

## eNOS gene polymorphisms in paraffin-embedded tissues of prostate cancer patients

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**Background/aim:** The purpose of the present study was to investigate whether endothelial nitric oxide synthase (eNOS) gene polymorphisms play a role in prostate cancer (PCa).

**Materials and methods:** We examined three eNOS gene polymorphisms (T-786C promoter region, G894T, and Intron 4 VNTR 4a/b) at extracted DNAs from 50 formalin-fixed paraffin-embedded tissues of PCa patients. For the controls, blood samples obtained from 50 healthy men were studied. Genotyping of molecular variants was performed by PCR-RFLP technique.

**Results:** We found that the TC genotype of the T-786C polymorphism was associated with PCa risk (OR: 3.325, CI: 1.350–8.188, P = 0.008). The eNOS G894T polymorphism was also associated with PCa. The frequency of the 894T allele was significantly higher in PCa patients. No association was identified between intron 4 VNTR polymorphism and PCa.

**Conclusion:** We found significant differences in genotypic and allelic frequencies between PCa patients and controls for eNOS T-786C and G894T polymorphisms. The presence of the T-786C genotype and 894T allele in carriers increased the risk of PCa. No association was found between intron 4 VNTR polymorphism and PCa patients.

**Key words:** Polymorphism, prostate cancer, eNOS

### 1. Introduction

Prostate cancer (PCa) incidence and mortality have increased dramatically after 1970 (1). PCa, which is the most common organ cancer in men, is the second leading cause of death by tumor. It progresses silently, invades locally or metastasizes in most patients with cancer. Most frequently patients are diagnosed between ages 65 and 75 (2).

Nitric oxide synthase (NOS), which is an enzyme family, catalyzes the conversion of L-Arginine to L-Citrulline and produces reactive nitric oxide (NO) molecules. There are 3 isoenzymes of NOS: neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), and eNOS. These enzymes are named according to their location (3,4).

With genome-wide studies, some chromosome regions, which are thought to have a role in PCa, have been identified. Examples for these regions may be given as 3p, 7q, 9q, 10q, 11p, 13q, 16q, 17p, and 18q (5,6). The

eNOS gene is located on 7q35-36 and consists of 26 exons. eNOS is a constitutively expressed enzyme of 135kDa in vascular endothelial cells. Three polymorphisms of the eNOS gene have been widely studied. The polymorphism at codon 298 (rs1799983) in exon 7 of the eNOS gene, also called G894T, is a functional polymorphism that results in a Glu-Asp substitution during protein synthesis. eNOS enzyme activity is found to be reduced in carriers with this substitution when compared with GG homozygous individuals. The second functional polymorphism of the eNOS is the T/C point mutation (rs2070744) located at nucleotide 786 close to 5' terminus. Mutation at this site causes a significant decrease in promoter activity. The third functional polymorphism of the eNOS is 27-bp VNTR (eNOS4a/b) in intron 4 and causes basal NO production (4,7).

Studies on the relationship between eNOS gene polymorphism and PCa are very scarce. Sanli et al. (8) reported that the eNOS 4a/b allele was not associated with

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PCa. Safarinejad et al. (4) investigated the relationship between eNOS gene polymorphisms (T-786C, G894T, and intron 4 VNTR a/b) and clinical parameters of PCa; they found association of PCa with T-786C or 4a/b eNOS polymorphisms, but not with G894T. In their experimental procedure, they extracted genomic DNA from peripheral blood leukocytes. With regard to polymorphisms T-786C, G894T, and intron 4 VNTR a/b, the present study is the first to identify eNOS gene polymorphisms in the formalin-fixed paraffin-embedded tissues of PCa patients. The purpose of this study was to investigate whether eNOS gene polymorphisms play any role in PCa (T-786C, G894T, intron 4 VNTR a/b).

## 2. Materials and methods

### 2.1. Patients and controls

This study was performed with 50 formalin-fixed paraffin-embedded tissues of PCa patients (35–72 years of age). The samples with adenocarcinoma were chosen with a combination of convenience and random sampling methods. Firstly, the Department of Pathology in the Faculty of Medicine at Cumhuriyet University was chosen as a convenient sample group for its access. Then, 50 samples were randomly selected from the tissue samples that were collected between 2009 and 2013 within the Department of Pathology in the Faculty of Medicine at the Cumhuriyet University Turkey. As the tissue samples had been irreversibly anonymized, no clinical or patient-related information was available for this study. From each block 10- $\mu$ m sections were cut on a standard microtome, placed in individual Eppendorf tubes, and stored at 4 °C until extraction.

A total of 50 individuals were evaluated as a control group in this study. The control group was hospital-based and selected from among healthy voluntary men whose ages matched those of the patient group. Genomic DNA was extracted from the whole blood treated with EDTA using the QIAamp DNA Blood Mini Kit, according to the manufacturer's guidelines. The extracted DNA was stored at –20 °C until analysis.

All procedures were performed in accordance with the guidelines of the Human Ethics Committee of the Kocaeli University School of Medicine (Ethic number: KOU KAEK 2013/23).

### 2.2. Extraction of DNA from paraffin-embedded material

Using the method described by Mattes and Miller, DNA was extracted from paraffin-embedded tissues of PCa patients: 1) Tissue was deparaffinized in 1 mL of xylene and incubated for 30 min at room temperature on rotary shaker. 2) The material was then centrifuged at top speed for 5 min and supernatant was discarded (this step was repeated twice). 3) Ethanol (100%) (0.5 mL) was added and mixed by inverting the tube 10 times. This mixture

was centrifuged at top speed for 5 min, and supernatant was discarded (this step was also repeated twice), and then air-dried until the ethanol evaporated. 4) Digestion buffer (50 mM Tris-HCl, pH: 8, 0.1 mM EDTA, and 0.5% Tween 20) (100  $\mu$ L) containing proteinase K (20mg/mL) was added and incubated for 3 h at 55 °C. 5) Samples were flash spun to remove the liquid from the cap, heated at 95 °C for 8 min to inactivate the proteinase K, and incubated for 1 h in ice. 6) The final DNA suspension was stored at –20 °C until used for PCR; 2–5  $\mu$ L in a 100  $\mu$ L reaction volume was used for PCR (9).

### 2.3. Molecular variants genotyping

The genotyping of eNOS gene polymorphisms was determined with PCR as described by Safarinejad et al (4).

#### 2.3.1. eNOS T-786C polymorphism

The eNOS T-786C genotypes were amplified by PCR with the primers 5'- AAGGCAGGAGACAGTGGATGGA-3' (forward) and 5'-CCCAGTCAATCCCTTTGGTGCTCA-3' (reverse) (4). The predicted PCR product size was 180 bp. The PCR products were digested with *MspI* (Thermo scientific) at 37 °C overnight, separated by electrophoresis on 2% agarose gels, and visualized under ultraviolet (UV) illumination after ethidium bromide staining. The wild-type allele (allele "T") contained two fragments, 140 and 40 bp. The Polymorphic variant (allele "C") contained three fragments, 90, 50, and 40 bp (Figure 1).

#### 2.3.2. eNOS G894T polymorphism

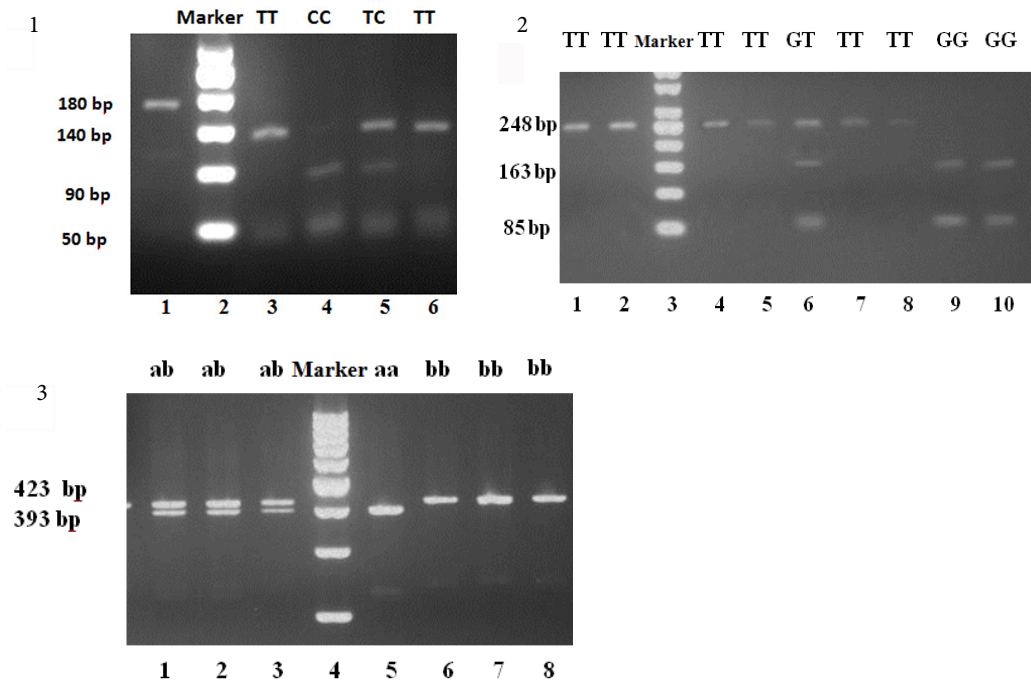
The G894T polymorphisms was determined by PCR followed by the restriction digestion using the following primers 5'-TGGAGAGTGCTGGTGTACCCCA-3' (forward) and 5'-GCCTCCACCCACCCTGTC-3' (reverse) (4). PCR products were restricted by *BanII* (Thermo scientific) at 37 °C overnight. The fragment sizes were 163 and 85 bp for the wild-type or no digestion for the variant allele. DNA fragments were separated on 2% agarose gel electrophoresis (Figure 2).

#### 2.3.3. 27 bp-VNTR polymorphism in intron 4

Genotypes of the 27-VNTR in intron 4 were determined by PCR amplification using primers 5'-AGGCCCTATGGTAGTGCCTT-3' (forward) and 5'-TCTCTTAGTGCTGTGGTCAC-3' (reverse) (4,10). The genotypes were determined by fragments visualized in 3% high-resolution agarose gel at 100 V for 75 min and visualized under UV after ethidium bromide staining. The wild type allele (allele "b") contained five tandem repeats of 27 bp and 420 bp; the mutant allele (allele "a") contained four tandem repeats of 27 bp and 393 bp band (Figure 3).

### 2.4. Statistical analysis

Statistical analysis was performed using SPSS version 18. The frequencies of homozygous and heterozygous eNOS gene mutations and the frequency of allelic mutations in PCa patients and controls were compared using chi-square



**Figure 1.** Detection of T-786C polymorphism. Lane 1: PCR product; lane 2: 50 bp DNA Marker; lane 3 and 6: control carrying wild-type alleles; lane 4: homozygote patient carrying a polymorphic allele; lane 5: heterozygote patient carrying both the polymorphic and wild-type alleles.

**Figure 2.** Representative agarose gel PCR products of G894T homozygote (TT, lanes 1 and 2, 4 and 5, 7 and 8), heterozygote (GT, lane 6), and wild-type (GG, lanes 9 and 10). A 50-bp marker was electrophoresed in lane 3.

**Figure 3.** Tandem 27 bp repeats in intron 4 VNTR polymorphism. Lanes 1–3: heterozygote genotype (intron 4 ab); lane 4: a standard 100-bp marker; lane 5: patient with 4 repeats of 27 bp (intron 4 aa genotype); lanes 6–8: patients with 5 repeats of 27 bp (intron 4 bb genotype).

analysis. For each polymorphism, unconditional logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (95% CI) for PCa. A value of  $P < 0.05$  was considered significant. The significant mutation profiles are discussed in the current report. Deviations from the Hardy-Weinberg equilibrium were analyzed by using the  $\chi^2$  test.

### 3. Results

In this study we analyzed 50 PCa patients and 50 healthy controls. The table shows the allele and the genotype frequencies of eNOS T-786C, G894T, and intron 4 VNTR (4a/b) polymorphisms in PCa patients and controls. When the genotype frequencies of these three polymorphisms were evaluated, no deviation from the Hardy-Weinberg equilibrium was observed for both cases and controls.

For the T-786C polymorphism of the eNOS gene, in the PCa group 32 patients (64%) were TT genotype, 11 patients (22%) were TC genotype, and 7 patients (14%) were CC genotype; in the control group, 21 healthy individuals (42%) were TT genotype, 24 individuals (48%) were TC genotype, and 5 individuals (10%) were

CC genotype. The TC genotype of the case group was also significant compared with that of the control group (Table, Figure 1), (OR: 3.325, CI: 1.350–8.188,  $P < 0.05$ ).

A significant difference in both genotype distribution and allele frequency between PCa patients and healthy controls was found for the eNOS G894T polymorphism. The GT and TT genotypes in PCa patients were higher than those in the controls (44% and 54% respectively,  $P < 0.05$ ). The T allele frequency was 0.760 for PCa and 0.250 for healthy individuals in the current study. The difference between the eNOS G894T polymorphism T allele frequency in PCa patients and that in controls was also significant (Table, Figure 2), (OR: 0.105, CI: 0.055–0.201,  $P < 0.05$ ).

No significant differences were observed between the groups for the intron 4 VNTR genotype and allele frequencies. The prevalence of genotypes of bb, ab, and aa profiles for the eNOS intron VNTR polymorphism were 82%, 14%, and 4% respectively in patients with PCa, and 72%, 24%, and 4% respectively in the control group. The “a” allele frequency was 0.110 in patients with PCa and 0.160 in the control group (Table, Figure 3).

**Table.** Distribution of the genotype and allele frequencies of eNOS T-786C, G894T, and intron 4 VNTR polymorphisms in patients with PCa and control subjects.

Gene/genotypes		Patients n=50 n/%	Control n=50n/%	P value	Odds ratio	95% (CI)
eNOS T-786C						
	TT	32 (64)	21 (42)	-	1	-
	TC	<b>11 (22)*</b>	24 (48)	0.008	3.325	1.350-8.188
	CC	7 (14)	5 (10)	0.896	1.088	0.305-3.887
Alleles	T	75 (75)	66 (66)	0.117	1.628	0.884-2.999
	C	25 (25)	34 (34)			
eNOS G894T						
	GG	1 (2)	29 (58)	-	1	-
	GT	22 (44)	17 (34)	0.0001	0.027	0.003-0.216
	TT	27 (54)	4 (8)	0.0001	0.005	0.001-0.041
Alleles	G	24 (24)	75 (75)	0.0001	0.105	0.055-0.201
	T	<b>76 (76)**</b>	25 (25)			
eNOS Intron 4 VNTR						
	bb	41 (82)	36 (72)	-	1	-
	ab	7 (14)	12 (24)	0.200	1.952	0.694-5.491
	aa	2 (4)	2 (4)	0.899	1.139	0.153-8.504
Alleles	b	89 (89)	84 (84)	0.301	1.541	0.676-3.512
	a	11 (11)	16 (16)			

\*The TC genotype in eNOS T-786C SNP was significant for the patient group in the current PCa cohort; Odds ratio: 3.325 (1.350–8.188),  $P < 0.05$ ; \*\*The T allele in eNOS G894T SNP was significant for the patient group in the current PCa cohort; Odds ratio: 0.105 (0.055–0.201),  $P < 0.05$ .

#### 4. Discussion

NO has a specific biological role in the human body. It is effective in physiological and pathological conditions, and therefore its construction is controlled in a very precise way. Its main physiological functions include vasodilation, inhibition of platelet aggregation, neurotransmission, and regulation of the immune response (11). Studies have highlighted the role of NO in the cardiovascular, nervous, and immune system. It has been shown that NO causes relaxation of the vascular smooth muscle cells in the cardiovascular system, functions as a signaling molecule in the nervous system, and is used by phagocytic cells of the immune system against pathogens. Some properties of NO, including its ability to kill circulating tumor cells and its ability to control disease progression, may contribute to these results (12).

The net effects of NO depend on its concentration, its target cell, and its interactions with metal ions, proteins,

and reactive oxygen species (ROS). In tumor cells, tumor-associated NO production may provide a selective growth advantage with an angiogenic stimulus and promote cancer progression by inducing DNA damage through the generation of free radicals on DNA. In addition, NO production stimulates hyperplasia in normal tissues (13).

To our knowledge, this is the first study showing the analysis of the T-786C, G894T and intron 4 VNTR (4a/b) polymorphisms of the eNOS gene identified by using paraffin-embedded tissues obtained from PCa patients. In a previous study by Sanli et al. (8), intron 4 VNTR (4a/b) polymorphism in Turkish patients with PCa was examined using DNA extracted from blood samples. There are also few studies on eNOS gene polymorphisms for PCa patients in different populations (4,12–15).

In the present study, we examined if three well-known polymorphisms of the eNOS gene were associated with the

risk of PCa in a Turkish cohort. According to the result of our study, there was a significant association between 2 eNOS gene polymorphisms (T-786C and G894T) and PCa (Table, Figures 1 and 2).

To our knowledge, there is only one other study examining eNOS gene polymorphisms (T-786C, G894T and intron 4 VNTR) in PCa patients (4). We performed a similar procedure on paraffin-embedded tissues in Turkish patients with PCa. We found that the TC genotype for the T-786C polymorphism was significant. Safarinejad et al. (4) found that the CC genotype for the T-786C polymorphism was associated with an increased risk of PCa and an increased rate of high grade and advanced disease. Some studies have shown that individuals with the C allele for the eNOS T-786C polymorphism have significantly decreased promoter activity compared with the T allele (16,17). According to Majumdar et al. (18), the T-786C polymorphism is able to reduce the promoter activity of the eNOS gene, which is responsible for the endothelial NO production. Safarinejad et al. (4) also showed that plasma NO levels were reduced in individuals with the -786C allele, and they indicated that this result was associated with high grade and advanced disease (4).

The T-786C polymorphism is located in the promoter region of the eNOS gene. The region where the RNA polymerase enzyme binds is important for transcription of the gene. Polymorphism in this region may result in increased or decreased NO synthesis. Although underlying mechanisms in PCa triggered by increased or decreased levels of NO are not known, this condition plays an important role in physiological functions and the disease states.

In the present study, we also examined whether the GT and TT genotypes for the eNOS G894T gene polymorphism were associated with PCa risk. Marangoni et al. (13) suggested that the GG and GT genotypes for the eNOS G894T polymorphism were associated with PCa risk, whereas in other population studies it was indicated that there is no association between G894T and PCa (4,15,19).

In a study on bladder cancer patients within the selected Turkish cohort, Verim et al. (20) found that the frequencies of the eNOS Glu298Asp heterozygous genotype and T allele were significantly different when compared with those of the controls. Although studies were performed on different cancer types, our results were concordant with those reported by Verim et al. (20).

NO production regulates blood flow specifically in coronary arteries. Decreasing the basal NO release was found to be associated with dispositions to hypertension, thrombosis, atherosclerosis, and vasospasm (21). G-T transversion at nucleotide position 894 within exon 7 of the eNOS gene results in a replacement of glutamic acid by aspartic acid at codon 298. This action causes

conformational changes, leading to decrease in the enzyme activity and changes in the enzyme structure. As a matter of fact, in a study by Majumdar et al. (18) it was shown that polymorphism in this region resulted in decreased eNOS enzyme activity.

As mentioned earlier, there are only a few studies on eNOS gene polymorphisms for patients with PCa. With regard to the Turkish population, VNTR polymorphisms in the intron 4 of Turkish PCa patients have been previously studied by Sanli et al. (8). We did not find any significant correlation between intron 4 VNTR (4a/b) polymorphism and PCa patients, and our results are concordant with those reported by Sanli et al. (8) ( $P > 0.05$ ). However, Sanli et al. (8) did not find any significant difference between intron 4 VNTR (4a/b) polymorphism and PCa with high-grade or low-grade tumors; they indicated that the VNTR allele was overrepresented among patients with advanced stage tumors and bone metastases, but this difference was not significant for histologic tumor grades between groups (8). Among populations, there may be differences between the phenotypic expression and distribution of eNOS polymorphisms with prevalence and etiology of PCa studies that included PCa patients from Iran (4) and Portugal (14) showing that the "a" allele for the intron 4 VNTR (4a/b) polymorphism was a significant and important risk factor for PCa ( $P < 0.05$ ).

There are ethnic differences in the distribution of eNOS variants. Frequencies of eNOS gene polymorphisms have been shown to vary among different ethnic groups such as Caucasian, African Americans, Asians, and Mexican Americans (10). Some genetic variants are more common in certain populations. Population specificity may reflect differences in allele frequency distribution. Results obtained both from our study and other studies revealed that the bb genotype appeared to be more frequent in the Turkish population (8,22–24).

With regard to the results from a study by Tsukada et al. (25) on plasma NO levels and NOS gene polymorphisms, their findings of the allele frequencies for intron 4 VNTR (89.8% for the b allele and 10.2% for the a allele) were parallel to our results (89% for the b allele and 11% for the a allele). Studies performed by Tsukada et al. (25) and Song et al. (26) showed that the plasma level of NO<sub>x</sub> was significantly lower in the group with an allele. Based on this observation, it can be suggested that the intron 4 VNTR polymorphism of the eNOS gene may affect enzyme levels of eNOS. According to these results, both bb genotype and elevated NO production may be effective to suppress the tumor growth, and thus it may have a protective role on prostatic carcinogenesis (25, 26).

In conclusion, we observed a significant difference between PCa patients and controls for both T-786C and G894T polymorphisms of the eNOS gene; on the other hand, the intron 4 VNTR (a/b) polymorphism of

eNOS did show any association with PCa. Both the TC genotype of the T786C polymorphism and the GT and TT genotypes of the G894T polymorphism of eNOS were previously found to be associated with PCa risk. Further studies are needed to clarify the roles of these genotypes on PCa development.

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