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Association of Toll-like receptor 9 haplotypes with chronic periodontitis in Czech population

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Abstract

Aim: Toll-like receptors (TLRs) belong to the pattern recognition receptors family of signal molecules that recognize conserved microbial structures. The aim of this study was to analyse polymorphisms in the TLR genes and their association with chronic periodontitis (CP).

Material and Methods: Two polymorphisms (2408G/A, i.e. Arg753Gln and − 16934A/T) in TLR-2 and three variants (− 1486C/T, − 1237C/T and+2848A/G) in the TLR-9 genes were studied in 222 patients with CP and 259 unrelated controls. All polymorphisms were detected using the polymerase chain reaction-restriction fragment length polymorphism methods. Subgingival bacterial colonization was investigated by the VariOr®Dento test.

Results: No significant differences were found in allele and genotype frequencies of all polymorphisms between patients and controls. Nevertheless, complex analysis revealed differences in TLR9 haplotype frequencies between both groups ($p = 0.001$). Specifically, the haplotype T(− 1486)/T(− 1237)/A(2848) was significantly more frequent (9.6% *versus* 2.8%, $p < 0.000001$) and the haplotype T(− 1486)/T(− 1237)/G (2848) of the TLR9 gene was less frequent (35.9% *versus* 43.3%, $p = 0.01$) in patients than in controls. However, no significant relationships between periodontal pathogens, TLR polymorphisms and CP were found.

Conclusions: In conclusion, although no significant role of the TLR2 gene in periodontitis was found, our results indicate that TLR9 haplotypes may be associated with susceptibility to CP.

Key words: gene polymorphism; haplotype; lipopolysaccharide; periodontitis; TLR

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Chronic periodontitis (CP) is a multifactorial disease caused by periodontal bacteria affecting the supporting structures of the teeth, which can lead to a loss of teeth (Haffajee & Socransky 1994). Although bacterial infection was regarded as the primary a etiological factor in periodontitis, it is commonly accepted that this disease also has an important genetic component (Loos et al. 2005, Nibali et al. 2007). The bacteria can trigger activation of the host immune system through a family of pattern recognition receptors called Toll-like receptors (TLRs) and induce inflammatory mediators, leading to the destruction of periodontal tissues (Takeda & Akira 2005).

To date, 11 different TLRs have been identified in the mammalian system (for review, see Arancibia et al. 2007), and their differential expression and distribution by the tissues determine the host immune interaction with microbes and their components (Mahanonda & Pichyangkul 2007). TLR4 belongs to the most frequently investigated member of human TLRs; this receptor has been shown to specifically recognize [in cooperation with several protein components, such as LPS-binding protein (LBP) and CD14 lipopolysaccharide (LPS)] Gram-negative bacteria. In contrast to TLR4, TLR2 recognizes a broad spectrum of different microbial components including peptidoglycan and lipoteichoic acids from Gram-positive bacteria and also lipoproteins and lipopeptides from Gram-negative cell walls. Interestingly, in contrast to most Gram-negative LPSs recognized by TLR4,

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Porphyromonas gingivalis LPS has been shown to stimulate TLR2 by human macrophages (Martin et al. 2001). TLR9, contrary to previous TLRs, is not located in the cell membrane; it however fulfils its function intracellularly. It is preferentially expressed in immune cell-rich tissues, such as lymph nodes, bone marrow and peripheral blood leucocytes (Du et al. 2000) and plays a role in the recognition of different PAMPs, such as bacterial unmethylated CpG (cytosine–phosphate–guanine) dinucleotide motifs (Bauer et al. 2001) and in the stimulation of macrophages and dendritic cells by several non-DNA components (Coban et al. 2005). TLR9 gene variability has recently been associated with an altered immune response in human diseases (Kikuchi et al. 2005, Lachheb et al. 2008).

Several polymorphisms in both TLR2 and TLR9 genes have been identified (Lazarus et al. 2003, Schröder et al. 2003) and some of them had a functional effect. Substitution of an A by a G at position 2408 (2408A/G, rs5743708) resulting in an amino acid exchange (Arg753Gln) diminishes the ability of TLR2 to respond to bacterial cell-wall components (Lorenz et al. 2000, Schröder et al. 2003). Promoter polymorphism (−16934T/A, rs4696480) in the TLR2 gene may influence transcription and has been associated with an increased risk of follicular lymphoma (Nieters et al. 2006). In the TLR9 promoter region, two polymorphisms were identified, −1486T/C (rs187084) and −1237T/C (rs5743836), which create a new binding site for Sp-1 and nuclear factor (NF)-kappa B, respectively, and may influence the transcriptional regulation of the TLR9 gene (Hamann et al. 2006). Also, polymorphism 2848A/G (rs352140) in the exon 2 was associated with altered gene expression (Kikuchi et al. 2005) and was recently found to play a role in the susceptibility to SLE in a Chinese population (Xu et al. 2009). However, to our knowledge, a study investigating the impact of TLR9 polymorphisms on the susceptibility to periodontitis has not been performed yet or, in case of TLR2 Arg753Gln variant, showed negative findings (Folwaczny et al. 2004, Berdeli et al. 2007, Emingil et al. 2007, Fukusaki et al. 2007).

We hypothesized *a priori* that TLR polymorphisms might modify the relative risk for the development of CP. On the basis of these findings, we evaluated two single-nucleotide polymorphisms (SNPs) in TLR2 and 3 variants in the TLR9 genes in groups of healthy and periodontitis subjects with three major goals: (i) to determine the TLR SNPs frequencies in the Czech population, (ii) to investigate a possible association of selected variants and their combinations (i.e. haplotypes) with susceptibility to and/or severity of CP, and (iii) to investigate the interaction of common bacterial pathogens with TLR variants.

Material and Methods

Subjects

All subjects were Caucasians of exclusively Czech ethnicity from the region of Southern Moravia. Patients with CP ($N = 222$) were examined at the Clinic of Stomatology, St. Anne's Faculty Hospital, Faculty of Medicine, Masaryk University, Brno. They had at least 20 remaining teeth and were of 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. The control group consisted of 259 unrelated subjects who did not have a 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 controls had at least 20 remaining teeth and were of good general health. Exclusion criteria were identical as in patients with CP. These subjects were screened using a WHO probe, and the Community Periodontal Index of Treatment Needs (CPITN) was assessed (Ainamo et al. 1982); values of the CPITN index in controls were < 3.

The diagnosis of CP was based on physical examination, medical and dental history, probing depth, assessment of attachment loss, tooth mobility and radiographs. All patients fulfilled the diagnostic criteria defined by the International Workshop for a Classification of Periodontal Diseases and Conditions for Chronic Periodontitis (Armitage 1999). Patients were classified according to the severity of their periodontal disease (on the basis of the amount of clinical attachment loss (CAL) into one of two disease categories. The ''mild/moderate'' ($n = 102$) classification required 3–5 mm CAL and the ''severe'' ($n = 120$) classification required $\geqslant 6$ mm CAL. The loss of alveolar bone was determined radiographically. We used the index of Mühlemann & Mazor (1955) to evaluate decreases in alveolar bone level. In order to adjust for the effect of smoking history on periodontal disease, the subjects (patients and controls) were classified into the following groups: subjects who never smoked (referred to as non-smokers) and subjects who were former smokers for $\geqslant 5$ 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.

The study was performed with the approval of the Committees for Ethics of the Medical Faculty, Masaryk University Brno and St. Anne's Faculty Hospital. Written informed consent was obtained from all participants, in line with the Helsinki declaration before inclusion in the study.

Molecular biological assessment of periodontal bacteria in the subgingival pockets

Microbial samples were taken from the subgroups of randomly selected controls ($N = 42$) and patients with CP ($N = 72$) before subgingival scaling was carried out. The control group included 31 non-smokers and one smoker (smoking status of the 10 remaining persons was not known). Of 72 periodontitis patients, 48 persons were non-smokers and 18 smokers (smoking status was not determined in six persons). Microbial samples were collected from the deepest pocket of each quadrant by inserting a sterile paper point for 20 s. Bacterial plaque samples, taken from each patient, were pooled in one tube. Detection of bacterial DNA was carried out using the VariOr®Dento kit (GenTrend, Ceske Budejovice, Czech Republic) according to the manufacturer's instruction. This test determined the individual pathogens semiquantitatively as follows: (−) undetected, which corresponds to the number of bacteria $< 10^3$, (+) slightly positive, corresponding to the number of bacteria from 10^3 to 10^4, (++) positive, this corresponds to the number of bacteria from 10^4–10^5 and (+++) strongly positive, with the number of bacteria higher than 10^5.

Genetic analyses

DNA for genetic analysis was obtained from leucocytes separated from peripheral blood samples using standard procedures with proteinase K. The TLR2 Arg753Gln gene polymorphism variant was identified using polymerase chain reactions (PCR) followed by restriction fragment length polymorphism (RFLP) analyses using AciI enzyme, according to methods published previously (Schröder et al. 2003). The promoter variant − 16934A/T of the TLR2 gene was analysed by a modified method according to Sutherland et al. (2005) with digestion of the PCR products using Hpy188III enzyme. The TLR9 polymorphisms were detected by PCR followed by the RFLP analysis with BstNI (T-1237C), AflII (T-1486C) and AciI (A2848C) enzymes as firstly described by Hamann et al. (2004). The primers, PCR conditions, and restriction enzymes used are listed in Table 1.

Statistical analyses

Comparisons were made between allelic and genotype frequencies in the patients and control population. The allele frequencies were calculated from the numbers of genotypes observed. The significance of differences in the allele frequencies among groups was determined by Fischer's exact test. χ^2 analysis was used to test for deviation of genotype distribution from Hardy–Weinberg equilibrium and for comparison of differences in genotype combinations among groups. Only the values of $p<0.05$ were considered significant. Variations in the quantity of subgingival bacteria corresponding to the particular genotypes were tested by Kruskal–Wallis ANOVA. Where appropriate, Bonferroni's correction was used to adjust the α level according to the number of independent comparisons to the overall value of 0.05. Adjusted p-values for particular analyses are denoted as p_{corr}. Pairwise linkage disequilibrium (LD) among polymorphisms in the TLRs genes was measured by the Lewontin standardized disequilibrium coefficient |D′|(Lewontin 1998).

Haplotype analysis was performed using the program PHASE v.2.1, developed by Stephens and colleagues (Stephens et al. 2001, Stephens & Donnelly 2003). It is provided free for non-profit use via the World Wide Web (http://www.stat.washington.edu/stephens/).

Table 1. PCR-RFLP conditions for analysis of the TLR genes

Polymorphisms	Primers (5′–3′)	Restriction enzyme
TLR2 G2408A (Arg753Gln) [rs 5743708]	GGGACTTCATTCCTGGCAAGT (sense) GGCCACTCCAGGTAGGTCTT (antisense)	*Aci*I
TLR2 A-16934T [rs 4696480]	CAGAAATTTATCCATTCATGGTTCTGG (sense) TGTTATCACCAAGGGAGCAGT (antisense)	*Hpy*188III
TLR9 T-1237C [rs5743836]	ATGGGAGCAGAGACATAATGGA (sense) CTGCTTGCAGTTGACTGTGT (antisense)	*BstN*I
TLR9 T-1486C [rs187084]	ATGGGAGCAGAGACATAATGGA (sense) CTGCTTGCAGTTGACTGTGT (antisense)	*Afl*II
TLR9 A2848C [rs352140]	TCTACCACGAGCACTCATTCAC (sense) GCTGTTGTAGCTGAGGTCCAG (antisense)	*Aci*I

Table 2. Demographic data in patients with chronic periodontitis and controls

	Controls	Patients with CP
Number of subjects	259	222
Mean age (years ± SD)	41.7 ± 9.2	42.7 ± 7.3
Age range (years)	From 33 to 55	From 35 to 55
Sex (M/F)	122/137	112/110
% of smokers	27.7	30.9

Table 3. Allele and genotype frequencies of TLR2 and TLR9 polymorphisms in control and chronic periodontitis (CP) groups

	Controls *N* (%)	CP *N* (%)	*p*-level (p_{corr})
TLR2 2408G/A	HWE: $p = 0.372$	HWE: $p = 0.501$	0.24
GG	244 (94.2)	203 (91.4)	
GA	15 (5.8)	19 (8.6)	
AA	0 (0.0)	0 (0.0)	
G allele	97.1	95.7	0.16
A allele	2.9	4.3	
TLR2 − 16934A/T	HWE: $p = 0.839$	HWE: $p = 0.936$	0.15
AA	66 (25.5)	42 (18.9)	
AT	138 (53.3)	121 (54.5)	
TT	55 (21.2)	59 (26.6)	
A allele	52.1	46.2	0.04 (0.20)
T allele	47.9	53.8	
TLR9 − 1237C/T	HWE: $p = 0.385$	HWE: $p = 0.325$	0.44
TT	208 (80.3)	168 (75.7)	
CT	49 (18.9)	51 (23.0)	
CC	2 (0.8)	3 (1.3)	
T allele	89.8	87.2	0.12
C allele	10.2	12.8	
TLR9 − 1486C/T	HWE: $p = 0.210$	HWE: $p = 0.363$	0.92
TT	82 (31.7)	74 (33.3)	
CT	126 (48.6)	106 (47.8)	
CC	51 (19.7)	42 (18.9)	
T allele	56.0	57.2	0.38
C allele	44.0	42.8	
TLR9 2848A/G	HWE: $p = 0.810$	HWE: $p = 0.895$	0.13
AA	76 (29.3)	80 (36.0)	
AG	136 (52.5)	114 (51.4)	
GG	47 (18.2)	28 (12.6)	
A allele	55.6	61.7	0.03 (0.15)
G allele	44.4	38.3	

N (%): Values represent numbers (%) of subjects.
HWE, Hardy–Weinberg equilibrium; TLR, Toll-like receptor.

Table 4. Allele and genotype frequencies (%) of TLR2 and TLR9 polymorphisms in subgroups of control and CP non-smokers and smokers and in patients with mild/moderate and severe CP

	Non-smokers*		Smokers#		CP	CP#
	Controls *N* (%)	CP *N* (%)	Controls *N* (%)	CP *N* (%)	Mild/Moderate *N* (%)	Severe *N* (%)
TLR2 2408G/A						
GG	150 (92.6)	129 (90.2)	60 (96.8)	62 (96.9)	96 (94.1)	107 (89.2)
GA	12 (7.4)	14 (9.8)	2 (3.2)	2 (3.1)	6 (5.9)	13 (10.8)
AA	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
G allele	96.3	95.1	98.4	98.4	97.1	94.6
A allele	3.7	4.9	1.6	1.6	2.9	5.4
TLR2-16934A/T						
AA	42 (25.9)	28 (19.6)	15 (24.2)	14 (21.9)	23 (22.5)	19 (15.8)
AT	89 (54.9)	80 (55.9)	27 (43.5)	34 (53.1)	52 (51.0)	69 (57.5)
TT	31 (19.1)	35 (24.5)	20 (32.3)	16 (25.0)	27 (26.5)	32 (26.7)
A allele	53.4	47.6	46.0	48.4	48.0	44.6
T allele	46.6	52.4	54.0	51.6	52.0	55.4
TLR9-1237C/T						
TT	128 (79.0)	105 (73.4)	51 (82.3)	50 (78.1)	76 (74.5)	92 (76.7)
CT	32 (19.8)	35 (24.5)	11 (17.7)	14 (21.9)	24 (23.5)	27 (22.5)
CC	2 (1.2)	3 (2.1)	0 (0.0)	0 (0.0)	2 (2.0)	1 (0.8)
T allele	88.9	85.7	91.1	89.1	86.3	87.9
C allele	11.1	14.3	8.9	10.9	13.7	12.1
TLR9-1486C/T						
TT	49 (30.2)	52 (36.4)	16 (25.8)	21 (32.8)	35 (34.3)	39 (32.5)
CT	85 (52.5)	66 (46.2)	28 (45.2)	30 (46.9)	45 (44.1)	61 (50.8)
CC	28 (17.3)	25 (17.5)	18 (29.0)	13 (20.3)	22 (21.6)	20 (16.7)
T allele	56.5	59.4	48.4	56.3	56.4	57.9
C allele	43.5	40.6	51.6	43.7	43.6	42.1
TLR9 2848A/G						
AA	51 (31.5)	50 (35.0)	20 (32.3)	22 (35.4)	37 (36.3)	43 (35.8)
AG	84 (51.9)	75 (52.4)	33 (53.2)	33 (51.5)	52 (51.0)	62 (51.7)
GG	27 (16.7)	18 (12.6)	9 (14.5)	9 (14.1)	13 (12.7)	15 (12.5)
A allele	57.4	61.2	58.9	60.2	61.8	61.7
G allele	42.6	38.8	41.1	39.8	38.2	38.3

*Smoking status was not known for 15 patients with CP and 35 controls.
#*p*-value = non-significant differences for all five polymorphisms between all subgroups.
TLR, Toll-like receptors; CP, chronic periodontitis.

The approach underlying PHASE is a Bayesian haplotype reconstruction method using coalescent-based models, to improve the accuracy of haplotypes for unrelated individuals, although extensions to related individuals are possible. The algorithm uses a flexible model for the decay of LD with distance and it incorporates an assumption about the recombination rate variation. PHASE uses Gibbs sampling, a Markov–Chain Monte Carlo algorithm for the estimation of the posterior distribution. Hence, individual haplotypes can be estimated from this posterior distribution by choosing the most likely haplotype reconstruction for each individual. Practically, PHASE was run with a single file including genotypes of all subjects; for c-option (two groups), 10,000 permutations were performed. Global differences in haplotype frequency profiles between both groups were determined by the permutation test and $p < 0.05$ was considered statistically significant.

Contingency table analysis, odds ratio (OR), 95% confidence intervals (CI) and significance values were estimated with the use of the Statistica v. 8.0 (Statsoft Inc., Tulsa, OK, USA) program package.

Results

A total of 259 healthy unrelated controls and 222 patients suffering from CP were included in the study. No significant differences were found in variables including gender, age and smoking between the CP group and control subjects (Table 2).

The genotype and allele frequencies of all the variants studied are presented in Table 3. Both groups were in Hardy–Weinberg equilibrium with non-significant χ^2 values comparing the observed and expected genotype frequencies of each of the tested polymorphisms. None of the polymorphisms was found to be significantly associated with CP after correction for multiple comparisons (Table 3).

In addition, when the patient population was divided into two groups according to the severity of CP, no differences in the frequencies of alleles or genotypes of all five variants between patients with mild/moderate ($N = 102$) and severe ($N = 120$) disease were detected (Table 4). The same analysis performed for smoking and non-smoking subjects showed similar frequencies of TLR variants in all groups (Table 4).

Possible links between genetic variants of TLR2 and TLR9 and microbiological findings (occurrence of periodontal bacteria in subgingival pockets, including *Aggregatibacter actinomycetemcomitans, P. gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis*, *Treponema denticola*, Peptostreptococcus *micros* and *Fusobacterium nucleatum*) were assessed in the subgroups of patients with CP ($N = 72$) and controls ($N = 42$). We found significant differences in the occurrence of *P. gingivalis* (74.2% in CP *versus* 15.6% in controls, $p < 0.0001$), *T. forsythensis* (92.4% in CP *versus* 53.1% in controls, $p < 0.0001$), *P. micros* (84.8% in CP *versus* 46.9% in controls, $p < 0.0001$), *P. intermedia* (78.8% in CP *versus* 50.0% in controls, $p < 0.005$), and marginal difference in the occurrence of *T. denticola* (66.7% in CP *versus* 46.9% in controls, $p < 0.05$) between patients with CP and healthy controls. However, no significant differences between both groups in the occurence of *A. actinomycetemcomitans* (22.7% in CP *versus* 31.3% in controls, $p = 0.25$) and *F. nucleatum* (98.5% in CP *versus* 93.8% in controls, $p = 0.24$) were detected. In addition, no significant links between TLR2 and TLR9 variants and subgingival bacterial colonization could be found in our study (Table 5). However, it may be due to a relatively low number of subjects ($N = 114$) included in this part of the study.

However, a combination of multiple SNPs sites and haplotype analysis showed only four TLR9 haplotypes with frequency $> 5\%$ because all three SNPs in the TLR9 gene exhibited variability in the degree of LD (Table 6). The frequencies of the TLR9 haplotypes are summarized in Table 7. Interestingly, while comparison of genotype or allele

Table 5. Occurrence of periodontal pathogens in relation to TLR2 and TLR9 polymorphisms

	TLR2 2408G/A		TLR2 − 16934A/T			TLR9 − 1486C/T			TLR9 − 1237C/T			TLR9 2848A/G		
	GG	GA	AA	AT	TT	CC	CT	TT	TT	CT	CC	AA	AG	GG
$N = 114$	104	10	27	56	31	17	55	42	84	30	0	32	62	20
Fusobacterium nucleatum														
Undetected	6	1	2	3	2	0	5	2	5	2	0	2	4	1
Slightly positive	32	2	7	17	10	4	12	18	23	11	0	7	21	6
Positive	42	3	13	22	10	8	23	14	38	7	0	9	27	9
Strongly positive	24	4	5	14	9	5	15	8	18	10	0	14	10	4
Aggregatibacter actinomycetemcomitans														
Undetected	75	8	21	39	23	13	42	28	60	23	0	26	42	15
Slightly positive	29	2	6	17	8	4	13	14	24	7	0	6	20	5
Positive	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Strongly positive	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Porphyromonas gingivalis														
Undetected	51	2	14	30	9	8	24	21	41	12	0	15	29	9
Slightly positive	9	2	3	3	5	0	6	5	7	4	0	1	6	4
Positive	5	1	2	2	2	1	5	0	5	1	0	1	5	0
Strongly positive	39	5	8	21	15	8	20	16	31	13	0	15	22	7
Tannerella forsythensis														
Undetected	25	1	6	16	4	3	9	14	19	7	0	5	16	5
Slightly positive	37	2	14	15	10	6	20	13	29	10	0	8	23	8
Positive	23	2	4	15	6	5	15	5	19	6	0	8	14	3
Strongly positive	19	5	3	10	11	3	11	10	17	7	0	11	9	4
Treponema denticola														
Undetected	43	3	10	25	11	7	18	21	32	14	0	11	28	7
Slightly positive	59	7	17	29	20	10	36	20	51	15	0	20	34	12
Positive	2	0	0	2	0	0	1	1	1	1	0	1	0	1
Strongly positive	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Peptostreptococcus micros														
Undetected	33	0	9	16	8	7	14	12	24	9	0	8	21	4
Slightly positive	45	4	12	22	15	7	24	18	40	9	0	11	26	12
Positive	17	3	3	13	4	2	11	7	13	7	0	8	10	2
Strongly positive	9	3	3	5	4	1	6	5	7	5	0	5	5	2
Prevotella intermedia														
Undetected	35	1	9	20	7	5	16	15	24	12	0	11	19	6
Slightly positive	33	4	11	16	10	7	15	15	28	9	0	10	20	7
Positive	17	1	1	10	7	2	10	6	12	6	0	5	11	2
Strongly positive	19	4	6	10	7	3	14	6	20	3	0	6	12	5

Undetected: number of bacteria $< 10^3$.
Slightly positive: number of bacteria from 10^3 to 10^4.
Positive: number of bacteria from 10^4 to 10^5.
Strongly positive: number of bacteria $> 10^5$.
TLR, Toll-like receptors.

Table 6. Pairwise linkage disequilibrium (LD) for all possible 2-way comparisons among 3 polymorphisms in the TLR9 gene in control and CP groups

	SNP1 − 1486	SNP2 − 1237	SNP3 +2848
SNP1 controls		0.9042	0.9772
CP patients		0.7871	0.8570
SNP2 controls			0.6373
CP patients			0.6951

LD was measured by Lewontin standardized disequilibrium coefficient |D′|.
CP, chronic periodontitis; SNP, single-nucleotide polymorphisms; TLR, Toll-like receptors.

distributions in healthy subjects with patients provided mostly negative results, the distribution of TLR9 haplotype frequencies between both groups showed significant differences ($p = 0.001$). Specifically, the haplotype *T*(− 1486)/*T*(− 1237)/A (2848) of the TLR9 gene was significantly more frequent in patients with CP than in controls (9.6% *versus* 2.8%, $p < 0.000001$, $p_{corr} < 0.00001$; OR = 5.80, 95% CI: 2.79–12.08) and in contrast, the haplotype T(− 1486)/T(− 1237)/G (2848) of the TLR9 gene was less frequent in patients *versus* healthy subjects (35.9% *versus* 43.3%, $p < 0.01$, $p_{corr} = 0.05$; OR = 0.73, 95% CI: 0.56–0.95) in this study (Table 7). No significant differences were found for distribution of TLR2 haplotypes between patients with CP and healthy controls (data not presented).

Discussion

CP is a multifactorial disease with complicated interaction of various genes and environmental factors (Borrell & Papapanou 2005, Yoshie et al. 2007). According to a current pathogenetic model, periodontitis is initiated and maintained in the first line by Gram-negative bacterial infection (Socransky et al. 1998). It has been demonstrated that some of the TLRs are involved in the recognition of periodontopathic bacteria, such as *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum* and expressed in different periodontal tissues (Sarah et al. 2006). Most studies of TLRs gene variability in periodontitis have focused on the TLR4 gene and its two cosegregating polymorphisms (Asp299Gly and Thr399Ile), which were found to have functional consequences (Arbour et al. 2000, Kinane et al. 2006). However, although the gene for TLR4 seems to be an appropriate ''candidate'' gene for periodontitis, the association of polymorphisms with periodontal diseases have shown conflicting results with some positive (Brett et al. 2005, Schröder et al. 2005, Zhu et al. 2008, Ozturk & Vieira 2009) but mostly negative findings (Folwaczny et al. 2004, Laine et al. 2005, Berdeli et al. 2007, Emingil et al. 2007, Izakovicova Holla et al. 2007, James et al. 2007, Tervonen et al. 2007, Imamura et al. 2008, Noack et al. 2008, Schulz et al. 2008).

Similarly, no significant association between TLR2 Arg753Gln mutation and CP was found in this study. Our results are in accordance with several previously published negative studies (Folwaczny et al. 2004, Berdeli et al. 2007, Emingil et al. 2007, Fukusaki et al. 2007, Zhu et al. 2008). The other investigated functional polymorphism in the TLR2 gene at position − 16934A/T did not display any association with susceptibility to nor with the severity of CP in our study either, suggesting that there was no involvement of the TLR2 gene in the pathogenesis of periodontal disease. However, polymorphisms in TLR2 gene have been previously associated with increased susceptibility to other inflammatory and autoimmune diseases (Lorenz et al. 2000, Ogus

Table 7. Estimated frequencies of the reconstructed TLR9 haplotypes characterized by the three polymorphisms analysed by PHASE v. 2.1. in both groups

Haplotypes			Healthy subjects	CP patients	*p* level	OR (95% CI)
−1486C/T	−1237C/T	+2848A/G				
T	T	G	0.4335	0.3586	**0.01**	**0.73 (0.56–0.95)**
C	T	A	0.4292	0.3973	NS	0.78 (0.60–1.00)
T	C	A	0.0951	0.1136	NS	1.22 (0.81–1.86)
T	T	A	0.0282	0.0959	**<0.000001**	**5.80 (2.79–12.08)**
Four others*			0.0141	0.0344	NS **$p = 0.001^{\dagger}$**	NA

Haplotypes are ordered according to decreasing haplotype frequency in the healthy subjects; those with frequencies <1% in both groups were pooled together as ''others''. Separate one-degree of freedom tests were conducted for a series of 2 × 2 contingency tables testing the frequency of each specific haplotype vs. all others between particular groups (two-tail Fisher exact test, five independent observations, Bonferroni's-corrected *p*-value for single test equals to 0.01). Global differences in haplotype frequency profiles between both groups were tested by permutation testing (PHASE output, 10,000 permutations).

*Four pooled remaining haplotypes with very low frequencies of occurrence.

†Omnibus *p*-value assessed by permutation testing.

Bold values are statistically significant.

NA, non-applicable; TLR, Toll-like receptors.

et al. 2004, Berdeli et al. 2005, Sutherland et al. 2005).

In contrast to TLR2 variants that were investigated in periodontal disease previously, there is no study of TLR9 polymorphisms with periodontitis in the literature to date. TLR-9 is expressed in the gingival lesions of gingivitis and periodontitis, in addition to TLR2 and TLR4. However, the expression of TLR9 was significantly up-regulated in periodontitis compared with gingivitis lesions (Kajita et al. 2007). In the present study, we inferred haplotypes in TLR9 from genotype data and performed haplotype-based association analysis with CP. Although, isolated SNPs showed no significant associations with CP in single-locus analyses, we identified significant association of the specific TLR9 haplotype [−1486T/−1237T/+2848A] with CP. How haplotypes formed by these polymorphisms might be related to a potentially increased risk of periodontitis remains unclear as the only functional data documenting variable effects come from studies of isolated variants. Two polymorphisms within the promoter region create new binding sites for NF-κB (−1237C/T) and Sp1 (−1486C/T) (Hamann et al. 2006). Novak et al. (2007) showed that the TT allelic variant sequence of the −1237C/T TLR9 gene had significantly higher promoter activity than the CC genotype. The combination of the C allele at position −1486 with a G allele at position+1174 (variant not investigated in our study) has the ability to down regulate TLR9 expression (Tao et al. 2007). In addition, Kikuchi et al. (2005) demonstrated that the AA genotype of the 2848A/G polymorphism in exon 2 was associated with significantly enhanced gene expression and higher frequency of intracellular IgM(+) B cells following CpG stimulation. Hence, the risk haplotype identified in our study contains the A allele of the+2848A/G together with T alleles of the −1237C/T and −1486C/T variants that exhibited higher promoter activity than the opposite alleles. We can assume that bacterial DNA represents a microbial component that can trigger the pathogenesis of periodontal disease, and the difference in transcriptional regulation of the TLR9 gene (determined by the presence of more active alleles) can be important for periodontitis susceptibility. This assumption is supported by findings observed in an animal model. TLR9-deficient mice did not show any response to CpG DNA, including inflammatory cytokine production from macrophages and dendritic cells (Hemmi et al. 2000). Nevertheless, neither differences in TLR9 protein expression of dendritic cells or in functional interferon (IFN)-α/IFN-β production after TLR9 stimulation related to the allelic variations at the SNP sites could be observed previously (Berghöfer et al. 2005). Similarly, no significant relationships between the presence of periodontal pathogens, TLR polymorphisms and CP were found in this study. So far, several studies have only investigated the role of TLR9 polymorphisms in human diseases, such as Crohn's disease, systemic lupus erythematosus (SLE), atopic eczema (AE) and asthma (Lazarus et al. 2003, Török et al. 2004, Novak et al. 2007, Tao et al. 2007). In an exploratory case-control study, an association of the C allele at −1237C/T SNP with asthma has been found in European Americans, but not in the Hispanic or African Americans (Lazarus et al. 2003). In contrast, Novak and colleagues described an association between −1237 T allele and AE, especially intrinsic AE. Török et al. (2004) studied TLR9 −1237C/T and+2848A/G variants in German patients with Crohn's disease, ulcerative colitis and healthy controls and found that the allele −1237C carrier status was associated with Crohn's disease. Finally, Tao et al. (2007) described a relationship between G allele at position +1174 and C allele at position −1486 (both down-regulating TLR9 expression) and a decreased risk of SLE in Japanese.

There are some limitations to this study that need to be considered. First, the major complicating factor in the study of isolated loci is the nature of the periodontitis as a multifactorial disease in which interaction between multiple genes (and environmental factors) plays a role and the effect of every single gene is rather small. Second, the case-control approach used is generally quite vulnerable to population stratification, e.g. due to different ethnic origin. The present sample, however, is exclusively of Czech Caucasian origin, restricted to the limited geographical area populated by a quite homogenous population with little admixture. Third, we did not measure RNA expression or

protein levels of TLR2 or TLR9, and thus we do not know the functional consequences of the polymorphisms of these genes in periodontitis.

In conclusion, this study represents the first work that analyses the distribution of several TLR9 polymorphisms and their combinations (e.g. haplotypes) with respect to CP. Our data suggest that the TLR9 haplotype ''−1486T/−1237T/+2848G'' has a protective effect against the development of CP and, in contrast, haplotype ''−1486T/−1237T/+2848A'' can increase the susceptibility of this disease in the Czech population. These findings may have important diagnostic implications and their molecular basis needs to be addressed in further studies. Nevertheless, the design of the association study can be sensitive to type II statistical error and our results require confirmation by other studies on different populations.

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Clinical Relevance

Scientific rationale for the study: Previous research has shown an inconsistent effect of TLR gene polymorphisms on susceptibility to CP. The TLR2 and TLR9 polymorphisms are hypothesized to be associated with a risk of CP in Czech population.

Principal findings: Although no significant differences in alleles or genotypes were found for all five polymorphisms of both genes, three SNPs in the TLR9 gene act in a cooperative fashion and their haplotype frequencies were significantly different between patients with CP and controls.

Practical implications: The TLR9 haplotypes might be one of the risk factors for CP, at least in our population.