

eNOS gene polymorphisms and transitional cell cancer of the bladder

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Abstract

Aim: In this case-control study, we examined the association between two types of eNOS gene polymorphisms (intron 4 a/b and Glu298Asp) and transitional cell carcinoma (TCC) of the bladder.

Nitric Oxide (NO) is a free radical that plays a key role in various physiological and pathophysiological processes, particularly in the circulatory system. Endothelial Nitric Oxide Synthase (eNOS), as a member of NOS family, is the enzyme, responsible for the physiological production of nitric oxide in the endothelium. Polymorphisms of eNOS have been related to increased risk for different types of cancers.

Material and Methods: A total of 64 patients with bladder TCC and 80 controls with similar epidemiological characteristics of patients group were evaluated and compared in terms of eNOS gene polymorphisms of two different types ((intron 4 a/b and Glu298Asp). Following DNA isolation from the blood samples, eNOS gene was replicated via PCR. The genomic distribution of two types of eNOS polymorphism was determined by gel electrophoresis following the process of restriction, for both groups.

Results: The alleles most commonly observed in patient group were "ab" for intron 4 (OR= 1.80, 95% CI: 0.71-4.64; P=0.20) and "GT" for Glu298Asp polymorphisms (OR= 1.65, 95% CI: 0.85-3.22; P=0.14). Neither bladder cancer risk nor disease grade was associated with these polymorphisms.

Conclusions: This study suggests that the intron 4 a/b and Glu298Asp eNOS gene polymorphisms are not associated with bladder cancer susceptibility. However, it is mandatory to conduct further trials with more patients to confirm these findings and to evaluate the association between bladder cancer and eNOS gene polymorphisms.

Keywords: Bladder cancer; Glu298Asp polymorphism; intron 4 a/b polymorphism; Nitric oxide synthase; Nitric oxide synthase polymorphism

INTRODUCTION

Transitional cell carcinoma (TCC) of the bladder is one of the most common types of urinary tract malignancies, and is the seventh most common type of cancer in men, with an annual incidence of 5.3 of 100.000 in the world population with the highest occurrence rate in industrialized and western countries. Although bladder cancer is observed at all ages, its incidence is elevated with aging and is the second most common urologic malignancy following prostate carcinoma in middle and advanced aged males (1).

Nitric oxide (NO) is a small, reactive free radical molecule, which has both intracellular and extracellular regulatory effects. Various cellular processes are regulated by NO, secreted from endothelium, platelets, vascular smooth muscle cells, neurons and other NO-producing cells (2).

Nitric oxide synthase (NOS) enzyme family consists of neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) enzymes. These isoenzymes are similar in terms of amino acid sequence and biochemical properties, however, they are encoded with different genes on different chromosomes and are distinguished from their locations and activators (3). The role of NO in the cancer pathogenesis is controversial. Experimental studies have shown both inhibitory and activatory effects of NO in tumor development (4,5). However, the evidence demonstrated indirect effects of eNOS enzyme on tumor cell proliferation, apoptosis, and angiogenesis. Nevertheless, the most emphasized property of eNOS is its function on tumor angiogenesis (6).

Previous studies have reported the involvement of eNOS in the pathogenesis of colorectal, breast, central nervous

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system and pancreatic cancers (7-10). The gene encoding eNOS is located on the long arm of the chromosome 7 and contains 26 exons. Research to date reported assorted single nucleotide polymorphisms (SNPs) resulting from the substitution of an individual base and variable number tandem repeats (VNTR). There are 3711 polymorphisms described in the eNOS gene to date (available at the URL: <http://www.ncbi.nlm.nih.gov/snp> [as of March 2019]).

Altered distributions of eNOS gene polymorphisms on patient groups of lung, prostate, and breast cancer have been shown previously. The most common investigated variants of eNOS polymorphisms on cancer subjects are 27 base pair (bp) VNTR in intron 4 and G to T transversion leading to the Glu298Asp in exon 7 (11-13).

It has been shown that, circulating NO concentrations are determined by the different types of eNOS gene polymorphisms. For instance, intron 4 VNTR polymorphism is responsible for the production of more than 25% of total NO in plasm, showing the genotype may be facilitating the role of NO in the pathogenesis of tumor development (14).

The 27-bp polymorphism in the intron 4 has two allele isoforms; 4 repeats are termed for "a" genotype and, 5 repeats are the "b" genotype (15). The highest activity of the compound is seen in the individuals with "bb" genotype of intron 4 VNTR(a/b) polymorphism, whereas the "aa" genotype is related with the lowest activity (16).

Thus, the aim of our study is to determine the allelic frequencies of two eNOS polymorphisms (Glu298Asp; intron 4 VNTR) in Turkish bladder TCC patients. Since eNOS polymorphisms are shown in a wide range of tumor types, we attempted to evaluate the incidence of these alleles in histopathologically confirmed TCC cases.

MATERIAL and METHODS

Subjects

The study group consisted of 64 patients (54 male, 10 female) with histopathologically confirmed bladder TCC with an age on 64.48 ± 11.52 years, and 80 cystoscopically proven cancer-free control subjects (69 male, 11 female; age 63.6 ± 10.63 years).

None of the patients initiated chemotherapy or radiotherapy before the study. The control group includes patients who had admitted to our clinic due to different causes and undergone cystoscopy. Following the cystoscopy procedure, the subjects without a finding of TCC of the bladder and who had equivalent population distribution in terms of smoking, geographical origin and gender were included. The patients and control subjects with a family history of any kind of cancer were excluded. Also, control subjects with a former or new diagnosis of cancer were not included.

The study protocol was approved by the Medical Ethics Committee of the Istanbul Education and Research Hospital (2011/0068). All study participants provided informed consent before the study, according to Helsinki declaration.

Evaluation

A detailed individual and family history of the participants were taken following the physical examination. The study and control subjects were questioned for height, weight, smoking status, drug use and accompanying health issues.

The information about tumor stage and grade, and chemotherapy and/or radiotherapy history were obtained from medical records. According to the 2010 TNM staging system of the American Joint Committee on Cancer, all the patients with TCC of the bladder had superficial tumors (pTa-pT1). The patients were subdivided into the low-grade and high-grade groups according to the grading systems of the 2004 World Health Organization (WHO)/ International Society of Urologic Pathologists (ISUP): Classification of Nonmuscle Invasive Urothelial Neoplasia.

Genotype determination

Blood samples were collected from all participants and genomic DNA was extracted from the peripheral blood leukocytes using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany). The extracted DNA was stored at -20°C until analysis.

Intron 4 VNTR 4a/b polymorphism

DNA samples were amplified using the primers: 5'- AGG CCC TAT GGT AGT GCC TTT-3' (sense) and 5'- TCT CTT AGT GCT GTG GTC AC-3' (antisense). PCR was performed in a final volume of 25 μL containing DNA (1 μL) and PCR mix consisting of dNTP (0.2 mM each), primer pair (10 pmol of each), 5U of DNA Taq polymerase, and 2.5 mL of 10X PCR buffer including 20 mM MgCl_2 . Following the initial denaturation at 95°C for 5 minutes, the reaction was resumed 38 cycles at 95°C for 1 minute for denaturation, 60°C for 1,5 minutes for annealing and 72°C for 2 minutes for extension. A final extension phase was performed for 5 minutes at 72°C .

420 bp amplified PCR products were then subjected to electrophoresis on 3% agarose gel at 80V for 80 minutes. Two allele isoforms were determined on the intron 4 of eNOS gene. 420 bp band corresponding to the "b" allele consisted of 5 copies of the 27 bp repeat, whereas 393 bp "a" allele spans for 4 copies (Figure 1A).

Thus, when electrophoresed with 100 bp DNA ladder, 420 bp band corresponds "bb" homozygous for intron 4 VNTR (4a/b) polymorphism, while the togetherness of both the 420 bp and 393 bp bands was evaluated as "ab" heterozygous. Only 393bp band on a lane was homozygous "aa" for the intron 4 VNTR (4a/b) polymorphism.

Glu298Asp polymorphism

The eNOS Exon7 Glu298Asp polymorphism was detected by PCR using the primer pair: 5'-CAT GAG GCT CAG CCC CAG AAC-3' (sense) and 5'-AGT CAA TCC CTT TGG TGC TCA-3' (antisense). The reaction mixture (a total volume of 25 μL) containing DNA (1 μL) and PCR mix consisting of dNTP (0.2 mM each), primer pair (10 pmol of each), 5U of DNA Taq polymerase, and 2.5 mL of 10X PCR buffer including 20 mM MgCl_2 . Reaction mixtures were heated at 95°C for 5 minutes for initial denaturation followed by 38

cycles of amplification including the phases, denaturation at 95°C for 1 minute, annealing at 62°C for 1,5 minutes and extension at 72°C for 2 minutes. A final extension phase was performed for 5 minutes at 72°C at the end of 38 cycles.

The PCR products were then digested with MboI at 37°C for 2 hours. The resulting 206 bp amplification product was cleaved into 2 smaller fragments of 119 and 87 bp in the presence of a T nucleotide (corresponding to Asp 298) but not in its absence (Figure 1B). Digested DNA fragments were separated by electrophoresis on 3% agarose gel stained with ethidium bromide.

Statistical analysis

Chi-square tests were used for the comparison of the distribution of demographic variables. The genotype frequencies among the patients and control subjects were calculated. Differences in genotype distribution were examined using the chi-square test. Odds ratio was calculated using a two-by-two contingency within the 95% CI. Results are expressed as means \pm SD for the continuous variables, and number and percentage for the categorical variables. Results with p-values less than 0.05 were considered statistically significant.

RESULTS

Study population characteristics

Sixty-four bladder cancer patients (54 male, 10 female) and 80 healthy controls (69 male, 11 female) were investigated in the context of the study. The clinical and demographic characteristics of the study population are presented in Table 1. The differences in age, BMI, gender, and smoking status were not statistically significant (p=0.63, 0.21, 0.75 and 0.11, respectively).

Table 1. Demographic characteristics of the study group and control subjects

Characteristics	Cancer patients (n=64)	Controls (n=80)	p value
Age (year)	64.48 \pm 11.52	63.60 \pm 10.63	0.63
BMI (kg/m ²)	27.4 \pm 2.4	26.8 \pm 3.2	0.215
Female	10 (15.6%)	11 (13.8%)	0.75
Male	54 (84.4%)	69 (86.2%)	0.75
Smoking status			
Yes	50 (78.1%)	53 (66.3%)	0.11
No	14 (21.9%)	27 (33.8%)	
Tumor grade			
Low grade	39 (61%)	-	NA
High grade	25 (39%)	-	NA

The histologic grades were classified as high grade and low grade using the grading system of the WHO and ISUP. Twenty-five patients with stage T1 and over and with the high-grade tumor in the pathological examination were categorized as "high grade", whereas 39 patients with stage Ta and lower were categorized as "low grade" group. Regarding the association between the tumor grade and gender, no statistically significant difference was found.

The genotype distributions of the studied eNOS polymorphisms for bladder cancer cases and healthy controls are demonstrated in Table 2.

Table 2. Distribution of Glu298Asp and intron 4 VNTR (a/b) genotypes in the study group and healthy control subjects

Variables	Cases (n=64)		Controls (n=80)		OR (95% CI)
	Number	Frequency	Number	Frequency	
Glu298Asp polymorphism					
Genotype					
GG	30	0.469	46	0.575	Referent
GT	31	0.484	29	0.363	1.65 (0.85- 3.22)
TT	3	0.047	5	0.063	0.73 (0.17- 3.21)
Alleles					
G	91	0.710	121	0.756	Referent
T	37	0.290	39	0.244	1.26 (0.75-2.13)
Intron 4 VNTR (a/b) polymorphism					
Genotype					
aa	0	0	3	0.038	NA
ab	12	0.188	9	0.113	1.80 (0.71-4.64)
bb	52	0.812	68	0.850	Referent
Alleles					
a	12	0.094	15	0.094	1.0 (0.45-2.22)
b	116	0.906	145	0.906	Referent

Table 3. Combined genotype distribution for eNOS Glu298Asp and intron 4 VNTR (a/b) polymorphisms in the study population and healthy controls

Variables	Cases (n=64)		Controls (n=80)		Crude OR (95% CI)
	Number	Frequency	Number	Frequency	
Glu298Asp / Intron 4 VNTR (a/b) polymorphism					
Genotype					
GG+aa	0	0	3	0.038	NA
GG+ab	4	0.063	5	0.063	1.0 (0.26-3.89)
GG+bb	26	0.406	38	0.475	Referent
GT+aa	0	0	0	0	NA
GT+ab	8	0.125	4	0.05	2.70 (0.78-9.46)
GT+bb	23	0.313	25	0.359	1.23 (0.62-2.48)
TT+aa	0	0	0	0	NA
TT+ab	0	0	0	0	NA
TT+bb	3	0.047	5	0.063	0.73 (0.17- 3.21)

Table 4. Distribution of Glu298Asp and intron 4 VNTR (a/b) genotypes and combined eNOS haplotypes in the study group with different pathological grades

Variables	High grade (n=25)		Low grade (n=39)		Crude OR (95% CI)
	Number	Frequency	Number	Frequency	
Glu298Asp polymorphism					
Genotype					
GG	12	0.480	18	0.460	Referent
GT	12	0.480	19	0.490	0.97 (0.36-2.65)
TT	1	0.040	2	0.050	0.77 (0.06-8.98)
Alleles					
G	36	0.720	55	0.705	Referent
T	14	0.280	23	0.295	0.93 (0.42-2.04)
Intron 4 VNTR (a/b) polymorphism					
Genotype					
aa	0	0	0	0	NA
ab	4	0.160	8	0.210	0.74 (0.19-2.77)
bb	21	0.840	31	0.790	Referent
Alleles					
a	4	0.080	8	0.103	0.76 (0.21-2.67)
b	46	0.920	70	0.897	Referent
Glu298Asp / Intron 4 VNTR (a/b) polymorphism					
Genotype					
GG+aa	0	0	0	0	NA
GG+ab	0	0	4	0.100	NA
GG+bb	12	0.480	14	0.370	Referent
GT+aa	0	0	0	0	NA
GT+ab	4	0.160	4	0.100	1.66 (0.38-7.38)
GT+bb	8	0.320	15	0.380	0.75 (0.26-2.17)
TT+aa	0	0	0	0	NA
TT+ab	0	0	0	0	NA
TT+bb	1	0.040	2	0.050	0.77 (0.07-8.97)

While Glu298Asp genotypes and allele frequencies were evaluated, the patients ($X^2=2.04$, $p=0.15$) and the controls ($X^2=0.02$, $p=0.88$) were in Hardy-Weinberg equilibrium. The number of GG and TT homozygous alleles were higher in the control group when compared to the patients, however there was not a statistically significant difference. The heterozygous GT alleles were relatively higher in the patient group ($p=0.14$). Regarding allele frequencies, we also did not find a statistically significant relationship between controls and the cancer patients.

Intron 4 genotypes were in Hardy-Weinberg equilibrium for the patients ($X^2=0.68$, $p=0.41$), whereas there is a deviation for the control subjects ($X^2=9.14$, $p=0.002$). Intron 4 ab allele was in higher rate in the tumor group when compared to the control group, however, this rate was not in statistically significant level ($p=0.2$). Analyzing two different homozygous alleles of Intron 4 VNTR(a/b), there was not a statistically significant difference between the patients and the controls.

A

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AGGCCCTATGGTAGTGCCTTGCTGGAGGAGGGGAAAAGTCTAGACCTGCT
GCAGGGGTGAGGAAGTCTAGACCTGCTGCAGGGGTGAGGAAGTCTAGACCTGC
TCAGGGGTGAGGAAGTCTAGACCTGCTGCGGGGTGAGGAAGTCTAGACCTG
CTCGGGGGTGAAGACAGCTGAGCGGAGCTTCCCTGGGCGGTGCTGTCACTAG
CAGGAGCAGCCTCTGAAAAAGCCCTGGCTGCTTCTCCCCAAGAGAGAA
GGCTTCTCCCGCAGGGCAGTCCAGTGCAGCCCTCACCCACACCCACTGTAC
CCAGTTCCTGCTTTCGGCCGACCCCTCCCTCACACCCAGCCACAGACTC
GGGGCTGGCCTTAGTTACTGGAACGCTGTGACCACAGCACTAAGAGA

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B

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CATGAGGCTCAGCCCAGAAACCCCTTGCCCACTCCCCACAGCTCTGCATTCA
GCACGGTGGACCCAGGAAACGGTCGCTTCGACGTGCTGCCCTGCTGTGACG
GCCCCAGATGATCCCCAGAACTTCTTCTGCCCCCGAGCTGCTCCTTGAGGT
GCCCTGGAGCACCCCACTGAGCACCAAGGGATTGACT

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Figure 1. eNOS gene intron 4 and Exon 7 nucleotide sequence. (A) Tandem repeats on the intron 4 of the eNOS gene. Oligonucleotide sequence depicted with darkest highlight shows the primer sequence. 27 bp repeats are the underlined nucleotide sequences. (B) Nucleotide sequence of Exon 7 of eNOS gene. Darkest highlighting shows the primer sequence, whereas the grey highlighted portion stands for the nucleotide sequence of the Exon 7. The single bold and underlined nucleotide (T) is the location of the transversion

Six of nine possible genotypic combinations were present in our study samples. The odds ratios and p-values of the combined genotype combinations were described in Table 3. Of the four statistically analyzable types, the genotype combination GT+ab was seemed to be associated with the tendency to bladder cancer, however the comparison lack the statistical significance ($OR=2.70$, $95\% CI=0.78-9.46$; $p=0.11$).

Both of the two eNOS genotypes were not associated with tumor grade. While two different alleles for each polymorphism were investigated, the allele frequencies were not statistically different between low grade and high-grade patients. A statistical evaluation could be possible on only three of the genotype combinations (for detailed information see Table 4) (Figure 1).

DISCUSSION

In this study, we attempted to determine the association between two eNOS gene polymorphisms and TCC of the bladder in a Caucasian population. Various case-control studies evaluated eNOS polymorphisms in different types of cancer. Genetic polymorphisms of NOS enzyme family genes and the involvement of functional differences in neoplastic processes have been a popular area of research with recent simultaneous evidence on NO biochemistry and genetics. We did not detect an association between the two polymorphisms mentioned and bladder TCC. In the context of the study, analyzing the patient and the control groups for eNOS intron 4 polymorphism, "bb" genotype was dominant within both groups as 81.2%, and 85%, respectively.

The ratio of "ab" genotype was higher in the patient group with an OR of 1.80. However, a statistically significant relationship was not detected between the groups in terms of intron 4 polymorphism. The polymorphism genotype ratios were similar with the rates of the previous report from Turkey for eNOS intron 4 polymorphism [17]. When the study group and the patient group were compared for the Glu298Asp polymorphism, "GG" and "TT" homozygous alleles were higher in the control group with the percentage of 57.5% vs 46.9% and 6.3% vs 4.7%, respectively. The ratio of "GT" heterozygous allele was higher in the patient group with a percentage of 48.4% vs 36.3% and an OR of 1.65; nonetheless, a statistically significant increase for the bladder cancer risk could not be reported. This finding is compatible with the previous study in the Turkish population which reported a 1.7-fold increase in bladder cancer patients carrying heterozygous GT allele [18]. Although there are similarities between two studies in terms of sample size, sample distribution and ethnical origin, the lack of statistical significance in our study might be due to the randomization of the study group in both studies. Similarly, Ryk et al. studied eNOS Glu298Asp polymorphism in a larger population of Caucasians and found no significant relationship between any of the alleles and the disease [19]. While genotypes are combined, we found a non-significant, 2.7-fold, risk for the GT+ab heterozygous allele combination.

With the sub-categorization of the patients as high grade and low grade, we were not able to detect statistical significance between these sub-groups despite the OR of 1.66 for the combined GT+ab allele in the high-grade group. Although this study comprises these two eNOS polymorphisms, more data from different reports of larger cohorts including different ethnical groups are required for a more precise conclusion.

These polymorphisms are the most commonly studied polymorphisms in different cancer types, causing several variations on the gene, thus leading to conformational changes of NOS protein and enzyme activity. It has been demonstrated that presence of TT homozygous allele

at for the Glu298Asp polymorphism may result in a decrease in eNOS activity [20]. As there are three different genes encoding NO synthases of various functions conserved in the human genome and NO is one of the most reactant transmitter substance on vessels, its effect on carcinogenesis may be a result of its involvement in the tumors' angiogenic and thus metastatic processes. Different NOSs might be functioning in the pathogenesis of distinct types of cancer. Supporting this theory, in a study, recruited 159 bladder cancer cases and 150 controls, iNOS (CCTTT)_n pentanucleotide repeat polymorphism was shown to be related with bladder cancer in a differing manner according to the number of tandem repeats [21].

Additionally, in a study by Ryk et al., 359 bladder cancer patients from a population-based cohort and 164 population controls were genotyped and TT homozygotes for the iNOS Ser608Leu polymorphism were found to be related with three-fold higher odds for bladder cancer. However, the risk of developing muscle-invasive disease was lower in the patients with the indicated polymorphism [22]. These findings support the hypothesis that NO effects on tumor cells on both cytotoxic and angiogenetic way depending on its concentration [23].

Consequently, taken together, previous studies and our report suggest a role for polymorphisms of NOSases in the pathogenesis of urinary bladder tumors and other types of cancers. Since, examinations of sole polymorphisms may exhibit a small view in understanding the pathogenesis and prognosis of the disease, better understanding of these topics would be available with mass genotype scanning of 1430 known polymorphisms of eNOS known thus far and other polymorphisms of different NOS types supported with microarray expression pattern determinations in larger patient and control groups.

CONCLUSION

It has been suggested that different eNOS polymorphisms yield different enzyme physiological activities [3]. In the present study, two frequent polymorphisms of eNOS were evaluated in patients with bladder TCC and a statistically significant relationship was not detected. In order to gain an insight into a full understanding of the relationship between nitric oxide and NOS genes, simultaneous measurement of NO and NOS synthesis in molecular level with these polymorphisms will possibly reveal the physiology of these molecules and give more substantial and practical information. Additionally, studies involving control subjects with similar risk factors can provide more satisfactory results for objective-oriented determination of isolated genetic factors.

Competing interests: The authors declare that they have no competing interest.

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REFERENCES

1. Ferlay J, Soerjomataram I, Ervik M, et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: <http://globocan.iarc.fr>, accessed on 23/03/2020.
2. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991;43:109-42.
3. Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J*. 1994;298:249-58.
4. De Wilt JHW, Manusama ER, van Etten B, et al. Nitric oxide synthase inhibition results in synergistic anti-tumour activity with melphalan and tumour necrosis factor alpha-based isolated limb perfusions. *Br J Cancer Suppl* 2000;83:1176-82.
5. Hussain SP, Trivers GE, Hofseth LJ, et al. Nitric oxide, a mediator of inflammation, suppresses tumorigenesis. *Cancer Research* 2004;64:6849-53.
6. Fukumura D, Gohongi T, Kadambi A, et al. Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. *Proc Natl Acad Sci U S A*. 2001;98:2604-9.
7. Chhatwal VJ, Ngoi SS, Chan ST, et al. Aberrant expression of nitric oxide synthase in human polyps, neoplastic colonic mucosa and surrounding peritumoral normal mucosa. *Carcinogenesis* 1994; ;15:2081-5.
8. Reveneau S, Arnould L, Jolimoy G, et al. Nitric oxide synthase in human breast cancer is associated with tumor grade, proliferation rate, and expression of progesterone receptors. *Lab Invest*. 1999;79:1215-25.
9. Cobbs CS, Brenman JE, Aldape KD, et al. Expression of Nitric Oxide Synthase in Human Central Nervous System Tumors. *Cancer Res* 1995;55:727-30.
10. Franco L, Doria D, Bertazzoni E, et al. Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in pancreatic cancer. *Prostaglandins Other Lipid Mediat* 2004;73:51-8.
11. Cheon KT, Choi KH, Lee HB, et al. Gene polymorphisms of endothelial nitric oxide synthase and angiotensin-converting enzyme in patients with lung cancer. *Lung* 2000;178:351-60.
12. Lee KM, Kang D, Park SK, Berndt SI, Reding D, Chatterjee N, et al. Nitric oxide synthase gene polymorphisms and prostate cancer risk. *Carcinogenesis* 2009;30:621-5.
13. Lu J, Wei Q, Bondy ML, et al. Promoter polymorphism (-786t>c) in the endothelial nitric oxide synthase gene is associated with risk of sporadic breast cancer in non-Hispanic white women age younger than 55 years. *Cancer* 2006;107:2245-53.
14. Wang XL, Mahaney MC, Sim AS, et al. Genetic contribution of the endothelial constitutive nitric oxide synthase gene to plasma nitric oxide levels. *Arterioscler Thromb Vasc Biol* 1997;17:3147-53.

15. Wang XL, Sim AS, Badenhop RF, et al. A smoking dependant risk of coronary artery disease associated with a polymorphism of the endothelial nitric oxide synthase gene. *Nat Med* 1996; 2:41-5.
16. Tsukada T, Yokoyama K, Arai T, et al. Evidence of Association of the ecNOS Gene Polymorphism with Plasma NO Metabolite Levels in Humans. *Biochem Biophys Res Commun* 1998;245:190-3.
17. Erkan E, Toktas G, Unluer E, et al. Expression of NOS isoforms in internal spermatic veins of infertile men with varicocele. *Syst Biol Reprod Med*. 2012;58:268-73.
18. Verim L, Toptas B, Ozkan NE, et al. Possible relation between the NOS3 gene GLU298ASP polymorphism and bladder cancer in Turkey. *Asian Pac J Cancer Prev* 2013;14:665-8.
19. Ryk C, Wiklund NP, Nyberg T, de Verdier PJ. Polymorphisms in nitric-oxide synthase 3 may influence the risk of urinary-bladder cancer. *Nitric Oxide* 2011;25:338-43.
20. Lembo G, De Luca N, Battagli C, et al. A common variant of endothelial nitric oxide synthase (Glu298Asp) is an independent risk factor for carotid atherosclerosis. *Stroke* 2001;32:735-40.
21. Huang ZM, Chen HA, Chiang YT, et al. Association of polymorphisms in iNOS and NQO1 with bladder cancer risk in cigarette smokers. *J Chin Med Assoc* 2014;77:83-8.
22. Ryk C, Wiklund NP, Nyberg T, et al. Ser608Leu polymorphisms in the nitric oxide synthase-2 gene may influence urinary bladder cancer pathogenesis. *Scand J Urol Nephrol* 2011;45:319-25.
23. Andrade SP, Hart IR, Piper PJ. Inhibitors of nitric oxide synthase selectively reduce flow in tumor-associated neovasculature. *Br J Pharmacol* 1992;107:1092-5.