Endothelial nitric oxide synthase polymorphisms and susceptibility to high-tension primary open-angle glaucoma in an Egyptian cohort

Wafaa A. Emam,1 Haidy E. Zidan,1 Bahaa-Eldin H. Abdulhalim,2 Sherif A. Dabour,2 Manar A. Ghali,2 Aliaa T. Kamal1

1Department of Medical Biochemistry, Faculty of Medicine, Zagazig University, Zagazig, Egypt; 2Department of Ophthalmology, Faculty of Medicine, Zagazig University, Zagazig, Egypt

Purpose: To analyze the association of polymorphisms of the endothelial nitric oxide synthase (NOS3) gene and nitric oxide (NO) levels with high-tension primary open-angle glaucoma (POAG) in an Egyptian population.

Methods: This case-control study included 160 patients who had high-tension POAG (76 men and 84 women; age range 41–75 years) and 110 controls (56 men and 54 women; age range 55–78 years). Genotyping of T-786C (rs2070744), Glu298Asp (rs1799983), and the 27-bp insertion variable number tandem repeat (VNTR) in intron 4 of the NOS3 gene was performed with an amplification refractory mutation system PCR assay. The NO level was determined by measuring the total nitrate/nitrite (NO\textsubscript{3}) plasma level.

Results: The CC genotype of the T-786C polymorphism was significantly associated with POAG (odds ratio [OR] = 2.54, 95% confidence interval [CI] = 1.26–5.13, p = 0.007). The C allele was significantly associated with POAG (OR = 1.86, 95% CI = 1.29–2.69, p<0.001). After stratification by sex, the CC genotype and the C allele were significantly associated with POAG in women only (OR = 3.06, 95% CI = 1.07–8.74, p = 0.03 for the CC genotype, and OR = 2.09, 95% CI = 1.24–3.53, p = 0.005 for the C allele). The genotype and allele frequencies of Glu298Asp and intron 4 were not significant between the patients with POAG and the controls, and after stratification by sex. The mean NO\textsubscript{3} plasma level was significantly lower in patients with POAG than in the controls (p = 0.01) and low in the (TC+CC) genotype compared to the TT genotype of T-786C in the patients and controls (p<0.001).

Conclusions: The results suggest that the CC genotype of T-786C NOS3 may be associated with an increased risk of developing high-tension POAG in Egyptians, particularly women. In addition, decreased NO levels may play a role in the development of POAG.

Primary open-angle glaucoma (POAG) is a complex disease that if untreated properly may lead to irreversible blindness. Intraocular pressure (IOP) plays a major role in the pathogenesis of POAG, but elevated IOP alone does not explain many of the clinical and experimental observations [1]. As many as half of patients do not have elevated IOP when POAG is detected [2,3]. In addition, in a group of patients with glaucoma who had normal IOP (normal tension glaucoma) elevated IOP was never detected [1]. Considerable evidence suggests that compromise of the microvasculature of the optic nerve may have a role in the damage seen in glaucoma [1,4-6]. POAG appears to have a genetic or familial component. Currently, most authorities believe that the genetic influence occurs through polygenic or multifactorial transmission [7].

Nitric oxide (NO) is synthesized in the vascular endothelium through the action of endothelial nitric oxide synthase (eNOS). NO maintains the basal vasodilator tone [8]. Endothelium-derived NO released under basal conditions or stimulated by bradykinin significantly regulates flow to the porcine ophthalmic microcirculation [9]. Systemic inhibition of NOS reduces the pulsatile choroidal blood flow [10]. Among the most important identified polymorphisms of the NOS3 (OMIM 163729) locus are a T to C single nucleotide polymorphism (SNP) in the promoter region (T-786C, rs2070744), a G to T SNP in exon 7 (Glu298Asp, rs1799983), and 27-bp variable number of tandem repeats (27 bp-VNTR-a/b) in intron 4 [11]. T-786C has been shown to reduce mRNA expression [12,13], Glu298ASP may alter eNOS function [14], and VNTR-a/b may reduce NO production [15].

Thus, since compromise of the microvasculature of the optic nerve may have a role in POAG and NO plays a role in regulating ocular circulation, we studied the association of the following NOS3 polymorphisms: T-786C, Glu298ASP, and 27 bp-VNTR-a/b and high-tension POAG in an Egyptian population. We also assessed the relationship of these
polymorphisms and NO levels in patients with POAG and controls.

METHODS

This case-control study included 160 unrelated patients with high-tension POAG and 110 sex-matched control subjects of an older age group than the patients. The patients were recruited from three main hospitals serving three governorates in Egypt: Zagazig University Hospital in Sharqia Governorate in Lower Egypt and Aswan and Edfo in Upper Egypt. All patients were Caucasian and older than 40 years and had anterior chamber open angle (grade III or IV on gonioscopy) and optic nerve and visual field changes compatible with glaucomatous damage and initial IOP >21 mmHg (before treatment). Patients were excluded if a) they had any other forms of glaucoma, e.g., congenital glaucoma, secondary glaucoma as pigmentary glaucoma, pseudoexfoliation syndrome, complications of uveitis or trauma, and steroid-induced glaucoma; b) they had ocular hypertension (IOP above 21 mmHg with optic nerve and/or visual field changes not typical of glaucomatous damage); and c) if glaucoma had developed before 40 years of age. All controls were older than 55 years, had IOP below 21 mmHg, and had an optic nerve without any abnormalities suggestive of glaucoma with normal visual fields.

All patients were subjected to complete ophthalmological evaluations that included a full medical history, best-corrected visual acuity, slit-lamp biomicroscopy (Haag-Streit BQ900, Bern, Switzerland), IOP was measured using applanation tonometer, gonioscopy, dilated fundus examination that stressed the optic disc, computerized visual field using the Humphrey Field Analyzer: HFA SITA 24–2 white on white program (HFA; Carl Zeiss Meditec, Dublin, CA), and central corneal ultrasound pachymetry (Echoscan US-4000 pachymeter, Nidek Co, Tokyo, Japan). The study was performed adherent to the tenets of the Declaration of Helsinki and the ARVO statement on human subjects. The study was approved by the Ethical Committee of Faculty of Medicine, Zagazig University. A written informed consent form was signed by each participant before participation.

Blood sampling and DNA extraction: One ml venous blood sample from each participant was collected into two sets of EDTA-treated tubes for genomic DNA extraction and NO assessment. The samples were coded and analyzed in a blind manner. Genomic DNA was extracted from whole blood using QIAamp DNA Blood Mini Kit supplied by Qiagen GmbH (Hilden, Germany) as described by the user manual. DNA purity and concentration were determined spectrophotometrically at 260 and 280 nm. Plasma was separated after centrifugation of the other set of EDTA-treated tubes for the NO assay. The purified genomic DNA and plasma were stored at –20 °C until use.

Genotyping of NOS3 polymorphisms: Genotyping for the NOS3 polymorphisms was done with PCR-restriction fragment length polymorphism using the primers (BioVision, Gentaur, Belgium). The primer sequence was in accordance to Thomas et al. [16] for the T-786C polymorphism, Luizon et al. for the Glu298Asp polymorphism [17], and Ayub et al. [18] for the 27-bp insertional VNTR; see Table 1. PCR was performed in a total reaction of 25 μl containing 100 ng of template DNA, 1.0 μM of each primer, and 12.5 μl of DreamTaq Green PCR Master Mix (2X; Thermo Scientific, St. Petersburg, FL). The PCR protocol was 94 °C for 4 min for denaturation followed by 35 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min. Finally, extension was conducted at 72 °C for 5 min. A negative control sample was included in each run to check for contamination.

For the T-786C polymorphism (rs2070744), the PCR products were digested overnight with 1 μl of MspI restriction enzyme at 37 °C, producing fragments of 140 and 40 bp (T allele) or 90, 50, and 40 bp (C allele). For the Glu298Asp polymorphism (rs1799983), the resulting 248-bp band size was digested by the 2U BanII restriction enzyme (New England Biolab, Beverly, MA) for 6 h at 37 °C. This enzyme cleaves the PCR product into fragments of 163 and 85 bp fragments (G allele) or no digestion (T allele).

| Gene name          | Primer (5’-3’)                  |
|--------------------|---------------------------------|
| T-786C (rs2070744) | TGGAGAGTGCTGGTGTTACCCCA          |
|                    | GCCTCCACCCCCACCTGTGC            |
| Glu298Asp (rs1799983) | AAGGCAGGAGACAGTGGATGGA          |
|                    | CCCAGTCAATCCCTTTGTGCTCA          |
| VNTR-a/b intron 4  | AGGCCCTATGTTAGTGCTTT            |
|                    | TCTTTAGTGCTGCTCAG               |
For the 27-bp insertional VNTR in the intron 4 polymorphism, two alleles were obtained when this region was amplified: “eNOS4a,” which was 393 bp long and consisted of four 27-bp repeating units, and “eNOS4b,” which was 420 bp long and consisted of five 27-bp repeating units. The genotyping products of all polymorphisms were separated in a 2% agarose electrophoresis system (Maxicell, EC360M Electrophoretic Gel System, Thermo Scientific) and then visualized with ethidium bromide staining under ultraviolet (UV) trans-illumination with the 100-bp SiZeR™ DNA marker (iNtRON Biotechnology, Seongnam, Korea).

**Measurement of total nitrate/nitrite:** NO production was determined by measuring total nitrate/nitrite (NOx), the stable end product of NO metabolism, in plasma in a two-step process (nitric oxide colorimetric assay kit from BioVision). In the first step, nitrate was converted to nitrite using nitrate reductase. In the second step, Griess reagents were used to convert nitrite to a deep purple azo compound. The amount of the azochromophore accurately reflected the amount of NO in the samples. Briefly, 50 μl samples of plasma were mixed with equal volumes of 1% sulfanilamide and 1 mg/ml solution of N-1-naphthylethlenediamine dihydrochloride in 0.5% H₃PO₄. After 10 min at room temperature, the absorbance was measured at 540 nm.

**Statistical analysis:** Data were processed using the Statistical Package for Social Science version 13 (SPSS Inc., Chicago, IL). Continuous variables were expressed as mean ± standard deviation (SD) and compared with the Student t test. Categorical variables were expressed as a percentage. The chi-square ($\chi^2$) test was used to compare the gender distribution, to test the association between the genotypes and alleles in relation to the cases and controls, and to test for deviation of the genotype distribution from the Hardy–Weinberg equilibrium. The odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated to estimate the strength of the association between the polymorphism genotypes and the alleles of the patients and controls. A value of $p<0.05$ was considered statistically significant.

**RESULTS**

Table 2 shows the demographic data for the study groups. The ages of the controls ranged from 55 to 78 years with a mean of 63.8±6.3 years. There were 56 men and 54 women. The ages of the patients with POAG ranged from 41 to 75 years with a mean of 48.9±7.0 years. There were 76 men and 84 women. There was a statistically significant increase in the age of the controls compared to the patients with POAG ($p<0.001$), but no significant difference regarding sex ($p = 0.58$).

**Table 2. Demographic characteristics of the high-tension primary open-angle glaucoma (POAG) patient (n=160) and controls (n=110).**

| Parameters          | POAG patients | Controls | P value |
|---------------------|---------------|----------|---------|
| Age (years)         | 41–75         | 55–78    | <0.001* |
| Range               | 48.9±7.0      | 63.8±6.3 |         |
| Mean ± SD           | 63.8±6.3      | 63.8±6.3 | <0.001* |
| Sex, n (%)          |               |          |         |
| Male                | 76 (47.5)     | 56 (50.9)|         |
| Female              | 84 (52.5)     | 54 (49.1)| 0.58**  |

* Two-tailed Student t test **Two-tailed chi square ($\chi^2$) test $p$ is significant at <0.05
high-tension POAG compared with the controls (p = 0.01), but there were no significant results after stratification by genotype. There was a significant decrease in the mean NO\textsubscript{X} plasma level in the (TC+CC) genotype compared to the TT genotype in the patients (p<0.001) and in the controls (p<0.001), but the results for the other genotypes of the Glu298Asp and intron 4 polymorphisms were not significant in the patients or controls; see Table 5.

**DISCUSSION**

In the present work, the relations between the T-786C, Glu298Asp, and VNTR-a/b intron 4 polymorphisms of the NOS3 gene and the development of high-tension POAG among an Egyptian population were studied. The distribution of the minor alleles of the three polymorphisms was compatible with the Hardy–Weinberg equilibrium, indicating that the screening method was appropriate. Since age is not a factor that interferes with gene distribution, the controls chosen were older than the patients with POAG to decrease the chance of choosing control subjects who may develop POAG with increasing age.

The frequency of the CC genotype of the T-786C NOS3 gene (rs2070744) was significantly higher in the patients with high-tension POAG than in the controls (OR = 2.54). In addition, the C allele of the T-786C NOS3 gene frequency was significantly higher in the patients with POAG than in the controls (OR = 1.86). These results suggest an association between the presence of the CC genotype of the T-786C genotype and the prevalence of high-tension POAG, and this polymorphism may play a role in the development of the disease. Neither the Glu298Asp nor VNTR-a/b intron 4 polymorphism was associated with POAG.

Magalhães da Silva et al. found that the T-786C polymorphism was marginally associated with the risk of POAG in patients ≥52 years of age at diagnosis, but the results were not confirmed after adjustment for other confounders. The researchers also found no significant association between the polymorphism Glu298Asp and the risk of POAG in men or women [19]. Unlike our results, although the frequencies were comparable, a significant association between the VNTR-a/b intron 4 polymorphism of the NOS3 gene and POAG was found in a Pakistani cohort [18]. This may be due to the relative rarity of this genotype, and larger studies may be needed to determine such a relationship. Logan et al. found an association between the NOS3 gene and subjects with POAG who had a history of migraine in a British population [20].

*Table 3. Genotype and allele distributions of polymorphisms of endothelial nitric oxide synthase (NOS3) gene in primary high-tension open-angle glaucoma patients (n=160) and controls (n=110) in Egyptians.*

**NOS3 T-786C (rs2070744)**

| Genotype | Patients, No (%) | Controls, No (%) | Odds ratio (95%CI) | P value** |
|----------|-----------------|-----------------|-------------------|----------|
| TT       | 63 (39.4)       | 60 (54.5)       | 1 (reference)     | –        |
| TC       | 59 (36.9)       | 38 (34.5)       | 1.11 (0.67–1.84)  | 0.70     |
| CC       | 38 (23.8)       | 12 (10.9)       | 2.54 (1.26–5.13)  | 0.007    |
| T allele | 185 (57.8)      | 158 (71.8)      | 1 (reference)     | <0.001   |
| C allele | 135 (42.2)      | 62 (28.2)       | 1.86 (1.29–2.69)  | <0.001   |

**NOS3 Glu298Asp (rs1799983)**

| Genotype | Patients, No (%) | Controls, No (%) | Odds ratio (95%CI) | P value** |
|----------|-----------------|-----------------|-------------------|----------|
| Glu/Glu  | 81 (50.6)       | 63 (57.3)       | 1 (reference)     | –        |
| Glu/Asp  | 59 (36.9)       | 37 (33.6)       | 1.15 (0.69–1.92)  | 0.58     |
| Asp/Asp  | 20 (12.5)       | 10 (9.1)        | 1.43 (0.64–3.18)  | 0.38     |
| Glu allele | 221 (69.1)    | 163 (74.1)      | 1 (reference)     | 0.21     |
| Asp allele | 99 (30.9)      | 57 (25.9)       | 1.28 (0.87–1.88)  | 0.33     |

**Intron 4 polymorphism of NOS3**

| Genotype | Patients, No (%) | Controls, No (%) | Odds ratio (95%CI) | P value** |
|----------|-----------------|-----------------|-------------------|----------|
| b/b      | 110 (68.8)      | 70 (63.6)       | 1 (reference)     | –        |
| b/a      | 41 (25.6)       | 32 (29.1)       | 0.84 (0.49–1.45)  | 0.53     |
| a/a      | 9 (5.6)         | 8 (7.3)         | 0.76 (0.28–2.03)  | 0.58     |
| b allele | 261 (81.6)      | 172 (78.2)      | 1 (reference)     | 0.33     |
| a allele | 59 (18.4)       | 48 (21.8)       | 0.81 (0.53–1.24)  | <0.001   |

*Hardy–Weinberg equilibrium among controls (p=0.12 for T-786C, 0.19 for Glu298Asp, and 0.12 for intron 4 genotypes) **Two-tailed chi square (χ\textsuperscript{2}) test significant at <0.05; CI: confidence interval*
Table 4. Genotype and allele distributions of polymorphisms of endothelial nitric oxide synthase (NOS3) gene in high-tension primary open-angle glaucoma patients and controls in Egyptians.

| Parameters | Males | | | | Females | | | |
|------------|-------|---|---|---|-------|---|---|---|
|            | Cases; n=76 | Controls; n=56 | Odds ratio (95%CI) | P value* | Cases; n=84 | Controls; n=54 | Odds ratio (95%CI) | P value* |
| NOS3 T-786C (rs2070744) | | | | | | | | |
| TT | 32 (42.1%) | 30 (53.6%) | Reference | – | 31 (36.9%) | 30 (55.6%) | Reference | – |
| TC | 26 (34.2%) | 19 (33.9%) | 1.01 (0.49–2.10) | 0.97 | 33 (39.3%) | 19 (35.2%) | 1.19 (0.59–2.42) | 0.63 |
| CC | 18 (23.7%) | 7 (12.5%) | 2.17 (0.84–5.63) | 0.11 | 20 (23.8%) | 5 (9.3%) | 3.06 (1.07–8.74) | 0.03 |
| T allele | 90 (59.2%) | 79 (69.6%) | Reference | – | 95 (57.6%) | 79 (73.1%) | Reference | – |
| C allele | 62 (40.8%) | 33 (30.4%) | 1.65 (0.98–2.77) | 0.06 | 73 (43.5%) | 29 (26.9%) | 2.09 (1.24–3.53) | 0.005 |
| NOS3 Glu298Asp (rs1799983) | | | | | | | | |
| Glu/Glu | 41 (53.9%) | 32 (57.1%) | Reference | – | 40 (46.6%) | 31 (57.4%) | Reference | – |
| Glu/Asp | 24 (31.6%) | 19 (33.9%) | 0.90 (0.43–1.87) | 0.78 | 35 (41.7%) | 18 (33.3%) | 1.43 (0.70–2.91) | 0.33 |
| Asp/Asp | 11 (14.5%) | 5 (8.9%) | 1.73 (0.56–5.28) | 0.33 | 9 (10.7%) | 5 (9.3%) | 1.18 (0.37–3.72) | 0.78 |
| Glu allele | 106 (69.7%) | 83 (74.1%) | Reference | – | 115 (68.5%) | 80 (74.0%) | Reference | – |
| Asp allele | 46 (30.3%) | 29 (25.9%) | 1.24 (0.72–2.14) | 0.44 | 53 (31.6%) | 28 (26.0%) | 1.32 (0.77–2.26) | 0.32 |
| Intron 4 polymorphism of NOS3 | | | | | | | | |
| b/b | 53 (69.7%) | 38 (67.9%) | Reference | – | 57 (67.9%) | 32 (59.3%) | Reference | – |
| b/a | 18 (23.7%) | 14 (25.0%) | 0.93 (0.42–2.08) | 0.86 | 23 (27.4%) | 18 (33.3%) | 0.75 (0.36–1.58) | 0.46 |
| a/a | 5 (6.6%) | 4 (7.1%) | 0.92 (0.23–3.58) | 0.90 | 4 (4.8%) | 4 (7.4%) | 0.63 (0.15–2.61) | 0.52 |
| b allele | 124 (81.6%) | 90 (80.3%) | Reference | – | 137 (81.6%) | 82 (75.9%) | Reference | – |
| a allele | 28 (18.4%) | 22 (19.6%) | 0.92 (0.50–1.72) | 0.80 | 31 (18.5%) | 26 (24.1%) | 0.71 (0.40–1.29) | 0.26 |

*Two-tailed chi square ($\chi^2$) test significant at <0.05; CI: confidence interval.
Unlike our results, in a case-control study, no association was found between the NOS3 T786C polymorphism and POAG in a Taiwanese population [21].

No difference was found in the allele or genotype distribution of T-786C in the NOS3 gene between POAG and normal tension glaucoma in Caucasian patients by Weiss et al. [22]. Unlike our study design, they did not compare patients with POAG with sex-matched controls, and most of their patients who were referred to tertiary centers had advanced disease.

In this study, after stratification by sex, we found the CC genotype and the C allele of the T-786C NOS3 gene were associated with high-tension POAG in women but not in men. The CC genotype has been previously found to be linked with women in the United States [23], and TC+CC genotypes have been found to be linked with women in Brazil [19]. This might suggest that female sex hormones may interact with these genotypes resulting in the development of POAG.

Other polymorphisms of the NOS3 gene were studied for possible association with POAG, and therefore are not comparable with our study. One study found the C-T haplotype established by rs3793342 and rs11771443 may be genetic markers of POAG in a Han Chinese population [24].

The 786CC polymorphism in the promoter region of the NOS3 gene is associated with lower eNOS mRNA and serum nitrite/nitrate levels, and individuals carrying the 786C allele have lower eNOS mRNA and serum nitrite/nitrate levels than those with the wild-type allele [13]. Some authors demonstrated that NO inhibits the release of potent vasoconstrictors such as endothelin and angiotensin II [25,26]. Because eNOS availability is regulated at the transcriptional and posttranscriptional levels and owing to its role in the production of NO, the NOS3 gene is a potential candidate for many vascular diseases [27]. Polak et al. compared the perfusion of the optic nerve head and fundus pulsation amplitude during NOS3 inhibition and found a significantly lower perfusion and pulsation amplitude in patients with POAG compared to controls, suggesting altered NOS3 activity in these patients [28]. Therefore, the decrease of NO synthesis in patients with the T-786C polymorphism could predispose the individuals to endothelial dysfunction, with the consequent loss of basal vasodilator tone and reduced blood flow to the ocular tissues, including the optic nerve head, which may be a principal factor in the development of POAG.

To investigate the functional aspects of the three polymorphisms, we measured the NOx plasma levels for the patients and controls. The levels were significantly decreased in the (TC+CC) genotype compared to the TT genotype of the T-786C polymorphism in the patients and the controls but not in other genotypes of the NOS3 gene (Glu298Asp and intron 4 polymorphisms). In addition, the levels were significantly decreased overall in patients with high-tension POAG compared to the controls. These results may be explained by the CC genotype, and the C allele of the T-786C polymorphism may be the cause of the decreased NOx levels. The significant decrease in the NOx levels in patients with POAG is because the (TT+CC) genotypes are more frequent in the patients with POAG than in the controls (60.6% versus 44.0%). In accordance with our results, Galassi et al. found lower NO plasma levels in patients with POAG [29]. Previous studies have also found lower NOx levels in the TC+CC group than in the TT group.

### Table 5. Nitrite/nitrate (NOx) plasma level (μM) in high-tension primary open-angle glaucoma patients (n=160) and controls (n=110) in Egyptians.

| Parameters                           | Patients                      | Controls                     | P value* |
|--------------------------------------|-------------------------------|------------------------------|----------|
|                                      | Number | NOx level | P value** | Number | NOx level | P value** |
| NOS3 T-786C (rs2070744)              |        |           |          |        |           |          |
| TT                                   | 63     | 26.3±4.2  | <0.001   | 60     | 26.4±4.1  | 0.89     |
| TC+CC                                | 97     | 21.1±3.9  | <0.001   | 50     | 21.8±3.7  | <0.001   |
| NOS3 Glu298Asp (rs1799983)           |        |           |          |        |           |          |
| Glu/Glu                              | 81     | 23.6±4.0  | 0.15     | 63     | 24.6±4.1  | 0.14     |
| Glu/Asp+Asp/Asp                      | 79     | 22.7±3.9  | 0.15     | 47     | 23.9±3.9  | 0.37     |
| NOS3 intron 4 polymorphism            |        |           |          |        |           |          |
| b/b                                  | 110    | 23.4±3.9  | 0.15     | 70     | 24.5±4.1  | 0.07     |
| b/a+a/a                              | 50     | 22.6±4.2  | 0.24     | 40     | 24.0±4.1  | 0.54     |
| Total                                | 160    | 23.1±3.8  | 0.15     | 110    | 24.3±4.1  | 0.01     |

* Two-tailed Student t test between patients and controls ** Two-tailed Student t test between genotypes of the same polymorphism.
The decrease in the plasma concentration of NO could be implicated in the deregulation of IOP [28,35].

This study has several limitations. The sample size seems small, and other risk factors for glaucoma, e.g., myopia, systemic factors, and other risk genes were not considered. Larger studies with a larger sample size that consider other risk factors for high-tension POAG may be needed to establish the association between the NOS3 gene and POAG.

In conclusion, we have studied the genotype and allele frequencies of T-786C (rs2070744), Glu298Asp (rs1799983), and VNTR-a/b intron 4 polymorphisms of the NOS3 gene in relation to high-tension POAG and found the CC genotype and the C allele of the T-786C genotype were associated overall in patients with POAG and in female patients, but not in male patients. The Glu298Asp and VNTR-a/b intron 4 polymorphisms were not associated with overall high-tension POAG or after sex stratification.

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