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Research Report

Toll-like receptor–4 gene polymorphisms in Javanese aggressive and chronic periodontitis patients

Chiquita Prahasanti
Department of Periodontic
Faculty of Dental Medicine, Universitas Airlangga
Surabaya - Indonesia

ABSTRACT

Background: *Tool-like receptor-4 (TLR4) gene polymorphisms affect the ability of the host in response to pathogenic bacteria, and can also be associated with the severity of periodontitis. TLR4 gene polymorphisms (Asp299Gly and Thr399Ile) are ones of gene mutations that occur in patients with aggressive periodontitis.* ***Purpose:*** *To investigate the involvement of TLR4 gene polymorphism as a risk factor of aggressive and chronic periodontitis of Javanese population in Surabaya.* ***Method:*** *This research can be considered as an analytic observational study, with a case-control study design in patients with aggressive periodontitis and chronic periodontitis. DNA samples were derived from peripheral blood. TLR4 gene polymorphisms (Asp299Gly and Thr399Ile) were then observed by PCR-RFLP.* ***Result:*** *There was no TLR4 gene polymorphism (Asp299Gly) in the whole samples. And, based on the results of simple logistic regression analysis on TLR4 gene polymorphisms (Thr399Ile), mutants heterozygote and homozygote obtained had OR value about 0.25.* ***Conclusion:*** *In Surabaya, there was no heterozygote and homozygote mutant in TLR4 gene polymorphisms, (Asp299Gly) and (Thr399Ile), that can be considered as risk factors of chronic periodontitis.*

Keywords: *gene polymorphisms; TLR4; aggressive periodontitis; chronic periodontitis*

Correspondence: Chiquita Prahasanti, c/o: Departemen Periodonsia, Fakultas Kedokteran Gigi Universitas Airlangga. Jl. Mayjend. Prof. Dr. Moestopo No. 47 Surabaya 60132, Indonesia. E-mail: chiquita_prahasanti@yahoo.com

INTRODUCTION

There is a close relationship in periodontal disorder between genetic, environmental, patient age factor when exposed to the severity of the disorder, and the course of their illness. Individual responses to infection, however, vary widely, and genetic factor plays an important role in triggering abnormality, such as periodontitis. Host genotype may influence the composition of subgingival bacteria, and its data even state that host gene polymorphism affects the host response to infection. Changes in gene structure can have an impact on both response quality and polymorphism setting, and can also alter the patient's response mechanism.[1]

Aggressive periodontitis, a specific type of periodontitis due to its own character, is characterized by a progressive course of the disease with severe periodontal tissue damage. In many cases, the disease occurred in apparently healthy individuals can be considered as a multifactorial disease caused by periodontal pathogenic bacteria. The incidence and severity of the disease, moreover, is determined by the host response to infection. Inflammation that occurs in periodontitis is a complex process begun with tissue damage and continued with repair process.[2,3] Genetic and environmental factors have implications for the cause of periodontal disease. Patients with aggressive periodontitis seem to have abnormal immunological factors, expected to be influenced by genetic factors.[4]

Patients with aggressive periodontitis will have inadequate host response to periodontal pathogenic bacteria seen in the increased expression of a wide variety of immunological factors and genetic risk factors. *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*) bacteria were commonly found

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in aggressive periodontitis. Yang *et al*.[5] even stated that 84.3% of aggressive periodontitis was caused by *A. actinomycetemcomitans* serotype b bacteria found in all regions of aggressive periodontitis, especially in patients with aggressive periodontitis not treated, as well as in the domain which has a pocket inside (> 5mm). Lipopolisakarida (LPS) is a product of *A. actinomycetemcomitans* bacteria that will trigger TLR4. The initiation of *LPS* and *TLR4* signal was caused by a complex process, which involves several additional proteins.[6,7]

Toll-like receptors (TLRs) is suspected as the starting point of immunity in which extracellular environment factors continuously give information to the cells to respond to infection and to facilitate cellular responses through the top of signaling pathways in new gene transcription.[2] The mechanism of innate immunity has always responded quickly to infection that occurs in the patients' body and to the spread of bacterial pathogens in order to evolve an effective and adaptive immunity well.[8] TLR4 located on chromosome 9 (9q32-33) has complex regulation, involving specific different tissues and cells, and the regulation largely determines innate immune system.[9] Polymorphisms that occur in TLR4 gene will disrupt the function of TLR4 receptor against LPS germs in bodies, causing interference to the cell membrane transport and also to both ligand binding and protein interaction, as a result, the expression of *TLR4* protein will be affected.[10,11]

TLR4 actually has an ability to protect from inflammation, and there is a significant relationship between TLR4 gene polymorphism, Asp299Gly, and aggressive periodontitis in adult *Caucasian* population. Previous studies reported that TLR4 gen polymorphism in *Caucasian* was 5 percent.[12,13] Amino acid changes that occur in the extracellular domain of TLR4 are related to LPS that is hypo-responsive in human epithelial cells and alveolar macrophages in vitro. Functionally, it will make TLR4 hypo-responsive against lipopolysaccharide of periodontopathogen germs, *A. actinomycetemcomitans*, so it becomes less susceptible to infections caused by gram-negative bacteria. TLR4 can also be related to the severity of aggressive periodontitis. TLR4 polymorphism will disrupt the function of TLR4 receptor against LPS, then causing transport disorder to the cell membrane, ligand binding disruption, and also protein interaction interference, so the expression of TLR4 protein will be affected.[11,14] Thus, genetic aggressive periodontitis, especially genetic pattern of the host needs to be studied to determine the location of specific polymorphism gene. It is important that the number and type of modifying disease genes for the same disease may not be same in different ethnic population. Therefore, some studies should be done to reveal the etiologies that may involved. Thus, the aim of the study to investigate the involvement of TLR4 gene polymorphismas a risk factor of aggressive and chronic periodontitis of Javanese population in Surabaya.

MATERIAL AND METHODS

This research was an observasional analytic study with case control study design in patients with aggressive periodontitis and chronic periodontitis. Thus, polymorphism test must be conducted on TLR4 gene (Asp299Gly and Thr399Ile) by PCR-RFLP.

The subject for this study were 40 cases of aggressive, and 40 cases of chronic periodontitis as control group who visited the periodontic clinic, Faculty of Dental Medicine Airlangga University. All subject were Javanese and subject with a history of systemic disease or smoking were excluded from the study. The nature of the study was explained to all subject verbally and in writing and all signed a content form.

Genomic DNA derived from peripheral blood of patients was taken about 2ml. DNA isolation principle was conducted by lysing leukocyte cell membrane using lysis buffer solution, cell membrane lysis buffer (CMLB), DNA extraction was conducted by using lysis buffer solution, NMLB (Nuclear Membrane Lysis Buffer).

Primer sequence used in the reaction was TLR4 (Asp299Gly) used were forward 5'-AGCATACTTAGACTACTACCTCCATG-3 and reverse 5'-GAGAGATTTGAGTTTCAATGTGGG-3'. PCR condition had to be with pre-denaturation 95^0C for 3 minutes, and then 35 cycles were performed at a temperature of 94^0 C for 30 seconds, at a temperature of 62^0 C for 30 seconds, and at a temperature of 72^0 C for 30 seconds. Enzyme restriction endonucleases used were NcoI, which will produce two fragments 80bp and 22bp (G allele) and 102bp (A allele). TLR4 (Thr399Ile) used were forward 5'-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3' and reverse 5'-GGAAATCCAGATGTTCTAGTTGTTCTAAGCC-3'. PCR condition had to be be with pre-denaturation at 95^0 C for 3 minutes, and then a total of 35 cycles was performed at a temperature of 94^0 C for 30 seconds, at a temperature of 60^0 C for 30 seconds, and at a temperature of 72^0 C for 30 seconds. Restriction endonuclease enzyme used was HinfI, which will produce two fragments 121bp and 25bp (T allele) and 146bp (C allele). Finally, visualization of PCR products for TLR4 was conducted by agarose gel electrophoresis using 4% Agar at 120V, 70 MAMP, for 40 minutes.

RESULTS

Based on the entire samples of this research, there was no TLR4 gene polymorphism (Asp299Gly) found in patients with aggressive periodontitis and chronic periodontitis. In the other hand, there was an TLR4 gene polymorphism (Thr399Ile) found in the whole samples as seen in the following Figure 1.

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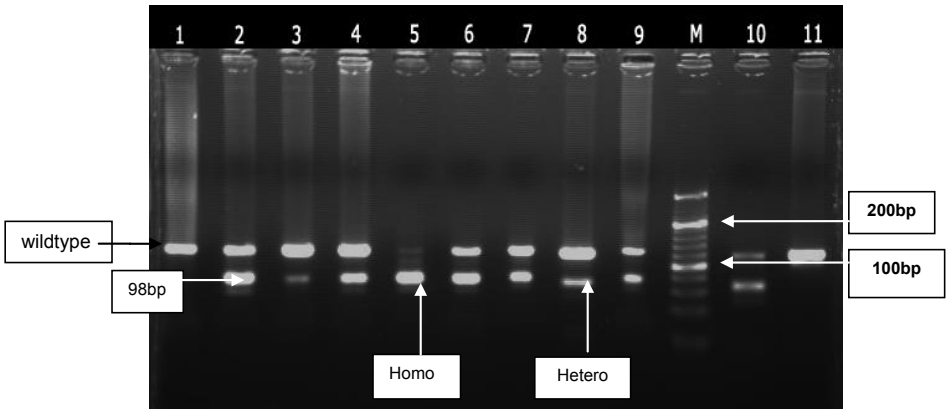

Figure 1. Results of PCR-RFLP in TLR4 gene (Thr399Ile).
Amplicon Line 1 and 11 at 124bp (normal homozygote); Amplicon Line 2, 3, 4, 6, 7, 8, 9, 10 at 124 bp + 98 bp + 26 bp (not visible) (heterozygote mutant); Amplicon Line 5 hydrolyzed at 98bp + 26bp (not shown) (homozygote mutant); Line M = DNA marker at 20bp.

Table 1. Distribution of TLR4 polymorphism genotype (Thr399Ile) in patients with aggressive periodontitis (AP) and chronic periodontitis (CP)

Genotype	Periodontitis			
	AP		CP	
	n	%	n	%
CC (allele 1)	20	(50%)	8	(20%)
CT (allele 2)	1	(2.5%)	0	(0%)
TT (allele 1+2)	19	(47.5%)	32	(80%)
Total	40	(100%)	40	(100%)

Table 2. Distribution of TLR4 gene alleles (Thr399Ile) in patients with aggressive periodontitis (AP) and chronic periodontitis (CP)

Allele	Periodontitis			
	AP		CP	
	n	%	n	%
C	59	(73.75%)	48	(60%)
T	21	(26.25%)	32	(40%)
Total	80	(100%)	80	(100%)

Distribution of genetic sequences of TLR4 gene (Thr399Ile) (genotype distribution and allele frequency) on the AP and CP can be seen in Table 1 and Table 2. Table 1 shows the frequencies of TLR4 genotype polymorphism (Thr399Ile) in patients with AP and CP. Based on the table, the frequencies of TLR4 genotype (Thr399Ile) were 50% CC (1 = wild-type allele), 47.5% CT (allele 1 + 2 = mutant heterozygote), and 2.5% TT (2 mutant allele homozygote). The frequency distribution, moreover, shows that the frequency of mutant heterozygote of TLR4 polymorphism (Thr399Ile) was 47.5% almost equivalent to the wild-type genotype (50%). Meanwhile, the frequency of the mutant allele (T) in patients with AP was 26.25% (Table 2). Based on these results, it can be said that aggressive periodontitis as variable of TLR4 gene polymorphisms (Thr399Ile), especially allele 1+ 2 and allele 2, was not a risk factor for aggressive periodontitis process.

Furthermore, the frequencies of TLR4 genotype (Thr399Ile) in patients with chronic periodontitis were 20% CC (allele 1 = wild type), 80% CT (allele 1 + 2 = mutant heterozygote) and 0% TT (not found 2 = mutant allele homozygote). The frequency distribution shows that the frequency of mutant heterozygote of TLR4 polymorphism (Thr399Ile) was 80%, 4 times higher than that of the wild-type genotype (20%). And, the frequency of the mutant allele (T) in this research was 40% (Table 2), so the variables of TLR4 gene polymorphisms (Thr399Ile) in CP, especially allele 1+ 2 and allele 2, can be considered as risk factors (4x normal) of CP abnormalities.

DISCUSSION

TLR4 gene has two missense polymorphisms, namely Asp299Gly and Thr399Ile, affecting extra cellular proteins and essential to reduce both LPS signal strength and inflammation. In this research, polymorphism occurs only in TLR4 gene (Thr399Ile). TLR4 gene polymorphism (Asp299Gly/Thr399Ile) can cause susceptibility to aggressive periodontitis. It means that polymorphisms occurred will increase the severity of disorder, and will also cause variations in the structure of tissues (innate immunity), antibody response (adaptive immunity), as well as inflammatory mediators (non-specific inflammation).[16]

The occurrence of polymorphism, moreover, is associated with response to endotoxin exposure. In other words, TLR4 polymorphism (Asp299Gly and Thr399Ile) can affect the extracellular domain of TLR4 protein in term of its expression and function causing a failure to respond to LPS since TLR4 will fail to capture LPS signal. Changes in the extracellular domain and the amino acid, playing a role in receptor function to capture the LPS signal, then will be disrupted and will result in a reduced ability to inhibit the

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occurrence of inflammation.[10,13] Mutations that occur will facilitate the occurrence of sepsis, leading to the failure of LPS signaling in individuals with mutant homozygote and heterozygote. The type and number of genetic abnormalities in the same disease manifestations that occur are not similar to different ethnic populations ranging from 0.1% to 15%.[12] Therefore, various polymorphisms that occur are likely to have contributed and considered as one of the risks to a person's susceptibility to the severity of aggressive periodontitis. It means that genetic and environmental factors have implications in the etiology of periodontitis.[17,18]

Based on the data in the previous researches, it is known that the allele frequency of TLR4 polymorphisms, especially Asp299Gly and Thr399Ile, is very different between the populations of Asia, Africa and Caucasia. A research on population related to geography even shows that the frequency of TLR4 polymorphism, especially Asp299Gly haplotypes, in African populations was 10-20 times different from the frequency of TLR4 polymorphisms, especially Asp299Gly, Thr399Ile and TLR4 Asp299Gly/Thr399Ile haploptype in Asian populations.[16] A research conducted by Laine *et al*.[19] aimed to see the relationship of TLR4 polymorphism and periodontitis severity shows that the prevalence of polymorphisms, especially Asp299Gly and Thr339Ile, was 5% in Dutch citizens belonging to the Caucasoid race.

The results of studies analyzing the correlation of gene polymorphism in chronic periodontitis and aggressive periodontitis, unfortunately, are less clear although TLR4 is biologically an important gene considered as the etiology of periodontal disease.[4] Genetic factors influencing the immune response to bacterial infection play an important role in individual susceptibility to inflammation caused by periodontal pathogens. It is because TLR4 plays a role as a receptor in responding to LPS signal from gram-negative bacteria.

Polymorphisms that occur will increase the severity of abnormality. According to genetic variants that occur can produce variations in the structure of tissue (innate immunity), antibody response (adaptive immunity), and inflammatory mediators (non-specific inflammation). [20] In other words, susceptibility to periodontitis is influenced by genetic and environmental factors, such as the influence of periodontopathogen bacteria. It means that genetic factors influencing the immune response to bacterial infections play an important role and affect a person's vulnerability reflected in the increased expression of TLR4 protein. Therefore, impaired function of TLR4 will disrupt homeostasis in a person's body so that the body is easy to be exposed to a disease.

Conclusion, in Surabaya there was no heterozygote and homozygote mutant in TLR4 gene polymorphisms, (Asp299Gly) and (Thr399Ile) in Javanese population, that can be considered as risk factors of chronic periodontitis. Therefore, others factors that may involved in the etiology of chronic periodontitis should be investigated.

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