RESEARCH ARTICLE

T-786C, G894T, and Intron 4 VNTR (4a/b) Polymorphisms of the Endothelial Nitric Oxide Synthase Gene in Bladder Cancer Cases

Fikriye Polat[1], Songül Budak Diler[2], İrfan Azazi[3], Artun Öden[4]

Abstract

The aim of the present study was to determine whether endothelial nitric oxide synthase (eNOS) gene polymorphisms play a role in development of bladder cancer in the Turkish population. The study was performed on 75 patients (64 men, 11 women) with bladder cancer and 143 healthy individuals (107 men, 36 women) with any kind of cancer history. Three eNOS gene polymorphisms (T-786C promoter region, G894T and intron 4 VNTR 4a/b) were determined with polymerase chain reaction and restriction fragment lenght polymorphism methods. In our study, GT and TT genotypes for eNOS G894T polymorphism were found to significantly vary among patients with bladder cancer and control group (OR: 0.185, CI: 0.078-0.439, p=0.0001 and OR: 0.324, CI: 0.106-0.990, p=0.026). Also, the frequency of the 894T allele was significantly higher in patients with bladder cancer (51%). No association was identified for eNOS T-786C and intron 4 VNTR 4a/b polymorphisms between patients with bladder cancer and control groups in our Turkish population.

Keywords: eNOS - polymorphism - bladder cancer - Turkish population

Asian Pac J Cancer Prev, **16 (6)**, 2199-2202

Introduction

Bladder cancer is the most frequent malignancy of urinary tract cancer and the fourth most incident cancer in males and seventh most incident in females in the world. Men have a higher risk of bladder cancer than women, approximately 3:1 (Crawford, 2008). Bladder cancer is diagnosed at any age including children. The incidence of bladder cancer is strongly associated with occupational exposure, tobacco smoking, lifestyle habits such as coffein intake, artificial sweeteners, and hair dyes. On the other hand, m olecular factors alterations like p53, Rb, p21, p27, p16 contribute to bladder cancer risk (Buyru et al., 2003; Soussi and Lozano, 2005; Crawford, 2008; Colombel 2008; Akca and Tokgun, 2012).

Nitric oxide (NO) is a free radical molecule which is generally produced in various tissues from the conversion of L-Arginine to L-Citrulline by different forms of nitric oxide synthase. There are three isoenzymes of NOS and named as neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), and endothelial nitric oxide synthase (eNOS). NO plays a very important effect on regulating the central and peripheral nervous systems, the cardiovascular system, and the immune system (Safarinejad et al., 2013; Liu et al., 2014).

Three genetic polymorphisms of eNOS have been widely studied. The G894T polymorphism (rs1799983) in exon 7 is a functional polymorphism that results in a Glu-Asp substitution during the protein synthesis. T-786C point mutation (rs2070744) causes a significant decrease in promoter activity of eNOS enzyme. Other polymorphism of the eNOS is intron 4 VNTR (27-bp) which causes basal NO production (Glueck et. al., 2010; Safarinejad et al., 2013).

The studies on the relationship between eNOS gene polymorphism and bladder cancer are scarce. Verim et al. (2013) reported that the eNOS G894T heterozygotes genotypes and T allele were significantly associated with bladder cancer in Turkish population. Amasyali et al. (2012) investigated the correlation between eNOS intron 4 VNTR polymorphism and Turkish patients with superficial bladder cancer. They found associations between bladder cancer and the aa plus ab genotype of the eNOS intron 4 VNTR. Ryk et al. (2011) analyzed the eNOS T-786C and Glu298Asp polymorphisms in a population-based urothelial bladder-cancer patients material of Caucasian origin. They reported that the C allele of T-786C promoter polymorphism in homozygous carries in the patient group was significantly associated but this was not the case with G894T.

The purpose of this study was to investigate whether three eNOS polymorphisms (T-786C, G894T, and VNTR intron 4) play any role in development of bladder cancer in Turkish patient group.

*[1]Department of Primary Education, Elementary Sciences Education, Faculty of Education, Kocaeli University, Kocaeli, [2]Department of Biology, Faculty of Science and Letters, University of Niğde, [4]Niğde State Hospital, Niğde, Department of Urology Clinic, [3]Luleburgaz State Hospital, Kırklareli, Turkey *For correspondence: fikriyepolat@gmail.com*

Materials and Methods

Subjects

The patients and controls were selected among the ones from urology clinic of Luleburgaz and Niğde State Hospital, Turkey. Eleven women and sixty four men included in this study. We investigated the eNOS gene polymorphisms in seventy five bladder cancer patients and one hundered-fourty three healthy controls. Patients with primary bladder cancer were included in as the main case group in this study. The control group was selected from among voluntaries without bladder cancer and individuals with any kind of cancer history in same population. The distribution of age was matched in our study groups. All procedures were performed in accordance with the guidelines of the Human Ethics Committee of the Cukurova University School of Medicine (KAEK 2013-29).

Isolation of DNA

Genomic DNA was extracted from the whole blood treated with EDTA using the QIAamp DNA Blood Mini Kit (Maryland, USA), according to the manufacturer's guidelines. The extracted DNA was stored at -20˚C until analysis.

Molecular variants genotyping

The genotyping of eNOS gene polymorphisms was determined with PCR as described by Safarinejad et al. (2013).

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) (Safarinejad et al., 2013). The predicted PCR product size was 180 bp. The PCR products were digested with MspI (Thermo scientific) at 37˚C for over-night, separated by electrophoresis on 2% agarose gels, and visualized under ultraviolet (UV) illumination after ethidium bromide staining. The wild-type allele (allele "T") contains two fragments, 140 and 40 bp. The Polymorphic variant (allele "C") contains three fragments, 90, 50 and 40 bp.

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'-GCCTCCACCCCCACCCTGTC-3' (reverse) (Safarinejad et al., 2013). PCR products were restricted by BanII (Thermo scientific) at 37˚C for over-night. 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.

27 bp-VNTR polymorphism in intron 4

Genotypes of the 27-VNTR in intron 4 were determined by PCR amplification using primers 5'-AGGCCCTATGGTAGTGCCTTT-3' (forward) and 5'-TCTCTTAGTGCTGTGGTCAC-3' (reverse) (Thameem et al., 2008; Safarinejad et al., 2013). The genotypes were determined by fragments visualized in 3% high-resolution agarose gel at 100V for 75 minutes and visualized under UV after ethidium bromide staining. The wild type allele (allele "b") contained five tandem repeats of 27 bp and 420 bp and the mutant allele (allele "a") four tandem repeats of 27 bp and 393 bp band.

Statistical analysis

Statistical analysis was performed by using SPSS version 18. The frequencies of homozygous and heterozygous eNOS gene mutations, the frequency of allelic mutations in urinary bladder cancer patients and controls were compared using chi-square analysis. For each polymorphism, unconditional logistic regression was used to calculate odds ratios (OR) and 95% confidence intervals (95% CI) for bladder cancer. A value of $p<0.05$ was considered as statistically significant. The statistically significant mutation profiles are discussed in the current report. Deviations from Hardy-Weinberg equilibrium were analyzed by using Michael H. Court's (2005-2008) online calculator

(http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20-%20HW%20calculator.xls).

Results

Table 1 shows the demographic characteristics of patients with bladder cancer and controls. In this study, we analyzed 75 bladder cancer patients and 143 healthy controls. No significant association was found between demographic characteristics such as age, sex, and smoking status. Genotypes and allele frequencies for eNOS T-786C, G894T and intron 4 VNTR (4a/b) polymorphisms in the bladder cancer patients and controls are listed in Table 2. When genotype frequencies of the T-786C and intron 4 VNTR polymorphisms were evaluated, no deviation from Hardy-Weinberg equilibrium was observed for neither cases and nor controls.

For the T-786C polymorphism of the eNOS gene, in the bladder cancer group 24 patients (32%) were TT genotype, 40 patients (53%) were TC genotype, and 11 patients (15%) were CC genotype; in the control group, 56 healthy individuals (39%) were TT genotype, 72 individuals (50%) were TC genotype, and 15 individuals (11%) were CC genotype. No significant differences were observed between groups for T-786C genotype and allele frequencies.

Table 1. Characteristics of Patients with Bladder Cancer and Controls

Parameters	Bladder Cancer (n=75)	Control (n=143)	p value
Age	61.2 ± 10.53	60.16 ± 13.90	0.43
Sex			
Male	64 (63.2 ± 8.77)	107 (61.8 ± 2.98)	0.45
Female	11 (52.2 ± 4.85)	36 (55.2 ± 15.47)	0.56
Smoking status			
Yes	38 (60.8 ± 9.77)	41 (58.5 ± 12.03)	0.35
No	37 (62.5 ± 1.32)	102 (60.8 ± 4.58)	0.54

*p<0.05; significantly different from control group

Table 2. Distribution of Genotype and Allele Frequencies of eNOS T-786C, G894T, and intron 4 VNTR Polymorphisms in Patients with Bladder Cancer and Control Subjects

Gene/Genotypes		Patients (n=75) n%	Control (n=143) n/%	P value	Odds ratio	95% (CI)
T-786C	TT	24 (32)	56 (39)	-	1	-
	TC	40 (53)	72 (50)	0.408	0.771	0.417-1.427
	CC	11 (15)	15 (11)	0.246	0.584	0.234-1.457
Allelles	T	88 (59)	184 (64)			
	C	62 (41)	102 (36)	0.246	0.787	0.525-1.180
G894T	GG	7 (9)	48 (34)	-	1	-
	GT	59 (79) *	75 (52)	0.0001	0.185	0.078-0.439
	TT	9 (12) *	20 (14)	0.042	0.324	0.106-0.990
Alleles	G	73 (49)	171 (60)			
	T	77 (51) *	115 (40)	0.026	0.638	0.428-0.949
VNTR	bb	50 (67)	97 (68)	-	1	-
	ab	24 (32)	43 (30)	0.797	0.924	0.504-1.691
	aa	1 (1)	3 (2)	0.707	1,546	0.157-15.251
Allelles	b	124 (83)	237 (83)			
	a	26 (17)	49 (17)	0.958	0.986	0.585-1.663

*The GT and TT genotype in eNOS G894T SNP was significant for patient group in the current bladder cancer cohort; Odds ratios:0.185 (0.078-0.439) and 0324 (0.106-0.990), p<0.05; The T allele in eNOS G894T SNP was significant for patient group in the current bladder cancer cohort; Odds Ratio: 0.026 (0.428-0.949), p<0.05

A statistically significant difference in both the genotype distribution and allele frequency between patients with bladder cancer and healthy controls was found for eNOS G894T polymorphism. The GT genotype was higher than control (79% and 52% respectively, p<0.05). However, the TT genotype was lower than control (12% and 14% respectively, p<0.05). The T allele frequency was 0.51 for patient group and 0.40 for healthy individuals in the current results. The difference of eNOS G894T polymorphism T allele frequency was also statistically significant when compared to the control group (Table 2), (OR: 0.638, CI: 0.428-0.949, p=0.026). Genotype distributions for eNOS G894T polymorphism in control group were in agreement with Hardy-Weinberg equilibrium (χ^2=1.178, p=0.277) but not in patient group (χ^2=24.7, p<0.0001).

No significant differences were observed between groups for the intron 4 VNTR genotype and allele frequencies. The prevalence of genotypes of bb, ab and aa profiles for eNOS intron 4 VNTR polymorphism were 67%, 32% and 1% respectively in patients with bladder cancer, and 68%, 30% and 2% respectively in control group. "a" allele frequency was 0.17 for in patients with bladder cancer and the control group (Table 2).

Discussion

NO is an important biological messenger, and a short-lived, pleitropic molecule that plays complicated roles in tumor biology. NO formation is catalyzed by eNOS, nNOS and iNOS isoforms. The eNOS is expressed in endothelial cells, including a variety of tumors such as bladder tumor vessels. Also, the eNOS expression may be involved in the development and progression of bladder cancer (Lin et al., 2003; Ryk et al., 2011; Wu et al., 2014).

In this study we analyzed if the eNOS T-786C promoter polymorphism, the G894T polymorphism in exon 7, and intron 4 VNTR (4a/b) polymorphism play any role in Turkish patients with bladder cancer. There are only few studies on eNOS gene polymorphisms for bladder cancer patients in literature (Ryk et al., 2011; Amasyali et al., 2012; Verim et al., 2013).

To our knowledge there is only one published report of the relationship between the T-786C polymorphism and bladder cancer. Ryk et al. (2011) found that the C allele of the -786>C promoter polymorphism was associated with an increased threefold odds ratio for bladder cancer in Sweden. However, we didn't find any statisticially significant correlation between T-786C polymorphism and patients with bladder cancer. There may be ethnic differences in the distribution of eNOS gene variants. In a meta-analysis study on three eNOS polymorphisms and cancer, Wu et al. (2014) detected that Caucasians in four genetic models have elevated cancer risk for eNOS T-786C polymorphism, while that was not detected in Asians (Wu et al., 2014).

The second of the eNOS gene polymorphisms is G894T, in which thymidine is substituted for guanine at nucleotide 894. G-T transversion at this position results in replacement of glutamic acid by aspartic acid at codon 298, and decreased eNOS enzyme activity (Wang et al., 2000, Majumdar et al., 2010). In the present study, we also examined if the eNOS G894T gene polymorphism were associated with bladder cancer risk. We found that the GT and TT genotypes and T allele for eNOS G894T polymorphism were associated with bladder cancer risk (p<0.05). Verim et al. (2013) reported that the eNOS G894T heterozygotes genotypes and T allele were significant associated with bladder cancer. Their study suggested an increased risk role of NOS3 GT genotype in bladder cancer susceptibility in Turkish population. Our results were concordant with the study of Verim et al. (2013). In a previous study, Ryke et al. (2011) didn't find increased risk of bladder cancer with G894T polymorphism, but there was an association between the Glu298Asp and tumor grade (p=0.04) (Ryk et al., 2011).

In the current case-control study, we found no increased risk of bladder cancer with the eNOS intron 4 VNTR polymorphism. However, in a recent study, Amasyali et al. (2012) reported that the a allele of the

eNOS intron 4 VNTR polymorphism as a pottential risk factor for bladder cancer in a Turkish patient group. Our eNOS intron 4 VNTR results, especially controls, were resembl to previously reported the healthy Turkish individuals (Sinici et al., 2009; Akar et al., 1999; Olcay et al., 2006).

In our study, smoking status did not show statistically significant risk when patients with bladder cancer and controls compared to each other. However, we know that smoking is the most frequent risk factor associated with bladder cancer and is especially estimated to account for up to 50% of bladder cancer in men. Even, as a reported by Marcus et al. (2000), stopping smoking and avoiding exposure to smoke should be an effective method of decreasing the incidence of this disease (Marcus et al., 2000; Crawford, 2008). According to Verim et al. (2013), in such studies, creating a homogenized study group for age, gender and smoking status etc. is one of the most challenging issues (Verim et al., 2013).

In conclusion, we have observed a significant difference between patients with bladder cancer and controls for G894T polymorphism of the eNOS gene, on the other hand both T-786C or intron 4 VNTR (4a/b) polymorphism of the eNOS gene did show any association with bladder cancer. GT and TT genotypes of G894T polymorphism of the eNOS were previously found to be associated with bladder cancer risk. Also, we represent the first results on T-786C promoter polymorphism and bladder cancer in Turkish cohort. Further studies are needed to clarify the roles of these genotypes on bladder cancer development.

Acknowledgements

We would like to thank to Scientific Research Project Unit of Niğde University (Project No: FEB 2013-28)

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