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The influence of *KIR* gene presence/absence polymorphisms on the development of periodontal disease in smokers and non-smokers

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Abstract

Introduction: *Periodontal diseases are highly prevalent inflammatory, multifactorial diseases. Smoking is one of the most important environmental risk factors for the development and severity of periodontal disease. Killer cell immunoglobulin-like receptors (KIRs) are members of the immunoglobulin (Ig) superfamily and play an essential role in the regulation of NK cell activity, allowing natural killer (NK) cells to sense and respond to human leukocyte antigen (HLA) class I. The aim of this study was to evaluate the influence of KIR gene presence/absence polymorphisms on the development of periodontal disease in smokers and non-smokers.*

Material and methods: *This study enrolled 400 Caucasian subjects (age range 25-69 years) from the West Pomeranian region of Poland. The subjects were categorized into four subgroups (smoking and non-smoking patients with periodontal disease; smoking and non-smoking subjects without periodontal disease – control subjects).*

Results: *The differences of KIR gene frequencies between non-smoking patients and non-smoking control subjects as well as smoking patients and control subjects were not statistically significant. In multivariate regression analysis advanced age of patients and smoking were independent factors associated with increased frequency of periodontal disease.*

Conclusions: *The results of this study suggest that the main factor associated with increased risk of periodontal disease is smoking, whereas KIR presence/absence polymorphism is not a significant factor involved in the pathogenesis of periodontal disease.*

Key words: *killer immunoglobulin-like receptors (KIRs), natural killer cells, inhibitory/activating receptors.*

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Introduction

Periodontal diseases are highly prevalent inflammatory and multifactorial diseases [1]. They can affect up to 90% of the global population [2] and are a major cause of tooth loss in humans [1, 3]. Periodontal pathogens in the subgingival biofilm are necessary to develop the illness. The destructive processes of periodontitis are influenced by the interaction between pathogenic micro-organisms and host responses [4]. Genetic and environmental factors also have an effect on the initiation and progression of periodontal disease [1, 3-5].

In a questionnaire study of 4,908 twin pairs, Corey *et al.* [6] found that concordance rates for periodontitis were 0.23-0.36 for monozygotic (MZ) twins and 9.98-0.16 for dizygotic (DZ) twins. However, in this study, environmental factors such as smoking were not controlled, thereby creating bias toward establishing a correlation between twins. In a study of 64 MZ and 53 DZ adult twin pairs, Michalowicz *et al.* [7] used maximum likelihood estimation techniques to estimate, according to path models, genetic and environmental variances and heritability in chronic perio-

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dontitis. MZ twins were found to be more similar than DZ twins for all clinical measures. Statistically significant genetic variance was found for both the severity and extent of disease. Adult periodontitis was estimated to have 50% heritability, which was unaltered following adjustments for behavioral variables, including smoking.

Smoking is one of the most important environmental risk factors for the development and severity of periodontal disease [1, 8, 9]. This is strengthened by the fact that one-third of the world's adult population are smokers [1]. Smoking reduces the clinical signs of inflammation. The suppressive effect on the vasculature (lower bleeding on probing, less gingival redness) and impairment of revascularization are due to smoking. It also influences the healing potential of the periodontal connective tissues, the inflammatory response and fibroblast function. This risk factor plays its role in multiple functions of neutrophils, and affects the levels of cytokines [8]. Smoking also has immunosuppressive effects and changes the human microflora and immune response, which leads to the destruction of periodontal tissues [1, 10].

In smokers we can observe changes in some cytokines and chemokines, and also T-cells and NK cells [11]. In smokers a decreased number and impaired function of NK cells was observed [12].

Natural killer (NK) cells are important effectors that play a central role in innate immunity. Human NK cells recognize HLA class I molecules through surface receptors (killer cell immunoglobulin-like receptors – KIRs) [13]. KIRs are members of the immunoglobulin (Ig) superfamily and play an essential role in the regulation of NK cell activity, allowing NK cells to sense and respond to human leukocyte antigen (HLA) class I downregulation, an important hallmark of viral infections and tumor transformation. KIR polymorphism affects NK cells by influencing the KIR repertoire, KIR expression, the strength of KIR-ligand interactions and the capability to deliver signals [14].

The family of *KIR* genes exhibits allelic polymorphism and haplotypic variability defined as presence or absence of a particular *KIR* gene (different genes for inhibitory and activating receptors on individual chromosomes) which generates a specific KIR receptor repertoire on the NK surface [15]. The KIR receptors exhibit two or three extracellular domains and a cytoplasmic tail that can transduce either activating or inhibitory signals [16-18]. The *KIR* nomenclature is based on the number of extracellular domains (KIR2D or KIR3D) and on the presence of a long inhibitory (L) or a short activating (S) cytoplasmic tail. In general, long-tailed receptors transduce inhibitory signals while short-tailed receptors are activating. The exception is KIR2DL4, which transduces predominantly activating signals [19].

The ligands of KIR are some of the classical HLA class I molecules [18]. The HLA-C molecules of group C1 are bound by KIR2DL2/3 while group C2 is recognized by KIR2DL1. There are studies showing that activating KIR2DS1, KIR2DS2 and KIR2DS3 recognize the same ligands as the homologous KIR2DL1/2/3, but with lower affinity. HLA-A and HLA-B molecules that exhibit the Bw4 epitope are recognized by KIR3DL1 and possibly KIR3DS1. The other three-domain KIRs have other ligands, for example KIR3DL2 binds HLA-A3, HLA-A11, HLA-F and HLA-B27 [18].

KIR haplotypes may be subdivided in two groups, A and B, with haplogroup A characterized by the presence of *KIR2DS4* as the only short-tailed activating gene. Haplogroup B consists of a large number of haplotypes differing for combinations of inhibitory and activating genes [19].

Moreover, KIR may influence NK cell function during infections, autoimmune diseases, pregnancy and allogeneic transplantation [20, 21]. As NK cells are one of the most important components of innate immunity against bacteria and viruses, they can be involved in the development of periodontal disease. Through direct interaction with periodontal pathogens, NK cells produce pro-inflammatory cytokines that subsequently may lead to tissue destruction [22, 23].

Therefore, the aim of this study was to evaluate the influence of *KIR* gene presence/absence polymorphism on the development of periodontal disease in smoking and non-smoking patients.

Material and methods

Study subjects

This study enrolled 400 Caucasian subjects (age range 25-69 years) from the West Pomeranian region of Poland. The subjects were submitted to anamnesis and to clinical and periodontal examination. The studied group of subjects was divided into two subgroups: patients with periodontal disease and control subjects without periodontal disease. The first group comprised 250 patients (104 men, 146 women), aged from 26 to 69 years (mean 50.47 ±9.09), with chronic periodontal disease, diagnosed using the periodontal disease classification system of the American Academy of Periodontology [24]. Out of this group of 250 patients, 173 were non-smokers and 77 were smokers. Those with no evidence of clinical features of periodontal disease were considered as the control group (150 subjects, 56 men, 94 women, aged from 25 to 69 years (mean 42.97 ±11.22). In this control group, 117 subjects were non-smokers and 33 were smokers. Exclusion criteria included systemic disease, patients who used systemic or sub-gingival antimicrobial agents, and those who 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, antibiotic use within the past 6 months, or were pregnant. All patients were otherwise healthy and had not been subjected to periodontal treatment or antibiotics for at least 6 months before the study.

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 5 years without interruption, 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. The study was approved by the ethics committee of the 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

Clinical measurements were taken in homogeneous conditions in a dental clinic.

Pocket depth (PD) and clinical attachment level (CAL) were assessed at four sites per tooth (disto-, mesio-buccal and midbuccal and mid-lingual/mid-palatal), 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.

DNA isolation and *KIR* genotyping

Genomic DNA was extracted from peripheral blood leukocytes using an Invisorb Spin Blood Midi Kit following the manufacturer's instructions (Invitek, Germany).

Typing of *KIR* genes was performed using the method of polymerase chain reaction with sequence specific primers (PCR-SSP). Briefly, we used KIR-specific primers to amplify short fragments with sequences defined by Vilches *et al.* [25] in modified reaction conditions as described previously [26].

Statistical analysis

Fisher's exact test was used to compare genotype distributions between groups. A multivariate logistic regression model was used to find independent predictors of periodontal disease risk. The Mann-Whitney test was used to compare the number of activating and inhibiting *KIR* genes between groups. All analyses were applied using Statistica package v. 9.0 (StatSoft, OA, U.S.A.), and p values < 0.05 were considered significant.

Results

In this study we compared the frequencies of *KIR* genes in smoking patients with periodontitis vs smoking control subjects as well as non-smoking patients with periodontitis vs non-smoking control subjects (Tables 1 and 2).

As shown in Table 1, there were not statistically significant differences in frequency of *KIR* genes between smoking patients with periodontitis vs smoking control subjects, with only one exception. The frequency of *KIR3DS1* was increased in smoking patients (45.45% vs. 24.24). How-

Table 1. Frequency of *KIR* genes in smoking patients with periodontal disease and healthy controls

KIR gene	Periodontitis smokers, number positive (%), n = 77	Control smokers, number positive (%), n = 33	p value[#] – periodontitis smokers vs. control smokers	OR (95% CI)
2DL1	77 (100)	31 (93.9)	0.088	1.06 (0.59-1.91)
2DL2	45 (58.4)	21 (63.6)	0.67	0.80 (0.34-1.88)
2DL3	69 (89.6)	31 (93.9)	0.72	0.56 (0.11-2.83)
2DL5	42 (54.6)	19 (57.6)	0.84	0.88 (0.38-2.03)
2DS1	29 (37.7)	13 (39.4)	1.00	0.93 (0.40-2.17)
2DS2	38 (49.4)	19 (57.6)	0.53	0.72 (0.31-1.65)
2DS3	31 (40.3)	13 (39.4)	1.00	1.04 (0.45-2.41)
2DS4n	31 (40.3)	11 (33.3)	0.53	1.35 (0.57-3.20)
2DS4d	57 (74.0)	29 (87.9)	0.13	0.39 (0.12-1.27)
2DS5	25 (32.5)	6 (18.2)	0.17	2.16 (0.78-5.98)
3DL1	69 (89.6)	32 (97.0)	0.27	0.27 (0.03-2.30)
3DS1	35 (45.5)	8 (24.2)	0.054	2.60 (1.03-6.56)
Haplotype Bx	15 (19.5)	4 (12.1)	0.42	1.75 (0.53-5.83)

[#]*Fisher's exact test*

Table 2. Frequency of *KIR* genes in non-smoking patients with periodontal disease and healthy controls

KIR gene	Periodontitis non-smokers, number positive (%), *n* = 173	Control non-smokers, number positive (%), *n* = 117	*p* value[#] – periodontitis non-smokers vs. control non-smokers	OR (95% CI)
2DL1	169 (97.7)	113 (96.6)	0.72	1.50 (0.36-6.15)
2DL2	100 (57.8)	75 (64.1)	0.33	0.77 (0.47-1.25)
2DL3	151 (87.3)	103 (88.0)	1.00	0.93 (0.45-1.92)
2DL5	93 (53.8)	71 (60.7)	0.28	0.75 (0.47-1.22)
2DS1	65 (37.6)	56 (47.9)	0.09	0.66 (0.41-1.06)
2DS2	108 (62.4)	68 (58.1)	0.47	1.20 (0.74-1.94)
2DS3	57 (33.0)	48 (41.0)	0.17	0.71 (0.43-1.15)
2DS4n	52 (30.1)	38 (32.5)	0.70	0.89 (0.54-1.48)
2DS4d	144 (83.2)	95 (81.2)	0.75	1.15 (0.62-2.13)
2DS5	47 (27.2)	39 (33.3)	0.29	0.75 (0.45-1.24)
3DL1	169 (97.7)	114 (97.4)	1.00	1.11 (0.24-5.26)
3DS1	71 (41.0)	48 (41.0)	1.00	1.00 (0.62-1.61)
Haplotype Bx	32 (18.5)	23 (19.7)	0.88	0.93 (0.51-1.69)

[#]*Fisher's exact test*

Table 3. Multivariate logistic regression analysis for presence of periodontitis as the dependent variable

Parameter	OR (95% CI)	*p*
Age (years)	1.08 (1.05-1.10)	< 0.000001
Sex (male vs. female)	1.30 (0.83-2.04)	0.25
Smoking	1.98 (1.19-3.28)	0.0083
KIR3DS1	1.28 (0.82-2.00)	0.28

ever, this difference had borderline statistical significance ($p = 0.05$).

The differences of *KIR* gene frequencies between non-smoking patients and non-smoking control subjects were not statistically significant (Table 2).

Additionally we examined the differences in the number of activating and inhibiting *KIR* genes between patients with periodontal disease and control subjects.

No significant differences of the number of activating and inhibiting *KIR* genes were found between patients with periodontal disease and controls, both in non-smoking subgroups (3.14 ±1.55 vs. 3.35 ±1.60 activating genes, $p = 0.29$ and 3.94 ±0.77 vs. 4.07 ±0.85 inhibiting genes, $p = 0.16$, respectively) and smoking subgroups (3.19 ±1.55 vs. 3.00 ±1.46 activating genes, $p = 0.57$ and 3.92 ±0.77 vs. 4.06 ±0.93 inhibiting genes, $p = 0.37$, respectively).

We also analyzed the frequencies of haplogroups A and B between smoking patients with periodontitis vs. smoking control subjects as well as non-smoking patients with periodontitis vs non-smoking control subjects. As shown in Tables 1 and 2, these differences were not statistically significant.

To find independent factors associated with increased risk of periodontal disease development we performed multivariate regression analysis taking into the account age and sex of patients, smoking and presence of the *KIR3DS1* gene. In multivariate regression analysis advanced age of patients and smoking were independent factors associated with increased frequency of periodontal disease (Table 3).

Discussion

The aim of this study was to examine the influence of *KIR* gene polymorphisms on tobacco smoke-related development of periodontal disease. To the best of our knowledge, this is the first study to evaluate whether *KIR* presence/absence polymorphism interferes with susceptibility to periodontal disease. In our previous study, we evaluated the impact of *KIR* genes on the disease markers and clinical course of periodontal disease. In that study, no association between either the presence or absence of *KIR* genes and PD and CAL parameters was found [27]. In this study we compared presence of *KIR* genes between non-smoking patients with periodontal disease with non-smoking

control subjects as well as smoking patients with smoking controls. Moreover, we compared the distribution of haplogroups A and B and frequency of *KIR* activating and inhibiting genes between patients with periodontal disease and control subjects. These analyses did not show any differences between patients and controls. Previous studies have indicated that haplogroup A exhibits a lower number of activating genes, and differences between these groups have been implicated in other diseases [18]. Hiby *et al*. reported that pregnant women, homozygous for *KIR* haplotype A, and therefore lacking all short-tailed activating genes except *KIR2DS4*, have increased risk of pre-eclampsia when the fetus has HLA-C2 [28].

The results of our study suggest that *KIR* gene content possibly does not play a major role in periodontal disease. In multivariate regression analysis advanced age of patients and smoking were independent factors associated with increased risk of periodontal disease. This analysis suggests that smoking is the most important factor associated with development of periodontal disease (OR = 1.98). Previous studies identified smoking and bacterial infections as significant factors influencing the progression of periodontal disease [29].

The local host response to bacteria, which initiates periodontal disease, includes the recruitment of leukocytes and the subsequent release of inflammatory mediators and numerous pro-inflammatory cytokines [30, 31]. Previous studies have confirmed the relationship between smoking and cytokine production [32, 33]. Smoking is a major risk factor in the development and progression of periodontal diseases, and its effect on the extent and prevalence of periodontal destruction is dose-dependent. Measures of the association between smoking status and periodontal destruction have been quite strong and consistent across studies and populations. Tobacco use is an important risk factor in the incidence and progression of periodontal disease. It impairs various components of the host response and immune system, such as inhibition of neutrophil chemotaxis and phagocytosis, inhibition of cellular immunity and suppression of local antibody production [10, 34, 35].

Previous studies suggest that smoking increases the proteolytic activity of neutrophils [36]. Neutrophils are a major source of the elastase and metalloproteinases causing gingival destruction in periodontal disease. Tobacco may exacerbate progression of periodontal disease through the induction of release of proteolytic enzymes from periodontal neutrophils [37, 38]. The studies suggest that smoking also alters the function of various cells involved in the immune response, leading to development of periodontal disease. In studies in animals and humans, tobacco smoke affected both humoral immunity and cell-mediated immunity [39, 40]. These changes include function and proliferation of neutrophils, B lymphocytes, T lymphocytes and NK cells. NK cells have a regulatory role in periodontal disease. The study by Yamazaki *et al*. [41] suggests that an immune response to autoantigens may be well controlled by NK cells. Previous studies detected a relationship between a deficiency in NK cell activity and autoimmune diseases. An impairment of the balance could be involved in the pathogenesis of periodontal disease. The results, however, showed an increase in the number of NK cells in periodontal disease, suggesting a functional role for these cells, and because of their capacity for rapid secretion of large amounts of cytokines, they may influence the T helper cytokine response [42, 43]. The results of studies assessing the role of NK cells in periodontal disease are inconclusive. Although several studies have shown some correlation between periodontal disease and NK cell numbers and phenotype [44-46], others have failed to show any relationship [47]. Also the results of studies examining the effect of smoking on NK cells are different. Some studies have found no differences between NK cells in smokers and non-smokers [48]. Others have found that smoking may alter the function of NK cells [49].

The role of autoimmunity in chronic inflammation is still not clear. It is possible that autoimmunity is a feature of all chronic inflammation. In this context, it has been demonstrated that gingival fibroblasts are able to phagocytose collagen; antibodies may facilitate this phagocytosis and hence the removal of broken-down collagen [43].

The pathogenesis of periodontal disease is very complex and includes environmental factors such as bacterial infections and smoking as well as genetic and immunological factors including innate immune system, humoral and cell-mediated immunity [50].

KIRs are surface receptors modulating the function of NK cells. These receptors are responsible for the innate immunity involved in the mechanisms of defense against bacterial infection as well as immunological processes responsible for the development of diseases [51].

Although our study is limited by the number of subjects, the results suggest that KIR presence/absence polymorphism is not a significant factor involved in the pathogenesis of periodontal disease. However, there are other levels of *KIR* polymorphism, and it would be interesting to check in a larger cohort whether *KIR* allelic variation or combinations of KIR-HLA ligands impact the risk of periodontal disease. Our results confirm the results of previous studies suggesting that the most important factor leading to the development of periodontal disease is tobacco smoking.

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The authors declare no conflict of interest.

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