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ORIGINAL ARTICLE

Microsatellite GT polymorphism in intron 2 of human Toll-like receptor (TLR) 2 gene and susceptibility to periodontitis

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Abstract As pattern recognition receptor, Toll-like receptor (TLR) 2 is a signal molecule essential for the cellular response to *Porphyromonas gingivalis*. A recently described guanine-thymine (GT) repeat microsatellite polymorphism of the human TLR2 gene leads to impaired promotor activity. A total of 380 patients with chronic periodontitis and 590 unrelated healthy control individuals of Caucasian descent were genotyped for the GT repeat microsatellite polymorphism of the TLR2 gene. The mutations were identified with polymerase chain reaction followed by sizing of GT repeat length. The number of GT repeats ranged from 13 to 29 in both study groups. There was no significant difference between periodontitis patients and healthy controls regarding the frequency of GT repeats (p=0.365). Subclassification of alleles into three subclasses (S allele, M allele, and L allele) revealed no significant association (p=0.810). Moreover, also the comparison of the summarized prevalence of S and L alleles (high promotor activity) with that of the M allele (low promotor activity) was not significantly different among study groups (p=0.377). The susceptibility to chronic peridontitis was not associated with the functional effective GT repeat microsatellite polymorphism in the human TLR2 gene.

Keywords Periodontitis · TLR2 · Pattern recognition receptor (PRR) · Pathogen-associated molecular patterns (PAMPs) · Lipopolysaccharide · Microsatellite

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Introduction

The early host defense against bacterial infection is executed by innate immunity activated by highly conserved molecular structures, *pathogen associated molecular patterns* (PAMPs) that are found on or in the cell walls of bacteria but not on human cells. Gram-negative bacteria are recognized by shed or membrane bound lipopolysaccharide, Gram-positive bacteria are recognized by the host immune system through the presence of membrane peptidoglycans, lipoteichoic acid, or extracellular toxins [1]. For the recognition of PAMPs the immune system provides a wide array of pattern recognition receptors (PRRs). The human Toll-like receptors (TLR) are part of the PRR family and play a central role in the initial immune response to bacterial infection [2].

In humans 11 toll-like receptors have yet been identified among which specifically the expression of TLR2 and TLR4 is upregulated in chronic periodontitis [3]. TLR4 has been postulated to function as the primary signaling receptor for lipopolysaccharide from enterobacterial species [4]. TLR2 has been shown to be involved in the recognition of lipoteichoic acid and bacterial lipoproteins [5]. Moreover, TLR2 was suggested as the primary receptor for most of the periodontopathogenic bacteria, e.g., *Porphyromonas gingivalis*, *Tannerella forsythensis*, or *Prevotella intermedia* [6, 7].

Recently, a polymorphic dinucleotide guanine-thymine (GT) repeat polymorphism has been described in intron II of the human TLR2 gene [8]. This microsatellite polymorphism has functional impact since it leads to changes in promotor activity depending on the number of GT repeats. Consistently, a significantly higher susceptibility for rheumatoid arthritis, tuberculosis, and infection with nontuberculous mycobacteria was found for individuals with smaller numbers (<16) of GT repeats in two different Korean populations [9–11].

The etiology of chronic periodontal disease implies environmental factors as microbial infection and smoking as well as genetic factors [12, 13]. Albeit the pathogenic Gram-negative bacterial infection was regarded as the primary pathophysiologic factor in periodontitis, the heritability of this entity was shown in twin studies to reach 50% [14, 15]. Hence, a strong genetic background has been suggested for the pathogenesis of chronic periodontitis. According to recent etiologic models genetic influences might affect different functions, e.g., periodontal tissue or oral hygiene behavior [16]. Alternatively, the genetic impact on the individual susceptibility and/or severity of periodontitis might be mediated by either an inappropriate or exaggerated immune response against a given bacterial stimulus [17, 18].

Referring to this etiologic model, genes encoding for receptors that are involved in the activation and regulation of immune responses against bacteria are excellent candidates for the elucidation of the genetic background of chronic periodontitis [19]. Since TLR2 was shown to play a pivotal role in the recognition of two highly virulent bacterial species that are commonly associated with chronic periodontitis, *P. gingivalis* and *P. intermedia*, functional effective polymorphisms affecting this receptor are of specific interest.

The present study aimed to assess the association between the functional effective GT repeat microsatellite polymorphism in intron II of the human TLR2 gene and the susceptibility and severity of chronic periodontitis.

Materials and methods

Patient population

The study was approved by the local ethics committee (No. 290/01) and conformed to the ethical guidelines of the Helsinki Declaration. All participants provided written informed consent prior to their enrolment into the study. All individuals included into the both study groups were adult Caucasians from the south of Germany (region of "Upper Bavaria"). In order to improve ethnic homogenicity of the study sample all subjects must have parents and grandparents of the German ethnic group. Individuals with severe medical disorders including diabetes mellitus, immunological disorders, increased risk for bacterial endocarditis, and pregnant females were excluded from the study.

Periodontitis group

Three hundred eighty patients with periodontitis were included in the study. The male to female ratio within the periodontitis group was 47.4%: 52.6%. The median±SD age was 54.1±11 years (age range 18-85 years).

All patients presented the diagnosis of generalized chronic periodontitis, which was assessed according to a standard evaluation procedure:

- determination of probing pocket depth measured with a Michigan type 'O' probe at six locations on each tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and distolingual);
- determination of furcation involvement by means of a Naber type probe;
- bleeding on probing registered as present or absent;
- bone loss estimated by orthopantomographs.

The probing pocket depth was defined as the distance from the base of the periodontal pocket to the free gingival margin. The furcation involvement was assessed by horizontal probing from the furcation entrance to the base of the defect. The furcation involvement was graded according to the classification of Nyman et al. [20].

Following characteristics had to be presented by a patient in order to be classified as generalized periodontitis:

- a total of at least 15 teeth in situ;
- ≥8 teeth with a probing pocket depth of≥5 mm at least at one location and/or a furcation involvement≥ class II;
- evidence of bone loss manifested as the distance between the alveolar crest and the cemento-enamel junction of≥3 mm around the affected teeth.

Extended clinical data was available for 228 patients with periodontitis. These patients were assigned to one of three groups of disease severity on basis of the following criteria:

(1) mild group (maximum probing depth: 6 mm; attachment loss >30%: ≤5 teeth; no attachment loss >50%);
(2) moderate group (maximum probing depth: 8 mm; attachment loss >30%: ≤8 teeth; attachment loss >50: ≤5 teeth);
(3) severe group (maximum probing depth: >8 mm; attachment loss >30%: >8 teeth; attachment loss >50%: >5 teeth).

Table 1 Demographic data of periodontitis and healthy control group

	Control (n=590)	Periodontitis (n=380)	p value
Age, years (median)	18-73 (45.7)	18-85 (54.1)	0.035
Sex (male-female)	49.8%:50.2%	47.4%:52.6%	0.254
Smokers	24.8%	75.2%	0.000
Non-smokers	60.4%	39.6%	0.000

According to these criteria, 86 (36.8%) of patients were classified as mild periodontitis, 81 (35.5%) as moderate periodontitis, and 63 (27.7%) of patients were classified as severe periodontitis.

Control group

A total of 590 unrelated, healthy blood donors of Caucasian genetic descent were included in the control group. The male to female ratio within the control group was 49.8%:50.2%. The median±SD age was 45.7±10.7 years (age range 18–73 years).

In order to be classified as no periodontitis controls, subjects had to meet the following criteria:

(1) ≥22 teeth in situ
(2) no more than one site with probing pocket depth≥ 3 mm
(3) no furcation involvement at any tooth.

Information regarding smoking habit was available for 347 of the patients with periodontitis and 396 of the control persons. Individuals have been classified as smokers if they had a self reported history of smoking of at least ten cigarettes per day for at least 1 year.

Blood samples and DNA isolation

A total of 9 ml peripheral venous blood was drawn from each individual by standard venipuncture. Blood samples were collected in sterile tubes containing 15% K_3EDTA solution. DNA was isolated using partly the QIAamp® DNA Blood Midi Kit (Qiagen, Hilden, Germany), partly the salting out procedure [21].

Genotyping of the microsatellite polymorphism in intron II of the TLR2 gene

For genotyping of the microsatellite polymorphism in intron II of the TLR2 gene we used polymerase chain reaction (PCR) to amplify a region of 131-163 bp surrounding the GT repeat microsatellite as described [8]. The number of GT repeats was identified by sizing of PCR products using an automatic sequencer. The total volume of the PCR was 10 µl containing 50 ng of genomic DNA, 1× PCR buffer (Qiagen, Hilden, Germany), 0.2 mM of each dNTP (Sigma, Taufkirchen, Germany), 0.25 units of HotStar-Taq™ DNA polymerase (Qiagen) and 0.25 µM of each primer 5′FAM-GCATTGCTGAATGTATCAGGGA-3′ (forward, containing the fluorescein marker 6-carboxyfluorescein (FAM)) and 5′-CTTGAGAAATGTTTTCTAGGC-3′ (reverse; TIB MOLBIOL, Berlin, Germany). The final concentration of $MgCl_2$ was 2 mM. Following an initial denaturation step at 95°C for 15 min, samples were subjected to 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. This temperature regime was followed by a final elongation step at 72°C for 10 min. The resulting fragments were run on an ABI 3700 sequencer. Samples for which genotypes were previously confirmed by sequencing: $(GT)_{13}$, $(GT)_{19}$, $(GT)_{23}$, and $(GT)_{24}$ were used as "gold standards" and were run in each gel separately.

Statistical analysis

Statistical analysis was performed using SPSS software, Version 17.0. Minimum sample size for each group was calculated on the basis of an alpha level of 0.05 and a power level of 0.80. For power calculation, the frequency of the S allele was assumed on basis of two previous studies [9, 10]. The X^2test or Fisher's exact test was used to

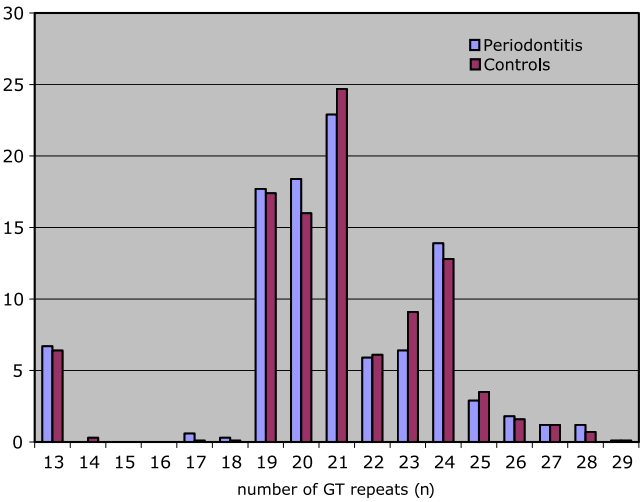

Fig. 1 Frequency distribution (%) of the number of GT repeats in periodontitis patients and healthy control individuals (p=0.365)

Table 2 Frequency of subclasses of alleles according to the number of GT repeats: S allele (GT≤17); M allele (GT 18≤22); L allele (GT≥23)

	S allele (%)	M allele (%)	L allele (%)	*p* value
Control (*n*=1,180)	79 (6.7)	760 (64.4)	341 (28.9)	-
Periodontitis (*n*=760)	51 (6.7)	499 (65.7)	210 (27.6)	0.810
Gender				
Periodontitis (male; *n*=348)	24 (6.9)	223 (64.1)	101 (29.0)	0.909
Periodontitis (female; *n*=386)	25 (6.5)	262 (67.9)	99 (25.6)	0.438
Severity of disease				
Periodontitis (mild; *n*=168)	10 (6.0)	106 (63.1)	52 (30.9)	0.858
Periodontitis (moderate; *n*=162)	13 (8.0)	106 (65.4)	43 (26.6)	0.658
Periodontitis (severe; *n*=126)	11 (8.7)	75 (59.5)	40 (31.8)	0.442
Smoking				
Periodontitis (non-smoking; *n*=470)	32 (6.8)	300 (63.8)	138 (29.4)	0.976
Periodontitis (smoking; *n*=224)	14 (6.2)	154 (68.8)	56 (25.0)	0.446

compare the allele and genotype distribution. Multiple logistic regression analysis was done to assess the association of the different genotypes with susceptibility to chronic periodontitis, adjusted for age, gender, and smoking status. The level of significance was set at $p<0.05$.

Results

Study sample

The demographic data are presented in Table 1. A total of 380 patients with chronic periodontitis and 590 healthy control individuals have been genotyped for the GT repeat microsatellite polymorphism. The power calculation revealed that the minimum sample size for patients with periodontitis required 256 individuals to test for associations with allele frequencies and 316 patients for analysis of genotype frequencies. For the healthy controls, the required minimum sample size was 383 (allele frequencies) and 473 (genotype frequencies), respectively.

Frequency distribution of the numbers of GT repeats

The number of GT repeats ranged from 13 to 29 in both study groups. The most common numbers were 19, 20, and 21 in both, the periodontitis group ($(GT)_{19}$=17.6%; $(GT)_{20}$=18.4%; $(GT)_{21}$=22.9%) and the healthy control group ($(GT)_{19}$=17.4%; $(GT)_{20}$=16.0%; $(GT)_{21}$=24.7%). The frequency distribution of the numbers of GT repeats was not significantly different between both study groups (p=0.365; Fig. 1).

Frequency distribution of alleles and genotypes

Dividing the alleles into three subclasses as described by Yim et al. [11] the lower component (S allele; (GT) ≤17) showed a prevalence of 6.7% in both, periodontitis patients

Table 3 Frequency of two subclasses of six genotypes (L/L, L/M, L/S, M/M, M/S, and S/S) according to the presence or absence of the S allele

	Genotypes with S allele (%)	Genotypes without S allele (%)	*p* value
Control (*n*=590)	77 (13.1)	513 (86.9)	-
Periodontitis (*n*=380)	50 (13.2)	330 (86.8)	0.962
Gender			
Periodontitis (male; *n*=174)	23 (13.2)	151 (86.8)	0.954
Periodontitis (female; *n*=193)	25 (13.0)	168 (87.0)	1.000
Severity of disease			
Periodontitis (mild; *n*=84)	9 (10.7)	75 (89.3)	0.548
Periodontitis (moderate; *n*=81)	13 (16.0)	68 (84.0)	0.458
Periodontitis (severe; *n*=63)	11 (17.5)	52 (82.5)	0.330
Smoking			
Periodontitis (non-smoking; *n*=235)	31 (13.2)	204 (86.8)	0.957
Periodontitis (smoking; *n*=112)	14 (12.5)	98 (87.5)	0.874

Table 4 Frequency of three subclasses of six genotypes (L/L, L/M, L/S, M/M, M/S, S/S) according to the presence or absence of the S allele (other/other vs. S/other vs. S/S)

Genotype	Others/others (%)	S allele/others (%)	S allele/others (%)	*p* value
Control (*n*=590)	513 (86.9)	75 (12.7%)	2 (0.3)	-
Periodontitis (*n*=380)	330 (86.6)	49 (12.9)	1 (0.3)	0.976
Gender				
Periodontitis (male; *n*=174)	151 (86.8)	22 (12.6)	1 (0.6)	0.909
Periodontitis (female; *n*=193)	168 (87.0)	25 (13.0)	0	0.719
Severity of disease				
Periodontitis (mild; *n*=84)	75 (89.3)	8 (9.5)	1 (1.2)	0.396
Periodontitis (moderate; *n*=81)	68 (84.0)	13 (16.0)	0	0.621
Periodontitis (severe; *n*=63)	52 (82.5)	11 (17.5)	0	0.518
Smoking				
Periodontitis (non-smoking) (*n*=235)	204 (86.8%)	30 (12.8%)	1 (0.4%)	0.982
Periodontitis (smoking; *n*=112)	98 (87.5%)	14 (12.5%)	0	0.824

and healthy controls (Table 2). The frequency of the M allele ((GT) 18≤22) was 65.7% in periodontitis patients and 64.4% in healthy individuals. The L allele ((GT) ≥23) was found in periodontitis in 27.6% and in control individuals in 28.9%. The distribution of the three subclasses of alleles was not significantly different between both study groups (p=0.810).

The summarized frequency of alleles associated with high promotor activity (S and L alleles) and low promotor activity (M allele) in the periodontitis group was 34.3% (high activity) and 65.7% (low activity). In the healthy control group, the frequency was 35.6% (high activity) and 64.4% (low activity; p=0.377). For the determination of the effect of genotype on the susceptibility of periodontitis the six genotypes (L/L, L/M, L/S, M/M, M/S, and S/S) have been divided into two subgroups according to the presence or absence of the S allele. The frequency of genotypes with or without the S allele was not significant different between the both study groups (p=0.962). These results were not changed after stratification according to the severity of periodontitis (Table 3). There was also no significant difference when comparing the distributions of three genotype subgroups (S/S vs. S/others vs. others/others; p=0.976; Table 4). Multiple regression analysis revealed no significant association between the different alleles and genotypes and the diagnosis of periodontitis after adjustment for gender, age, and smoking (Table 5).

Discussion

In the first line, periodontal disease is initiated and maintained by Gram-negative bacterial infection of the gingival sulcus [22]. Specific PAMPs, i.e., bacterial lipopolysaccharide, are expressed by the pathogenic microflora subsequently stimulating an inflammatory cascade that finally results in the characteristic periodontal tissue destruction [23, 24].

As evidenced by twin studies apart from bacterial infection, genetic determinants are of paramount importance for the susceptibility to periodontal disease [12, 15, 25]. Numerous direct, population-based association studies on different biologically effective single nucleotide polymorphisms have been recently performed for the delineation of the genetic background (for review, see [19]). For direct genetic association studies, particularly genes encoding for PRRs are excellent candidates referring to the bacterial origin of periodontitis. Previous studies on functionally effective polymorphisms within the human TLR2 and TLR4 did not give evidence for associations [26].

In periodontal disease Gram-negative pathogenic bacteria such as *Aggregatibacter actinomycetemcomitans*, *P. gingivalis*, and *T. forsythensis* are of particular relevance [22]. Although there exist still some controversy as to which TLR is primarily involved in the recognition of periodontopathogenic bacteria a recent study has clearly demonstrated that these bacteria stimulate TLR 2 in the first

Table 5 Multiple regression analysis of associations between alleles and genotypes and manifestation of periodontitis after adjustment for age, gender, and smoking

	p value
Number of GT repeats	0.590
Allele S/M/L	0.663
Allele S/L vs. M	0.974
Genotype (S/S; S/other; other/other)	0.902
Genotype (with S-allele/without S-allele)	0.693

line [7]. Thus, functional effective polymorphims within the human TLR2 gene might significantly influence the individual predisposition for chronic periodontitis.

Microsatellite polymorphisms in several human genes have been shown to be associated with the development or the clinical features of various diseases. For example, pulmonary tuberculosis and graft vs. host disease after bone marrow transplantation appears to be correlated with the number of cytosine-adenine repeats in intron 1 of the interferon-γ gene [27, 28]. Moreover, the clinical manifestation of rheumatoid arthritis revealed an association with adenine-thymine repeats in the cytotoxic T-lymphocyte antigen 4 gene [29].

Previous data on the impact of the amount of GT repeats in the human TLR2 gene on the promotor activity are contradictory. It was first suggested that shorter (<18) or longer (>22) GT repeats might lead to higher promotor activity following stimulation by interferon-γ than middle sized GT repeats [8]. More recently, it was postulated that the length of GT repeats is positively correlated with the promotor activity when stimulated with bacterial antigens [10]. According to this later work, shorter GT repeats lead to less TLR2 promotor activity and to less expression of TLR2 receptors. However, it must be emphasized that the differences in promotor activity might be attributed to different stimuli as used in both studies. Recent findings among individuals of Dutch Caucasian descent could also demonstrate a significant relationship between the number of GT repeats and the expression of proinflammatory cytokines [30]. However, in this study, smaller numbers of GT repeats were linked to higher production of inflammatory mediators, i.e., TNFα and IL-6. Although the precise functional effects of the GT microsatellite polymorphism on the expression of TLR2 receptors yet remain to be elucidated, recent studies have shown linkage with the susceptibility for several inflammatory diseases, i.e., tuberculosis, sarcoidosis, and rheumatoid arthritis [9, 10, 30].

Herein, the frequencies of GT repeats in both study groups have been determined. The most common numbers of GT repeats among healthy controls was 19 (17.4%), 20 (16.0%), and 21 (24.7%) indicating that the results presented herein are in accordance with previous observations on Caucasian subjects [8]. Considering the frequency distribution of the length of GT repeats in the TLR2 gene among periodontitis patients and healthy individuals, no difference was found. When dividing the alleles into three subclasses, as previously described in the analysis of the GT microsatellite polymorphism in the TLR2 gene, the distribution of allele frequencies was not significantly different between periodontitis subjects and healthy control individuals. Comparison of the summarized prevalence of S and L alleles with that of the M allele, which was suggested to be associated with high or low TLR2 promotor activity [8], revealed also no difference among both study groups. Finally, also after stratification of periodontitis patients according to gender, smoking, or their respective disease severity no association with the prevalence of chronic periodontitis was obsereved. It can thus be assumed that the GT repeat microsatellite polymorphism has no influence on the individual susceptibility for periodontal disease. Different reasons might be assumed for the lack of association between the microsatellite polymorphism and periodontal disease.

First, a large body of evidence indicates that lipopolysaccharides of many periodontopathogenic bacteria stimulate TLR2 and not TLR4 [31, 32]. In spite of these observations, it was assumed that TLR4 is at least involved in the recognition of lipopolysaccharide from *P. gingivalis* [33, 34]. It was postulated that the cell wall of the same bacterial species, i.e., *P. gingivalis*, presents at least two distinct molecular isoforms of lipid A which opens the possibility that both receptor types become activated. In fact, recent data provide evidence that stimulation by whole *P. gingalis* bacteria lead to signaling through both subtypes of Toll-like receptors, but the activation was considerably stronger for TLR2 than for TLR4 [35].

Second, chronic periodontitis has a complex genetic background. Hence, the analysis of polymorphisms within only one single gene might be insufficient for the delineation of the genetic background of chronic periodontitis. For further studies, linkage analysis or a genome-wide "map-based" association study might probably comprise an attractive alternative to direct population-based direct genetic association studies as performed herein [36].

In conclusion, no correlation was found between the functional effective GT repeat polymorphism in intron 2 of the human Toll-like receptor 2 gene and the susceptibility for chronic periodontitis.

Conflict of interest The authors declare that they have no conflict of interest.

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