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Endothelial nitric oxide synthase gene polymorphisms associated with periodontal diseases in Turkish adults

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Endothelial nitric oxide synthase (NOS3) is involved in key steps of immune response. Genetic factors predispose individuals to periodontal disease. This study's aim was to explore the association between NOS3 gene polymorphisms and clinical parameters in patients with periodontal disease. Genomic DNA was obtained from the peripheral blood of 23 subjects with aggressive periodontitis (AgP), 26 with chronic periodontitis (CP), 31 with gingivitis (G) and 50 healthy controls. Probing depth (PD), clinical attachment loss (CAL), plaque index (PI) and gingival index (GI) were recorded as clinical parameters. We genotyped NOS3 polymorphisms using the PCR and/or PCR-RFLP method. Genotype frequencies differed significantly among periodontal diseases and controls for these polymorphisms. A significant association was detected between NOS3 +894 polymorphism and PD and CAL in the CP and AgP patient groups; whereas NOS VNTR analysis detected no associations with clinical parameters in the CP and AgP groups. However, a significant association was detected between the AA genotype and both PI and GI in patients with gingivitis; and a significant association was shown between the BB genotype and PI. The present study shows that two common polymorphisms of the NOS3 gene cluster are significantly associated with the occurrence of periodontal diseases.

Key words: Periodontal diseases, nitric oxide synthases gene, DNA, PCR.

INTRODUCTION

Periodontal disease is one of most common chronic inflammatory disorders that has a primarily bacterial etiology and affects teeth and supporting tissue, including bone (Brown and Løe, 1993). Previous clinical studies have proven the genetic origin of periodontitis (Michalowicz et al., 2000; Loss et al., 2005).

Nitric oxide (NO) is an important mediator of immunity and inflammation; it is the most powerful endogenous vasodilator known and it also regulates cells' adhesion to the endothelium and inhibits platelets' aggregation and vascular smooth muscle cells' proliferation (Andrew and Mayer, 1999; Freedman et al., 1997). It is implicated in various diseases' pathogenesis because of its dual role

as a modulator of immunity and a key mediator in host-mediated destruction (Moilanen and Vapaatalo, 1995). Nitric oxide is a free radical synthesized from L-arginine by a member of the family of nitric oxide synthases (NOS) enzymes. Endothelial NOS (NOS3) is one of the three isoforms of NOS (Hattori et al., 1994). The NOS3 gene is mapped to the human chromosome 7q35-36 and encodes for NOS3, an enzyme that catalyzes the production of the free radical NO by converting L-arginine to L-citrulline within endothelial cells and platelets (Walch et al., 2008). The exon 7 Glu298Asp missense variant and the intron 4 27-base pair (bp) variable number of tandem repeat (VNTR) polymorphism of NOS3 are postulated to be associated with altered NOS3 function, leading to impaired NO synthesis (Walch et al., 2008). These polymorphisms are reportedly associated with complications of preeclampsia (Serrano et al., 2004), Behcet's disease (Karasneh et al., 2005), diabetes (Monti

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Table 1. NOS3 +894 and intron 4 VNTR primer sequences and PCR conditions.

NOS3 regions	Primer sequence	Annealing
+894	CATGAGGCTCAGCCCCAGAAC AGTCAATCCCTTTGGTGCTCAC	58 °C and 30 cycles
Intron 4 VNTR	AGGCCCTATGGTAGTGCCTTT TCTCTTAGTGCTGTGGTCAC	60 °C and 30 cycles

et al., 2003) and several cardiovascular diseases (Casas et al., 2004). Considering the proposed mechanisms of linkages between periodontal disease and systemic diseases, NOS3 polymorphism might provide evidence of the association between periodontal and systemic diseases (Berdeli et al., 2006).

The present study's purpose was to evaluate: 1) the genotype distribution of NOS3 polymorphisms and 2) its relation to different forms of periodontal disease severity in Turkish patients with AgP, CP and gingivitis.

MATERIALS AND METHODS

Study population

This study was performed at the Department of Periodontology, Gaziantep University Faculty of Dentistry. The Gaziantep University School of Medicine's Ethical Committee approved the study. The study group included 23 subjects with AgP, 26 subjects with CP, 31 subjects with gingivitis and 50 healthy controls. All of the healthy controls were systemically healthy and non-smokers. The patients and controls were informed of the study's purpose and method and they all agreed to participate. The exclusion criteria were pregnancy, lactation period and intake of antibiotics or anti-inflammatory drugs in the previous 6 months, systemic diseases with influence on periodontitis. A detailed medical, oral and family medical history was taken, followed by a complete periodontal examination. For periodontitis patients, measurements of probing depth (PD) and clinical attachment loss (CAL) were made at six sites (mesiobuccal, buccal, distobuccal, mesiolingual/palatal, palatal/lingual and distolingual/palatal) for each tooth using a manual periodontal probe (Carl Martin, Solingen, Germany). Gingival index (GI) (Löe and Silness, 1963) and plaque index (PI) (Silness and Löe, 1964) were recorded at four sites (mesiobuccal, buccal, distobuccal and palatal/lingual). The patients were diagnosed according to the criteria of the IWC 1999 International Workshop for Classification of Periodontal Diseases and Conditions (Armitage, 1999) and were assigned as having aggressive periodontitis (AgP), chronic periodontitis (CP), or gingivitis (G). The patients and controls were from the same geographic areas.

DNA isolation

Genomic DNA was extracted from mononuclear cells obtained from EDTA-treated peripheral venous blood using the salting-out method (Miller et al., 1988).

NOS3 894 G > T genotyping

A polymerase chain reaction (PCR) was used to amplify a 206 bp fragment (Table 1). The resulting fragment was digested with MboI restriction endonuclease (Invitrogen, Carlsbad, CA, USA) overnight

at 37 °C. Digestion was resolved on 3% agarose gel and visualized using ultraviolet light. The 206 bp PCR products had a consistent restriction site resulting in a 119 and 87 bp fragment. Twenty percent of the samples were duplicated as an internal quality control to avoid sampling or reading errors (Hingorani et al., 1999).

NOS3 intron 4 VNTR genotyping

Primers were designed to amplify a 393 and/or 420 bp segment of the NOS3 intron 4 VNTR region containing the microsatellite repeat sequence (Table 1) (Walch et al., 2008). The products were then separated on 4% NuSieve GTG agarose. The experimental process was repeated twice for each sample.

Statistical analysis

Data were analyzed using the computer software SPSS for Windows (version 13.0; SPSS, Inc., Chicago, IL, USA). The statistical significance of the differences between the patient and control groups was estimated by logistic regression analysis. Adjusted odds ratios (ORs) were calculated with a logistic regression model that controlled for gender and age and were reported to be at 95% confidence intervals (CI). Differences in NOS3 allele frequencies between the control group and patients were compared with a chi-square test and when needed, Fisher's exact test was used. The Hardy-Weinberg equation was used to calculate estimated genotype frequency and experienced genotype frequency. For statistical comparisons between groups, a Mann-Whitney U-test was used. A p value less than 0.05 was considered statistically significant.

RESULTS

The study group included 23 subjects with AgP, 26 subjects with CP, 31 subjects with G and 50 healthy volunteers. The patients and controls were from the same geographical areas. Demographic and clinical data of patients with AgP, CP, G and control groups are given in Table 2.

Table 3 shows the frequencies of the NOS3 gene polymorphisms in patients in the AgP, CP, G and C groups. Tables 4, 5 and 6 show comparisons between polymorphisms of the NOS3 gene and clinical parameters in the AgP, CP, G and control groups.

DISCUSSION

Periodontal diseases affect millions of people around the world. Current knowledge of these diseases' pathogenesis

Table 2 Clinical feature with periodontal diseases and controls.

Clinical feature		CP	AgP	G	Healthy control
		n ^a	n ^b	n ^c	n ^d
Age*		47 (36-68)	27 (18 - 38)	35 (14 - 58)	41 (20 - 65)
Gender ^{&}	Female/ Male	16/10	15/8	14/17	28/22
PD	mm	3.9 (± 0.54)	5.3 (± 0.73)	3.1 (± 0.23)	1.85 (± 0.27)
CAL	mm	4.5 (± 0.93)	5.6 (± 0.86)	2.8 (± 0.75)	1.90 (± 0.20)
PI		2.2 (± 0.20)	1.9 (± 0.26)	2.1 (± 0.23)	0.27 (± 0.25)
GI		2.0 (± 0.16)	2.0 (± 0.25)	2.0 (± 0.23)	0.49 (± 0.36)

n^a = 26, n^b = 23, n^c = 31, n^d = 50, ± sdt. deviation, *median, [&]years.

CP; chronic periodontitis, AgP; aggressive periodontitis, G; gingivitis, PD; probing depth, CAL; clinical attachment loss, PI; plaque index, GI; gingival index.

Table 3. Comparison of frequencies of NOS (+894) and NOS VNTR gene polymorphisms between patients with periodontal diseases and healthy controls.

	Genotype	CP	AgP	G	Healthy control	HWE	OR	95% CI	p
		n ^a (%)	n ^b (%)	n ^c (%)	n ^d (%)				
NOS VNTR	AA	16 (61.5)	15 (65.2)	17 (54.8)	36 (72)	0.001 ^a 0.263 ^b	1.794 ^{ad*} 0.807 ^{bd*} 1.529 ^{cd*}	0.630 - 5.109 ^{ad*} 0.225 - 2.902 ^{bd*} 0.562 - 4.161 ^{cd*}	0.274 ^{ad*} 0.743 ^{bd*} 0.405 ^{cd*}
	AB	4 (15.4)	6 (26.1)	11 (35.5)	13 (26)	0.545 ^c 0.889 ^d	0.605 ^{ad*} 0.649 ^{bd*} 1.210 ^{cd*}	0.167 - 2.185 ^{ad*} 0.168 - 2.500 ^{bd*} 0.431 - 3.397 ^{cd*}	0.443 ^{ad*} 0.529 ^{bd*} 0.717 ^{cd*}
	BB	6 (23.1)	2 (8.7)	3 (9.7)	1 (2)		14.897 ^{ad*} 1.905 ^{bd*} 3.317 ^{cd*}	1.620 - 136.947 ^{ad*} 0.102 - 35.502 ^{bd*} 0.298 - 36.891 ^{cd*}	0.017 ^{ad*} 0.666 ^{bd*} 0.329 ^{cd*}
NOS (-894)	GG	19 (73.1)	11 (47.8)	18 (58.1)	33 (66)	0.000 ^a 0.598 ^b	0.801 ^{ad*} 1.519 ^{bd*} 1.099 ^{cd*}	0.272 - 2.365 ^{ad*} 0.449 - 5.135 ^{bd*} 0.409 - 2.952 ^{cd*}	0.688 ^{ad*} 0.501 ^{bd*} 0.852 ^{cd*}
	GT	3 (11.5)	9 (39.1)	8 (25.8)	17 (34)	0.037 ^c 0.147 ^d	0.287 ^{ad*} 0.966 ^{bd*} 0.457 ^{cd*}	0.073 - 1.122 ^{ad*} 0.283 - 3.294 ^{bd*} 0.154 - 1.355 ^{cd*}	0.073 ^{ad*} 0.956 ^{bd*} 0.158 ^{cd*}
	TT	4 (15.4)	3 (13.1)	5 (16.1)	0 (0)		3.273 ^{ad&} 0.870 ^{bd&} 0.839 ^{cd&}	2.310 - 4.636 ^{ad&} 0.742 - 1.019 ^{bd&} 0.719 - 0.979 ^{cd&}	0.012 ^{ad&} 0.028 ^{bd&} 0.007 ^{cd&}

n^a = 26, n^b = 23, n^c = 31, n^d = 50, *:OR (95%CI) was adjusted by age and sex, [&]Fisher's Exact Test. HWE: Hardy-Weinberg Equilibrium. [&], median test; *, OR (95% CI) was adjusted by age and sex; CP; chronic periodontitis, AgP; aggressive periodontitis, G; gingivitis, ^{ad}, comparison of genotypes frequencies between CP and healthy control groups; ^{bd}, comparison of genotypes frequencies between AgP and healthy control groups; ^{cd} comparison of genotypes frequencies between Gingivitis and healthy control groups.

suggests that they are multifactorial diseases resulting from the interaction between hosts and microbial factors (Reichert et al., 2008). Recent studies have improved our knowledge of an association between certain cytokine gene polymorphisms and periodontal disease (D'Aiuto et al., 2007; Feng et al., 2006). Individual factors that have a genetically determined hyper inflammatory response might increase the risk of severe periodontal disease (Kornman and di Giovine, 1998). NOS3 gene polymor-

phisms have been associated with preeclampsia (Serrano et al., 2004). Behcet's disease (Karasneh et al., 2005), cardiovascular diseases (Casas et al., 2004), cancer (Lee et al., 2009) and diabetes (Monti et al., 2003), possibly, are linked with periodontal disease. Available data, however, provide evidence that the development of periodontal diseases is complex, is regulated by multiple genetic pathways and is modified by several exogenous factors. Berdeli et al. evaluated the genotype

Table 4. Association between polymorphisms of the NOS gene and clinical parameters in chronic periodontitis.

NOS VNTR	n	PD*	p ^{&}	CAL*	p ^{&}	PI*	p ^{&}	GI*	p ^{&}
AA ^a	16	3.81 (± 0.53)	0.294 ^{ac}	4.38 (± 0.69)	0.494 ^{ac}	2.11 (± 0.18)	0.590 ^{ac}	1.95 (± 0.17)	0.115 ^{ac}
AB ^b	4	4.07 (± 0.76)	0.617 ^{ab}	4.77 (± 1.55)	0.963 ^{ab}	2.1 (± 0.21)	0.892 ^{ab}	1.97 (± 0.17)	0.892 ^{ab}
BB ^c	6	4.03 (± 0.44)	0.762 ^{bc}	4.68 (± 1.14)	0.352 ^{bc}	2.15 (± 0.25)	0.762 ^{bc}	2.08 (± 0.09)	0.257 ^{bc}
NOS (+894)									
GG ^d	19	3.76 (± 0.39)	0.001 ^{de}	4.27 (± 0.53)	0.001 ^{de}	2.11 (± 0.19)	0.523 ^{de}	1.96 (± 0.17)	0.787 ^{de}
GT ^e	3	5.03 (± 0.15)	0.725 ^{df}	6.63 (± 0.72)	0.409 ^{df}	2.0 (± 0.3)	0.366 ^{df}	2.0 (± 0.1)	0.162 ^{df}
TT ^f	4	3.72 (± 0.82)	0.057 ^{ef}	4.07 (± 0.22)	0.057 ^{ef}	2.22 (± 0.15)	0.229 ^{ef}	2.1 (± 0.11)	0.400 ^{ef}

n = 26, * Mean mm, p[&]: Mann-Whitney U test, ± sdt. deviation, * PD; probing depth, CAL; clinical attachment loss, PI; plaque index, GI; gingival index, ^{ab}: Comparison of AA genotype and AB genotype, ^{ac}: Comparison of AA genotype and BB genotype, ^{bc}: Comparison of AB genotype and BB genotype, ^{de}: Comparison of GG genotype and GT genotype, ^{df}: Comparison of GG genotype and TT genotype, ^{ef}: Comparison of GT genotype and TT genotype.

Table 5. Association between polymorphisms of the NOS gene and clinical parameters in aggressive periodontitis.

NOS VNTR	n	PD*	p ^{&}	CAL*	p ^{&}	PI*	p ^{&}	GI*	p ^{&}
AA ^a	15	5.31 (± 0.86)	0.941 ^{ac}	5.77 (± 0.99)	0.368 ^{ac}	1.96 (± 0.27)	0.441 ^{ac}	2.04 (± 0.22)	0.088 ^{ac}
AB ^b	6	5.18 (± 0.42)	1.000 ^{ab}	5.20 (± 0.26)	0.178 ^{ab}	1.85 (± 0.29)	0.470 ^{ab}	1.88 (± 0.32)	0.569 ^{ab}
BB ^c	2	5.40 (± 0.71)	0.857 ^{bc}	5.05 (± 0.35)	0.643 ^{bc}	1.80 (± 0.14)	0.857 ^{bc}	1.85 (± 0.07)	1.000 ^{bc}
NOS (+894)									
GG ^d	10	4.91 (± 0.55)	0.105 ^{de}	5.08 (± 0.43)	0.052 ^{de}	1.85 (± 0.28)	0.481 ^{de}	1.87 (± 0.29)	0.190 ^{de}
GT ^e	10	5.27 (± 0.48)	0.007 ^{df}	5.58 (± 0.62)	0.007 ^{df}	1.9 (± 0.21)	0.371 ^{df}	2.02 (± 0.13)	0.112 ^{df}
TT ^f	3	6.60 (± 0.53)	0.014 ^{ef}	7.10 (± 0.90)	0.014 ^{ef}	2.0 (± 0.40)	0.811 ^{ef}	2.23 (± 0.21)	0.112 ^{ef}

n = 23, * Mean mm, p[&]: Mann-Whitney U test, ± sdt. deviation, * PD; probing depth, CAL; clinical attachment loss, PI; plaque index, GI; gingival index, ^{ab}: Comparison of AA genotype and AB genotype, ^{ac}: Comparison of AA genotype and BB genotype, ^{bc}: Comparison of AB genotype and BB genotype, ^{de}: Comparison of GG genotype and GT genotype, ^{df}: Comparison of GG genotype and TT genotype, ^{ef}: Comparison of GT genotype and TT genotype.

and allele distribution of the Glu298Asp polymorphism of the NOS3 gene and its relation to different forms of periodontal diseases' severity in Turkish patients with AgP and CP (Berdeli et al., 2006). Their study shows that NOS3 Glu298Asp polymorphism is associated with bleeding after probing in AgP patients and that the NOS3 gene's 298Asp allele might be related to CP (Berdeli et al., 2006). In the present study, we investigated to establish for the first time, to the best of our knowledge, an association between two well-established polymorphisms of the NOS3 gene cluster and clinical parameters in different forms of periodontal disease.

When the results of the G and control groups with respect to the NOS3 +894 polymorphism; were compared in terms of HWE, a deviation from HWE appeared in the G patient group; whereas no deviations were observed in the control groups (Table 3). An increase in the TT genotype with a decrease in the GG genotype in the patient group revealed that it might be related to susceptibility to G. In conclusion, patients with the T allele had an 8.7 times greater risk of gingivitis than patients without this allele (Table 3). No deviations from HWE in NOS3 VNTR polymorphism were observed in either the patient or the control group and no associations were detected in

terms of allele or genotype frequency (Table 3).

When the NOS3+894 polymorphism in the CP and control groups were compared in terms of HWE, a deviation from HWE was present in the CP group, but no deviations from HWE were present in the control group (Table 3). Although no significant associations were detected between the CP and control groups in terms of allele frequency, the results show that the TT genotype significantly increased in the CP group and that it might be associated with susceptibility to CP (Table 3). When the NOS3 VNTR polymorphism in the CP and control groups was compared in terms of HWE, a deviation from HWE was noted in the CP group, but no deviations were present in the control group (Table 3). A significant association was present in terms of allele and genotype frequencies. The B allele was associated with susceptibility to periodontal disease and patients with that allele had about 8 times greater risk for CP than patients without it (Table 3).

No significant associations were detected in terms of HWE in the analysis of the NOS3+894 polymorphism in either the AgP or the control group; whereas a significant association was present in terms of allele and genotype frequencies (Table 3). The TT genotype or the T allele

Table 6. Association between polymorphisms of the NOS gene and clinical parameters in gingivitis.

NOS VNTR	n	PD*	p ^{&}	CAL*	p ^{&}	PI*	p ^{&}	GI*	p ^{&}
AA ^a	17	3.12 (± 0.23)	0.689 ^{ac}	2.80 (± 0.88)	0.305 ^{ac}	2.04 (± 0.19)	0.002 ^{ac}	1.95 (± 0.21)	0.028 ^{ac}
AB ^b	11	3.03 (± 0.22)	0.487 ^{ab}	2.83 (± 0.32)	0.122 ^{ab}	2.14 (± 0.23)	0.191 ^{ab}	2.03 (± 0.22)	0.611 ^{ab}
BB ^c	3	3.07 (± 0.25)	0.885 ^{bc}	2.33 (± 1.15)	1.000 ^{bc}	2.43 (± 0.57)	0.022 ^{bc}	2.30 (± 0.20)	0.088 ^{bc}
NOS (+894)									
GG ^d	18	3.08 (± 0.23)	0.935 ^{de}	2.77 (± 0.76)	0.683 ^{de}	2.14 (± 0.24)	0.724 ^{de}	2.03 (± 0.24)	0.849 ^{de}
GT ^e	8	3.10 (± 0.24)	0.638 ^{df}	2.79 (± 0.75)	0.801 ^{df}	2.10 (± 0.25)	0.325 ^{df}	2.02 (± 0.26)	0.403 ^{df}
TT ^f	5	3.04 (± 0.21)	0.724 ^{ef}	2.70 (± 0.87)	0.943 ^{ef}	2.04 (± 0.19)	0.622 ^{ef}	1.92 (± 0.16)	0.354 ^{ef}

n = 31, * Mean mm, p[&]: Mann-Whitney U test, ± sdt. deviation, *PD; probing depth, CAL; clinical attachment loss, PI; plaque index, GI; gingival index, ^{ab}: Comparison of AA genotype and AB genotype, ^{ac}: Comparison of AA genotype and BB genotype, ^{bc}: Comparison of AB genotype and BB genotype, ^{de}: Comparison of GG genotype and GT genotype, ^{df}: Comparison of GG genotype and TT genotype, ^{ef}: Comparison of GT genotype and TT genotype.

played a role in susceptibility to AgP and patients with the T allele had a 13.5 times greater risk for AgP than patients without it (Table 3). No deviations from HWE were observed in the NOS3 VNTR polymorphism in either the patient or control group, or no significant associations were detected in terms of allele or genotype frequency (Table 3).

The analysis of NOS3-894 found a significant association between PD, CAL and the GG and TT genotype in the CP group; and an association was demonstrated between the GT and TT genotypes and PD and CAL in the AgP group (Tables 4 and 5). No associations between genotypes and these clinical parameters were detected in the gingivitis group (Table 6). The analysis of NOS VNTR detected no associations with clinical parameters in the CP or AgP groups (Tables 4 and 5). However, a significant association was detected between the AA genotype and both PI and GI in the gingivitis group; whereas the findings show a significant association between the BB genotype and PI (Table 6).

The alterations in the NOS3 protein's functional aspects might lead to exaggerated inflammation and periodontal tissue destruction because NOS3-mediated NO production possibly protects tissues and possesses anti-inflammatory features, particularly by conferring anti-adhesive properties to the endothelium (Berdeli et al., 2006). Therefore, the increased inflammation indicated by the significantly elevated percentage of GI and PI in VNTR subjects in the gingivitis group might be a result of insufficient vasoprotective NO production. Nevertheless, an association was detected between PD and CAL in the CP and AgP groups in NOS3+894, but no associations were detected in the gingivitis group.

Within this study's limitations, NOS3 gene polymorphisms demonstrated a significant association with AgP, CP and gingivitis. The different associations of AgP, CP and gingivitis with these gene polymorphisms might help to explain these different periodontal diseases' differing aetiopathogeneses. However, these data can explain only a small portion of the genetic susceptibility to periodontal disease. Furthermore, NOS3's possible role in

different forms of periodontal diseases, including any genetic variation, therefore might be worthy of additional investigation.

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