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Original article

Endothelial nitric oxide synthase polymorphisms/haplotypes are strong modulators of oral cancer risk in Serbian population

Jelena Carkic[1)], Nadja Nikolic[1)], Jelena Nisevic[2)], Milos Lazarevic[1,3)], Jovana Kuzmanovic-Pficer[4)], Drago Jelovac[3)], and Jelena Milasin[1)]

[1)] Department of Human Genetics, School of Dental Medicine, University of Belgrade, Belgrade, Serbia
[2)] Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia
[3)] Clinic for Maxillofacial Surgery, School of Dental Medicine, University of Belgrade, Belgrade, Serbia
[4)] Department of Medical Statistics and Informatics, School of Dental Medicine, University of Belgrade, Belgrade, Serbia

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Abstract: Oral carcinoma is the sixth most common malignancy worldwide, with survival rates of approximately 50%. The major type of oral cancer, present in 90% of the cases, is oral squamous cell carcinoma (OSCC). The genetic background predisposing an individual to OSCC is complex and largely unknown. Studies have suggested that endothelial nitric oxide synthase (eNOS) gene polymorphisms modulate the cancer risk, prompting us to assess the impact of three functional eNOS gene polymorphisms on OSCC risk. The present study included 50 patients with OSCC and 110 controls. Polymerase chain reaction and restriction fragment length polymorphism analysis were used for genotyping of single-nucleotide polymorphisms −786 T/C (rs2070744) and 894 G/T (rs1799983) and variable number of tandem repeats (VNTR) intron 4b/a polymorphism. Homozygous carriers of −786 T/C and intron 4b/a VNTR variant alleles paired with a significant increase of oral cancer risk [odds ratio (OR): 3.63, 95% confidence interval (CI): 1.08-12.21; $P = 0.045$ and OR: 11.29, 95% CI: 2.71-47.11; $P < 0.001$, respectively]. When combined, CC and 4b4a genotypes together led to a 21-fold OSCC risk increase (OR: 21, 95% CI: 2.07-213.29; $P = 0.006$). Haplotype analysis showed that the C-G-4b haplotype conferred an 11-fold increase in OSCC risk. In conclusion, eNOS polymorphisms considerably influence levels of OSCC risk in the Serbian population.

Keywords; eNOS, gene polymorphisms, haplotype, oral cancer

Introduction

Oral carcinoma is a common malignancy that accounts for 2-4% of all cancer cases worldwide [1]. It is the sixth most common neoplasm, and despite continued therapeutic advances, approximately 50% of oral squamous cell carcinoma (OSCC) patients still die within 5 years of disease onset [2,3].

Studies have suggested that nitric oxide (NO) has an important role in cancerogenesis, but the exact mechanisms by which NO contributes to tumor development remain unclear. Some researchers have proposed that NO represents a "double-edged sword" in cancer biology [4], owing to the contrasting effects of different NO concentrations, with low levels generally leading to tumor progression and higher levels showing antitumor effects [5]. The dual role of NO in cancer is achieved via the promotion of tumor angiogenesis, cell proliferation, and dissemination [4] as well as the mediation of tumor cell lysis [6] and the induction of apoptosis [7]. Apart from variations in overall NO levels, the role of NO can also be attributed to its bioavailability, cellular microenvironment, genetic profile of the individual, and localization and activity of NO synthase (NOS) enzyme isoforms [8,9].

NOS are enzymes essential for the synthesis of NO. Endothelial NOS (eNOS) is one of the three classes of NOS enzymes and is expressed by certain cancers, including OSCCs [10]. Numerous polymorphisms are distributed throughout the eNOS gene; some of which have been found to have functional and, consequently, clinical significance in malignancies. Those polymorphisms in the eNOS gene appear to mainly regulate its transcription and mediate NO production, but the results of available studies are inconsistent [11-13].

This study aimed to assess the possible association of the eNOS single-nucleotide polymorphisms (SNPs) −786 T/C and 894G/T and variable number of tandem repeats (VNTR) intron 4b/a polymorphism and their corresponding haplotypes with OSCC risk in the Serbian population.

Correspondence to Dr. Jelena Milasin, Department of Human Genetics, School of Dental Medicine, University of Belgrade, Dr Subotica 8, 11000 Belgrade, Serbia
Fax: +381-11-2685288 E-mail: jelena.milasin@stomf.bg.ac.rs

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Materials and Methods

Study participants and sample collection

The study was performed in full accordance with the ethical principles governing medical research and human subjects as laid down in the Declaration of Helsinki (2002 version, http://www.who.int/bulletin/archives/79%284%29373.pdf) and with the approval of the ethics committee of the School of Dental Medicine (no. 36/7). All study participants were informed of the study procedures and signed a written consent form. The study included 50 patients with diagnosed OSCC treated at the Clinic for Maxillofacial Surgery, School of Dental Medicine, University of Belgrade, and 110 cancer-free controls recruited at the School of Dental Medicine, University of Belgrade. The demographical characteristics of both study groups are summarized in Table 1. The histopathological diagnosis of OSCC was established according to the World Health Organization guidelines, whereas tumor staging was performed using the tumor-node-metastasis (TNM) classification [14]. The clinicopathological characteristics and tumor localization of OSCC patients are given in Table 1.

Swab samples were taken from the buccal mucosa of all participants after careful mouth disinfection [15]. DNA was extracted using the PureLink Genomic DNA Purification Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturers' recommendations.

Genotyping

The following three eNOS polymorphisms were genotyped using polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis: promoter polymorphism −786 T/C (rs2070744); 894 G/T (rs1799983) polymorphism, also known as Glu298Asp, located in exon 7; and intron 4b/a VNTR. Primer sequences, restriction enzymes, and product lengths are given in Table 2. PCR analyses were performed under standard amplification conditions, using PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA), 200 nM of each primer, and 300 ng of template DNA. Amplification products for the intron 4b/a VNTR and digestion products for SNPs were loaded on 8% polyacrylamide gel and stained with ethidium bromide, and the bands were then visualized using an ultraviolet fluorescence imaging system. The specificity of the obtained results was confirmed by random regenotyping of about 20% of the samples; no discrepancies were observed.

Statistical analysis

All statistical calculations were completed using the SPSS Statistics for

Table 1 Distribution of demographical characteristics in 50 OSCC patients and 110 healthy controls

Variable	Patients $n = 50$ (%)	Controls $n = 110$ (%)	P value*
Age (years)			
≤55	15 (30)	41 (37.3)	0.371
>55	35 (70)	69 (62.7)	
Gender			
Male	31 (62)	60 (54.5)	0.395
Female	19 (38)	50 (45.5)	
Cigarette smoking			
No	22 (44)	73 (66.4)	0.009
Yes	28 (56)	37 (33.6)	
Alcohol consumption			
No	35 (70)	89 (80.9)	0.153
Yes	15 (30)	21 (19.1)	
Cancer site			
Tongue	11 (22)		
Floor of the mouth	11 (22)		
Buccal mucosa	4 (8)		
Alveolar ridge mucosa	4 (8)		
Lip	17 (34)		
Oropharynx	3 (6)		
Stage			
I + II	25 (50)		
III + IV	25 (50)		
Lymph node status			
N0	27 (54)		
N1 + N2 + N3	23 (46)		
Metastasis			
M0	48 (96)		
M1	2 (4)		

*Chi-squared test, significant at $P < 0.05$

Table 2 Sequence of primers and restriction enzymes used for the detection of eNOS −786 T/C, 894 G/T, and intron 4b/a VNTR polymorphisms

Polymorphism	Primer sequence	Annealing temperature (°C)	PCR product (bp)	Restriction enzyme	Wild-type	Heterozygote	Mutant
−786 T/C	F 5′-CCCCTGTGGACCAGATGC-3′	55	379	Msp I	233/146	233/187/146/46	187/146/46
	R 5′-ACATTAGGGTATCCCTTCC-3′						
894G/T	F 5′-AAGGCAGGAGACAGTGGATG-3′	55	246	MboI	246	246/159/87	159/87
	R 5′-CAGTCAATCCCTTTGGTGCT-3′						
Intron 4b/a VNTR	F 5′-AGGCCCTATGGTAGTGCCTT-3′	55	420, 394	-	420	420, 394	394
	R 5′-TCTCTTAGTGCTGTGGTCAC-3′						

bp, base pair

Windows version 22.0 software program (IBM Corp., Armonk, NY, USA). Descriptive statistics are presented as frequencies and percentages. Differences in the genotype and allele frequency distributions were determined by using Pearson's chi-squared test and Fisher's exact test. The association of gene variants with the risk of disease was examined by unconditional logistic regression analysis, and odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated. The expected frequency of variants among controls was analyzed using the Hardy-Weinberg equilibrium test. Pairwise linkage disequilibrium (LD) (D′ and r^2 values) and haplotype frequencies were determined using the Haploview version 4.2 software program (Broad Institute, Cambridge, MA, USA) [16]. CI block definition was used for haplotype block identification. P values of less than 0.05 were considered to be statistically significant.

Results

Association of individual SNPs with clinicopathological characteristics and OSCC risk

Demographic and lifestyle characteristics of the 50 OSCC patients and 110 controls together with the clinicopathological features of the tumors are given in Table 1. The most frequent tumor localization was the lip ($n = 17$; 35%), followed by the tongue ($n = 11$; 22%) and the floor of the mouth ($n = 11$; 22%). None of the analyzed polymorphisms were associated with the clinicopathological characteristics of the tumors (data not shown).

A significant difference was observed between OSCC patients and controls regarding smoking ($P = 0.009$), implicating this lifestyle choice as an important factor in the onset of oral carcinogenesis (Table 1). Logistic regression analysis demonstrated that smoking alone led to a 2.5-fold increase in the risk of oral cancer development (OR: 2.51, 95% CI: 1.27-4.98).

The genotype and allele distributions of eNOS −786 T/C, 894 G/T, and intron 4b/a VNTR polymorphisms among the cases and controls were consistent with expectations under the Hardy-Weinberg principle ($P > 0.05$) and are displayed in Table 3. Homozygous carriers of the variant allele for the −786 T/C SNP exhibited a 3.6-fold increase in the risk of OSCC development (OR: 3.63, 95% CI: 1.08-12.21; $P = 0.045$). Meanwhile, the variant allele of the intron 4b/a VNTR polymorphism led to a significant increase in cancer risk ranging from threefold for heterozygotes up to 11-fold for homozygous carriers (Table 3).

The adjusted ORs with their 95% CIs estimated by multiple logistic regression modeling after controlling for smoking did not differ significantly from the crude ORs for the analyzed polymorphisms, which suggests the independent influence of smoking and the patient's genetic constitution on oral carcinogenesis (adjusted ORs not shown).

The 894 G/T SNP did not present significant differences in allelic and genotype distribution between patients and controls (Table 3).

The results of the combined effect of two polymorphisms showing an association with OSCC are given in Table 4. Combinations of eNOS −786T/C and intron 4b/a VNTR polymorphisms involving heterozygotes for the VNTR were significantly more frequent in patients than in controls (Table 4, upper section). The combination CC/4b4a exhibited the highest increase in risk as compared with the double wild-type combination (OR: 21, 95% CI: 2.07-213.29; $P = 0.006$). Among the −786 T/C and 894 G/T combination genotypes, several were significantly more frequently observed in patients than in controls, with the CC/GT combination showing the highest increase in OSCC risk (OR: 6.67, 95% CI: 1.27-35.04; $P = 0.032$) (Table 4, middle section). Double heterozygotes for the 894 G/T and intron 4b/a VNTR combination were more frequent among

Table 3 The genotype and allelic distribution of eNOS −786 T/C, 894 G/T, and intron 4b/a VNTR polymorphisms in patients and controls

Variable	Patients $n = 50$ (%)	Controls $n = 110$ (%)	*P* value[a]	OR (95% CI)[†]
−786 T/C				
TT	18 (36)	56 (50.9)	Ref.	
TC	25 (50)	48 (43.6)	0.186	
CC	7 (14)	6 (5.5)	0.037	3.63 (1.08-12.21)
TC + CC	32 (64)	54 (49.1)	0.080	
T allele	61 (61)	160 (72.7)	Ref.	
C allele	39 (39)	60 (27.3)	0.035	1.70 (1.03-2.81)
HWE *P* value[b]	0.719	0.294	-	-
894 G/T				
GG	21 (42)	61 (55.5)	Ref.	
GT	24 (48)	42 (38.2)	0.157	
TT	5 (10)	7 (6.3)	0.302	
GT + TT	29 (58)	49 (44.5)	0.115	
G allele	66 (66)	164 (74.5)	Ref.	
T allele	34 (34)	56 (25.5)	0.115	
HWE *P* value[b]	0.623	0.949	-	-
intron 4b/a VNTR				
4b4b	17 (34)	72 (65.5)	Ref.	
4b4a	25 (50)	35 (31.8)	0.003	3.03 (1.45-6.32)
4a4a	8 (16)	3 (2.7)	<0.001	11.29 (2.71-47.11)
4b4a + 4a4a	33 (66)	38 (34.5)	<0.001	3.68 (1.82-7.44)
4b allele	59 (59)	179 (81.4)	Ref.	
4a allele	41 (41)	41 (18.6)	<0.001	3.03 (1.80-5.12)
HWE *P* value[b]	0.813	0.606	-	-

HWE, Hardy-Weinberg principle; OR, odds ratio; CI, confidence interval; Ref, reference. [a]Chi-squared test; significant at $P < 0.05$. [†]Calculated if significant. [b]Calculated by the Hardy-Weinberg principle test

Table 4 Combination analysis of eNOS −786 T/C, intron 4b/a VNTR, and 894 G/T polymorphisms in OSCC patients and controls

−786 T/C / intron 4b/a VNTR	OSCC $n = 50$ (%)	Controls $n = 110$ (%)	*P* value*	OR (95% CI)
TT / 4b4b	8 (16)	42 (38.2)	Ref.	
TT / 4b4a	5 (10)	14 (12.7)	0.491	
TT / 4a4a	5 (10)	2 (1.8)	0.005	13.13 (2.16-79.86)
TC / 4b4b	7 (14)	23 (20.9)	0.417	
TC / 4b4a	16 (32)	22 (20.0)	0.006	3.82 (1.41-10.31)
TC / 4a4a	2 (4)	1 (0.9)	0.088	
CC / 4b4b	1 (2)	5 (4.5)	1.000	
CC / 4b4a	4 (8)	1 (0.9)	0.006	21 (2.07-213.29)
CC / 4a4a	2 (4)	0 (0)	ND	
−786 T/C / 894 G/T	OSCC $n = 50$ (%)	Controls $n = 110$ (%)	*P* value	OR (95% CI)
TT / GG	9 (18)	45 (40.9)	Ref.	
TT / GT	7 (14)	10 (9.1)	0.048	3.50 (1.05-11.65)
TT / TT	2 (4)	1 (0.9)	0.092	
TC / GG	10 (20)	16 (14.5)	0.032	3.13 (1.08-9.07)
TC / GT	13 (26)	29 (26.4)	0.098	
TC / TT	2 (4)	3 (2.7)	0.230	
CC / GG	2 (4)	0 (0)	ND	
CC / GT	4 (8)	3 (2.7)	0.032	6.67 (1.27-35.04)
CC / TT	1 (2)	3 (2.7)	0.446	
894 G/T / intron 4b/a VNTR	OSCC $n = 50$ (%)	Controls $n = 110$ (%)	*P* value	OR (95% CI)
GG / 4b4b	8 (16)	37 (33.6)	Ref.	
GG / 4b4a	11 (22)	21 (19.1)	0.096	
GG / 4a4a	2 (4)	3 (2.7)	0.569	
GT / 4b4b	7 (14)	28 (25.5)	0.806	
GT / 4b4a	12 (24)	14 (12.7)	0.010	3.96 (1.34-11.74)
GT / 4a4a	5 (10)	0 (0)	ND	
TT / 4b4b	2 (4)	7 (6.4)	1.000	
TT / 4b4a	2 (4)	0 (0)	ND	
TT / 4a4a	1 (2)	0 (0)	ND	

ND, not determined; OR, odds ratio; CI, confidence interval. *Chi-squared test, significant at $P < 0.05$

OSCC patients relative to among controls (OR: 3.96, 95% CI: 1.34-11.74; $P = 0.010$) (Table 4, lower section).

Linkage disequilibrium and haplotype analysis

It was hypothesized that the three polymorphisms mentioned herein are in a state of LD. However, after pairwise LD values (D′ and r^2 values) for the analyzed polymorphisms were calculated, it appeared that none of the polymorphisms were in LD ($D' < 1$, $0 < r^2 < 1$) in OSCC patients alone, controls alone, or both groups taken together.

A haplotype analysis of OSCC patients and controls was carried out to evaluate the combined effect of all three polymorphisms on OSCC susceptibility (Table 5). Eight haplotypes could be distinguished resulting from the six alleles of the analyzed polymorphisms (−786 T/C–894 G/T–intron 4b/a VNTR) in the eNOS gene. The results of the present study indicated that the C-G-4b, T-T-4b, and T-G-4a haplotypes were associated with an increased OSCC risk. In particular, the haplotype C-G-4b was associated

Table 5 Haplotype frequencies of eNOS −786 T/C, 894 G/T, and intron 4b/a VNTR polymorphisms in OSCC patients and controls

Haplotype −786 T/C–894 G/T–intron 4b/a VNTR	Patients (%)	Controls (%)	*P* value	OR (95% CI)
C–G–4b	20.3	4.1	<0.001	11.52 (3.54-37.49)
C–T–4b	5.3	16.6	0.493	
C–G–4a	2.9	5.6	1.000	
C–T–4a	10.6	0	ND	
T–G–4b	22.5	52.7	Ref.	
T–T–4b	10.9	7.9	0.025	3.17 (1.13-8.91)
T–G–4a	20.3	12.1	0.002	3.84 (1.61-9.14)
T–T–4a	7.2	0	ND	

ND, not determined; OR, odds ratio; CI, confidence interval

with the highest risk for oral cancer development (OR: 11.52, 95% CI: 3.54-37.49; $P < 0.001$).

Discussion

NO is known to play a key role in various physiological and pathological processes; further, numerous studies have reported a connection between this small short-lived molecule and malignant diseases [17]. NOS endothelial constitutive isoform is pivotal for regulating NO synthesis [18], and elevated levels of eNOS expression have been reported in different types of cancer, including OSCC [19,20].

This is only the second study to deal with the possible association between eNOS polymorphisms and oral cancer. The results of the present study show that eNOS −786 T/C functional polymorphism located in the promoter region of the eNOS gene confers a 3.6-fold increase of OSCC risk on CC homozygotes. Previous investigations have correlated this SNP with an overall elevated risk of cancer [21-23], including, in particular, gastric [24], colorectal [25], and prostate [26] cancers. Interestingly, a meta-analysis from 2010 showed that this genetic variant could reduce the risk of breast cancer [27]. A recent study on OSCC did not report significant differences in genotype and allelic distributions between patients and controls; however, the variant allele was associated with advanced clinical stages of the disease and exhibited a synergistic effect with environmental carcinogens [28]. This eNOS SNP was previously found to reduce the rate of mRNA transcription, resulting in decreases of eNOS and NO levels [10,29]. Since low NO concentrations have been implicated in tumor promotion, it is therefore plausible that the decreased expression of eNOS due to the presence of a variant allele, particularly in the homozygous form, could affect oral cancer progression, as suggested by the present findings.

Another SNP shown to modify eNOS transcription and the endogenous production of NO is 894 G/T, located in exon 7 of the eNOS gene. This single-nucleotide variation has been shown to retard the entrance of inactive eNOS to the caveolae and decrease enzyme activity and NO production [11,12]. It has also been found that the minor allele T induces proteolytic cleavage of the eNOS protein and, rather than affecting its production, downregulates NO bioavailability [9,11], which could influence tumor progression. The present study reported no association between 894 G/T SNP and OSCC risk, which is partially in accordance with the results of Su et al. who observed no association of this variant alone with OSCC except when it was combined with smoking and betel nut chewing [28]. A meta-analysis from 2015 stated that this SNP does not represent a risk modulator for cancer overall [13]. Yao et al., in contrast, suggested that 894 G/T could even reduce breast cancer risk [27]. Some studies, however, have associated 894 G/T with individual susceptibility for cancer [23]. Other research also linked this SNP to colorectal [25] and prostate cancers [26] as well as to the risk of developing large tumors in patients with urothelial cancer [9]. Yanar et al. indicated a potential relationship between this polymorphism, the risk of laryngeal cancer, and impaired redox homeostasis due to the reduced production of NO, which acts as an antioxidant [30]. A recent study involving women with breast cancer found the TT genotype constitutes both a risk and a protective factor depending on the patient's menopausal state [31], emphasizing the simultaneous role of other tumor-modulating factors and explaining to some extent the conflicting results of previous studies.

The 27 bp VNTR eNOS polymorphism in intron 4 has two allelic forms: 4b with five repeats and 4a with four repeats. In the present study, the variant 4a allele led to a significant increase in cancer risk, from threefold for heterozygotes up to 11-fold increase for homozygous carriers. However, although some studies have reported similar findings to ours related to this polymorphism as a possible risk factor for cancer development [22,32], others found no such association [13,25,26]. It has been suggested that this polymorphism could impact the expression of eNOS by the formation of 27 bp long, so-called short intronic repeat RNAs (sirRNAs) during RNA splicing, with 4a variant cells generating lower quantities of sirRNAs and higher levels of eNOS messenger RNA relative to wild-type 4b cells [33]. Although higher eNOS expression and NO levels have generally been found to exhibit antitumor effects, it has been proposed that these higher NO concentrations owing to 4b/a polymorphism could lead to the overproduction of reactive oxygen species, genetic instability, and tumor progression [32].

Such inconsistencies between studies regarding eNOS polymorphisms and cancer risk could partly be attributed to differences in sample sizes, participants' ethnicities, and types of cancer. Additionally, since cancer has a complex polygenic background, it is evident that numerous genes and alleles contribute to its formation and progression and that knowledge of a single genetic variant is usually not enough to predict the risk for this disease [13]. Therefore, the concomitant effects of two-by-two eNOS polymorphisms on OSCC risk were examined, finding that seven different combinations were associated with significantly elevated risk, with CC/4b4a in particular exhibiting a 21-fold risk increase as compared with the double wild-type combination.

It is also important to take into consideration the fact that one polymorphism, although it does not influence carcinogenesis per se, could still be used as a genetic marker to locate or implicate other functional polymorphisms influencing the course of the disease. Although the present study did not show the analyzed eNOS polymorphisms to be in LD, three different haplotypes (T-G-4a, T-T-4b, and C-G-4b) were associated with a significantly elevated risk for OSCC. The C-G-4b haplotype, which exhibited the highest risk for oral cancer development, has been previously correlated with an increased risk for colorectal cancer in the Korean population [25]. Since eNOS haplotypes were found to affect NO levels [34,35], it could be hypothesized that eNOS haplotypes may contribute to oral cancer development by altering NO production. The present study demonstrated that smoking alone led to a 2.5-fold increase in the risk of oral cancer development; however, combined effects of eNOS polymorphisms and tobacco on OSCC risk were not present. Since the relationship between oral cancer and environmental factors, including tobacco and alcohol use, is well-established, the combined contribution of eNOS genetic variants and lifestyle choices on OSCC development should be further investigated. An increasing body of evidence suggests the importance of different polymorphisms as risk-factor modifiers for head and neck tumor development [36-38]. Consequently, assessing genotypes and haplotypes will become a necessity in the field of personalized diagnosis and therapy of neoplastic diseases.

In conclusion, the results of the present study suggest that the presence of eNOS polymorphisms—separately and, especially, concomitantly—as well as eNOS haplotypes represent possible risk factors for OSCC in the Serbian population. However, additional studies are necessary to validate these findings.

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Conflict of interest

None of the authors declare any conflict of interest exist.

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