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Original Article

Toll-like receptor 4 gene polymorphism is associated with chronic periodontitis

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Abstract: Toll-like receptors (TLRs) contribute to the immune response by recognizing patterns presented by bacteria and other pathogens. These receptors have been implicated in the inflammatory response that contributes to gingivitis and periodontitis. Conflicting reports have suggested that variations in the genes encoding TLRs, particularly TLR2 and TLR4, may influence susceptibility to periodontitis. In this study, the contribution of variations in the genes encoding TLR2 and TLR4 in the context of periodontitis was examined in 254 patients with moderate periodontitis, 418 patients with severe periodontitis, and 260 healthy controls free of gum disease. Genomic DNA was extracted from participants' whole blood, and genotyping of *TLR2/TLR4* as performed using real-time polymerase chain reaction with TaqMan MGB primer. The genotype, allele, and haplotype frequencies were compared among control, moderate periodontitis, and severe periodontitis groups. Statistical analysis was performed using chi-square and logistic regression analyses. Of the 9 polymorphic loci detected in the two genes, one, rs11536889 (G>C) in *TLR4*, displayed a statistically significant difference in distribution between individuals with moderate periodontitis and severe periodontitis ($P<0.05$). The distribution of the GG genotype in moderate periodontitis was higher than in the severe periodontitis group ($P<0.05$). Further, for the haplotype rs7873784, rs1927907, and rs1153688 of *TLR4,* the distribution of haplotype GCG was statistically different between moderate periodontitis and severe periodontitis ($P<0.05$, $OR=1.501$). These findings indicate that variation in *TLR4* may affect chronic periodontitis susceptibility in a Han Chinese population.

Keywords: Periodontitis, TLR2, TLR4, polymorphism

Introduction

Periodontitis manifests as chronic inflammation of the tissues supporting the teeth, called the periodontium, in which inflammation of the gums spreads to the deeper tissue layers to reach the periodontal ligaments, alveolar bones, and cementum [1]. The condition arises when one of the most common oral diseases, gingivitis, goes untreated [1]. Indeed, periodontitis has become a serious disease that endangers both the teeth and the overall health. A leading cause of the loss of masticatory organ functions, periodontitis can also lead to increased risk of heart attack and stroke [2, 3].

Inflammation that leads to periodontitis begins with plaque and tartar buildup at the gum line. This buildup contains bacteria that are recognized as pathogens by the immune system, which determines subsequent immune responses to the inflammation. Individuals have different inflammatory responses and immune responses to bacteria; indeed, not all bacterial infections lead to periodontitis. Therefore, the host genetic susceptibility, in addition to the pathogens, plays an important role in the pathology of periodontitis.

Toll-like receptors (TLRs) are important immune receptors that bind to ligands and activate cells to trigger the release of inflammatory cytokines, promoting inflammation [4, 5]. TLRs can recognize pathogen-associated molecular patterns, i.e., the conserved structure and composition of pathogenic microorganisms; in addition, they

can activate adaptive immunity [6]. TLR2 and TLR4 play a crucial role in mediating tissue immune responses to periodontitis [7, 8]. These two receptors are expressed in the cells of periodontal tissues such as gingival epithelial cells, and highly-expressed TLR2 and TLR4 are detected in infected gingival tissues [9, 10]. When virulence factors produced by periodontal pathogens, such as fimbriae and lipo-polysaccharide (LPS), bind to TLR2 and TLR4, host cells are induced to produce proinflammatory cytokines [11, 12]. Thus, TLRs participate in inflammatory responses and innate immune responses following stimulation by bacteria in periodontal tissues.

However, some debate exists about the extent to which these receptors contribute to individual differences in susceptibility to periodontitis. Some studies have indicated that variations in the genes encoding TLR2 and TLR4 may affect susceptibility to and/or severity of periodontitis, but the results have been conflicting [13, 14]. To help understand the potential contribution of genetic variation in TLR2 and TLR4 to periodontitis, the current study used a case-control approach to explore the correlation between polymorphisms in *TLR2* and *TLR4* and the degree of susceptibility to and severity of chronic periodontitis.

Participants and methods

Participants

Patients visiting the School of Stomatology, Jiamusi University between Dec. 2012 and Sept. 2014 were recruited as study participants. Patients were included if having moderate or severe chronic periodontitis according to the classification criteria related to periodontal health in the Third National Health and Nutrition Examination Survey in the United States [15, 16]. Additional inclusion criteria were as follows: Han nationality; no prior periodontal treatment; absence of chronic systemic diseases including cardiovascular diseases, diabetes, and endocrine disorders; no prior use of antibiotics or immunosuppressant agents within the preceding six months; absence of fever, cough, and inflammatory lesions of other regions in the preceding month; not pregnant or lactating; and having at least 15 remaining teeth. A total of 672 patients with periodontitis were included (254 cases had moderate periodontitis, 418 cases had severe periodontitis). An additional 260 individuals receiving physical exams in the same period were selected as a healthy control group; they were free of periodontal disease. The participants included a total of 932 individuals; 641 were males (68.8%) and 291 were females (31.2%). The mean participant age was 62.2±9.7 years. This study was approved by the Hospital Ethics Committee, and informed consent was obtained from all participants.

Periodontal health examination

Periodontal health was evaluated for all participants by periodontal specialists. Two sites of each remaining tooth were examined. Clinical indexes comprised the sulcus bleeding index (SBI), probing depth (PD), periodontal attachment loss (AL), and plaque index (PLI), as well as the status of bone resorption and loosening of teeth. The median value of distal lingual sides and the median value of proximal buccal sides were recorded.

Genomic DNA extraction

To identify genetic variation in *TLR2* and *TLR4*, DNA was obtained from blood samples of each participant. Four mL of venous blood was drawn from the forearm of each participant, treated with EDTA anticoagulant, and stored at -20°C. Blood samples were thawed, and red blood cells were lysed using an erythrocyte lysate (Boster Biotech, Wuhan, China). Samples were centrifuged, then precipitated white blood cells were collected and evenly mixed with 10% sodium lauryl sulphate (Sangon Biotech, Shanghai), Te buffer [10 mM Tris-HCl (pH 7.8); 1 mM EDTA (pH 8.0)], and proteases. The mixture was stored overnight at 37°C. After salt fractionation and centrifugation, the supernatant was collected, treated with chloroform, and centrifuged for extraction. DNA was precipitated with ethanol, washed with 70% Ethanol, and transferred to an Ependorf tube (Generay Biotech, Shanghai). After the DNA was dried in the open air, 200 μL of TE buffer were added to resuspend it, and then samples were stored at -80°C.

Genotyping

Single nucleotide polymorphisms (SNPs) in *TLR2* and *TLR4* were genotyped with a high-throughput real-time quantitative PCR method

Table 1. Genotype and allele frequencies of *TLR2* and *TLR4* variants in individuals with periodontitis and healthy controls [n (%)]

SNP locus	Genotype	Control	Moderate periodontitis	Severe periodontitis	x^2	*P*
TLR2						
rs1898830	AA	62 (24.31)	69 (27.94)	121 (28.95)	3.638	0.457
	AG	131 (51.38)	132 (53.44)	207 (49.52)		
	GG	62 (24.31)	46 (18.62)	90 (21.53)		
	Allele A	255 (50.00)	270 (54.66)	449 (53.71)	2.551	0.279
	Allele G	255 (50.00)	224 (45.34)	387 (46.29)		
rs13150331	AA	64 (24.90)	68 (27.20)	109 (26.08)	1.790	0.774
	AG	129 (50.20)	128 (51.20)	222 (53.11)		
	GG	64 (24.90)	54 (21.60)	87 (20.81)		
	Allele A	257 (50.00)	264 (52.80)	440 (52.63)	1.083	0.582
	Allele G	257 (50.00)	236 (47.20)	396 (47.37)		
rs3804100	CC	63 (25.20)	66 (28.21)	110 (26.96)	3.230	0.520
	CT	108 (43.20)	94 (40.17)	151 (37.01)		
	TT	79 (31.60)	74 (31.62)	147 (36.03)		
	Allele C	234 (46.80)	226 (48.29)	371 (45.47)	0.967	0.617
	Allele T	266 (53.20)	242 (51.71)	445 (54.53)		
TLR4						
rs10759930	CC	33 (12.84)	40 (16.00)	50 (12.11)	2.151	0.708
	CT	123 (47.86)	114 (45.60)	197 (47.70)		
	TT	101 (39.30)	96 (38.40)	166 (40.19)		
	Allele C	189 (36.77)	194 (38.80)	297 (35.96)	1.092	0.579
	Allele T	325 (63.23)	306 (61.20)	529 (64.04)		
rs11536879	AA	205(79.77)	198(79.84)	328 (80.00)	2.324	0.676
	AG	44 (17.12)	47 (18.95)	72 (17.56)		
	GG	8 (3.11)	3 (1.21)	10 (2.44)		
	Allele A	454 (88.33)	443 (89.31)	728 (88.78)	0.248	0.883
	Allele G	60 (11.67)	53 (10.69)	92 (11.22)		
rs10983755	AA	24 (9.37)	25 (10.00)	35 (8.54)	0.768	0.943
	AG	96 (37.50)	95 (38.00)	164 (40.00)		
	GG	136 (53.13)	130 (52.00)	211 (51.46)		
	Allele A	144 (28.13)	145 (29.00)	234 (28.54)	0.095	0.954
	Allele G	368 (71.87)	355 (71.00)	586 (71.46)		
rs1927907	AA	29 (11.60)	30 (12.15)	40 (10.31)	0.959	0.961
	AG	103 (41.20)	95 (38.46)	155 (39.95)		
	GG	118 (47.20)	122 (49.39)	193 (49.74)		
	Allele A	161 (32.20)	155 (31.38)	235 (30.28)	0.540	0.763
	Allele G	339 (67.80)	339 (68.62)	541 (69.72)		
rs7873784	CC	0 (0.00)	2 (0.81)	2 (0.49)		0.084*
	CG	49 (19.60)	32 (12.96)	54 (13.24)		
	GG	201 (80.40)	213 (86.23)	352 (86.27)		
	Allele C	49 (9.80)	36 (7.29)	58 (7.11)	3.439	0.179
	Allele G	451 (90.20)	458 (92.71)	758 (92.89)		
rs11536889	CC	11 (4.28)	11 (4.40)	23 (5.61)	8.800	0.066
	CG	97 (37.74)	69 (27.60)	151 (36.83)		
	GG	149 (57.98)	170 (68.00)	236 (57.56)		
	Allele C	119 (23.15)	91 (18.20)	197 (24.02)	5.841	0.051
	Allele G	395 (76.85)	409 (81.80)	623 (75.98)		

Note: *Fisher's exact test.

Table 2. Genotype and allele frequencies of *TLR4* rs11536889 [n (%)] in individuals with periodontitis

rs11536889	Moderate periodontitis	Severe periodontitis	*P*	*P*1=GG *vs* GC + CC	*P*2=CC *vs* GC + GG
CC	11 (4.40)	23 (5.61)	0.028		0.362
CG	69 (27.60)	151 (36.83)			
GG	170 (68.00)	236 (57.56)		0.011	
Allele C	91 (18.20)	197 (24.02)	0.013		
Allele G	409 (81.80)	623 (75.98)			

Table 3. Haplotype distributions for *TLR4* (rs1927907-rs11536889-rs7873784) in individuals with periodontitis

Haplotype	Moderate periodontitis	Severe periodontitis	*OR* (95% *CI*)	*P*
AGC	0.024	0.014		
AGG	0.259	0.267		
ACG	0.006	0.012		
GGC	0.041	0.049		
GGG	0.478	0.420		
GCG	0.169	0.230	1.501 (1.067-1.986)	0.021

OR-odds ratio; 95% CI, 95% confidence interval.

using the 7900HT instrument (Applied Biosystems, Foster City, CA, USA) and the TaqMan MGB probe (Biocoen Biotechnology, Beijing). This method is suitable for large-scale screening for SNPs. The reaction was performed in a total volume of 25 μL, comprising 0.5 μL DNA template, 2.5 μL TaqMan universal PCR master mix 2× (Applied Biosystems, Foster City, CA, USA), 0.125 μL 40× TaqMan genotyping assay mix, and 1.875 μL ultrapure water. The reaction was performed under the following conditions: predenaturation at 95°C for 10 min; and denaturation at 95°C for 15 s, and annealing at 60°C for 1 min, for a total of 50 cycles. SDS2.3 software was then used for genotyping. Multiple experiments were conducted on certain samples, where a blank control was designed to improve the accuracy of genotyping, and the results of repeated genotyping were consistent with that of original genotyping.

Statistical analysis

Double data entry was performed using EpiData version 3.1 to create a data bank, and logic checks were performed. SAS 9.2 (SAS Institute, Cary, NC, USA) was used to analyze data. Chi-squared, Hardy-Weinberg equilibrium, and unconditional logistic regression analyses were used to assess distributions and variables. Odds ratio (OR) and 95% confidence intervals (95% CI) were used to assess the risk of periodontitis. Testing Haplotype Effects In Association Studies (THESIAS) software (http://genecanvas.ecgene.net/downloads) was used to analyze haplotype differences between affected participants and healthy controls. $P<0.05$ was considered to indicate a difference was statistically significant.

Results

Distribution of genotypes at 9 loci of TLR2 and TLR4

Nine loci (3 in *TLR2*; 6 in *TLR4*) were identified to have genetic variation among individuals in the study. Hardy-Weinberg equilibrium was tested for the genotype frequencies of these polymorphic loci (**Table 1**). The distributions of genotypes and alleles at each locus did not significantly differ (each *P* value >0.05) between the control group and the periodontitis group. However, a significant difference in genotype distributions was detected between patients with moderate periodontitis and patients with severe periodontitis for the SNP rs11536889 (*TLR4*) ($P<0.05$). Specifically, the frequency of the GG genotype compared to the combined GC + CC genotypes was significantly higher in moderate periodontitis than in severe periodontitis ($P<0.05$) (**Table 2**).

Haplotype analysis and population characteristics

There were 6 haplotypes at 3 adjacent polymorphic loci (rs7873784, rs1927907 and rs1153688) of *TLR4*, wherein the GCG haplotype (at rs7873784, rs1927907, and rs1153688) showed statistical significance ($P<0.05$, $OR=1.501$) in terms of its frequency distributions in patients with moderate periodontitis and patients with severe periodontitis (**Table 3**). All participants were compared regarding their status of smoking and toothbrushing. Smoking status was significantly different in patients with periodontitis ($P<0.05$) (**Table 4**). A stratified analysis on the smokers found no dif-

Table 4. Relationship between smoking and periodontitis [n (%)]

Smoking status	Control	Moderate periodontitis	Severe periodontitis	x^2	*P*
Current smoker (n=143)	22 (8.46)	27 (10.63)	94 (22.49)	32.280	<0.001
Ever smoker (n=362)	102 (39.23)	102 (40.16)	158 (37.80)		
Non-smoker (n=427)	136 (52.31)	125 (49.21)	166 (39.71)		

Table 5. Relationship between the degree of smoking and periodontitis [n (%)]

Number of cigarettes		Control	Moderate periodontitis	Severe periodontitis	x^2	*P*
<1 pack/day	Current smoker	25 (26.04)	22 (22.45)	64 (35.36)	5.870	0.053
	Ever smoker	71 (73.96)	76 (77.55)	117 (64.64)		
≥1 and ≤2 pack/day	Current smoker	2 (9.52)	6 (26.09)	15 (31.25)	3.697	0.158
	Ever smoker	19 (90.48)	17 (73.91)	33 (68.75)		
>2 pack/day	Current smoker	1 (14.29)	1 (12.50)	7 (30.43)		0.653*
	Ever smoker	6 (85.71)	7 (87.50)	16 (69.57)		

Note: *Fisher's exact test.

Table 6. Relationship between the frequency of brushing and periodontitis [n (%)]

Frequency of brushing	Control	Moderate periodontitis	Severe periodontitis	*P*
1 time/day	85 (32.69)	97 (38.19)	167 (39.95)	0.372*
2 times/day	139 (53.46)	133 (52.36)	209 (50.00)	
3 times/day	25 (9.62)	21 (8.27)	33 (7.89)	
1 time/several days	2 (0.77)	1 (0.39)	2 (0.48)	
Never brushing	9 (3.46)	2 (0.79)	7 (1.67)	

Note: *Fisher's exact test.

ference in the number of packs of cigarettes smoked per day between groups (*P*>0.05) (**Table 5**). Additionally, no difference was detected in the frequency of toothbrushing between groups (P>0.05) (**Table 6**).

Discussion

The occurrence of periodontitis is mediated, at least in part, by the host response to pathogenic bacteria. Bacteria, particularly bacterial LPS, can be recognized by TLRs, which are pattern recognition receptors. TLR2 and TLR4 thereby mediate the innate immune responses of related host cytokines. TLR gene polymorphisms may change the structural domains of receptors and alter their responses to pathogenic bacteria. TLR4, upon recognizing Gram-negative bacteria, binds to LPS and subsequently induces cells to produce inflammatory cytokines including tumor necrosis factors and interleukins [9, 10]. This stimulation leads to more serious inflammatory responses; in the case of gum tissue inflammation, a chronic and heightened response produces more serious periodontal damage [17-19]. Interestingly, no responses to stimulation by LPS are observed in TLR4-deficient mice [20]. Further, the expression levels of TLR2 and TLR4 on the surface and interior of gingival epithelial cells are higher in periodontitis patients than in healthy individuals. After TLR ligands stimulate the gingival epithelial cells, they produce inflammatory cytokines [9, 10, 21]. A study in European populations found that *TLR4* polymorphisms were risk factors for periodontitis [22].

This study found that one SNP locus, rs11536889, of *TLR4* displayed different genotype distributions between patients with moderate periodontitis and patients with severe periodontitis. Patients carrying the GG genotype at rs11536889 are more prone to developing severe periodontitis. There were 6 haplotypes at 3 adjacent polymorphic loci (rs7873784, rs1927907, and rs1153688) of *TLR4*, wherein the GCG haplotype differed in distribution between patients with moderate periodontitis and patients with severe periodontitis. This finding suggests that *TLR4* polymorphisms are closely correlated with moderate and severe chronic periodontitis. Indeed, *TLR4* SNPs appear to be a genetic susceptibility fac-

tor for periodontal diseases, influencing the progression of periodontal diseases and leading to more serious inflammatory responses. The production of additional inflammatory cytokines and bacterial products leads to more serious damage to periodontal tissues.

In addition, this study detected a difference in the smoking status between controls and those with periodontitis, in that the proportion of smokers in the periodontitis group was higher. However, a stratified analysis did not detect a difference in the number of packs of cigarettes smoked per day. Interestingly, although toothbrushing is a good approach to mechanical plaque control, no difference was found here in the frequency of toothbrushing between individuals with periodontitis and healthy controls. We will further explore this finding in following-up studies.

In summary, this study found that *TLR4* polymorphisms were correlated with periodontitis susceptibility in a Han Chinese population, and the GCG haplotype (at rs7873784, rs1927907, and rs1153688) may be a risk factor for periodontitis development. Genetic susceptibility to periodontitis might be correlated with other polymorphic loci of TLRs, but those regions were not explored in this study. Further, chronic periodontitis may involve multiple gene interactions and synergy effects of factors including surrounding environments and individual health conditions. Currently, a more in-depth study is required to investigate the degree of the impact of TLR polymorphisms on susceptibility to periodontal diseases, and we will further investigate the impact of TLR polymorphisms on protein functions in follow-up studies.

Disclosure of conflict of interest

None.

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