it has synergistic effect with 4a/b, G894T polymorphisms on the development of diabetic nephropathy.

**References**


الملخص العربي

يعتبر داء السكري مرض متعدد العوامل يتأثر بأسباب بيئية ووراثية وهو إضطراب التمثيل الغذائي الأكثر انتشارا.

يعتقد حاليا أن النوع الثاني من داء السكري يحدث في الأشخاص المؤهلين وراثيا الذين يتعرضون لسلسلة من التأثيرات البيئية التي تجعل بظاهر أعراض المرض. تمت هذه المجموعة من المرضى ما يقرب من 90% من جميع حالات السكري. قد تكون تركيزات الأنسولين طبيعية أو منخفضة أو زائدة ومعظم هؤلاء المرضى لديهم مقاومة للأنسولين.

إعتلال الكلية السكري هو إعتلال مزمن للشعيرات الدموية للكليتين ويحدث في كل من النوع الأول والنوع الثاني من داء السكري، وهو السبب الرئيسي لوفيات الكلية في النهاية. يعتقد العديد أيضا أن متلازمة المشكلة قد تعتمد على عوامل وراثية.

يعتبر الإنزيم المكون لأكسيد التيتريك سبب محتمل لحدث إعتلال الكلية السكري منذ اكتشاف الموقع (35q7) على الكروموسومات كنحد أساليب مضاعفات داء السكري.

قد أجريت هذه الدراسة على 120 شخص من عبادة داء السكري في العيادات الخارجية للمعهد القومي لأمراض السكر والغدد الصماء. تم تقسيمهم إلى 2 مجموعات (40 مريض من النوع الثاني لمريض السكري دون اعتلال الكلية و40 مريض من النوع الثاني لمريض السكري يعانون من اعتلال الكلية و40 شخص طبيعي للمقارنة).

تم دراسة التنميط الجيني متوسط الاشكال (4a/b) بواسطة تفاعل سلسلة البلمرة المتضخم وكذلك دراسة التنميط الجيني متعدد الأشكال G-894T (G-894T) بواسطة تفاعل سلسلة البلمرة المقيد وذلك للإنزيم المكون لأكسيد التيتريك.

أظهرت الدراسة أن لا يوجد علاقة بين التنميط الجيني متوسط الأشكال (4a/b) في الإنزيم المستقل عن تكوين أكسيد التيتريك وإعتلال الكلية السكري.