Eur J Clin Microbiol Infect Dis (2016) 35:1871–1876
DOI 10.1007/s10096-016-2741-6

ORIGINAL ARTICLE

Alveolar bone loss in relation to toll-like receptor 4 and 9 genotypes and *Porphyromonas gingivalis* carriage

U. K. Gursoy[1] · Q. He[2] · P. Pussinen[3] · S. Huumonen[4,5] · E. Könönen[1,6]

Received: 30 May 2016 / Accepted: 24 July 2016 / Published online: 4 August 2016

Abstract Toll-like receptors (TLRs) are highly developed sensors to detect microbe-associated molecular patterns. Functional polymorphisms of the genes *TLR4* and *TLR9* were found to be associated with alveolar bone loss in a *Porphyromonas gingivalis*-induced periodontitis model in mice. Our aim was to examine whether such an association can be detected in a group of Finnish adults. Polymorphisms of *TLR4* Asp299Gly (rs4986790) and *TLR9* rs187084 (1486 T/C) were genotyped by pyrosequencing and PCR from the saliva samples of 223 adults (age range 40–60 years). Alveolar bone loss, measured from panoramic radiographs, were compared between *TLR* genotype groups according to subjects' salivary carriage of *P. gingivalis*, measured using a single copy gene–based real-time PCR. The frequencies of *TLR4* wild type and heterozygote variants were 87.4 % and 12.6 %, respectively, while those of *TLR9* wild type, heterozygote, and homozygote variants were 25.6 %, 39.1 %, and 35.3 %, respectively. In the *TLR4* heterozygote group, *P. gingivalis*-positive subjects had more alveolar bone loss than *P. gingivalis*-negative subjects ($p = 0.027$), while no difference was observed in the wild type group. *P. gingivalis*-negative individuals with *TLR9* heterozygotes exhibited significantly less alveolar bone loss compared to those with *TLR9* wild type ($p = 0.007$). Polymorphisms of *TLR4* in *P. gingivalis* carriers seem to expose to alveolar bone loss. Polymorphisms of *TLR9* can be protective against alveolar bone loss in the absence of *P. gingivalis*.

✉ U. K. Gursoy
ulvi.gursoy@utu.fi

[1] Department of Periodontology, Institute of Dentistry, University of Turku, Turku, Finland

[2] Department of Medical Microbiology and Immunology, University of Turku, Turku, Finland

[3] Department of Oral and Maxillofacial Diseases, University of Helsinki, Helsinki, Finland

[4] Department of Oral Radiology, Institute of Dentistry, University of Turku, Turku, Finland

[5] Department of Diagnostic Imaging, Turku University Hospital, Turku, Finland

[6] Oral Health Care, Welfare Division, City of Turku, Turku, Finland

Introduction

Periodontitis is a chronic inflammatory disease of tooth-supporting tissues induced by pathogenic bacteria, which are often found in biofilms attached to tooth surfaces [1, 2]. Periodontitis is a polymicrobial disease, where *Porphyromonas gingivalis*, a Gram-negative anaerobic bacterium, acts as a pathogen and promotes dysbiosis by regulating the biofilm composition qualitatively and quantitatively [3]. Moreover, several virulence factors of this periodontopathogen, i.e., gingipains and lipid A phosphatases, can activate or inhibit innate and adaptive immune responses of the host [4]. Although the carriage of *P. gingivalis* does not necessarily predict the development or presence of periodontitis, it indicates an increased risk for periodontitis in susceptible subjects [5].

Host innate and adaptive immune response mechanisms use pattern recognition receptors (PRR) to sense microbe-associated molecular patterns. Among the PRRs, toll-like receptors (TLRs) are the most studied ones. Up to now, ten TLRs (TLR1-10) have been characterized in humans. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are located on cell membrane, while TLR3, TLR7, TLR8, and TLR9 reside in

endosomes [6]. Against the constant challenge of bacteria present in the oral cavity, resident cells activate innate immune response via TLR-dependent mechanisms [7]. Periodontal ligament cells and gingival tissue cells can recognize bacterial lipopolysaccharides (LPS) by their TLR4 [8, 9] and bacterial DNA by endoplasmic TLRs [10]. During periodontal infection, there is an increased TLR gene expression leading to elevated protein levels [11, 12]. Nevertheless, when bacterial LPS is detected by TLR, it does not necessarily result in a successful elimination of the pathogen and suppression of the disease by immune response. For example, LPS impairs the differentiation of periodontal ligament stem cells to osteoblasts through the activation of TLR4, disturbing new bone formation [9].

In knock-out mice models, it was observed that disturbances in function of TLR4 and TLR9 may cause disrupted immune response against *P. gingivalis*, which can lead to impaired inflammatory response and, eventually, alveolar bone loss [13–15]. To our knowledge, only two studies have demonstrated simultaneous interactions of *P. gingivalis* and *TLR4* single nucleotide polymorphism (SNP) with periodontitis [16, 17]. Concerning *TLR9*, the findings of mice studies have not been confirmed in humans. In the present study, we hypothesized that being positive for *P. gingivalis* is a risk factor of advanced alveolar bone loss in subjects with functional polymorphisms of *TLR4* and *TLR9*. Therefore, we aimed to determine the associations between carriage of *P. gingivalis*, functional polymorphisms of *TLR4* and *TLR9*, and alveolar bone loss.

Material and methods

Salivary samples

Salivary samples originated from 223 subjects (part of the national Finnish health survey Health 2000), and their information came from the data on questionnaires, interviews, and clinical health examinations. All participants gave a written informed consent, and study protocols were approved by the Ethical Committee for Epidemiology and Public Health of the Hospital District of Helsinki and Uusimaa, Finland.

Paraffin-stimulated whole saliva samples had been collected into Eppendorf tubes, which were transported in carbonic acid ice and immediately frozen at −70 °C until further use. When the saliva samples were thawed for the first time, they were centrifuged at 10,000 rpm for 5 min, and the pellets were used for the assays of genotyping and microbiology.

Genotyping

For the present study, the genotyping of *TLR4* Asp299Gly (rs4986790) from saliva was performed by pyrosequencing (PSQ™96MA Pyrosequencer, Biotage, Uppsala, Sweden), using the PSQ™96 Pyro Gold Q96 reagent kit according to the manufacturer's instruction as described previously [18]. *TLR9* rs187084 (1486 T/C) genotyping was performed [19] by using BspTI restriction enzyme (ThermoFischer Scientific, Waltham, USA) for digestion of the PCR product.

Microbiological analysis

Salivary *P. gingivalis* levels were available from our previous study [20]. The analysis was performed using a single copy gene–based real-time PCR as published in detail [21]. The viability or salivary levels of *P. gingivalis* were unknown to the researchers at the time of the saliva collection.

Radiographic analysis

Alveolar bone loss (Fig. 1) was measured from radiographs that were taken with a dental panoramic X-ray machine (PM 2002 CC proline apparatus, Planmeca, Helsinki, Finland) using imaging values between 58–68 kV and 4–10 mA. A specialist in oral and maxillofacial radiology (S.H.) analyzed alveolar bone loss from the digital panoramic radiographs using an inbuilt measuring tool of the Dimaxis software (Planmeca, Helsinki, Finland). The mesial and distal surfaces

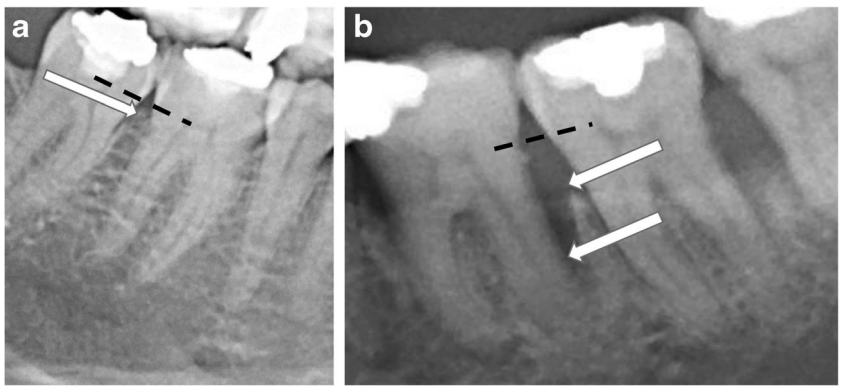

Fig. 1 In a periodontally healthy condition (**a**), the distance between the alveolar bone crest (marked with an *arrow*) and cemento-enamel junctions of neighbouring teeth (marked with a *dashed line*) is about 2 mm. With the development of periodontitis (**b**), alveolar bone loss becomes visible in vertical and/or horizontal forms on radiographs (by courtesy of Güliz N. Güncü, Associate Professor, University of Hacettepe, Ankara, Turkey)

of each tooth and the furcation areas of molar teeth were evaluated. Total alveolar bone loss of a subject was calculated as a sum of horizontal (the distance between the cemento-enamel junction and the alveolar bone crest), vertical (the distance from the alveolar bone crest to the bottom of alveolar bone pocket), and furcation (the distance from the furcation to the alveolar bone crest) bone losses. All measurements were recorded in millimeters [22]. The Advisory Board for Radiation Safety approved radiographical examination protocols, and the Radiation and Nuclear Safety Authority and the Ethics Committees for Human Studies of the National Public Health Institute and the Institute of Epidemiology and National Health, Finland granted the safety licenses.

Statistical analyses

All data analyses were performed with the SPSS statistical program (version 23.0; IBM Armonk, New York, USA). The data distributions of alveolar bone loss were skewed, therefore, non-parametric tests were applied. The Kruskal-Wallis H (for multiple comparisons) and Mann–Whitney U tests were used for comparing alveolar bone loss between the *TLR* genotype groups. All other parameters were compared with the ANOVA test (with Bonferonni correction). A statistical significance was defined as a p value <0.05.

Results

Of the 223 salivary samples, *TLR4* genotypes were determined in all samples, while *TLR9* genotypes were detected in 215 samples. In eight samples, *TLR9* genotype was not detected due to the low quality or quantity of saliva samples and their DNA. The frequencies of *TLR4* wild type and heterozygote variants were 87.4 % and 12.6 %, respectively, while those of *TLR9* wild type, heterozygote, and homozygote variants were 25.6 %, 39.1 %, and 35.3 %, respectively.

The distribution of age, gender, the percentage of smokers, number of teeth, alveolar bone loss, salivary levels of *P. gingivalis*, and percentage of *P. gingivalis* carriers did not differ between the genotypes of *TLR4* and *TLR9* (Table 1). Seventy-three subjects had mild (0–2.9 mm), 74 subjects had moderate (3–13.4 mm), and 76 subjects had advanced (over 13.5 mm) alveolar bone loss.

In *TLR4* heterozygotes, *P. gingivalis*-positive subjects had more alveolar bone loss than *P. gingivalis*-negative subjects ($p = 0.025$). Moreover, among *P. gingivalis*-positive subjects, individuals with *TLR4* heterozygotes had elevated alveolar bone loss in comparison to individuals with *TLR4* wild type ($p = 0.054$). Alveolar bone loss was not different between the *P. gingivalis*-positive ($n = 111$) and -negative ($n = 84$) subjects with *TLR4* wild types (Fig. 2).

Among the *P. gingivalis*-negative subjects ($n = 91$), individuals with *TLR9* heterozygotes exhibited significantly less alveolar bone loss in comparison to those with *TLR9* wild type (Fig. 3).

Discussion

In the present study, it was shown that in subjects with *TLR4* Asp299Gly (rs4986790) SNP, the carriage of *P. gingivalis* in saliva is related to advanced alveolar bone loss. We also present evidence that *TLR9* (rs187084) SNP may be protective against alveolar bone loss in the absence of *P. gingivalis*. Since periodontitis is a multifactorial disease and multispecies-biofilms are responsible for periodontal infection, a single SNP and the presence of a single pathogen cannot fully explain the initiation and development of periodontitis. However, our results can be used to characterize susceptible patients (subjects with *TLR4* Asp299Gly SNP and positive for *P. gingivalis*) to define risk groups of periodontitis.

Alveolar bone loss was used as an outcome variable in the present study. The extent of periodontal destruction was then related to *TLR* polymorphisms and *P. gingivalis* carriage that

Table 1 Characterization of the subjects in *TLR* genotype groups with age, gender, smoking status, number of teeth, alveolar bone loss, and carriage of *P. gingivalis*

Characteristic	*TLR4*			*TLR9*			
	Wild type ($n = 195$)	Heterozygote variants T/C ($n = 28$)	p value	Wild type ($n = 55$)	Heterozygote variants C/T ($n = 84$)	Homozygote variants T/T ($n = 76$)	p value
Age in years [mean (SD)]	48.5 (5.3)	49.8 (5.7)	0.231	48.4 (6.0)	49.1 (5.4)	48.5 (5.1)	0.733
% female	53.3	60.7	0.463	50.9	60.7	48.7	0.273
% smokers	37.9	35.7	0.954	47.3	32.3	35.5	0.478
No. of teeth [mean (SD)]	26.9 (2.4)	26.9 (2.6)	0.959	27.2 (2.4)	26.8 (2.5)	26.9 (2.3)	0.672
Alveolar bone loss (mm) [median (range)]	5.9 (196.2)	10.0 (183.8)	0.194	8.2 (113.9)	3.9 (196.2)	6.8 (184.1)	0.099
% subjects with Pg	56.9	53.6	0.738	63.6	54.8	56.6	0.568

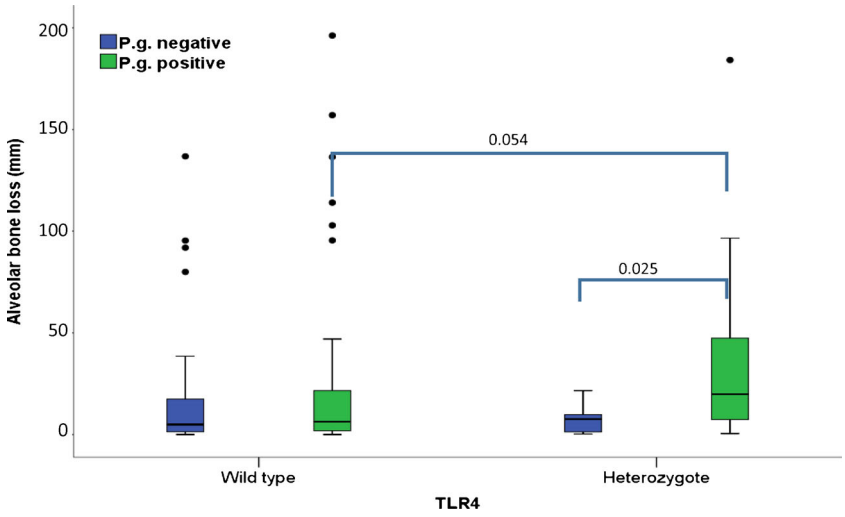

Fig. 2 Alveolar bone loss in subjects with wild type and heterozygote *TLR4* genotypes according to their *P. gingivalis* (P.g.) carriage. Significant differences between the groups are marked with *p* values above the connector lines. *Black dots* indicate outliers

were measured from saliva. However, the use of alveolar bone loss had some shortcomings, such as distortion and missing information on buccal and palatal sides of the teeth. Yet, readily available panoramic radiographs serve more efficiently than clinical periodontal measurements in large population surveys for evaluating periodontal health. *P. gingivalis* carriage was determined by using stimulated salivary samples. It was recently demonstrated that in terms of bacterial profiles, stimulated and unstimulated saliva samples give comparable results [23]. Moreover, the use of saliva in detection of oral bacteria has superiority against that of plaque samples from a few subgingival sites, since in the latter case the absence of the target bacterium may indicate the absence of its colonization on the selected region, despite its presence elsewhere in the oral cavity [17]. Finally, the reliability of saliva in genetic studies in humans has been successfully demonstrated before [24].

In the present study, the *TLR4* coding region Asp299Gly (rs4986790) was investigated. The frequency of *TLR4* Asp299Gly polymorphism has been found to be 18 % in another Finnish study population [18], which is close to our present finding (12.6 %). Previous reports on the associations of *TLR4* Asp299Gly with periodontitis are controversial. A high [24] or low [25] periodontitis prevalence has been associated with *TLR4* Asp299Gly SNP, while there are also studies showing no association between the *TLR4* Asp299Gly SNP and periodontitis [26, 27]. One explanation of the controversial results can be the simultaneous activation of TLR4 and TLR2 by LPS. The cooperative function of these TLRs may hinder the dysfunction of TLR4. Another explanation can be the role of pathogenic bacteria in the initiation of periodontitis. It was previously demonstrated that subjects with *TLR4* Asp299Gly SNP were more likely to be affected by osteomyelitis, a serious bone infection with an inflammatory character and relation to Gram-negative bacteria [28]. *TLR4* heterozygous subjects may be prone to infection-induced inflammatory bone destruction, where specific Gram-negative pathogens are involved in initiating the disease process. A recent study

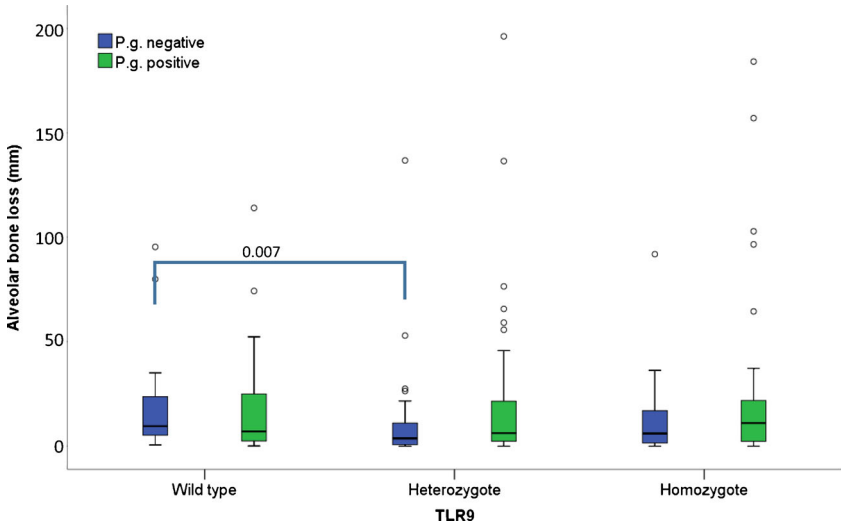

Fig. 3 Alveolar bone loss in subjects with wild type, heterozygote, and homozygote *TLR9* genotypes according to their *P. gingivalis* (P.g.) carriage. A significant difference between the groups is marked with a *p* value above the connector line. *Empty circles* indicate outliers

suggested that *TLR4* Asp299Gly polymorphism is protective against chronic periodontitis in an American population [17]. According to their results, there is a negative interaction between the carriage of *P. gingivalis* and *TLR4* Asp299Gly SNP. In a study on Dutch Caucasians, *P. gingivalis* showed an association between the *CD14*-260T/T genotype and periodontitis, while the frequency of the *TLR4* 299Asp>Gly gene polymorphism did not differ between the periodontitis and control groups [16]. Our findings are not in line with the results of these two studies. The contradiction can be attributed to the applied methods or ethnic background of the study populations; for example, the prevalence of *TLR4* Asp299Gly SNP was 5.0 % among Dutch periodontitis patients and controls, while it was 12.6 % in the present Finnish study population.

TLR9 is an endosomal PRR and major sensor for microbial DNAs [14]. SNPs in the *TLR9* gene have been related to the susceptibility to periodontitis, suggesting that *TLR9* plays a role in the pathogenesis of periodontitis [29, 30]. In a recent study, where *TLR9* knockout ($TLR9^{-/-}$) and *TLR9* wild type mice were infected with *P. gingivalis*, the *TLR9* knockout mice exhibited significantly less alveolar bone loss in comparison to the wild type mice [14]. In the present study, however, no significant difference was observed in alveolar bone loss between different *TLR9* genotype groups. Notably, *P. gingivalis*-negative individuals with *TLR9* heterozygotes had less alveolar bone loss than those with wild type. It can be speculated that in the absence of dysbiosis-regulating *P. gingivalis*, *TLR9* could protect from alveolar bone loss. The SNP in *TLR9* (rs187084) tested in the present study occurs in the promoter and affects the expression of the receptors. No information is available on the impact of this SNP on the pathogenesis of periodontitis; however, it functions as a susceptibility factor for rheumatoid arthritis [19].

In conclusion, the carriage of *P. gingivalis* in subjects with functional polymorphisms of *TLR4* is related to alveolar bone loss, whereas polymorphisms of *TLR9* can be protective in the absence of *P. gingivalis*.

Compliance with ethical standards The authors report no conflicts of interest related to this study. This study was partly funded by the Finnish Dental Society Apollonia (UKG) and University of Turku joint research grant fund and the Academy of Finland (EK, PP). The study protocols have been approved by the Ethical Committee for Epidemiology and Public Health of the Hospital District of Helsinki and Uusimaa, Finland (407/E3/2000). All participants gave a written informed consent.

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