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Lack of association between chronic periodontitis and the Toll-like receptor 4 gene polymorphisms in a Czech population

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Background and Objective: Periodontitis is a bacterially induced chronic inflammatory disease and a major cause of tooth loss among adults. Toll-like receptors are signal molecules essential for the cellular response to bacterial cell wall components. The aim of this study was to investigate relationships between chronic periodontitis and variations in the *TLR4* gene.

Material and Methods: A total of 171 patients with chronic periodontitis and 218 unrelated controls were genotyped for the Asp299Gly (896A > G) and Thr399Ile (1196C > T) polymorphisms of the *TLR4* gene.

Results: Both variants were in nearly complete linkage disequilibrium. No homozygotes for the less common alleles, 299Gly and 399Thr, respectively, were found. The prevalence of the Asp299Gly and the Thr399Ile heterozygotes was 5.3% and 5.0% in controls, and 7.0% and 7.0% in periodontitis patients.

Conclusion: In conclusion, *TLR4* gene polymorphisms were not significantly associated with the susceptibility to, or severity of, chronic periodontitis in our population.

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Periodontal diseases are considered to be multifactorial disorders, where putative periodontopathogens trigger chronic inflammatory and immune responses (1). Periodontitis is characterized by irreversible loss of alveolar bone and/or connective tissue attachment in the periodontium, which ultimately results in the loss of teeth. The magnitude of the inflammatory responses to plaque microorganisms, particularly their lipopolysaccharide component, is crucial in determining the extent of periodontitis (2).

Toll-like receptor 4 is the principal receptor for lipopolysaccharide. In the extracellular compartment of the body, lipopolysaccharide binds to the lipopolysaccharide-binding protein and CD14 (3), followed by coupling to the transmembrane receptor, Toll-like receptor 4 (4). This complex initiates a complex signaling cascade, resulting in systemic inflammation mediated by monocytes and macrophages (5). Increasingly, data are accumulating to suggest that the ability of certain individuals to respond appropriately to Toll-like receptor ligands may be impaired by single nucleotide polymorphisms within Toll-like receptor genes, resulting in an altered susceptibility to inflammatory disease, such as chronic periodontitis (6). Most studies have focused on two cosegregating single nucleotide polymorphisms within the gene encoding Toll-like receptor 4, which has been mapped to chromosome 9 (9q32-q33) (7,8). One of these, an A > G transition situated +896 bp downstream of the translation start site of *TLR4*, results in replacement of an

aspartic acid residue with glycine at amino acid 299. The second polymorphism is in strong linkage disequilibrium with *TLR4* Asp299Gly and represents a C > T transition at +1196, resulting in amino acid exchange of Thr399Ile. Both variants were found to have functional consequences as they attenuate the Toll-like receptor 4 signaling pathway and lead to a blunted inflammatory response (9). In addition, these polymorphisms were recently associated with the risk of periodontitis in German (10) and British (11) populations. As frequency of polymorphisms, lifestyle and gene–environment interactions differ among populations, the risk of a particular gene variant for a given genetic trait might be different in different populations.

Therefore, the aims of our study were to test (i) whether chronic periodontitis is associated with the Asp299Gly or Thr399Ile polymorphisms in the *TLR4* gene in the Czech population, and (ii) whether there is an association between the severity of periodontitis and the polymorphisms mentioned above.

Material and methods

Subjects

All subjects were Caucasians of exclusively Czech nationality. Phenotype status was assigned without knowledge of genotypes by two independent investigators. One hundred and seventy-one Czech patients with chronic periodontitis (88 men and 83 women; age range, 35–50 years; mean age ± standard deviation: 43.7 ± 6.7 years), referred to the Periodontal Clinic of Masaryk University and St Anne Hospital, were included in the study. Consecutive subjects who met the diagnostic criteria for chronic periodontitis were recruited into the study in 2000–2005. All subjects had to have at least 18 remaining teeth and to be in good general health. Exclusion criteria were history of cardiovascular disorders (such as coronary artery diseases or hypertension), diabetes mellitus, malignant diseases, immunodeficiency, current pregnancy or lactation. In addition, no patients had a previous history of periodontal surgery in the past 6 mo and were not taking any medication. Patients were classified during their first visit at the specialist before treatment. The control group consisted of 218 unrelated Caucasian individuals (110 men and 108 women; mean age ± standard deviation: 44.4 ± 13.13 years), residing in the same geographic area as the patients, and who had no clinical history of periodontal disease. Controls were selected randomly during the same period as patients and matched for age, gender and smoking status. Similarly as in patients, all subjects had to have at least 20 remaining teeth and to be in good general health. Exclusion criteria were identical as in patients with chronic periodontitis. These subjects were screened using a World Health Organization probe, and the Community Periodontal Index of Treatment Needs was assessed (12); values of the Community Periodontal Index of Treatment Needs in controls were less than 3.

The diagnosis of chronic periodontitis was based on physical examination, medical and dental history, probing depth, assessment of attachment loss, tooth mobility and radiographs, as described previously (13). All patients fulfilled the diagnostic criteria defined by the International Workshop for a Classification of Periodontal Diseases and Conditions for Chronic Periodontitis (14). Patients were classified according to the severity of their periodontal disease (on the basis of the amount of clinical attachment loss) into one of two disease categories. The 'moderate' ($n = 35$) classification required 3–5 mm clinical attachment, loss and the 'severe' ($n = 136$) classification required 6 mm clinical attachment loss. The loss of alveolar bone was determined radiographically. We used the index of Mühlemann & Mazor (15) to evaluate decreases in alveolar bone level. In our patients with chronic periodontitis, the mean maximum values of Mühlmann's bone index (15), calculated from all remaining teeth except for the 3rd molars, were 3.20 ± 0.86 for the upper jaw and 2.92 ± 0.80 for the lower jaw (2.12 ± 0.65 for the upper jaw and 2.12 ± 0.59 for the lower jaw in patients with moderate chronic periodontitis, and 3.50 ± 0.61 for the upper jaw and 3.14 ± 0.68 for the lower jaw in patients with severe periodontitis). In order to adjust for the effect of smoking history on periodontal disease, the subjects (patients and controls) were classified into the following two groups: subjects who never smoked (referred to as non-smokers); and subjects who were former smokers for ≥ 5 pack years or current smokers (referred to as smokers). The pack years were calculated by multiplying the number of years of smoking by the average number of cigarette packs smoked per day (16). The study was performed with the approval of the Committee for Ethics of the Medical Faculty, Masaryk University Brno, and informed consent was obtained from all participants (in line with the Helsinki declaration) before inclusion in the study.

Genotyping

Genomic DNA samples were isolated from peripheral blood leukocytes by a standard method using proteinase K.

The Toll-like receptor 4 Asp299Gly (896A > G; NCBI SNP Cluster ID: rs4986790, GenBank accession No. AF177765) polymorphism in the *TLR4* gene was detected using our modification of methods, as described previously (17). Briefly, a 152-bp product of this polymorphism was generated using primers 5′-gattgcatacttagactactacctccatg-3′ and 5′-gttaactaattctaaatgttgccatcc-3′. *Nco*I digestion resulted in two fragments of 126 bp and 26 bp (G allele) or 152 bp (A allele).

Genotyping of the Toll-like receptor 4 Thr399Ile (1196C > T; NCBI SNP Cluster ID: rs4986791, GenBank accession No. AF177765) variant was conducted according to Lorenz and colleagues (17).

All analyses were performed without prior knowledge of the subject's case/control status.

Statistical analysis

Comparisons were made between allelic and genotype frequencies in the disease and control populations, as

well as between patients with different severity of chronic periodontitis. Allele frequencies were calculated from the observed numbers of genotypes. The significance of differences in allelic and genotype frequencies between each two groups was determined by Fisher's exact test. Chi-square analysis was used to test for deviation of the genotype distribution from Hardy–Weinberg equilibrium. Linkage disequilibrium was evaluated by Lewontin's measure of linkage disequilibrium (D′) (18). Contingency table analysis, odds ratio, 95% confidence intervals and significance values were estimated with the use of the program package STATISTICA, version 6.0 (Statsoft Inc., Tulsa, OK, USA).

Results

The demographic features of patients with chronic periodontitis and healthy controls are shown in Table 1. No significant differences were found in the variables gender, age, and smoking between the chronic periodontitis group and control subjects.

In Tables 2 and 3, *TLR4* genotype and allele frequencies in periodontitis and control groups are presented. Both groups were in Hardy–Weinberg equilibrium with nonsignificant chi-square values comparing the observed and expected genotype frequencies of each of the tested polymorphisms. Overall, within the group of patients with chronic periodontitis, the allele frequency of the 299Gly variant was 7.0% (24/171), as well as for the 399Ile polymorphism. In comparison, in the control group it was 5.3% (23/218) for the 299Gly allele ($p = 0.19$) and 5% (22/218) for the 399Ile allele ($p = 0.15$). None of the study subjects in the periodontitis or control groups showed homozygosity for the Toll-like receptor 4 mutant alleles. Both variants were in complete linkage disequilibrium (D′ = 1.00 for both groups). In all subjects, heterozygous combination of the Toll-like receptor 4299 alleles was associated with heterozygosity at position 399; only one control subject, who was homozygous for the 399Ile allele, was a carrier of the heterozygote combination Asp299Gly.

Furthermore, the relationship between the *TLR4* genotype and allele frequencies, and the severity of periodontitis, was investigated. Distributions were compared between patients with moderate disease and patients with severe disease. No significant skewing was observed in the distribution of both Toll-like receptor 4 variants between these subgroups. Heterozygosity for both polymorphisms was found in 12.5% of 136 patients with severe disease compared with 20.0% of 35 patients with moderate disease (Table 4). When the same analysis was performed for smoking and nonsmoking subjects, similar frequencies of both variants were observed in all groups (Table 4). The comparison of nonsmoking patients, with a moderate form of periodontitis, vs. nonsmoking patients, with a severe form of periodontitis, (Table 4) did not show any significant differences. It was difficult to evaluate smokers in our study as a result of the small numbers of persons in individual subgroups, especially in the group of patients with moderate periodontitis, therefore this analysis was not conducted.

Discussion

According to a current pathogenetic model, periodontitis is initiated and maintained initially by a gram-negative bacterial infection. The presence of specific pathogen-associated molecular patterns stimulates a limited number of pattern recognition receptors and initiates an inflammatory cascade that finally results in periodontal tissue destruction (19). Examples of pattern recognition receptors are Toll-like receptors (e.g. Toll-like receptor 4, which is increasingly expressed on the

Table 1. Demographic data in patients with chronic periodontitis and controls

	Controls	Patients with periodontitis
No. of subjects	218	171
Mean age (years) ± SD	44.4 ± 13.13	43.7 ± 6.7
Age range (years)	33–55	35–50
Gender (male/female)	110/108	88/83
% of smokers	28.8	29.5

Table 2. Distribution of genotype and allele frequencies of the Asp299Gly polymorphism in the groups of control and periodontitis subjects

		Genotype Asp299Gly			Alleles		
	n	Asp/Asp (%)	Asp/Gly (%)	Gly/Gly (%)	Asp	Gly	*p*-value
Controls	218	195 (89.4)	23 (10.6)	0 (0.0)	0.947	0.053	
Patients with CP	171	147 (86.0)	24 (14.0)	0 (0.0)	0.930	0.070	NS

The *p*-value represents statistical significance for the comparison of genotype or allele frequencies between the two groups by Fisher's exact test.
CP, chronic periodontitis; NS, nonsignificant.

Table 3. Distribution of genotype and allele frequencies of the Thr399Ile polymorphism in the groups of control and periodontitis subjects

		Genotype Thr399Ile			Alleles		
	n	Thr/Thr (%)	Thr/Ile (%)	Ile/Ile (%)	Thr	Ile	*p*-value
Controls	218	0 (0.0)	22 (10.1)	196 (89.9)	0.050	0.950	
Patients with CP	171	0 (0.0)	24 (14.0)	147 (86.0)	0.070	0.930	NS

The *p*-value represents statistical significance for the comparison of genotype or allele frequencies between the two groups by Fisher's exact test.
CP, chronic periodontitis; NS, nonsignificant.

Table 4. Prevalence of the Toll-like receptor 4 homozygotes vs. heterozygotes in different subgroups of patients with chronic periodontitis and controls

	TLR4 Asp299Asp vs. Asp299Gly		TLR4 Ile399Ile vs. Thr399/Ile	
	Nonsmokers[a] (%)	Smokers[a] (%)	Nonsmokers[a] (%)	Smokers[a] (%)
Controls	127/18 (87.6,/12.4)	57/6 (90.5/9.5)	128/17 (88.3/11.7)	57/6 (90.5/9.5)
Patients with CP	94/16 (85.5/14.5)	40/6 (87.0/13.0)	94/16 (85.5/14.5)	40/6 (87.0/13.0)
Moderate CP	17/5 (77.3/22.7)	10/1 (90.9/9.1)	17/5 (77.3/22.7)	10/1 (90.9/9.1)
Severe CP	77/11 (87.5/12.5)	30/5 (87.5/14.3)	77/11 (87.5/12.5)	30/5 (87.5/14.3)
All moderate CP		28/7 (80.0/20.0)		28/7 (80.0/20.0)
All severe CP		119/17 (87.5/12.5)		119/17 (87.5/12.5)

[a]In the remaining 15 patients with chronic periodontitis and 9 controls, smoking status was not determined.
p = nonsignificant for all comparisons (comparison of groups with moderate chronic periodontitis-smoker and severe chronic periodontitis-smoker was not performed owing to a small number of subjects).
CP, chronic periodontitis; TLR4, Toll-like receptor 4.

surface of macrophages and gingival fibroblast within inflamed periodontal tissues) (20). It was very recently described that the epithelial cells heterozygous for the Toll-like receptor 4 polymorphism, Asp299Gly, are functionally hyporesponsive and therefore less susceptible to gram-negative infections (21).

There is convincing evidence that bacteria play a role in driving the inflammatory response in periodontitis and that genetic factors contribute not only to the pathogenesis but also to the course and extent of these disorders. Therefore, we investigated whether polymorphisms in the *TLR4* gene, encoding protein involved in innate immunity, were associated with periodontal disease, or its severity.

In this study, we found heterozygosity for the Asp299Gly and the Thr399Ile *TLR4* gene in $\approx$10–11% of the healthy controls and in 14% of the periodontitis patients. Data from previous studies revealed carriage rates for the Asp299Gly variant of 7–12% among healthy Caucasian subjects, indicating that our results are in accordance with the data from the literature (22,23). While analysis of our data revealed no positive association of both variants with the risk of developing chronic periodontitis in the Czech population, this is the first report on the prevalence of *TLR4* polymorphisms in patients with chronic periodontitis from a Central Eastern European country.

To our knowledge, to date, only four studies have investigated a role of the *TLR4* gene in patients with periodontitis. The association study of the variants in *TLR4* and *TLR2* genes with chronic periodontitis in the German population found no significant relationship of Toll-like receptors with disease (22). In contrast, other German authors, in a later study, found significant differences in distribution of *TLR4* variants between patients with chronic periodontitis and controls, but not between patients with aggressive periodontitis and healthy subjects (10). Brett *et al*. found an association of periodontitis (both in a chronic and aggressive form) with the *TLR4* gene polymorphisms in Great Britain (11). Finally, no relationship between *TLR4* polymorphisms and adult periodontitis has been published by Laine and colleagues in the Netherlands (24).

Many factors could account for the observed discrepancies among these studies. The racial and environmental differences among the populations might be a highly significant factor. Another factor could be the selection criteria of the studied population. Our population comprised quite a large number of patients with periodontitis, as well as control subjects (nearly 400 persons in total). Thus, in terms of the size of sets it is comparable only with the study of Schröder *et al*. (10) but it contains almost double the numbers of persons compared with the other three studies published to date (11,22,24). The controls in our study were selected so that they were slightly older than the patients, whereas in the previous studies controls were younger. Use of younger controls can lead to selection bias. Differences between the individual studies are also evident in the selection of the patients with chronic periodontitis when different criteria (e.g. number of afflicted teeth, number of examined localities, severity of bone loss, etc.) were used for their inclusion in this group. Patients with total disease of the cardiovascular system, diabetes, malignancies and immunodeficiencies were excluded, as in the study of Schröder *et al*. (10), where patients with malignant disease and diabetes were excluded, and in the study of Folwaczny *et al*. (22), which omitted persons with diabetes mellitus, immunodefects or pregnant women. Discrepancy in the prevalence of systemic diseases could affect the conclusions of the association studies of the effect of Toll-like receptor 4 variants on the pathogenesis of periodontitis. Composition of subgingival bacterial plaque may be one of the most important factors for this type of study. To date, only one study has analyzed the presence of bacterial species in subgingival plaque in relation to the variability of the *TLR4* gene (24), with negative results obtained. In our study, however, bacterial profiles were not investigated, so it is not clear which bacterial species were present in our chronic periodontitis patients. Recent data suggest that *Porphyromonas gingivalis*, one of the most important periodontopathogens, in contrast to other gram-negative bacteria, exhibits an untypical variant of lipopolysaccharide, interacting with Toll-like

receptor 4 and also Toll-like receptor 2 (25,26). Toll-like receptor 2 is a receptor generally recognizing cell wall compounds distinct from lipopolysaccharide, such as lipoproteins (27). Thus, Toll-like receptor 2 may be involved in immune responses against *P. gingivalis*, and variations in its gene can be important for susceptibility to chronic periodontitis.

In conclusion, the present findings indicate that the susceptibility to periodontitis is not influenced by either of the *TLR4* gene polymorphisms that have been investigated in this study. However, as differences in the genetic background greatly influence allele frequencies of a disease-associated gene, further studies in different races are needed to provide us with more information on the contribution of the *TLR4* gene in the etiopathogenesis of periodontitis.

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References

1. Kantarci A, Hasturk H, Van Dyke TE. Host-mediated resolution of inflammation in periodontal diseases. *Periodontol 2000* 2006;**40:**144–163.
2. Bainbridge BW, Coats SR, Darveau RP. *Porphyromonas gingivalis* lipopolysaccharide displays functionally diverse interactions with the innate host defense system. *Ann Periodontol* 2002;**7:**29–37.
3. Guha M, Mackman N. LPS induction of gene expression in human monocytes. *Cell Signal* 2001;**13:**85–94.
4. Chow CJ, Young DW, Golenbock DT, Christ WJ, Gusovsky F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* 1999; **274:**10689–10692.
5. van Deventer SJH, Büller HR, ten Cate LA, Hack CE, Sturk A. Experimental endotoxemia in humans: analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. *Blood* 1990; **76:**2520–2526.
6. Schroder NWJ, Schumann RR. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. *Lancet – Infectious Dis* 2005; **5:**156–164.
7. Medzhitov R, Presto-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila toll protein signal activation of adaptive immunity. *Nature* 1997;**388:**394–397.
8. Hoshino K, Takeuchi O, Kawai T *et al*. Cutting edge: toll-like receptor 4 (TLR-4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR-4 as the Lps gene product. *J Immunol* 1999;**162:** 3749–3752.
9. Arbour NC, Lorenz E, Schulte BC *et al*. TLR4 mutations are associated with endotoxin hyporresponsiveness in humans. *Nat Genet* 2000;**25:**187–191.
10. Schröder NWJ, Meister D, Wolff V *et al*. Chronic periodontal disease is associated with single-nucleotide polymorphisms of the human TLR-4 gene. *Genes Immun* 2005;**6:**448–451.
11. Brett PM, Zygogianni P, Griffiths GS *et al*. Functional gene polymorphisms in aggressive and chronic periodontitis. *J Dent Res* 2005;**84:**1149–1153.
12. Ainamo J, Barmes D, Beagrie G, Cutress T, Martin J, Sardo-Infirri J. Development of the World Health Organization (WHO) community periodontal index of treatment needs (CPITN). *Int Dent J* 1982;**32:**281–291.
13. Holla LI, Buckova D, Fassmann A, Halabala T, Vasku A, Vacha J. Promoter polymorphisms in the CD14 receptor gene and their potential association with the severity of chronic periodontitis. *J Med Genet* 2002;**39:**844–848.
14. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 1999;**4:**1–6.
15. Mühlemann HR, Mazor ZS. Röntgendiagnostik. *Schweiz Mschr Zahnheilk* 1955;**65:**1005–1013.
16. McDevitt MJ, Wang HY, Knobelman C *et al*. Interleukin-1 genetic association with periodontitis in clinical practice. *J Periodontol* 2000;**71:**156–163.
17. Lorenz E, Hallman M, Marttila R, Haataja R, Schwartz DA. Association between the Asp299Gly polymorphisms in the Toll-like receptor 4 and premature births in the Finnish population. *Pediatr Res* 2002;**52:**373–376.
18. Lewontin RC. On measures of gametic disequilibrium. *Genetics* 1988;**120:**849–852.
19. Graves DT, Jiang Y, Genco C. Periodontal disease: bacterial virulence factors, host response and impact on systemic health. *Curr Opin Infect Dis* 2000;**13:**227–232.
20. Mori Y, Yoshimura A, Ukai T, Lien E, Espevik T, Hara Y. Immunohistochemical localization of Toll-like receptors 2 and 4 in gingival tissue from patients with periodontitis. *Oral Microbiol Immunol* 2003;**18:**54–58.
21. Kinane DF, Shiba H, Stathopoulou PG *et al*. Gingival epithelial cells heterozygous for Toll-like receptor 4 polymorphisms Asp299Gly and Thr399Ile are hypo-responsive to *Porphyromonas gingivalis*. *Genes Immun* 2006;**7:**190–200.
22. Folwaczny M, Glas J, Török HP, Limbersky O, Folwaczny C. Toll-like receptor (TLR) 2 and 4 mutations in periodontal disease. *Clin Exp Immunol* 2004;**135:**330–335.
23. Lammers KM, Ouburg S, Morre SA *et al*. Combined carriership of TLR9–1237C and CD14–260T alleles enhances the risk of developing chronic relapsing pouchitis. *World J Gastroenterol* 2005;**11:**7323–7329.
24. Laine ML, Morre SA, Murillo LS, van Winkelhoff AJ, Pena AS. CD14 and TLR4 gene polymorphisms in adult periodontitis. *J Dent Res* 2005;**84:**1042–1046.
25. Bainbridge BW, Darveau RP. *Porphyromonas gingivalis* lipopolysaccharide: an unusual pattern recognition receptor ligand for the innate host defense system. *Acta Odontol Scand* 2001;**59:**131–138.
26. Darveau RP, Pham TT, Lemley K *et al*. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infect Immun* 2004;**72:**5041–5051.
27. Kirschning CJ, Schumann RR. TLR2: cellular sensor for microbial and endogenous molecular patterns. *Curr Top Microbiol Immunol* 2002;**270:**121–144.