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

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Abstract

Background: The aim of this study was to investigate *vitamin D receptor* (VDR) gene polymorphism as a risk factor associated with chronic periodontitis (CP) and to determine the effect of VDR gene polymorphism on phenotypic CP.

Methods: This study is a case-control design that included 162 adults 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 gene polymorphism was determined using Restricted Fragment Length Polymorphism-Polymerase Chain Reaction (RFLP-PCR) and DNA sequencing to identify endonuclease restrictions in exon 9 (TaqI). The data were analyzed using an independent t-test and Fisher's exact test. The odds ratio (OR) was used to calculate the risk of VDR gene polymorphism in CP.

Results: VDR gene polymorphism was detected in patients with CP and a TT genotype (86.4%), Tt genotype (12.4%), and tt genotype (1.2%). The case group with TT and Tt genotypes had an OR of 12.5 (95% CI:1.6–99.8) of having CP compared to the control group (P<0.05).

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

Background

Chronic periodontitis (CP) is one of the most common inflammatory diseases affecting the tooth-supporting tissues and is characterized by progressive resorption of the alveolar bone [1–3]. CP is also one of the ultimate causes of tooth loss in adults and can 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 an imbalance of periodontal pathogens, host immunity, and environment, as well as local and systemic factors [7, 8]. Most evidence has emphasized that genetic factors have a vital role in disease pathogenesis and that these factors influence the unique reaction that characterizes the susceptibility of each individual [1, 5]. Approximately half of the clinical severity of CP may be associated with the host genetics [3, 8]. However, the genetic effects may differ among different ethnicities due to the diversity of people in a population [3, 7]. Hence, understanding the pathophysiology of this disease from a genetic standpoint is essential for early detection and diagnosis of CP disease.

Over the past decade, considerable efforts have been made to identify the influence of various genetic factors, 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], interleukin-4 receptor-α (IL-4RA) [13], Fc gamma receptor (FcγR) [14], cluster of differentiation-14 (CD14) [15], and the vitamin D receptor (VDR), on the severity of different diseases [16, 17]. The VDR, in particular, is a promising candidate for its role in periodontitis since it affects both bone metabolism and immunological function. [4, 18]. The VDR occurs in various cell types and can act as a transcription regulator [2]. It is located on chromosome 12 q and has 14 exons, 6 of which are located in the 5' region that is not translated (1a-1f) [7, 18]. The untranslated 3'

region of the VDR gene comprises a polymorphism cluster in TaqI, ApaI, and BsmI [19, 20]. If VDR gene polymorphism affects the degree or function of VDR, then a high probability exists that this polymorphism is critical for the pathogenesis of systemic diseases related to bone tissues, such as periodontal disease [19, 20].

Many studies have attempted to elucidate a 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 identified between the susceptibility to periodontal disease and a certain number of single-candidate gene polymorphisms [21]. Other studies have also shown a connection between periodontal disease and vitamin D [2, 22, 23]. Although extensive evidence has been obtained, only a few studies have focused on the association between the risk of CP and VDR variants. A relationship between VDR gene polymorphisms and the susceptibility to CP also remains to be established. The aim of the present study was therefore to determine possible correlations between VDR gene polymorphism, especially on exon 9 (TaqI), and the occurrence of CP through a case-control study in a Makassar-based population.

Materials And Methods

Subjects

The subjects were recruited from the Periodontology Department of the Dental Hospital, Hasanuddin University, Indonesia. All methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols in this study were reviewed and approved by Ethics Commission for Biomedical Research in Humans, Faculty of Medicine, Hasanuddin University. And informed consent was obtained from all subjects participate in this study. (Register Number UH09090137). The inclusion criteria for the subjects were age 25–60 years; no diabetes, tuberculosis (TBC), hepatitis, or human immunodeficiency virus (HIV); no smoking habit; no anti-inflammatory/contraception medications; and not pregnant/breastfeeding. Prior to the study, written informed consent was obtained from all subjects. A clinical examination, including the periodontal pocket depth (PPD) and clinical attachment loss (CAL) records, was performed on all subjects. The study group was divided into 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, Makassar, Indonesia. Genomic DNA was isolated from peripheral leukocytes following a standard protocol. Polymorphisms were determined based on endonuclease restriction in exon 9 (TaqI) of the examined VDR gene using Restricted Fragment Length Polymorphism-Polymerase Chain Reaction (RFLP-PCR) and direct sequencing.

Examination method

A 0.5–1 mL sample of venous blood was withdrawn, and DNA was extracted and purified using the Boom method [24]. A 900 µL volume of L6 buffer solution was added to 1.5 mL tubes, followed by 100 µL of patient blood, and the mixture was homogenized for 30 min. A 20 µL diatomaceous earth suspension was added to the tube. The mixture was vortexed and stirred using a gyratory shaker at 100 rpm for 10 min. The mixture was vortexed again in an Eppendorf microcentrifuge for 15 seconds at 12.000 rpm.

The supernatant was washed twice with 1 mL of L2 washing buffer, and centrifuged for 15 seconds, then the supernatant was removed. The supernatant was washed twice with 1 mL 70% ethanol, vortexed, and centrifuged for 15 seconds. The acetone in the supernatant was separated by opening the vial cover and heating the vial at 56°C in a water bath or a dry block heater for 10 min. A 60 mL volume of TE elution buffer was added and vortexed to dissolve any sediment from the suspension. The tubes were incubated for 10 minutes at 56°C in a water bath.

Each tube was centrifuged at 12.000 rpm for 30 s and 40–50 µL of the supernatant was carefully transferred to a new vial, taking care not to touch the tip of the pipette to the sediment from the suspension. A 40 µL volume of TE elution buffer was added into the sediment and re-vortexed so that the sediment would dissolve in the TE elution buffer. The vials were incubated again in the water bath at 65°C for 10 min. At the end of the Boom procedure, a small amount of diatomaceous earth will be retained (about 1 µL of diatomaceous earth suspension in 100 mL TE elution buffer); this amount will have no effect on the PCR results of the sample. The extract was stored at -20°C or -80°C.

Amplification by PCR

Amplification by PCR was done in 32 cycles, consisting of denaturation for 1 min at 94°C, annealing for 1.5 min at 59°C and, extension for 2 min at 72°C. After completion of 30 cycles, chemidians were followed by heating at 72°C for 7 min. The amplification results were analyzed by agarose gel electrophoresis.

RFLP-PCR procedure

VDR gene polymorphisms were detected on exon 9 using specific primers: forward (CTGGGGAGCGGGGAGTATGAAGGA) and reverse (GGGTGGCGGCAGCGGATGTA). DNA amplification, conducted for RFLP, used the TaqI restriction enzyme. As much as 22.5 µL of PCR mix was combined with 2.5 µL of primer and 2.5 µL of DNA extraction reagent with a final volume of 25 µL. 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 loaded onto 1.5% agarose gels along with ethidium bromide. The gel was soaked in a container containing TBE buffer, and then electrophoresis was conducted at a steady voltage of 80 V for 1 h. The gel was removed and observed under UV light. Fragment bands at different distances from the sample indicated genotypic differences of the VDR gene polymorphisms. The differences were marked with the letter t (a restriction

area is present) or the letter T (no restriction area is present). Genotypes based on the bands on agarose gels were classified as follows:

- (TT): no restriction area at 1398 bp
- (Tt): restriction areas are present at (946 + 452 bp) and 1398 bp.
- (tt): restriction areas are present at 946 bp and 452 bp.

DNA sequencing

Direct sequencing was carried out by Macrogen South Korea. Sequencing confirmed changes in the nucleotide base arrangement in exon 9 of the VDR gene. The Basic Local Alignment Search Tool (BLAST) and the NCBI database were used to analyze the sequencing results.

Statistical Analysis

SPSS version 11.5 was utilized for data analysis. Fisher's exact test was performed to determine the relationship between VDR gene polymorphisms and incidence of CP and to calculate the odds ratio (OR) variables as risk factors for CP. An independent t-test was conducted to detect phenotypic differences in CP.

Results

Distribution of the characteristics of the study subjects

The study consisted of 162 persons divided into two groups: 81 subjects with CP (case group; 14 males and 67 females) and 81 subjects without CP (control group; 38 males and 43 females). The detailed characteristics are shown in (Table 1). Subjects with CP had a higher oral hygiene index score-simplified (OHI-S), were more edentulous, and had more caries compared to the healthy subjects.

VDR gene polymorphisms

The VDR gene polymorphism occurs as three genotypes: TT (1 band), tt (2 bands), and Tt (3 bands). (Fig. 1A and Fig. 1B) show no restriction area in the TT genotype (1398 bp), but some restriction areas of 946 bp, 452 bp, and 1398 bp for the Tt genotype and two restriction areas of 946 bp and 452 bp for the tt genotype. (Fig. 1C) shows the quantification of the VDR genotype in both groups. The case group had a greater percentage of the TT genotype (86.4%) compared to either the Tt (12.3%) or the tt (1.2%) genotype. The group showed a TT genotype percentage of 98.8%, a Tt genotype percentage of 1.2%, and no tt genotype.

(Table 2) highlights the relationship between the VDR genotype and the incidence of CP. The case group showed a TT genotype percentage of 86.4% and a Tt/tt percentage of 13.6%. The relationship analysis of

VDR genotypes and the incidence of CP revealed an OR of 12.5 (95% CI: 1.6-99.8) for the case group with the TT and Tt genotype to contract CP compared to the control group (P<0.05).

(Table 3) shows the comparison between the VDR genotype in CP based on the OHI-S, PPD, and CAL. (Fig. 2) shows a comparison of the severity of CP based on the VDR genotype. Both (Table 3) and (Fig. 2) confirm that the mean of OHI-S for patients with CP was higher for the patients with the TT genotype (2.71) than with the Tt/tt genotype (2.12), although the difference was not statistically significant (P>0.05). Both the PPD status and CAL were higher for the Tt/tt genotype (PPD 5.14 and CAL 4.41) than for the TT genotype (PPD 3.52 and CAL 2.80). Both showed statistically significant differences, with P values of 0.006 and 0.001, respectively.

Polymorphism analysis of the VDR gene by sequencing

DNA sequencing confirmed a change in the sequence of the VDR gene nucleotides. (Fig. 3) shows the sequencing results of the three genotypes. The nucleotide sequence for the tt genotype at codon 352 changed from the initial sequence of AGGTCGA (Fig. 3A, 3B) to AGGCCGA (Fig. 3C), indicating a nucleotide substitution from T to C (GTC to GCC) due to the change in the amino acid valine (coded by GTC) to alanine (coded by GCC).

Discussion

Current periodontology research has been particularly focused on the influence of genetic factors on individual susceptibility to CP [25–27]. The tendency for early onset periodontitis can be inherited as either an autosomal recessive or autosomal dominant trait [28, 29]. In this study, VDR gene polymorphism as a risk factor associated with CP was investigated by determining the presence of polymorphisms on exon 9 of the VDR gene, followed by DNA sequencing to identify the nucleotide changes. The clinical features of periodontal tissue damage in the form of PPD and CAL were also examined. The results indicated that the frequency of the Tt and tt (t allele) genotypes is smaller than that of the TT genotype (T allele) in both the CP and control groups. This can be explained by the fact that the population in Makassar, Indonesia, is a part of the Asian race that has only a minor presentation of the Tt and tt genotypes in the VDR gene. In agreement with our results, a study by Zmuda et al. [30] reported a frequency of the Tt and tt genotypes of only 2% in Asians, 5% in African Americans, and 17% in Caucasians. Similarly, research by Sun et al. [28] and Tachi et al. [31] reported that the t allele was present in only 4% of a Chinese ethnic group and 11% in a Japanese ethnic group [28, 31]. A small distribution of t alleles (5%) in the Asian race was also found by Zhao et al. [32].

The differences in the frequency of genotypes or alleles in a population can be explained by the concept that all polymorphisms begin as mutations that occur as a result of DNA damage and that the frequency of the allele then grows in the population and becomes a true polymorphism [33, 34]. Thus, the difference in the allele frequencies between ethnicities tends to be influenced by evolutionary processes and genetic traits of one population. Periodontitis is considered a complex multifactorial disease [21] with a relatively mild phenotype, as with other chronic diseases. The phenotype of a complex disease is determined by

genetics and the environment that affects the individual [21], but it can also be influenced by the diversity of people in the population, as well as geography, race, and ethnicity [33, 34]. In the present study, the detection of genotypic variations in exon 9 of the VDR gene with the TaqI enzyme confirms the influence of genetic factors on the incidence of CP.

In the current study, we used Fisher's exact test to establish the correlation between the VDR gene and CP. A statistically significant relationship was evident between the polymorphism in exon 9 of the VDR gene and the incidence of CP ($P < 0.005$). The OR for the TT/Tt genotype was 12.57 (95% CI, 1.58–99.83), which was larger than the unadjusted value. This finding confirms that VDR gene polymorphism may be a risk factor associated with the incidence of CP in the Makassar-based population. Similar association studies regarding the VDR polymorphism and periodontal disease have been performed in other ethnic populations and races [26, 35, 36]. For example, a study by Borges [37] reported an OR of 4.57 for the association between VDR and CP in a Brazilian population. In 2004, de Brito from Brazil reported the same result and 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 [38]. Similarly, Brett et al. [39] found an association between VDR gene polymorphism and CP in the Caucasian race. Likewise, Tachi et al. [31] reported an OR value as high as 2.3 in the Japanese population.

The OR value of 12.57 in the present study indicated that subjects with VDR gene polymorphisms were 12.57 times more likely to suffer from CP than were those without VDR gene polymorphisms. This is understandable considering that the VDR gene is involved in various processes ranging from bone metabolism to the regulation of the immune response. Interestingly, the mechanism by which VDR gene polymorphisms affect the occurrence of CP is still unclear. The fundamental etiology of CP is inflammation caused by bacterial infection, which promotes alveolar bone resorption; therefore, the VDR gene appears to be a good candidate for predicting CP susceptibility. 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 [40, 41]. VDR, which is involved in controlling calcium and phosphate concentrations in the blood, is disrupted by the presence of variations in DNA or polymorphisms, resulting in a decrease in BMD across the body, including the mandible and maxilla [42]. A decrease in jaw bone density will increase the alveolar porosity through changes in the trabecular pattern and will increase the bone resorption rate after the invasion of periodontal pathogens [40, 41].

We investigated the clinical features of periodontal tissue damage accompanying CP by examining the CP phenotype based on OHI-S, PPD, and CAL. Patients with CP who had the Tt/tt genotype showed a greater severity of periodontal issues than those with the TT genotype. We assume that the Tt/tt genotype of exon 9 of the VDR gene affects mRNA stability or increases the expression of mRNA damage in osteoblast cell structures. This would decrease the osteoblast function and increase the osteoclast function, thereby leading to severe alveolar trabecular bone resorption. In our study, when associated with the clinical features of CP, the samples with the tt genotype had the highest level of periodontal tissue

damage, with a PPD value of 8 mm, compared to the Tt (4.81 mm) and TT (3.52 mm) genotypes. Hence, 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 showed that samples with genotype TT and genotype Tt had no nucleotide changes. By contrast, patients with CP who had the tt genotype showed a nucleotide change from AGGTCGA to AGGCCGA at codon position 352 of the VDR gene. This substitution of T with C (GTC to GCC) would result in a change from the amino acid valine (GTC) to alanine (GCC). Changes in amino acids are likely to affect the level of VDR gene expression, the level of protein translation, or the degree of stability and translation of RNA; therefore, patients with these alleles are susceptible to decreased bone density or decreased immune system function. However, further study is needed to analyze the mechanism underlying this phenomenon.

Conclusions

The findings in our study suggest the possibility that VDR gene polymorphism is a leading factor associated with CP in the Makassar-based population. Indirect evidence suggests that the severity of periodontal tissue damage may be a consequence of the presence of the t allele or the tt genotype. Moreover, a genetic variation was detected in patients with periodontitis in which the amino acid valine (GTC) was substituted by alanine (GCC). This change is likely to affect the level of VDR gene expression, causing a greater susceptibility in patients with these alleles to decreased bone density or decreased immune system function. However, more research is required before a definite conclusion can be drawn regarding the specific disease pathogenesis and the functional significance of RFLP in the VDR gene.

Abbreviations

VDR: Vitamin D Receptor; CP: Chronic Periodontitis; RFLP-PCR: Restricted Fragment Length Polymorphism-Polymerase Chain Reaction; DNA: Deoxyribonucleic Acid; IL-1: Interleukin-1; TNFA: Tumor Necrosis Factor-α; IL10: Interleukin-10; IL4: Interleukin-4; IL-4RA: interleukin-4 receptor-α; FcγR: Fc Gamma Receptor; CD14: Cluster of Differentiation-14; TBC: Tuberculosis; PPD: Periodontal Pocket Depth, CAL: Clinical Attachment Loss; mL: Milliliter; TBE: Tris-borat EDTA; EDTA: Ethylenediaminetetraacetic acid; bp: Base Pair; BLAST: Basic Local Alignment Search Tool; NCBI: National Center for Biotechnology Information; SPSS: Statistical Packages for the Social Sciences; OR: Odds Ratio; N: Number; OHI-S: Oral Hyigiene Indeks-Simplified; BMD: Bone Mineral Density; mRNA: *Messenger* Ribonucleic Acid; AGGTCGA: Adenine Guanine Guanine Thymine Cytosine Guanine Adenine; AGGCCGA: Adenine Guanine Guanine Cytosine Cytosine Guanine Adenine; GTC: Guanine Thymine Cytosine; GCC: Guanine Cytosine Cytosine;

Declarations

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Author Contributions

N.H., S.O. and E.M., conceptualization, methodology and writing—original draft preparation; M.R. and A.S.H.Y., review, editing and formal analysis; T.S. and K.L.O., validation. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The datasets generated during and/or analysed during the current study are available in the dbVar (human variations greater than 50bp) repository.

Ethics approval and consent to participate

This research was approved by Ethics Commission for Biomedical Research in Humans, Faculty of Medicine, Hasanuddin University. (0189/H.04.8.4.5.31/PP36-KOMETIK/2010).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Distribution characteristics of subjects

Parameter	Case (n=81)		Control (n=81)	
	Mean	SD	Mean	SD
Age (year)	38,90	9,24	37,61	11,82
Height (cm)	155,53	6,55	157,33	7,33
Weight (kg)	55,46	6,45	56,19	9,22
BMI $(kg/m)^2$	22,47	2,90	23,18	3,40
Edentulous	2,48	2,69	1,06	1,07
Caries	2,17	2,3	2,04	1,69
OHI-S	2,63	0,96	2,29	0,69
PPD (mm)	4,54	1,27	-	-
CAL (mm)	3,02	1,48	-	-

Note: SD=Standard Deviation, BMI=Body Mass Index, OHI-S= Oral Hygiene Index-Simplified, PPD=Probing Pocket Depth, CAL=Clinical Attachment Loss

Table 2 Relationship between genotype VDR and the incidence of chronic periodontitis

Genotype VDR	Case		Control		*P*	OR	95% CI
	n	%	n	%			
TT	70	86,4	80	98,8	0.005*	12,5	1,6 – 99,8
Tt/tt	11	13,6	1	1,2			

**Fisher's Exact Test, significant P<0.05; OR=Odds Ratio, CI=Confidence Interval*

Table 3 Comparison between genotype VDR in chronic periodontitis based on the OHI-S, PPD, and CAL

Parameter	Genotype	n	Mean	SD	*P*
OHI-S	TT	70	2,71	0,95	0,060
	Tt/tt	11	2,12	0,93	
PPD (mm)	TT	70	3,52	1,49	0,006*
	Tt/tt	11	5,14	2,98	
CAL (mm)	TT	70	2,80	1,26	0,001*
	Tt/tt	11	4,41	2,06	

**Independent T-test, significant P<0.05;* OHI-S=*Oral Hyigiene Indeks-Simplified*, PPD=*Probing Pocket Depth,* CAL= *Clinical Attachment Loss*, n = Total of Sample

Figures

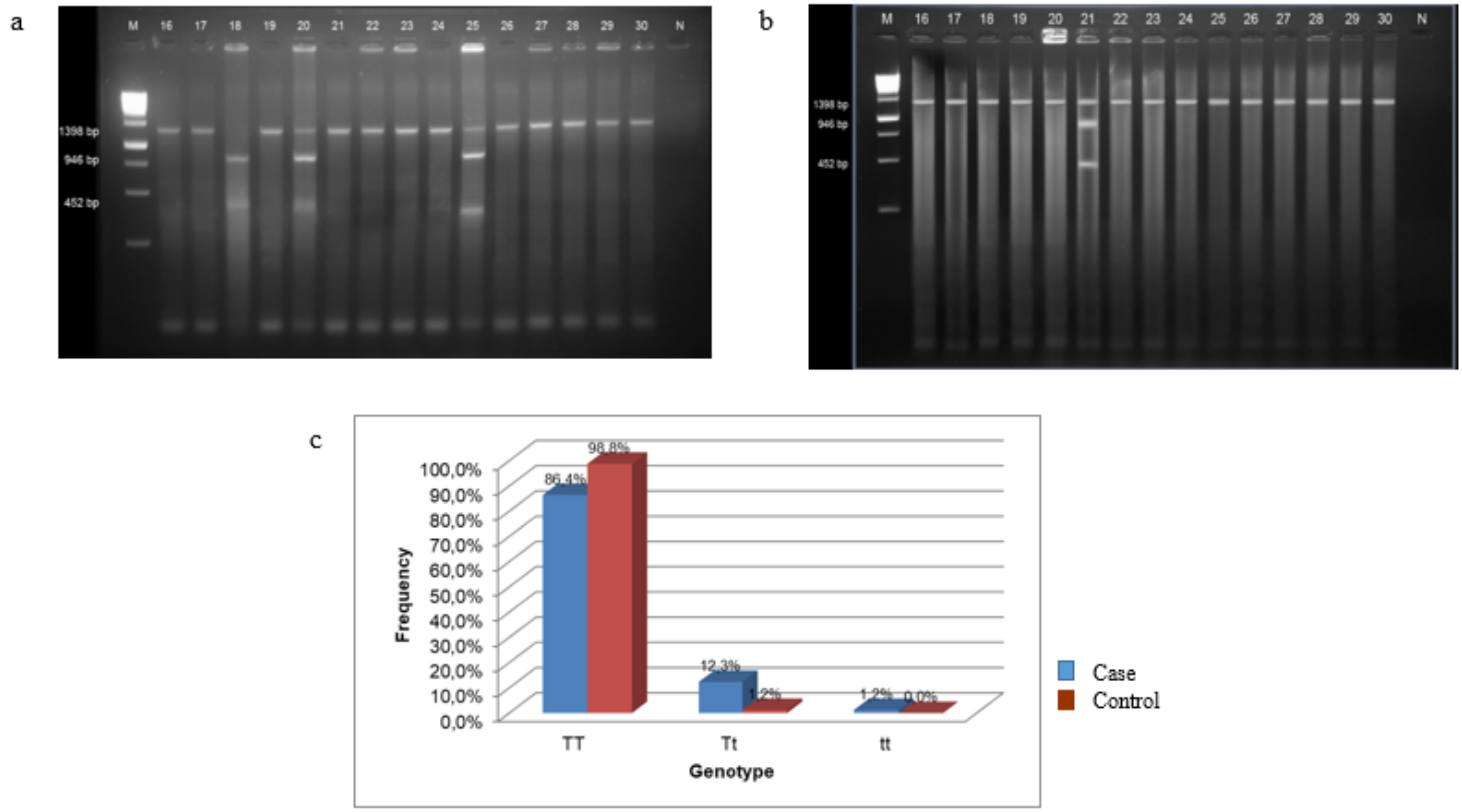

Figure 1

VDR gene polymorphism; a RFLP PCR in the case group; b RFLP PCR in the control group; and c comparison of the genotype VDR in case group and control group.

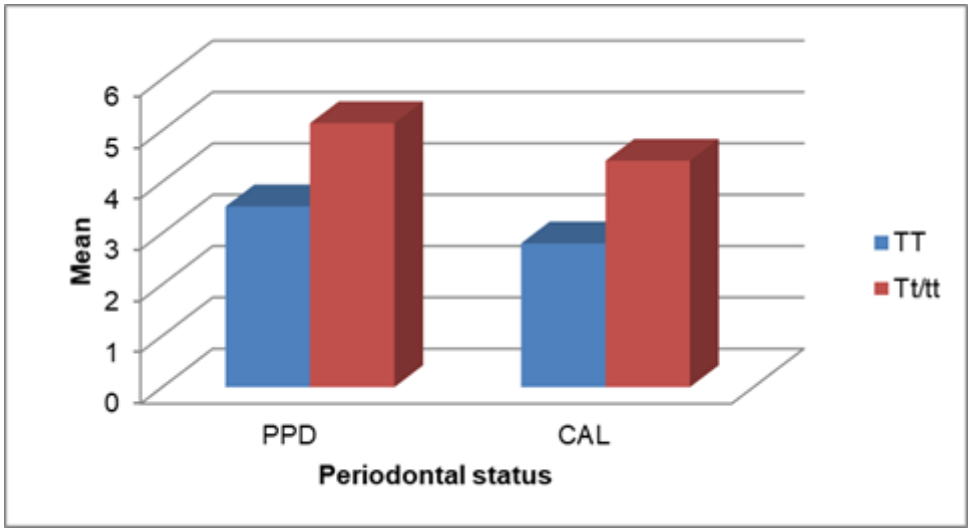

Figure 2

Comparison of the severity of chronic periodontitis based on the genotype VDR

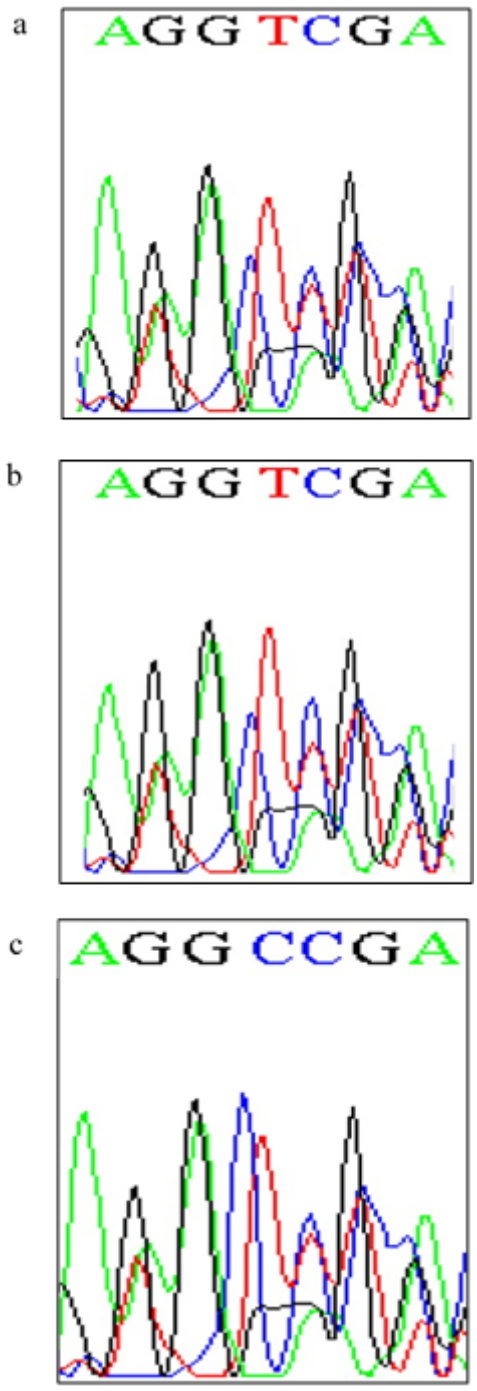

Figure 3

DNA sequencing of genotype VDR; a sequencing for TT genotype; b sequencing for Tt genotype; and c sequencing for tt genotype