Original Article

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Internal Medicine Section

# Correlation of Endothelial Nitric Oxide Synthase Gene Polymorphism (GG, TT and GT Genotype) with Proteinuria and Retinopathy in Type 2 Diabetic Patients

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## ABSTRACT

**Background:** Nephropathy is the most important leading cause of end stage renal failure in type 2 diabetic patients, so numerous studies were done to diagnose and evaluate risk factors of diabetic nephropathy (DN). Some gene polymorphisms may be associated with progression or regression of DN, so the aim of this study was to compare prevalence of eNOS gene polymorphism in diabetic patients with controls and its association with diabetic nephropathy.

**Materials and Methods:** In a cross-sectional study, 94 type 2 diabetic patients and 94 normal participants were enrolled. Patients without retinopathy were excluded from this study. For all of the patients, fasting blood sugar (FBS), 2 hours post-prandial (BS), Blood Urea Nitrogen (BUN), Creatinine (Cr), 24 hours urine protein were measured in the case group. Endothelial nitric oxide synthetase gene polymorphism was evaluated in the case and control groups.

**Results:** There was no significant difference based on age and sex between patients in case and control groups. GG genotype of eNOS was less common in the patient group compared to control group. There was no difference between prevalence of TT, GT or GG genotype based on age and sex. There was no correlation between diabetic retinopathy or proteinuria and genotypes of eNOs.

**Conclusion:** The study showed that in type 2 diabetic patients, NOS gene polymorphism was more common compared to normal population; however, there is no correlation between this gene polymorphism and proteinuria or retinopathy in these patients.

**Keywords:** eNOS, Nephropathy, Retinopathy

## INTRODUCTION

Diabetes mellitus is the most common cause of chronic renal failure and end stage renal disease worldwide. Microvascular and macrovascular complications of diabetes increase cardiovascular and overall mortality [1]. Diabetic nephropathy occurs in about 30%-35% of patients with type 1 and type 2 diabetic patients. After 5-10 years, some diabetic patients have micro albuminuria that means urine albumin is between 30 and 300 mg/day [2,3]. After additional 5-10 years, macroalbuminuria (urine albumin ≥300 mg/day) developed and at the time being glomerular filtration rate (GFR) began to decline at the rate of 10-12 ml/year. Advanced glycosylation end products (AGES), oxidative stress and hypertension are the main causes of pathophysiologic changes that lead to diabetic nephropathy [4,5]. In addition, inhibition of vascular dilation factors that decrease production or release of EDRF (Endothelium-derived relaxing factor) also may have a role in the intonation or augmentation of diabetic nephropathy. Deficiency of nitrous oxide (NO) that is a vasodilation factor releasing by vascular endothelium also have a role in this regard. Decrease in the production of nitrous oxide synthase (NOS) may lead to decline of NO and vascular dilation. ENOS is an important enzyme that contribute in vascular homeostasis, so eNOS gen located on chromosome 7 and has 26 exon [6,7]. Gen polymorphism of ACE (Angiotensin Converting Enzyme) was reported to have a basic role in diabetic nephropathy [8]. DD allele of ACE gene has been reported with development and severity of diabetic nephropathy and more rapid progression to end stage renal disease [9]. For example in a study on 109 type 2 diabetic patients, there was a positive association between the D allele of the ACE polymorphism and proteinuria [10]. Although there is a controversy in the results of studies, so some studies with large sample size could not find this correlation in specific races [11]. Correlation between polymorphism of some allele of eNOS gene and diabetic nephropathy and its severity has been also reported in other studies [12-14]. Based on our knowledge, there are a few studies in Iran especially in special races such as Lor on this issue. So, the aim of this study was to evaluate eNOS gene polymorphism with diabetic nephropathy and compare it among normal individuals.

## MATERIALS AND METHODS

In a cross-sectional study, in Imam Ali clinic of Shahrekord, Iran, 100 diabetic patients and 100 normal participants were enrolled. All of the patients were among Lor tribe (Bakhtiari) that are one of the great and noble people of Iran. Inclusion criteria were: age greater than 40 year and presence of diabetes mellitus based on American Diabetes Association definition [15]. Diabetic patients with hypothyroidism, congestive heart failure and patients who had contraindication for consumption of angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARBs) were excluded from this study. All of the patients evaluated based on diabetic retinopathy and patients without retinopathy were excluded from the study. For all of the patients, Fasting Blood sugar (FBS), 2 hours post-prandial BS, Blood Urea Nitrogen (BUN), Creatinine (Cr), 24 hours urine protein were measured in case group by using Biotechnica Instruments (BT 3000) and Flame Photometer (Corning 480) Nyocard Reader II. Body weights, high Body Mass Index (BMI) with formula of body weight/height$^2$ were also measured in the patients. Consent form was filled in by al the patients.

### DNA Extraction and Polymerase Chain Reaction Experiment

Three ml of blood sample was drawn from patients and stored in 5ml EDTA vaccutainers at -20°c. Then DNA extraction was done using standard phenol/chloroform method and the final DNA pellet was dissolved in 20 Ul dd$H_2O$ or TE. The quality of extracted

DNA was analyzed either by 0.7% agarose gel electrophoresis visualized by gel documentation imaging or UV spectrophotometry at 260/280 nm. Characteristics of forward and reverse primer were eNOS3-F: 5'-AGATGAAGGCAGGAGACAGTGG-3' and eNOS3-R: CCATCCCACCCAGTCAATC-3' respectively.

Each PCR was performed in a 10 µL reaction containing the following reagents: 25 ng genomic DNA, 1 µL buffer (10 mMTris-HCl {pH 8.3}, and 1.5 mM $MgCl_2$), 250 µM $MgCl_2$, 1% DMSO (Sigma-Aldrich, St. Louis, MO), 200 µMdNTPs, 0.5 µM primer set, and 1U Taq DNA polymerase (sinagen, Iran). The condition of PCR cycles was included: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation 94°C for 40 s, annealing 62°C for 90 s, and extension 72°C for 50 s, and a final extension at 72°C for 3 min. After confirming the achievement of the expected 262 bp band on 0.7% gel electrophoresis and visualization, the PCR products were subjected to MboI enzyme digestion and the products were then subjected to analysis on 12.5% polyacrylamide gel electrophoresis (PAGE). The expected restriction fragments of 162 bp and 100 bp were observed on the PAGE after visualization by silver staining. Sequence analysis of the PCR products was performed using the same primers as PCR by the ABI Big Dye Terminator Kit v.3.1 (Applied Biosystems, USA).

## STATISTICAL ANALYSIS

For continuous variables, data were presented as means ± SD as well as median; and for categorical ones, as frequency with percentage. Comparisons between groups were done using the Chi-square test for categorical variables and independent t-test for continuous ones. Kruskal-Wallis test was used to comparing the variables of interest among different genotype, because our data didn't meet the assumptions of the parametric analysis of variance. Statistical analysis was performed by SPSS and p-values <0.05 were statistically significant.

## RESULTS

As 6 cases of each group could not follow the study, so this study continued and was done by 94 patients in each group. Age of the patients was 42 to 85 years with mean of 61.8±9.5 years. Thirty six (38.3%) of the patients were male and 58 (61.7%) were female. There was no significant difference based on age and sex between patients and control groups (p>0.05). Twenty six (27.7%) of the patients had proteinuria less than 150 mg (mean of 105.5mg) and the others had greater than 150 mg proteinuria per day. Some characteristics of the patients were mentioned in the [Table/Fig-1].

Frequency of eNOS genotype in the two groups of study was summarized in [Table/Fig-2]. Based on data in [Table/Fig-2], GG genotype was significantly less frequent in the patients compared to control group.

There is no difference between prevalence of TT, GT or GG genotype based on age and sex in two groups of the patients [Table/Fig-3,4]. Diabetic retinopathy did not have any correlation with genotypes of NOs. We did not find any correlation among TT, GT or GG genotypes and 24 hours urine protein, FBS and 2 hours postprandial blood sugar [Table/Fig-5].

## DISCUSSION

The study showed that NOS synthase gene polymorphism in type 2 diabetes was more common compared to normal population but it was not found any correlation between this gene polymorphism and proteinuria or retinopathy in the patients.

T. Angeline in a study on 260 patients showed a correlation between NOS synthase and diabetes mellitus [16]. In a study on 400 diabetic patients, an association between 3 eNOS polymorphism (894G>T, -786T>C, and 27-bp-VNTR) and diabetic nephropathy was found [17]. Ze-jun Ma et al., in a meta-analysis found a significant association between the eNOS-4b/a polymorphism and DN in Chinese population, but not in non-Asian populations [18]. Khamaisi showed that decreasing renal NOS activity at the progressive phase of diabetes is associated with a decline in neuronal NOS activity and protein expression [19]. In the Rippin et al., study on Type 1 diabetic patients, there was no correlation between NOs polymorphism and diabetic nephropathy, so their results have some similarity with our study [20]. El-Din reported that TT genotype of eNOS had association with increased risk of end stage renal disease in type 2 diabetic patients, so it may be a useful marker for identification of high risk diabetic patients [21]. Association of NOs gen polymorphism and retinopathy that is another microvascular complication of diabetes was reported by Bazzaz et al., [22]. Zintzaras et al., in a meta-analysis, showed that G894T NOs gen polymorphism is associated with diabetic nephropathy in East Asians patients [12]. In a systemic review, association of DN with eNOS 4b/a and T-786C gen polymorphism was reported by Dellamea et al., [23]. Bernhard et al., in their study

| Variable | Minimum | Maximum | Mean ± SD |
|---|---|---|---|
| Age(year) | 42 | 85 | 61.80±9.50 |
| BMI(Kg/m$^2$) | 20.48 | 42.97 | 28.60±4.52 |
| SBP(mmHg) | 100 | 210 | 146.60±24.95 |
| DBP(mmHg) | 60 | 120 | 87.18±13.61 |
| Urine Protein(mg/day) | 59 | 2438 | 589.80±627.20 |
| FBS(mg/dl) | 62 | 489 | 59±67 |
| 2HPPBS(mg/dl) | 98 | 644 | 251.5±81.24 |
| Serum Cr | 0.50 | 5.90 | 1.41±0.799 |

**[Table/Fig-1]:** Some characteristics of the patients in the study

| Genotype | Patients (N (%) | Control (N (%) | p |
|---|---|---|---|
| TT | 58 (61.7) | 62 (66) | 0.047 |
| GG | 3 (3.2) | 10 (10.6) | |
| GT | 33 (35.1) | 22 (23.4) | |

**[Table/Fig-2]:** Prevalence of eNOS gen polymorphism in the patients and control groups.

| Diabetic | TT (N (%) | GG (N (%) | GT (N (%) | p |
|---|---|---|---|---|
| Retinopathy | 30 (58.8) | 2 (3.9) | 19 (37.3) | 0.863 |
| No Retinopathy | 28 (65.1) | 1 (2.3) | 14 (32.6) | |

**[Table/Fig-3]:** Gene polymorphism prevalence of the patients based on diabetic retinopathy.

| Sample | Variable | TT (N (%) | GG (N (%) | GT (N (%) | p |
|---|---|---|---|---|---|
| Patients | Male | 26 (72.2) | 1 (2.8) | 9 (25) | 0.25 |
| | Female | 32 (55.2) | 2 (3.4) | 24 (41.4) | |
| Healthy | Male | 27 (65.9) | 5 (12.2) | 9 (22) | 0.891 |
| | Female | 35 (66) | 5 (9.4) | 13 (24.5) | |

**[Table/Fig-4]:** Gene polymorphism prevalence of the patients and healthy groups based on sex.

| Variable | TT | GG | GT | p |
|---|---|---|---|---|
| | Mean ± SD (Median) | Mean ± SD (Median) | Mean ± SD (Median) | |
| Age | 61.6 ± 8.8 (61.5) | 65 ± 5 (61) | 61.9 ± 11.9 (61) | 0.722 |
| Urine protein | 616.4 ± 656.8 (358) | 459 ± 502.2 (221) | 554.8 ± 595.8 (399) | 0.91 |
| Serum Cr | 1.44 ± 0.81 (1.18) | 1.16 ± 0.07 (1.14) | 1.39 ± 0.78 (1.1) | 0.85 |
| FBS | 153.5 ± 55.8 (144) | 169.6 ± 50.5 (158) | 168.1 ± 85.5 (138) | 0.81 |
| 2hppBS | 253 ± 70.2 (245) | 256 ± 23.39 (269) | 248.5 ± 101.8 (221) | 0.53 |

**[Table/Fig-5]:** Association among some variables of interest and eNOS gene polymorphism in the patients group

on type 1 and type 2 diabetic patients did not find that eNOS gene polymorphism plays a significant role in the development of diabetic nephropathy [24]. Huo P in Chinese population found an association between ACE and eNOS and diabetic nephropathy [25]. Rahimi in a study on 173 diabetic patients and 101 healthy cases found the significantly increasing risk of macroalbuminuria in the presence of either eNOS 4a or 894T allele, however, he could not find any association between concomitant presence of both alleles with increasing risk of macroalbuminuria [26]. In a study on albuminuric and normoalbuminuric diabetic patients by Cheema; in normoalbuminuric patients, eNOS -786 CC and C-b-G and C-b-T genotype were associated with loweranti-proteinuric response to ACEi agents. However, in macroalbuminuric patients, eNOS -786 CC, C-b-G and C-b-T and 27VNTR aa genotypes were associated with higher response to ACEi or ARB drugs [27]. In the patients with proliferative diabetic retinopathy, higher frequency of the eNOS was minor '4a' allele than in control group that it was found by Cilenšek [28]. Cheema showed that eNOS gene polymorphism increase responsiveness in type 2 diabetic patients without nephropathy and decrease response in microalbuminuric patients [27].

In a study by Corapciogluin on 97 Turkish diabetic foot ulcer patients and 102 controls, it was reported a significant association between eNOS alleles in patients with atherosclerotic heart disease, because GT-TT alleles were significantly higher than the GG alleles [29].

Cause of the discrepancy between above studies may be due to different ethnicity of the study populations.

## LIMITATION

Cardinal limitation of the study was small sample size, so we recommended similar studies on larger number of the patients and evaluation of correlation between other gene polymorphisms and DM complications such as nephropathy, retinopathy, neuropathy and atherosclerosis.

## CONCLUSION

In conclusion, the study showed more common prevalence of eNOS synthase gene polymorphism in type 2 diabetic patients compared to normal population; however, there is no correlation between this gene polymorphism and proteinuria or retinopathy in these patients.

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