Original Article

Nos3 Gene Rs1799983 and Rs2070744 Polymorphisms in Patients with Periodontal Disease

(gene polymorphism / *NOS3* gene / periodontal disease)

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Abstract. Periodontal disease is a common oral disease. Inflammatory and immune responses to oral microorganisms initiate the development of periodontitis. Cigarette smoking is an important environmental risk factor for periodontitis. Another important inflammatory mediator is nitric oxide (NO). NO modulates vascular tone, microvascular permeability, leukocyte migration and oxidative activity, contributing to the direct killing of microorganisms. Several polymorphisms of the *NOS3* gene have been detected, which may alter gene expression and NO synthesis. The aim of this study was to examine the association between the *NOS3* rs1799983 and rs2070744 polymorphisms and periodontal disease. This study enrolled 200 patients with periodontal diseases (130 were non-smokers and 70 were smokers) and 160 control subjects (126 were non-smokers and 34 were smokers). Among the patients with periodontal disease, we observed a statistically increased frequency of patients with the CT genotype (TC vs. TT; 95%CI 1.83, OR 1.16–2.88, P = 0.011). There was a statistically significant increased frequency of CT genotype carriers among non-smoking patients with periodontal disease as compared with non-smoking controls, whereas there were no statistically significant differences between smoking patients with periodontal disease and smoking control subjects. The results of our study suggest an association between the *NOS3* rs2070744 polymorphism and periodontal disease.

Introduction

Periodontal disease is a common oral disease characterized by degradation of the periodontal attachment and alveolar bone (Otenio et al., 2012; Shin et al., 2015; Özdemir et al., 2016). Inflammatory and immune responses to oral microorganisms initiate the development of periodontitis. These responses protect gingival tissues against local microbial invasion, but also induce an inflammatory cascade (Graves and Cochran, 2003; Jung et al., 2013). The pathogens induce production of cytokines, such as interleukin (IL)-6, IL-1β and tumour necrosis factor α (TNF-α) in gingival tissue. These cytokines enhance inflammatory response and progression of the disease (Eley and Cox, 2003; Preshaw and Taylor, 2011; Nishikawa et al., 2012; Nebel et al., 2013).

Cigarette smoking is an important environmental risk factor for periodontal disease development (Johnson et al., 2007). Nicotine is the main component of cigarette smoke and is one of its most pharmacologically active agents (Shin et al., 2015). Another important inflammatory mediator is nitric oxide (NO) (Wang et al., 2002). It plays an essential role in many important physiological and pathophysiological processes such as modulation of microvascular permeability and vascular tone, and leukocyte migration. NO has potent antimicrobal activity and protects against bacterial infections (Lefer, 1997; Nebel et al., 2013). It can act as a cytotoxic molecule against the microorganisms and may have both beneficial and harmful effects leading to tissue damage (Jung et al., 2013). NO is a short-lived molecule having regulatory functions in inflammatory processes (Lefer, 1997). Furthermore, NO exerts significant antibacterial action in saliva (Hussain et al., 2016). It is synthesized from arginine by a family of isoenzymes called nitric

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Abbreviations: API – approximal plaque index, CAL – clinical attachment loss, eNos – endothelial NO (NOS2), HWE – Hardy-Weinberg equilibrium, iNOS – inducible NO (NOS3), nNOS – neuronal NOS (NOS1), NO – nitric oxide, NOS – nitric oxide synthases, PD – pocket depth, SBI – sulcus bleeding index, TNF-α – tumour necrosis factor α.

oxide synthases (NOS). Three isoforms of NOS have been detected in the tissues: neuronal NOS (nNOS), (NOS1); endothelial NOS (eNOS) (NOS2); and inducible NOS (iNOS) (NOS3). These isoforms are encoded by three different genes. The *NOS3* gene is located on chromosome 7q35–36 and contains 26 exons (Dosenko et al., 2006). Several polymorphisms have been detected in the *NOS3* gene that may alter gene expression and NO synthesis. Polymorphisms rs1799983 (894G>T, Glu298Asp) in exon 7 and rs2070744 (-786T>C) in the promoter region may influence mRNA transcription and gene expression, which may further lead to altered production of NO (Wang et al., 2002). These polymorphisms were considered to be a risk factor for various vascular and immune diseases. The aim of this study was to examine the association between the *NOS3* rs1799983 and rs2070744 polymorphisms and periodontal disease.

Material and Methods

Study subjects

This study enrolled 360 Caucasian subjects (with an age range of 25–69 years) from the West Pomeranian region of Poland. The subjects were submitted to anamnesis and to clinical and periodontal examination. The subjects were divided into two subgroups: patients with periodontal disease and healthy subjects without periodontal disease. The first group comprised 200 patients (84 men, 116 women), aged 26–69 years (mean 49.85 ± 8.71), with chronic periodontal disease, diagnosed using the periodontal disease classification system of the American Academy of Periodontology (2015). Patients diagnosed with chronic generalized moderate-advanced periodontitis had a periodontal involvement of at least 30 % and a clinical attachment loss of ≥ 3 mm (moderate 3–4 mm, advanced ≥ 5 mm). Out of this group of 200 patients, 130 were non-smokers and 70 were smokers. Those with no evidence of clinical features of periodontal disease (a periodontally healthy individual was defined by clinical attachment loss < 1 mm) were categorized as healthy and were considered the control group (160 subjects, 55 men, 105 women, aged 25–69 years, mean 45.28 ± 10.15). In this healthy group, 126 subjects were non-smokers and 34 were smokers.

Patients were enrolled by a clinical researcher based on the following inclusion criteria: four or more periodontal pockets with a probing depth ≥ 5 mm and bleeding on probing. Clinical parameters were recorded at six sites per tooth by a single calibrated examiner who was blinded to the type of treatment received by the patient.

Exclusion criteria included systemic disease, patients who used systemic or sub-gingival antimicrobial agents or made chronic use of anti-inflammatory medication. Subjects were also excluded from the study if they had a history of hepatitis, AIDS or HIV, recent radiation therapy, diabetes, uncontrolled hypertension, use of immunosuppressive medications, or were pregnant.

Additionally, the subjects were categorized into four subgroups: smoking and non-smoking with periodontal disease and smoking and non-smoking control. Patients who had smoked tobacco for at least five years without interruption and smoked 10 cigarettes per day or more were allocated to the smoking group with or without periodontitis. Patients who had never smoked were placed in the non-smoking group with or without periodontitis. All patients were otherwise healthy and were not subjected to periodontal treatment or antibiotics for at least six months before the study.

The study was approved by the ethics committee in Pomeranian Medical University, Szczecin, Poland, and written informed consent was obtained from all subjects. The study protocol was approved by the Committee of Ethical Affairs of the Pomeranian Medical University (BN-001/93/08).

Periodontal examination

Periodontal evaluation included probing pocket depth (PD), clinical attachment loss (CAL), the approximal plaque index (API) and modified sulcus bleeding index (percent SBI).

Clinical measurements were taken in homogeneous conditions in a dental clinic. PD and CAL were assessed at six sites per tooth (disto-, mesio-buccal and midbuccal and disto-, mesio-lingual/palatal, mid-lingual/midpalatal), using a periodontal probe calibrated with 1 mm. PD represents the distance from the gingival margin to the bottom of the periodontal pocket and CAL represents the distance from the cemento-enamel junction to the bottom of the periodontal pocket. A UNC-15 Color Coded Probe (Hu-Friedy Mfg Co Inc, Chicago, IL) (graduated 1-2-3-4-5-6-7-8-9-10-11-12-13-14-15) was used for all explorations. Pressure of approximately 20 g was applied for probing.

Genotyping

All samples were genotyped in duplicate using allelic discrimination assays with TaqMan® probes (Applied Biosystems, Carlsbad, CA) in a 7500Fast Real-Time PCR Detection System (Applied Biosystems). To discriminate *NOS3* rs1799983 and rs2070744 gene polymorphisms, TaqMan® Pre-Designed SNP Genotyping Assays were used with the assay IDs of C__15903863_10 and C___3219460_20, respectively, including appropriate primers and fluorescently labelled (FAM and VIC) MGB™ probes to detect the alleles.

Statistical analysis

The consistency of the genotype distribution with Hardy-Weinberg equilibrium (HWE) was assessed using the exact test. The χ^2 test and Fisher's exact test were used to compare genotype and allele distributions between groups. A multivariate logistic regression model was used to find independent predictors of periodontal disease risk. A P value of < 0.05 was considered to indicate a statistically significant result.

Results

The clinical periodontal parameters in the studied groups are shown in Table 1.

The distribution of *NOS3* genotypes among patients with periodontal disease and control subjects was in HWE and is shown in Table 2. Among patients with periodontal disease, we observed a statistically increased frequency of patients with the CT genotype (TC vs. TT; 95% CI = 1.83, OR 1.16–2.88, P = 0.011).

Additionally, we compared the distribution of the studied polymorphisms between smoking patients with periodontal disease and smoking control subjects, and between non-smoking patients with periodontal disease and non-smoking control subjects. As shown in Tables 2 and 3, there was a statistically significant increased frequency of CT genotype carriers among non-smoking patients with periodontal disease as compared with non-smoking controls, whereas there were no statistically significant differences between smoking patients with periodontal disease and smoking control subjects (Tables 3, 4).

In the multivariate logistic regression analysis, taking into account the patients' sex, age, smoking, and *NOS3*

Table 1. Clinical periodontal parameters of the studied subjects

Parameter	Controls (N = 160)	Periodontitis patients (N = 200)
SEX (M/F)	55/105	84/116
AGE (mean years ± SD)	45.28 ± 10.15	49.85 ± 8.71
API % (mean ± SD)	35.81 ± 20.66	72.98 ± 21.03
SBI % (mean ± SD)	6.53 ± 11.29	57.66 ± 25.45
PD mm (mean ± SD)	1.63 ± 0.54	4.36 ± 2.32
CAL mm (mean ± SD)	0.41 ± 1.18	5.04 ± 2.41

Table 2. Distribution of NOS3 rs1799983 and rs2070744 genotypes in periodontal disease patients and control group

	Periodontitis patients		Control group					
	N	%	N	%	P[a]		P[b]	OR (95 % CI)
***NOS3* rs1799983 Genotype**								
GG	99	49.50	85	53.13		TT+GT vs GG	0.53	1.16 (0.76–1.75)
GT	86	43.00	65	40.62	0.76	TT vs GT + GG	0.68	1.22 (0.53–2.79)
TT	15	7.50	10	6.25		TT vs GG	0.67	1.29 (0.55–3.02)
						GT vs GG	0.58	1.14 (0.74–1.75)
						TT vs GT	0.83	1.13 (0.48–2.69)
***NOS3* rs1799983 Allele**								
G	284	71.00	235	73.44				
T	116	29.00	85	26.56		T vs G	0.50	1.13 (0.81–1.57)
***NOS3* rs2070744 Genotype**								
TT	69	34.84	71	44.66		CC+TC vs TT	0.06	1.51 (0.98–2.31)
TC	110	55.56	62	38.99	0.006*	CC vs TC + TT	0.08	0.54 (0.29–1.02)
CC	19	9.60	26	16.35		CC vs TT	0.49	0.75 (0.38–1.48)
						TC vs TT	0.011*	1.83 (1.16–2.88)
						CC vs TC	0.010*	0.41 (0.21–0.80)
***NOS3* rs2070744 Allele**								
T	248	62.63	204	64.15				
C	148	37.37	114	35.85		C vs T	0.70	1.07 (0.79-1.45)

[a] χ^2 test, [b] Fisher's exact test, *P < 0.05

NOS3 rs1799983, HWE: examined group P = 0.61, control group P = 0.69

NOS3 rs2070744, HWE: examined group P = 0.01, control group P = 0.06

Table 3. Distribution of NOS3 rs1799983 and rs2070744 genotypes in periodontal disease patients and control group in the non-smokers' group

	Periodontitis patients (non-smokers)		Control group (non-smokers)					
	N	%	N	%	P[a]		P[b]	OR (95% CI)
***NOS3* rs1799983 Genotype**								
GG	67	51.54	71	56.35		TT+GT vs GG	0.45	1.21 (0.74–1.99)
GT	50	38.46	45	35.71	0.70	TT vs GT+GG	0.66	1.29 (0.54–3.06)
TT	13	10.00	10	7.94		TT vs GG	0.51	1.38 (0.57–3.35)
						GT vs GG	0.60	1.18 (0.70–1.99)
						TT vs GT	0.82	1.17 (0.47–2.93)
***NOS3* rs1799983 Allele**								
G	184	70.77	187	74.21				
T	76	29.23	65	25.79		T vs G	0.43	1.19 (0.81–1.75)
***NOS3* rs2070744 Genotype**								
TT	41	31.78	60	48.00		CC+TC vs TT	0.01*	1.98 (1.19–3.30)
TC	76	58.92	43	34.40	0.00041*	CC vs TC+TT	0.065	0.48 (0.23–1.02)
CC	12	9.30	22	17.60		CC vs TT	0.69	0.80 (0.36–1.79)
						TC vs TT	0.0007*	2.59 (1.50–4.46)
						CC vs TC	0.005*	0.31 (0.14–0.69)
***NOS3* rs2070744 Allele**								
T	158	61.24	163	65.20				
C	100	38.76	87	34.80		C vs T	0.36	1.19 (0.83–1.70)

[a] χ^2 test, [b] Fisher's exact test, *P < 0.05
NOS3 rs2070744, HWE: examined group P = 0.0091, control group P = 0.0097

rs2070744 polymorphism, we examined the independent risk factors of periodontal disease. In this analysis, more advanced age, smoking and CT genotype (*NOS3* rs2070744) were independent significant predictors of a higher risk of periodontal disease (Table 5).

Discussion

In this study, we examined the association between the *NOS3* rs1799983, rs2070744 polymorphisms and periodontal disease. We have revealed an increased frequency of the rs2070744 CT genotype among a group of patients with periodontal disease in comparison with a control group. This difference was also detected in a subgroup of non-smoking patients compared to non-smoking controls, whereas the differences between smoking patients with periodontal disease and smoking control subjects were not statistically significant. The multivariate regression analysis confirmed that the *NOS3*rs2070744 CT genotype and smoking were significant risk factors for periodontal disease.

Previous studies suggest that the *NOS3* rs2070744 polymorphism may be associated with an increased risk of vascular diseases and diseases with immune background. So far, the *NOS3* gene polymorphisms have not been investigated in patients with periodontal diseases; however, numerous studies indicate the involvement of iNOS and NO in the pathogenesis of periodontal diseases. Elevation of the iNOS activity in periodontal tissues has been reported in inflammatory periodontal disease, which suggests the involvement of NO in the disease process (Ozer et al., 2011). NO is also a highly reactive free radical inducing peroxynitrite and nitrotyrosine synthesis responsible for the NO-related cytotoxicity (Nishikawa et al., 2012). The endothelial NOS and neuronal NOS are constitutive enzymes, which produce low NO amounts for a short period after stimulation. iNOS produces high amounts of NO for a long period, and the expression of iNOS is stimulated by bacterial products and cytokines such as IL-1, IL-6 and TNF-α (Ozer et al., 2011; Özdemir et al., 2016). This process induces inflammatory responses in various tissues. NO plays a significant role in the defence against microbial infections, but also has a destructive role in tissues leading to tissue damage and development of periodontal disease (Pan et al., 2010).

Previous studies have indicated that increased concentrations of NO can inhibit osteoblastic or osteoclastic activity of cytokines in periodontal disease (Özdemir et al., 2016). It has been shown that NO prevents alveo-

Table 4. Distribution of NOS3 rs1799983and rs2070744 genotypes in periodontal disease patients and control group in the smokers' group

	Periodontitis patients (smokers)		Control group (smokers)					
	N	%	N	%	P[a]		P[b]	OR (95% CI)
***NOS3* rs1799983 Genotype**								
GG	32	45.71	14	41.18		TT+GT vs GG	0.68	0.83 (0.36–1.91)
GT	36	51.43	20	58.82	0.52	TT vs GT+GG	1.00	–
TT	2	2.86	0	0.00		TT vs GG	1.00	–
						GT vs GG	0.67	0.79 (0.34–1.81)
						TT vs GT	1.00	–
***NOS3* rs1799983 Allele**								
G	100	71.43	48	70.59				
T	40	28.57	20	29.41		T vs G	1.00	0.96 (0.51–1.82)
***NOS3* rs2070744 Genotype**								
TT	28	40.58	11	32.35		CC+TC vs TT	0.52	0.70 (0.30–1.66)
TC	34	49.28	19	55.88	0.72	CC vs TC+TT	1.00	0.85 (0.23–3.12)
CC	7	10.14	4	11.77		CC vs TT	0.71	0.69 (0.17–2.82)
						TC vs TT	0.50	0.70 (0.29–1.72)
						CC vs TC	1.00	0.98 (0.25–3.78)
***NOS3* rs2070744 Allele**								
T	90	65.22	41	60.29				
C	48	34.78	27	39.71		C vs T	0.54	0.81(0.45–1.47)

[a] χ^2 test, [b] Fisher's exact test, *P < 0.05
NOS3 rs2070744, HWE: examined group P = 0.60, control group P = 0.48

Table 5. Multivariate logistic regression analysis of NOS3 rs2070744 in periodontal disease patients with PD patients and control group as the dependent variable

Independent variables	OR (95% CI)	P
Sex (male gender)	1.39 (0.88–2.19)	0.16
Age (years)	1.06 (1.03–1.09)	4.08E-6
Smoking	2.08 (1.25–3.44)	0.0045
NOS3 rs2070744 (CT vs CC+TT)	2.14 (1.37–3.36)	0.0009

lar bone loss in experimental periodontitis, and also modulates the inflammatory response and bone turnover in periodontal disease (Stefanovic-Racic et al., 1993; Lohinai et al., 1998). NO plays a significant role in the defence against pathogenic microorganisms, but overproduction of NO exerts cytotoxic effects on the tissues. It has been shown that gingival fibroblasts from patients with chronic periodontal disease have significantly higher levels of the iNOS protein and NOS2 mRNA than those in healthy gingival tissues (Lohinai et al., 1998). Furthermore, iNOS induces activity of cyclooxygenase and metalloproteinases, which enhance synthesis of mediators leading to the development of inflammatory process and gingival tissue destruction (Lohinai et al., 1998). Increased iNOS expression was detected in patients with periodontal disease, especially those with diabetes (Shaker et al., 2013; Lucarini et al., 2016).

The results of the studies examining the effect of smoking on iNOS expression and NO production are inconsistent. Hoyt at al. (2003) observed that cigarette smoke extract decreases iNOS expression and NO production from lung epithelial cells. Özdemir et al. (2016) have shown that chronic smoking stimulates iNOS expression in periodontal tissues and enhances the inflammatory process in the tissues. Moreover, nicotine and LPS synergistically induced NO and PGE_2 production and increased iNOS and COX-2 expression in human periodontal ligament cells (Shin et al., 2015).

Conclusion

Our results suggest that the *NOS3* rs2070744 polymorphism may be associated with an increased risk of periodontal diseases especially in non-smoking patients. As mentioned above, smoking may increase iNOS expression, and therefore may reduce the effect of the gene polymorphism on iNOS expression in smoking patients. The results of our study suggest an association between the *NOS3* rs2070744 polymorphism and periodontal disease. Nevertheless, this hypothesis requires further investigation.

Discloser of conflict of interest

The authors declare that they have no conflict of interest.

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