
The analysis mutation of the CARD 15 gene variants in chronic periodontitis

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Abstract: Purpose of this study was to determine the variant of CARD 15 gene mutations with periodontitis chronic. Identified by using polymerase chain reaction (PCR) and followed by using restriction fragment length polymorphism analysis (RFLP). CARD 15 gene mutation with chronic periodontitis were found heterozygote mutation and homozygote mutation variants base on statistical analysis there were significant with control group ($p=0,005$) and also was founded genetics variation that changed the composition of C → T nucleotide at codon 802 in exon 4 amino acid, from alanine to valine.

Keywords: CARD 15 Gene, Mutation, Chronic Periodontitis, Heterozygote, Homozygote

1. Introduction

Chronic periodontitis is a chronic inflammatory disease that causes damage to the teeth supporting tissues, the pathogenesis of multifactorial disease caused by bacteria periodontopathogen attached to the teeth referred to as plaque, other factors: age, race, gender, socioeconomic, smoking habit, systemic disease, hormonal and genetic. The accumulation of bacterial metabolism on the oral hard tissue surface is considered as the primary cause of chronic periodontitis. More than 400 species have been isolated and characterized in dental plaque. Accumulation of bacteria on the teeth stimulate the inflammatory response is reversible in gingival tissue.^(1,2)

Periodontal pathogen bacteria commonly found in chronic periodontitis is *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans*. The products of bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA) which will cause tissue damage periodontium.^(3,4)

Microorganisms that have been found as the main cause of periodontal disease will, but endurance and immunity a person also contributed to the occurrence of chronic periodontitis. The results of another study report that genetic differences between individuals also play an important role in susceptibility to the occurrence of chronic periodontal

disease.⁽⁵⁾

Clinical and scientific fact showed that genetic factor is an important determinant of periodontitis susceptibility and the pathogenesis. It is supported by the study in human and animal that showed influence of genetic factors inflammatory response and immune system generally, and periodontitis specifically. Individual responds differently toward environmental change and those different response is influenced by the individual genetic profile. Various gene form (allele variant) could produce variation of tissue structure (acquired immune), antibody response (adaptive immune), and mediator inflammatory (non-specific inflammatory). Allele that is found in different gene locus may influence the susceptibility of periodontitis.⁽⁶⁾

Genetically, human body could detect the infection through). Caspase Recruitment Domain 2 (CARD 15) is the initial protein that function in bacterial detection and recognize the presence of lipopolysaccharide protein whether it is a negative gram or positive gram through introduction of peptide muramyl in the leucine-rich bacteria that is repeated on CARD 15.^(7,8)

The binding of peptide muramyl and CARD 15 will activate nuclear factor κB (NF-κB) and induce apoptosis, whereas this factor is one of transcription factor that is highly

effective in inflammatory support cytokines secretion. CARD 15 mutation will reduce body's capability to eliminate pathogen bacteria.⁽⁹⁻¹¹⁾

2. Materials and Methods

2.1. Collecting Samples

This research was conducted in Periodontology Department of Oral and Dental Hospital, Dentistry Faculty, Hasanuddin University, Makassar. Total sample was 162 people, there are 81 samples with diagnose of chronic periodontitis, and 81 samples as control group without chronic periodontitis. The age of the subject was 30 -60 years old. The age group is a reason to minimize the influence of age on the predisposition of individual genotip. Written and oral informed consent was obtained from all subject based on the Agreement of Ethical Committee Medical Faculty Hasanuddin University.

2.2. DNA Extraction

Boom methods⁽¹²⁾ were used for extraction and DNA purify. A total 2-3 cc of venous blood sample was added into the eppendorf tube with 900 μ l buffer lisis L6. (Hatta M et al 2007). The blood sample was rotated in centrifugated for 15 minutes and added 40 μ l of cellite suspension (diatoms) which already homogenized in vortex shaker. This solution was rotated in the Gerhardt at 100 rpm and 12.000 rpm for 15 second, respectively.

The formed Supernatant were separated with micropipe and rinsed, it was added by 1 ml Buffer L2, homogenized and centrifugated at 12.000 rpm for 15 seconds. The Rinsing process using Buffer L2 was done twice. Then, 1 ml etanol 70% was added then homogenized and centrifugated at 12.000 rpm for 15 seconds. Rinse with etanol 70% twice.

The supernatant was mixed with 1 ml acetone, homogenized and centrifugated. After that the supernatant removed and the tube was left opened. Then the tube was placed in the oven of 55°C for 10 minutes until it is dried, and added with 80 μ l Buffer TE solution, homogenized [up-down] for 15 times, centrifugated for 30 second to tied the DNA. The sample was homogenized with vortex shaker [up-down], centrifugated for 30 second. The formed supernatant was placed in the eppendorf tube and labeled.

2.3. PCR Amplification

The sample were placed in the PCR tube which contained 100 mg DNA genome mixed with PCR buffer (10mM Tris-HCl pH 8,3; 50 mM KCl; 1,5 mM MgCl₂) 200 uM dNTP, 1,25 enzyme DNA polymeraze and 10 pmol of primary pairs (which is, sense: 5'CCCAGCTCCTCCCTCTTC3' and Anti-sense 5' AAGTCTGTAATGTAAAG-CCAC 3'). The samples were incubated in a PCR machine, initial denaturation were done at 95°C for 5 minutes, followed with 35 PCR cycles which included denaturation at 95°C for 45 seconds, annealing at 53°C for 40 seconds, and extension at 72°C for 30 seconds.

2.4. Restriction Fragment Length Polymorphisms (RFLP) PCR

The PCR product of NOD 2 gene DNA was done using restriction fragment enzyme length polymorphism-polymerase chain reaction (RFLP-PCR) method by BamHI enzyme

2.5. Gel Electrophoresis

To recognize if the DNA target amplified in the correct way, the PCR product was placed in 2% agarose gel which already added with TAE 10x solutions mixed with ethium bromide, two μ l product samples of loading buffer (0,25% of bromophenol blue, 40% b/v sucrose) and 4 μ l dH₂O were mixed with 2 μ L PCR product samples. This solutions were mixed homogenically and aspirated 8 μ l to be placed in the agarose gel slot. The electrophoresis proces started at 220 volt for 40 to 45 minutes. The visualized DNA ribbons which marked by ethium bromide under the UV lights were documented and recorded. Then, 5 μ l PCR amplification result and 2 μ l buffer loading were mixed and added into the 1.5 agarose gel and ethidium bromide.

2.6. DNA Sequencing

DNA sequencing by Macrogen methods.

2.7. Statistical Analytic

All data was analyzed using a Chi square test

3. Result

3.1. Characteristics of Sample

Table 1. Distribution sample based on age

Variable	N	Sample (mean) \pm SD	N	Control (mean) \pm SD
Age	81	38,90 \pm 9,24	81	37,61 \pm 11,42

SD=Standard Deviation

The mean age of chronic periodontitis samples was 38.90 years old and the control group without chronic periodontitis was 37,61 years old (Table 1).

Table 2. Distribution severity of periodontal disease of the sample

Parameter	Sample	Mean \pm SD	Control	Mean \pm SD
OHI-S	81	1,63 \pm 0,96	81	2,29 \pm 0 69
PPD (mm)	81	4,54 \pm 1,27	81	-
CAL (mm)	81	3,02 \pm 1,48	81	-
Edentulous	81	2,48 \pm 2,69	81	1,06 \pm 1,07
Caries	81	2,17 \pm 2,30	81	2,04 \pm 1,69

Subject was assessed based on clinical parameter: probing pocket depth (PPD), attachment destruction/clinical attachment loss (CAL) on 6 surfaces of teeth (mesiobuccal, mesiolingual/palatal, distobuccal, distolingual/palatal, buccal and palatal/lingual, number of missing teeth (edentulous) and caries. (Table 2)

3.2. Result of RFLP- PCR

RFLP PCR examinations performed on 81 samples of patients with chronic periodontitis and 81 controls. PCR test was performed 162 samples of peripheral blood, using the BamH1 enzymes to determine the CARD15 gene mutation in exon 4 examined the RFLP method.

The electrophoresis showed of 162 samples obtained

observations of each band of DNA fragments, determined of position in bp (basepair) based on distance bands. RFLP-PCR result obtained with CARD15 gene variants showed homozygote mutation with two bands (104 bp and 83 bp) and heterozygote mutation with three bands (187bp, 104bp, and 83bp) (fig 1-2).

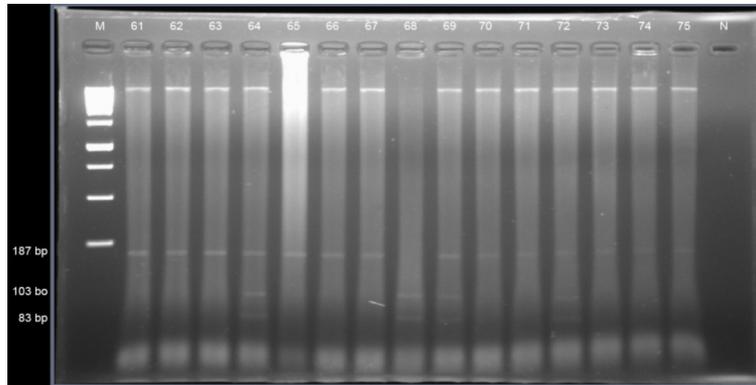


Figure 1. The RFLP-PCR Method with one of the case group

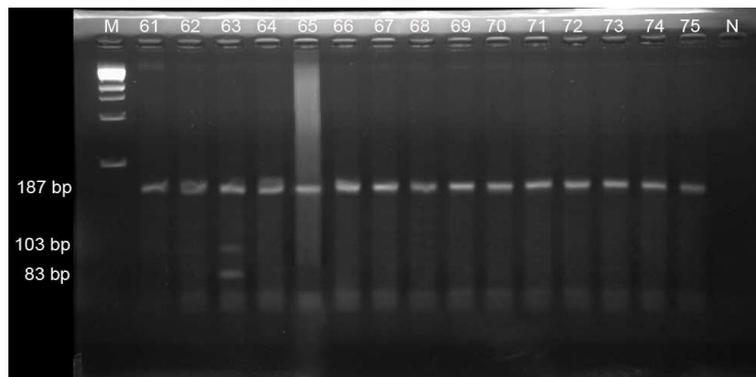


Figure 2. The RFLP-PCR Method with one of the control group

Table 3. Examination samples chronic periodontitis and control group without chronic periodontitis results with RFLP-PCR

NOD2 Gene	Case		Control		p
	N	%	N	%	
Not Mutation	71	87,7	80	98,8	0,005
Mutation	10	12,3	1	1,2	
Total	81	100	81	100	

Table 3 showed there was 12,3 % of periodontitis subject that had been mutated and in control group only 1,2 %, were statistically significant difference (p=0,005).

3.3. CARD15 Gene Mutation Analysis by Sequencing

Sequencing performed to ensure the order of nucleotides in CARD15 gene mutation of chronic periodontitis, based on gene sequences were confirmed from the National Center for Biotechnology Information (NCBI, Bethesda,MS,USA). The primer sequences used are forward: CAGTCTCGCTTCCTCAGTACC and reverse primer sequence: AGTGTCCGCATCGTCATTG and BamH1 enzymes will cut the sequence nucleotide C / ATC . figure 3-5.

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101130-29_K01_6-CARD 15_For.ab1Not mutated
gtgtctgctcagtctcgctt cctcagtacc tatgatggag cagagacgct ctgcctggag
gacatatacacagagaatgt cctggagggtc tgggcagatg tgggcatggc tgcacccccg
cagaagagcccagccacct gggcctggag gagctcttca gcaccctgg ccacctcaat
gacgatgctggacactgtgct ggtggtgggt gaggcgggca gtggcaagag cacgctcctg
    
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Figure 3. Nucleotide Sequence result of the samples was not mutation

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>101130-29_K01_15-CARD 15_For.ab1 HETEROZYGOTE MUTATION
gtgtctgctcagtcctcctt cctcagtacc tatgatggag cagagacgct ctgctggag gacatataca
cagagaatgt cctggaggtc tgggcagatg tgggcatggc tgcaccccc cagaagagcc
cagccaccct gggcctggag gagctcttca gcaccctgg ccacctcaat gacgatcgg
acactgtgct ggtggtgggt gaggcgggca gtggcaagag cacgctcctg
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Figure 4. Nucleotide Sequence of sequencing result heterozygote mutation

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>101130-29_K01_68-CARD15_For.ab1 HOMOZYGOTE MUTATION
gtgtctgctcagtcctcctt cctcagtacc tatgatggag cagagacgct ctgctggag gacatataca
cagagaatgt cctggaggtc tgggcagatg tgggcatggc tgcaccccc cagaagagcc
cagccaccct gggcctggag gagctcttca gcaccctgg ccacctcaat gacgatcgg
acactgtgct ggtggtgggt gaggcgggca gtggcaagag cacgctcctg
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Figure 5. Nucleotide Sequence of sequencing result homozygote mutation

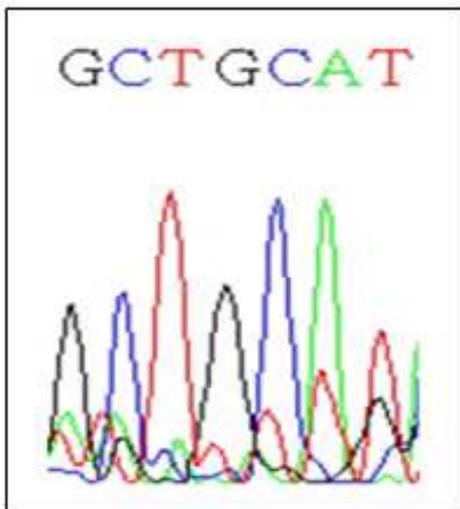


Figure 6. Not Mutated sequencing result

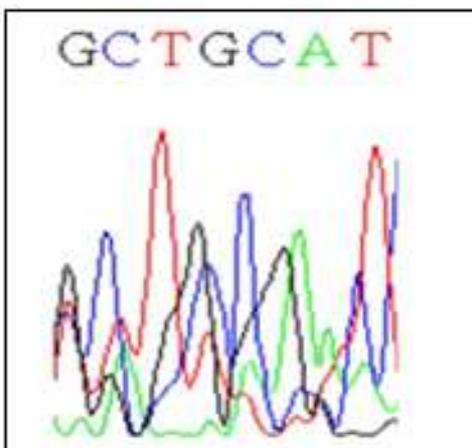


Figure 7. Heterozygote mutation sequencing result

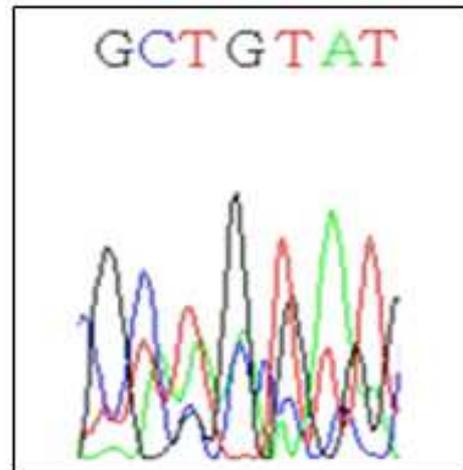


Figure 8. Homozygote mutation sequencing result

Sequencing followed by BLAST N an analysis showed not mutated and heterozygote mutation 100 % homology with the gene bank, whereas the homozygote mutation was found changes in composition C → T nucleotide of the codon at position 802

4. Discussion

The chronic periodontitis subject had OHI-s, PPD, CAL and edentulous and caries. This is relevant with the previous research that showed the role of OHI-s and caries toward the occurrence of chronic periodontitis. Edentulous showed the tooth loss history that also reflect high caries or poor oral hygiene. Haake suggest that the primary cause of periodontal disease is bacterial irritation that is caused by plaque.(5) Most of inflammatory periodontal disease is caused by bacterial infection. Major cause of periodontal disease is kolonized microorganisms on teeth surface (bacterial plaque and their product). Plaque accumulation is associated with the increase

of bacterial amount. But, the presence of bacterial are not enough to initiate the disease. The susceptibility of host immunity against periodontal disease play a role in the occurrence of periodontal tissue inflammation.⁽¹³⁾

Mean age of chronic periodontitis patient was 38.90 years old and the group of chronic periodontitis patient was consist of more woman than man. It is contrast with the longitudinal study about the risk factor of periodontitis by Taize⁽¹⁵⁾, that showed the higher frequency of this disease in man (35% higher). In some literature, it is mentioned that the frequency of chronic periodontitis is higher in man than woman. It is told that smoking habit is a predisposition factor of periodontitis. According to Manson, the risk of periodontal disease in smoking woman in the age of 20-39 years old and smoking man in the age of 30-59 is two times higher than those who not smoke at all. Some authors have made hypotesa that smoking could cause vasoconstriction of gingival because nicotine or another component of cigarette. Clarke et al,⁽¹⁶⁾ studied the direct effect of nicotine to the gingival microvascular and reported that perifer vasoconstriction lead to nicotine absorption in the gingival blood flow. So, the difference of this research result possibly caused by the exclusion of smokers in the sample requirement and patient that come to the hospital of Oral and Dental Dentistry faculty Hasanuddin University is dominated by woman.

The result of this study provide a relation between gene mutation CARD15 with chronic periodontitis insiden. Chronic periodontitis is a multifactorial inflammatory disease, resulted from complex interaction between bacterial and specific host. Several study at least since ten years ago support the hypothesis that immune host reaction, quality and quantity of response to inflammation is controlled by gene.⁽¹⁷⁾

There were a lot of supportive evidences to claim that gene play a role of the advance of periodontal disease, genetic factor is important to periodontitis incidence.⁽¹⁸⁾

This research found CARD 15 gene mutation in chronic periodontitis group was 12 (12,3%) and in the control group was found only 1 person. (1,2 %). This study also proves the existences of a significant relationship with the occurrence of CARD15 gene mutation of chronic periodontitis. (Table 3). Statistical test using Fisher Exact Test found a significant relationship CARD15 gene mutation to occurrence of chronic periodontitis.

CARD15 gene was identify as a gene which has a role to increase susceptibility the inflammatory disease. CARD15 gene coded intracellular protein that implicated in innate immune response, which followed by bacterial induction in inflammatory response⁽¹⁷⁾

There was a relation between CARD15 gene mutation as one of risk factor in chronic periodontitis incidence (p= 0.005).

One of the electrophoresis analysis in the subject group did not showed the restriction remains mutation at position 183 bp whereas 9 persons experiencing chronic periodontitis with heterozygote mutation, no change electrophoresis genotyping and the results showed restriction position of

187bp, 104bp,83bp, also found that a person with chronic periodontitis who had homozygote mutations and results showed restriction position of 103bp,83bp (Fig 1), that were indicates that nucleotide sequeance can be recognized by restriction enzyme BamHI enzyme. The results of the sequencing GCTGCAT nucleotide changes to GCTGTAT codon at position 802 indicates the type of variation of point mutation C / ATC which encodes amino acids from alanine to valine.

As conclusion, CARD 15 gene mutation with chronic periodontitis was found to have heterozygote mutation and homozygote mutation variants, and also found genetics variation that changed the composition of C → T nucleotide at codon 802 in exon 4 amino acid changed from alanine to valine.

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