Vitamin D Receptor Gene Polymorphism as a Risk Factor for Chronic Periodontitis

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Submission date: 21-May-2021 01:41PM (UTC+0800)
Submission ID: 1590423446
File name: Manuscript_-_The_Polymorphism_of_Vitamin_D_Receptor_20210521.pdf (154.29K)
Word count: 3562
Character count: 17906

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Abstract:

Background: This study aimed to investigate *Vitamin D Receptor (*VDR*)* gene polymorphism as one of the risk factors associated with chronic periodontitis (CP) and find out the effect of VDR gene polymorphism on phenotypic chronic periodontitis.

Materials and methods: This study is a case-control design in 162 adults who were divided into two groups: patients with CP (case group) and patients without CP (control group). Venous blood and DNA were obtained from individual samples. The polymorphism was determined based on the endonuclease restriction in the exon 9 (TaqI) by using the method of Restricted Fragment Length Polymorphism-Polymerase Chain Reaction (RFLP-PCR) and DNA sequencing. The data were analyzed using an independent T-test and Fisher exact test. Odds ratio (OR) was used to calculate the risk of VDR gene polymorphism in chronic periodontitis.

Results: VDR gene polymorphism was detected in CP with TT genotype 86.4%, Tt genotype 12.4%, and tt genotype 1.2%. The case group with TT and Tt genotype has OR 12.5 (95% CI: 1.6-99.8) to get CP compared to the control group ($P<0.05$).

Conclusion: VDR gene polymorphisms (TT and Tt genotype) are the risk factors associated with the individual susceptibility to CP.

Keywords: Polymorphism, vitamin D receptor, chronic periodontitis, risk factor.

Introduction

Chronic periodontitis (CP) is including as one of the most common inflammatory diseases affecting the tooth-supporting tissues characterized by progressive resorption of the alveolar bone (1-3). CP has been accounted as one of the ultimate causes of tooth loss in adults that will affect their quality of life if left untreated (4-6). CP is initiated by bacterial plaque on the tooth surface and can be compounded by multiple factors such as unbalance of the periodontal pathogen, host immunity, environment, as well as local and systemic factors (7, 8). Most evidence has emphasized that genetic factor has a vital role in disease's pathogenesis and influences the unique reaction that characterizes the susceptibility of each individual (1, 5). Approximately half of the clinical severity in CP may be associated with the host genetic (3, 8). However, the genetic effects may differ from different ethnicities due to the diversity of people in a population (3, 7). Hence, understanding this disease's pathophysiology on a genetic basis is highly requisite for early detection and diagnostic of the disease.

Over the past decade, considerable effort has been done to reveal the influence of various genetics on the severity of different diseases such as genes that code for interleukin-1 (IL-1) (9), tumor necrosis factor-α (TNFA) (10), interleukin-10 (IL10) (11), interleukin-4 (IL4) (12), and interleukin-4 receptor-α (IL-4RA) (13), Fc gamma receptor (FcγR) (14), cluster of differentiation-14 (CD14) (15), and Vitamin D receptor (VDR) (16, 17). The VDR, in particular, is a promising candidate for its role in periodontitis since it affects bone metabolism and immunological function. (4, 18). The VDR can act as a transcription regulator and occur in various cell types (2). It is located on chromosome 12 q and has 14 exons, 6 of which are located in the 5 region which is not translated (1a-1f) (7, 18). Region 3 untranslated of the VDR gene comprises a polymorphism cluster in TaqI, ApaI, and BsmI (19, 20). If the VDR gene polymorphism affects the degree or

function of VDR, then there is a high possibility that this polymorphism is critical in the pathogenesis of systemic diseases related to bone tissue specifically periodontal disease (19, 20).

In many studies, authors have tried to elucidate the link between VDR gene polymorphism and the pathogenesis of disease through a series of characterized VDR gene polymorphisms including FokI, ApaI, TaqI, and BsmI (2, 3, 5). To date, a relation has been pointed between susceptibility of periodontal disease and a certain number of single-candidate gene polymorphisms (21). Besides, some studies have also shown a connection between periodontal disease and vitamin D (2, 22, 23). Although extensive evidence have been obtained, only a few of them that focused on the association between the risk of chronic periodontitis and VDR variants. In addition, it is still indistinct to draw unequivocal conclusions whether there is a relationship between VDR gene polymorphisms and susceptibility of CP. In this regard, we attempt to study the correlation between VDR gene polymorphism especially on exon 9 (TaqI) and the occurrence of CP through a case-control study in Makassar based population.

Materials and methods

Subjects

Subjects were recruited in the Periodontology Department of Dental Hospital, Hasanuddin University, Indonesia after obtaining the approval from Ethics Commission for Biomedical Research in Humans, Faculty of Medicine, Hasanuddin University (0189/H.04.8.4.5.31/PP36-KOMETIK /2010). The inclusion criteria for the subjects including patient aged 25-60 years old, patient has no diabetes, tuberculosis (TBC), hepatitis and human immunodeficiency virus (HIV), patients with no smoking habit, patient who does not get anti-inflammatory/contraception and does not pregnant/breastfeeding. Prior to the study, written informed consent was filled by all subjects.

A clinical examination including the periodontal pocket deep (PPD) and clinical attachment loss (CAL) was performed in all subjects. The study group is divided in two groups; patients with CP (case group) and patients without CP (control group).

Laboratory Analysis

The laboratory analysis was conducted in the Laboratory of Immunology and Molecular Biology, Faculty of Medicine, Hasanuddin University, Indonesia. The genomic DNA was isolated from peripheral leukocytes in accordance with the standard protocol. The polymorphism was determined based on the endonuclease restriction in the exon 9 (TaqI) of the examined VDR gene by using the method of Restrictied Fragment Length Polymorphism-Polymerase Chain Reaction (RFLP-PCR) and direct sequencing.

Examination Method

As much as 0.5-1cc venous blood was isolated, extraction and purification of DNA was done with Boom method (Hatta M, Smits HL, 2002). L6 900 μl buffer solution was inserted into 1.5 tubes and 100 μl of patient blood was added, then homogenized for 30 minutes. A 20 μl diatom suspension was added to the tube. The mixture was vortexed and stirred using a gyrotary shaker, speed 100 rpm for 10 minutes. The mixture was vortexed again using a centrifuge in an Eppendorf microcentrifuge for 15 seconds with 12,000 rpm speed.

The supernatant was washed 2 times using 1 ml of washing buffer L2. The washing buffer L2 was added as much as 1 ml of vortex and centrifuge for 15 seconds, then the supernatant was sucked. The supernatant was washed again with 1 ml of 70% ethanol twice, vortexed and centrifuged for 15 seconds. The acetone in the supernatant is separated by opening the vial cover and heated at 56 ° C in a water bath or by using a dry block heater for 10 minutes. 60 ml of TE

elution buffer was added, then evenly vortexed so that the sediment from the suspension could dissolve. The vials were incubated for 10 minutes at 56 °C in the water bath.

The vial was centrifuged at 12,000 rpm for 30 seconds and carefully extracted about 40-50 μl from the supernatant and put into a new vial, with the tip of the pipette not touching the sediment from the suspension. Re-added 40 μl TE of elution buffer into the diatom sediment and re-cortex so that the sediment will dissolve in the TE elution buffer. The vials were incubated again in the water bath for 10 minutes at 65 ° C. At the end of the Boom procedure a small amount of diatom will be obtained (about 1 μl of diatom suspension in 100 ml TE of elution buffer), this amount will have no effect on the PCR results of the sample. The extract can be stored at -20 ° C or -80 ° C.

Amplification on the PCR Machine

Amplification on the PCR machine was done in 32 cycles, each cycle was subjected to denaturation for 1 minute with a temperature of 94ºC, annealing for 1.5 minutes at 59ºC and, extension for 2 minutes at 72ºC. After completion of 30 cycles, chemidians were followed by heating at 72ºC for 7 minutes. The amplification results were analyzed using electrophoresis in agarose gel.

RFLP- PCR procedure

VDR gene polymorphisms will be detected on exon 9 using specific primers; forward (CTGGGGAGCGGGGAGTATGAAGGA) and reverse (GGGTGGCGGCAGCGGATGTA). DNA amplification was used for RFLP, using the TaqI restriction enzyme. As much as 22.5 μl of PCR mix was put and added with 2.5 μl of primer and 2.5 μl of DNA extraction with a final volume of 25 μl, then paraffin was added to prevent evaporation and incubated at 37 °C for 1 hour.

After amplification, 5 μl of PCR amplification results and 2 μl of loading buffer were mixed and put into 1.5% agarose gel molds which had been given Ethidium Bromide. The gel is soaked in a container containing a TBE buffer. Then the electrophoresis was run with a steady voltage of 80 volts for 1 hour. After the electrophoresis process is completed, the gel is removed then observed under UV light. The results of observations of each DNA fragment/band were determined in its position in bp (basepair) based on the distance of the marker / ladder bands. Fragment bands at different distances from the sample indicate the genotypic differences of the VDR gene polymorphisms. Everything that results from this difference is marked with the letter t (there is a restriction area) or the letter T (there is no restriction area). Genotype analysis based on bands on agarose under UV light:

- (TT): there is no restriction area in 1398 bp
- (Tt): there is a restriction area in (946 + 452 bp) and 1398 bp.
- (tt): there is a restriction area in 946 bp and 452 bp.

DNA sequencing

Direct sequencing were carried out at Macrogen South Korea. Sequencing was performed to prove that there was a change in the nucleotide base arrangement of the exon 9 VDR gene. The Basic Local Alignment Search Tool (BLAST) was used to analyze the sequencing results based on the NCBI data base.

Statistical Analysis

The program of SPSS version 11.5 was utilized for data analysis. Fisher's exact test was performed to see the relationship of VDR gene polymorphisms with the incidence of chronic

periodontitis and calculating the odds ratio (OR) variables as risk factors for chronic periodontitis. The independent T-test was done to see differences in phenotypic of chronic periodontitis.

Results

Distribution characteristics of subjects

The study consisted of 162 people that divided into two groups; 81 subjects with chronic periodontitis (case group; 14 males and 67 females) and 81 subjects without chronic periodontitis (control group; 38 males and 43 females). The details characteristics can be seen in Table 1. Based on the result, we found that subjects with chronic periodontitis have a higher oral hygiene index score-simplified (OHI-S), edentulous, and caries compared to the healthy subjects.

VDR Gene Polymorphisms

The VDR gene polymorphism gives three types of genotype including TT (1 band), tt (2 bands), and Tt (3 bands). In Figure 1a and Figure 1b, we can see that there is no restriction area in TT genotype (1398 bp) while in Tt genotype, some restriction areas was seen in 946 bp, 452 bp, and 1398 bp. In tt genotype, there are two restriction areas in 946 bp and 452 bp. Figure 1c represents the quantification of genotype VDR in both groups. In case group, the TT genotype is higher (86.4%) compared to both Tt (12.3%) and tt (1.2%) genotype. In the control group, we found 98.8% of TT genotype, 1.2% of Tt genotype, and no tt genotype.

Table 2 highlights the relationship between genotype VDR with the incidence of chronic periodontitis. It was seen that in the case group, there are 86.4% of TT genotype and 13.6% Tt/tt genotype. In the relationship analysis of genotype VDR with the incidence of chronic periodontitis, we discovered that case group with TT and Tt genotype has OR 12.5 (95% CI: 1,6-99,8) to get chronic periodontitis compared to the control group (P<0.05).

Table 3 shows the comparison between genotype VDR in chronic periodontitis based on the OHI-S, PPD, and CAL. While Figure 2 represents the comparison of the severity of chronic periodontitis based on the genotype VDR. In Table 3 and Figure 2 we can see that the mean of OHI-S for the periodontitis patients in TT genotype (2.71) was higher than Tt/tt genotype (2.12), yet in statistical view, this difference is not significant ($P > 0.05$). For both PPD status and CAL, Tt/tt genotype has a higher mean (PPD 5.14 and CAL 4.41) compared to TT (PPD 3.52 and CAL 2.80). Both have a statistically difference with *P* value 0.006 and 0.001, respectively.

Polymorphism Analysis of VDR Gene by Sequencing

DNA sequencing was carried out to prove a change in the sequence of nucleotide VDR. Figure 3 shows the sequencing results of three types of genotype VDR; Figure 3a for (TT) genotype, Figure 3b for (Tt) genotype, and Figure 3c for (tt) genotype. It was found that, there is a chance in the nucleotide sequence for tt genotype for codon 352 with the initial sequence of AGGTCGA to AGGCCGA (Figure 3c). It means that there is a nucleotide substitution from T to C (GTC to GCC) with the change of valine amino acid (GTC) to alanine (GCC).

Discussion

Currently, research in periodontology has been much focused on the influence of genetic factors on the individual susceptibility to chronic periodontitis. It has been presumed that the early onset of periodontitis can be acquired hereditary in either autosomal recessive or autosomal dominant fashion (13, 14). In this study, to investigate the VDR gene polymorphism as a risk factor associated with CP, the presence of polymorphisms on exon 9 VDR genes was determined and followed by DNA sequencing to prove the changes in nucleotide. In addition, clinical features of periodontal tissue damage in the form of PPD and CAL were also examined. Based on the results,

we found that the frequency of genotypes Tt and tt (t allele) is smaller than that of the TT genotype (T allele) in both CP and control groups. This can be explained by the fact that the population in Makassar, Indonesia is a part of the Asian race with a minor presentation of Tt and tt genotype in the VDR gene. Supporting our results, a study by Zmuda JM et al (2000) found that the frequency of Tt and tt genotypes was only 2% in Asians, 5% in African and Americans, and 17% in Caucasians. Similarly, research by Sun et al (2002) and Tachi et al. (2003) consecutively reported that t allele was found only 4% in Chinese ethnic and 11% in Japanese ethnic. Furthermore, a small number of t alleles in the Asian race also found by Zhao et al (2004) with a presentation of 5%. C

The differences in the frequency of genotype or allele in the population can be explained with the concept that all polymorphisms begin as mutations that occur as a result of DNA damage, then the frequency of allele grows in the population and becomes pure polymorphisms. Thus, the difference in the allele frequencies between ethnicities tends to be influenced by evolutionary processes and genetic traits from one population. It has been well described that periodontitis considered a complex multifactorial disease. As with other chronic diseases, typical for this disease in humans are having a relatively mild phenotype. The phenotype of a complex disease is determined by two factors, namely genetics and environment that affect the individual. (Laine MJ, 2009). Besides, it could be influenced by the diversity of people in the population, geography, race, and ethnicity (Uitterlinden AG 2004, Naito M, 2007). In the present study, the detection of VDR genotypic variations in exon 9 with the TaqI enzyme proves that there was an influence of genetic factors on the incidence of chronic periodontitis.

In the current study, we performed Fisher's exact test to find out the correlation between the VDR gene and CP. Data indicated that there is a statistically significant relationship between the polymorphism on exon 9 gen VDR with the incident of CP ($P<0.005$). The OR for the genotype

(TT/Tt) was 12.57 (95% CI, 1.58-99.83), being larger than the unadjusted value. This finding asserts that the VDR gene polymorphism may be the risk factor associated with the incidence of CP in the Makassar-based population. In other different ethnic populations and races, various association studies regarding the VDR particularly the TaqI polymorphism and periodontal disease have been performed. The latest study reported by Borges MAT (2009) found an OR of 4.57 indicating the association between VDR and chronic periodontitis in a Brazilian population. In 2004, de Brito from Brazil reported the same result stated that the genotype and haplotype of the VDR gene polymorphisms had an OR of 2.41 and 4.32 respectively associated with the incidence of periodontal disease. In other similar topics, Brett et al. (2005) found that there is an association between VDR gene polymorphism and CP in the Caucasian race. Accordingly, Tachi et al (2003) reported the OR value as much as 2.3 in the Japanese population and Nibali H et al (2008) found the OR value as much as 15.24 in patients with aggressive and chronic periodontitis in the Caucasian race.

In the present study, the OR value of 12.57 explained that subjects with VDR gene polymorphisms were 12.57 times more likely to suffer from chronic periodontitis than those without VDR gene polymorphisms. This is understandable considering the function of the VDR gene is involved in various processes such as bone metabolism and regulation of the immune response. It is interesting to note that the mechanism through which the VDR gene polymorphism affects the occurrence of CP is still unclear. Because the fundamental etiology of CP is inflammation caused by bacterial infection, which promotes alveolar bone resorption, the VDR gene appears to be a good candidate for CP susceptibility (Haffajee and Socransky, 1994; Offenbacher, 1996). Previously published studies claimed that VDR plays a more important role in trabecular bone than in cortical bone and that the variation in the VDR allele is responsible for

the variation in BMD. VDR, which is involved in controlling calcium and phosphate concentrations in the blood, is disrupted if variation in DNA or polymorphisms are present, resulting in a decrease in BMD across the body, including the mandible and maxilla. A decrease in jaw bone density will increase alveolar porosity through changes in trabecular pattern and increase bone resorption rate after the invasion of periodontal pathogens. (Kuo LC, Polson AM 2008; Schoor NM, Visser M 2008).

In regard to the clinical features of periodontal tissue damage accompanying the CP, we investigate the phenotypic of CP from the mean level of OHI-S, PPD, and CAL. We discover that patients with CP who have the Tt/tt genotype show a higher severity of periodontal tissue than those who have TT genotype. We assume that the Tt/tt genotype detected on exon 9 VDR genes in this study affects mRNA stability or increases the expression of mRNA damage in osteoblast cell structures. This causes a decrease in osteoblast function and an increase in osteoclast function that finally leading to a very great alveolar trabecular bone resorption. In our study, when associated with the clinical features of CP, it appears that samples with the 'tt' genotype have the highest level of periodontal tissue damage with a PPD value of 8 mm, compared to the genotype Tt (4.81 mm), and genotype TT (3.52 mm). Hence, there is a strong suspicion that the difference in severity might be caused by the presence of the t allele or the tt genotype.

The DNA sequencing results in this study show that samples with genotype 'TT' and genotype 'Tt' were not found to have any changes in the nucleotide. The changes in the nucleotide were only found in patients with CP who had the tt genotype from AGGTCGA to AGGCCGA at codon position 352 of the VDR gene. It showed variations in the substitution of T to C (GTC to GCC) with changes in amino acids valine (GTC) to alanine (GCC). Changes in amino acids are likely to affect the level of VDR gene expression, affecting the level of translation proteins, or

cause changes in stability and translation of RNA so that samples with these alleles are susceptible to decreased bone density or decreased immune system. However, further study is required to analyze the mechanism of this phenomenon.

Conclusion

Findings in our study suggest a notable possibility that VDR gene polymorphism is a leading factor associated with chronic periodontitis in Makassar-based population. Indirect evidence suggests that the severity of periodontal tissue damage may be caused by the presence of the t allele or the tt genotype. Moreover, there is a genetic variation in patients with periodontitis in which amino acid valine (GTC) transformed to alanine (GCC). This change is likely to affect the level of VDR gene expression causing samples with this allele are more susceptible to decreased bone density or decreased immune system. However, more research is needed before a definite conclusion can be drawn on disease pathogenesis and functional significance of VDR RFLP.

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