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Analysis of vitamin D receptor gene polymorphisms in patients with chronic periodontitis

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***Background & objectives*: Genetic polymorphisms in the vitamin D receptor (VDR) gene are related to bone mineral density, bone turnover, and diseases with bone loss. Alveolar bone loss is a key feature in periodontitis. The aim of this study was to determine whether severe generalized chronic periodontitis (CP) in a Turkish population was associated with polymorphisms in the VDR gene.**

***Methods*: Samples of venous blood and DNA were obtained from 72 patients with severe generalized chronic periodontitis and 102 healthy controls. The polymorphic regions were amplified using PCR followed by digestion with restriction enzymes *Bsm*I A/G(rs1544410), *Apa*I G/T(rs11168271), *Taq*I T/C(rs731236), and analyzed electrophoretically. Genotype and allele frequencies were calculated.**

***Results*: There were no statistically significant differences in the frequencies of VDR *Bsm*I, *Apa*I, *Taq*I genotypes between the CP patients and healthy controls. The GTT haplotype, constructed from the three adjacent restriction fragment length polymorphisms was found to be over-represented among CP cases. This corresponded an OR of 2.4 (95% confidence interval, 1.12-5.18) for heterozygous carriers and 2.27 (95% confidence interval, 0.95-5.4) for homozygous carrier of the risk haplotype.**

***Interpretation & conclusions*: The present findings indicated that *Bsm*I, *Apa*I, *Taq*I polymorphisms of the VDR gene were not associated with the severe generalized CP in the studied Turkish patients. Moreover, the VDR genotypes based on haplotype analysis may be associated with chronic periodontitis. In the future, diagnostic periodontal risk assessments like polymorphisms may be useful in detection of individuals susceptible for periodontitis.**

Key words Chronic periodontitis - genotypes - polymorphism - vitamin D receptor

Chronic periodontitis (CP) is one of the most common diseases prevalent throughout the world, and it is the main cause of tooth loss in the elderly[1,2]. CP has a microbial aetiology and is known to be caused by intraoral inflammation after infection with specific bacteria[3-5]. The ultimate result of periodontal disease is a progressive alveolar bone resorption[3,4]. Both genetic and environmental factors are involved in this

inflammatory disease aetiology of which is influenced by interaction of periodontal pathogens and host responses[6]. Many studies have been attempted to identify genetic factors that may be related to enhanced susceptibility to periodontal disease[1,3,6-9].

Vitamin D plays an important role in skeletal muscle metabolism, including calcium absorption and bone loss, and has also been shown to play an important role in other metabolic pathways such as those involved in immune response and cancer[10]. Vitamin D receptor (VDR) gene (OMIM 601769) can have profound effects on mineral metabolism and bone mineral density[11-13]. The 3' untranslated region of the VDR gene includes a cluster of linked polymorphisms: *Bsm*I, *Apa*I, *Taq*I sites[11,14,15]. If VDR gene polymorphisms influence the level or function of the VDR, these polymorphisms may have roles in pathogenesis of periodontal and systemic diseases which affect the bone tissue.

Genetic polymorphisms in genes which encode mediators of bone homeostasis have been shown to be associated with parameters of bone mineral density and incidence of common disorders on metabolism, in particular osteoporosis[8]. Alveolar bone loss is a key feature in periodontitis. There are few studies on association of VDR polymorphism to CP[2,3,11]. Tachi *et al*[2] suggested that *Taq*I polymorphism was found to be significantly associated with the occurrence of CP in Chinese and Japanese population. de Brito *et al*[3] reported an association of *Taq*I and *Bsm*I polymorphisms of the VDR gene with CP in a Brazilian population[3]. Brett *et al*[16] found a statistically significant association between *Taq*I polymorphism and both chronic and aggressive periodontitis. Associations of VDR polymorphisms with CP are inconsistent in different studies conducted in various population groups, due to different linkage disequilibrium (LD) and haplotype blocks in populations, small sample sizes, population stratification, and variation in environmental factors between geographically separated areas. Because the genetic effect may be different in different ethnic groups[11,17,18], we undertook of this study to investigate the relationships between severe generalized CP, and the *Bsm*I, *Apa*I, *Taq*I polymorphisms of the VDR gene in patients with CP attending a tertiary care centre in Turkey.

Material & Methods

Subject selection: Seventy two patients with CP (30 men and 42 women; age range 31 to 70 yr, mean±SEM 47.51±1.14 yr) and 102 healthy controls (43 men and 59 women; age range 30 to 65 yr, 46.08±8.36 yr) attending at the Oral Diagnosis and Radiology and Periodontology Clinics of the Dentistry Faculty, Ondokuzmayis University, Samsun, Turkey, were enrolled in the study between September 2003 and November 2006. The study protocol was approved by the ethics committee for our other researches on periodontitis (No. DHF-041 and DHF-043) and we used the same subject group. Written informed consent was obtained from all subjects. None of the subjects had a history or current manifestation of systemic diseases, disease of the oral hard or soft tissues except dental caries and periodontal diseases, chronic usage of anti-inflammatory drugs, a history of diabetes, hepatitis or HIV infection, immunosuppressive chemotherapy, history of any disease known to severely compromise immune function, smoking, current pregnancy and lactation, and obesity. All subjects were of Turkish origin from the Black Sea Coastal Region and had similar socio-economic background.

All patients fulfilled the diagnostic criteria defined by the International Workshop for a Classification of Periodontal Diseases and Conditions for CP[19].

Clinical assessments: The same investigator performed clinical assessments of the patients in their first visit. The clinical parameters were: probing pocket depth (PPD), clinical attachment loss (CAL), and radiographs. PPD (the distance in millimeters from the free gingival margin to the bottom of the pocket) and CAL (the distance in millimeters from the cemento-enamel junction to the bottom of the pocket)[20,21] of all the teeth were assessed by using a William's Probe (Hu-Friedy, Chicago, IL, USA) at six sites of a tooth: mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, and distolingual.

The diagnosis of severe generalized CP was made according to the severity of periodontal disease on the basis of the amount of CAL, and according to the extent of disease sites on the basis of the number of sites with PPD >4 mm involved. Patients exhibiting CAL >5 mm and >30 per cent of the sites with PPD >4 mm were considered with severe generalized CP. Patients who were diagnosed as severe generalized CP, reported to be in good general health, and agreed to participate were included in the study. The healthy controls consisted of unrelated Turkish subjects residing in the same geographic area as the CP patients who did not have history of periodontitis. These periodontally healthy individuals did not show CAL, PPD >3 mm at more than one site, and radiographic evidence of bone loss[22].

Subjects who did not exhibit these clinical parameters were excluded from the study.

DNA extraction and determination of VDR genotype: Peripheral venous blood samples were obtained and genomic DNA was isolated by a salting out method from peripheral leukocytes[23].

The genotypes for three restriction fragment length polymorphisms of the VDR were determined by polymerase chain reaction (PCR) (Techne Gradient, Camridge, UK) and enzymatic digestion of the products with *Bsm*I, *Apa*I and *Taq*I restriction enzymes.

Primer sequences (Iontec, Bursa, Turkey) were: intron 8, *Bsm*I (rs1544410) polymorphic site: 5'-CAA CCA AGA CTA CAA GTA CCG CGT CAT GA-3' forward and 5'-AAC CAG CGG GAA GAG GTC AAG G G-3' reverse; intron 8 and exon 9, *Apa*I and *Taq*I polymorphic sites: 5'-CAG AGC ATG GAC AGG GAG CAA-3' forward, 5'-CAC TTC GAG CAC AAG GGG CGT TAG C-3' reverse (Fig.).

An 825-bp fragment encompassing the *Bsm*I polymorphic site was amplified. PCR reaction was performed in 25 µl 1xPCR buffer (MBI, Fermentas, Lithuania) containing 20 pmol of each primer, 2.5 mM $MgCl_2$, 200 mM of each dNTP (MBI, Fermentas, Lithuania), 50 ng DNA, and 1.25 U Taq polymerase (MBI, Fermentas, Lithuania). Following initial denaturation at 94ºC for 5 min, amplification was performed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 30 sec. Final extension was allowed to proceed at 72°C for 5 min[15]; 8 µl of the PCR products were digested overnight with 10 U *Bsm*I (MBI, Fermentas, Lithuania) at 37°C.

Amplification of the 490 bp fragment encompassing *Apa*I (rs11168271) *and Taq*I (rs731236) polymorphic site was performed in 25 µl 1xPCR buffer (MBI, Fermentas, Lithuania) containing 20 pmol of each primer, 2.5 mM $MgCl_2$, 200 mM of each dNTP (MBI, Fermentas, Lithuania), 50 ng DNA, and 1.25 U Taq polymerase (MBI, Fermentas, Lithuania). Following initial denaturation at 94 ºC for 5 min, amplification was performed by 35 cycles of denaturation at 94°C for 30 sec, annealing of at 64°C for 30 sec, and extension at 72°C for 30 sec[24-26]. The reaction was terminated by extension at 72°C for 5 min; 8 µl of the PCR products were digested overnight with 10 U *Apa*I (MBI, Fermentas, Lithuania) at 22°C and for 3 h with 10 U *Taq*I (MBI, Fermentas, Lithuania) at 65°C.

All digested products were resolved on 2 per cent agarose and analyzed in a video gel documentation system (Biolabs, Kyoto, Japan) after staining with ethidium bromide.

Then haplotype analysis is carried out as the single nucleotide polymorphisms (SNPs) are frequently inherited together. *Bsm*I, *Apa*I and *Taq*I SNPs were assessed in relation to each other by a direct molecular haplotyping procedure[27,28]. For the comparison of carriage rate of VDR haplotypes, the reference, homozygote and heterozygote groups for VDR alleles were made. Among the triple combinations, genotypes GAGTTT, and GAGGCC accounted each for less than 5 per cent of the study population. Thus these genotypes were excluded from the haplotype analysis. Because there was an over-representation of CP patients with the 'GTT' haplotype, the patients were grouped according to their carrier status for this VDR haplotype as homozygous carriers (GGTTTT) and heterozygous carriers (including GAGGTC and GAGTTC genotypes) of the risk haplotype and compared with patients not carrying the haplotype (including GGGGTT, GGGTTT, and AAGGCC genotypes).

Statistical analysis: The statistical analysis was performed using a commercially available software program (SPSS 12.0, SPSS Inc., Chicago, Illinois, USA). To determine whether any significant differences in polymorphisms frequencies occurred between the case and the control populations the allele and genotype frequencies were compared, using the Chi square method. Haplotype frequencies were inferred by using the fastPHASE 1.2 program (*http://www.stat.washington.edu/stephens/phase.html*). For comparisons of haplotypes, reference, heterozygote, and homozygote groups were made for VDR alleles. Where significant *P* values were generated, the odds ratio (OR) was calculated[29]. Associations between the disease and genotypes were assessed by calculating odds ratios and 95 confidence intervals (CI). The crude ORs were calculated and then adjusted for gender.

Results

According to the SNP database VDR BB, Bb, and bb genotypes are referred to as AA, AG, and GG; VDR AA, Aa and aa genotypes are referred to as GG, GT, and TT; and VDR TT, Tt, and tt genotypes as TT, TC, and CC.

The size of the PCR product for the *Bsm*I polymorphism was 825 bp. Following the digestion, two restriction fragments of 650 and 175 bp were observed

for GG homozygotes, a single 825 bp band was obtained for AA homozygotes, and AG individuals displaying all three bands.

The size of the reaction product for the *Apa*I and *Taq*I polymorphism was 490 bp fragment which was cut into 280 bp and 210 bp fragments with *Apa*I digestion or into 290 bp and 200 bp by *Taq*I digestion. The homozygous GG genotype displayed only 490 bp fragment, GT heterozygotes displayed all three fragments and TT homozygotes displayed 280 bp and 210 bp fragments. The homozygous TT genotype displayed only 490 bp fragment, TC heterozygotes all the three fragments and CC homozygotes displayed 290 bp and 200 bp fragments.

Table I. Genotypic and allelic frequencies of vitamin D receptor gene *Bsm*I, *Apa*I and *Taq*I polymorphisms in patients with CP (N=72), and healthy controls (N=102)

VDR allele genotypes	CP patients N (%)	Controls N (%)
*Apa*I		
GG	33 (45.8)	40 (39.2)
GT	23 (31.9)	43 (42.2)
TT	16 (22.2)	19 (18.6)
Frequency		
G	0.62	0.60
*Bsm*I		
AA	10 (13.9)	9 (8.8)
GA	33 (45.8)	51 (50.0)
GG	29 (40.3)	42 (41.1)
Frequency		
A	0.37	0.34
TaqI		
TT	36 (50.0)	48 (47.0)
TC	28 (38.9)	47 (35.2)
CC	8 (11.1)	7 (6.9)
Frequency		
T	0.69	0.70

There were no significant differences in any of the alleles between the severe generalized CP and control groups (Table I). There was no differences in age and gender distribution between the groups.

Considering the three SNPs independently, genotype distributions were in Hardy-Weinberg equilibrium (HWE)[30] among the controls. The *Bsm*I and *Taq*I polymorphisms did not deviate from HWE; slight departure was observed for *Apa*I polymorphism in CP patients.

As the three SNPs that we studied showed strong linkage disequilibrium[15], the association of composite genotypes between the CP patients and healthy controls was also investigated. There was no significant difference between the composite genotypes of the patients with CP and control groups (Table II). Polymorphisms are prevalent, are located in virtually all regions of the chromosome set, and have multiple alleles and so yield a high proportion heterozygous genotype. The most common haplotype was GTT (21%) for patients with CP and GAGTTC composite genotype (21.6%) for the controls. Subsequently, the GGGTTT, GGGGTT and AAGGCC haplotypes were taken as reference genotypes. The GAGGTC and GAGTTG haplotypes were considered as heterozygous carriers, while GGTTTT haplotype was taken as homozygous carriers and homozygous genotypes. The GGTTTT haplotype was the risk haplotype (Table III). Patients homozygous for the risk haplotype had risk for CP that was close to statistical significance (OR 2.27, 95% CI 0.951-5.40; P=0.065), heterozygous carrier had a statistical significant risk (OR 2.40, 95% CI 1.12-5.18; P=0.025) compared with the control group (Table III).

Table II. Genotype frequencies of vitamin D receptor gene in patients with CP (N=72) and healthy controls (N=102)

VDR genotype	CP patients N (%)	Estimated halotype frequencies* (SE)	Controls N (%)	Estimated halotype frequencies* (SE)
Haplotype I (GTT)	15 (20.8)	0.286 (5.33E-4)	17 (16.7)	0.249 (4.29E-4)
Haplotype II (AGC)	6 (8.3)	0.297 (5.39E-4)	8 (7.8)	0.347 (4.72E-4)
Haplotype III (GGT)	8 (11.2)	0.283 (5.31E-4)	7 (6.9)	0.294 (4.52E-4)
GAGTTC	11 (15.4)	0.078 (3.18E-4)	22 (21.6)	0.039 (1.91E-4)
GAGGTC	15 (20.2)	0.012 (1.27E-4)	21 (20.6)	0.025 (1.54-4)
GGGTTT	7 (9.8)	0.030 (2.03E-4)	16 (15.6)	0.020 (1.4E-4)
GAGTTT	3 (4.3)	0.0074 (1.01E-4)	5 (4.9)	0.019 (1.38E-4)
Others	7 (9.8)	0.0066 (8.30E-4)	6 (5.9)	0.07 (6.70E-4)
Total	72		102	

*Calculated by FastPHASE 1.2

Table III. Associations of VDR *Bsm*I-*Apa*I-*Taq*I haplotypes with chronic periodontitis

Genotype	No. of CP patients	No. of control	Adjusted OR* (%95 CI)	*P*
Reference[+]	21	31	1.0	
Heterozygotes	23	46	2.40 (1.12-5.18)	0.025
Homozygotes	15	17	2.27 (0.951-5.40)	0.065

*Each genetic marker is adjusted for gender
[+]Reference includes VDR genotypes GGGTTT, AAGGCC, and GGGGTT; heterozygotes include GAGGTC and GAGTTC; homozygotes include GGTTTT

Discussion

CP is a multifactorial disease, the onset and severity of which are influenced by both genetic and environmental factors[1,31]. Different genes may influence different aspects of the disease pathology[3,4].

The results of this study suggested that *Bsm*I, *Apa*I, *Taq*I polimorphisms of the VDR gene were not associated with severe generalized CP in the studied population. However, the GTT haplotype, constructed from the three adjacent restriction fragment length polymorphisms was found to be over-represented among CP cases. The difference in OR was statistically significant for heterozygous carriers and nearly statistically significant for homozygous carriers but difficult to explain biologically. There is extensive linkage disequilibrium at the 3' untranslated region of the VDR gene which can be measured accurately by the molecular haplotypes constructed from the cluster of linked polymorphisms *Bsm*I, *Apa*I, and *Taq*I sites. Thus these haplotypes, which themselves are not functional polymorphisms, can be used as markers for truly functional polymorphisms elsewhere in the 3' end of the VDR gene[11,14,15] . It should be noted that the sample size of our study was relatively small; therefore, this results need to be conformed on a larger sample size. Our results were different from those of other VDR studies[2,3] evaluated in different populations, regarding the genotype and allele frequencies of *Bsm*I, *Apa*I, and *Taq*I of the VDR gene indicating that different populations may have different frequencies.

In the present study no significant difference was observed between the VDR variant frequencies of *Bsm*I in severe generalized CP patients and healthy controls. de Brito *et al*[3] showed an association between the *Bsm*I polymorphism and CP in a Brazilian population with four different ethnic groups. The discrepancy between their findings and ours might be related to ethnicity as the VDR allele frequencies alter among different populations. Similar to the results of the present study, Yoshihara *et al*[20] found no association between the distribution of the VDR *Bsm*I genotypes in the groups of generalized early-onset periodontitis, CP and healthy controls.

The frequency of the *Apa*I allele was also not significantly different in the CP patients and controls. The deviation of HWE for *Apa*I polymorphism in CP patients may not be due to genotyping error because cases and controls were genotyped at same time on the same PCR, thus deviation from HWE would be expected to be seen equally in both cases and controls. Inagaki *et al*[4] compared the periodontal disease progression among polymorphisms of *Apa*I and *Taq*I of the VDR gene in a longitudinal study. They concluded that these polymorphisms of the VDR gene might be associated with periodontal disease progression and tooth loss.

The frequency of the *Taq*I allele was not significantly different in the CP patients and controls. Our results were different from those of other studies[2,3]. de Brito *et al*[3] reported that patients with 'C' allele (formerly t) were 2.4 times more susceptible to periodontal disease than patients who lacked this allele. Another study performed with a Japanese population showed a significant correlation between VDR *Taq*I genotypes and CP[2]. However, Sun *et al*[11] determined genotypes of the *Taq*I VDR gene in 24 cases of CP, 37 cases of early-onset periodontitis and 39 healthy controls and found no difference in the distribution of VDR *Taq*I genotypes between CP patients and controls. Likewise in the present study, *Taq*I VDR genotypes between the CP patients and healthy controls were not statistically different.

Our data showed that GAGGTC and GAGTTC composite haplotypes were more susceptible to periodontal disease than others. de Britto *et al*[3] showed that the haplotypes 'TB' and 'TB/tb' were associated with periodontal disease.

The frequencies of *Bsm*I, *Apa*I, and *Taq*I alleles may vary among different ethnic groups. We found the frequencies of A, G and T alleles as 34, 60 and 70 per cent, respectively. The frequencies of A and G alleles in the present study were in between the frequencies of Caucasian (74% for A allele, 44% for G allele) and Asian (42% for A allele, 7% for G allele) populations[32]. The T allele of the *Taq*I polymorphism had a higher frequency compared to those of Caucasians and Asians (43 and 8%, respectively).

Studies on the VDR gene polymorphisms associated with psoriasis, osteoporosis, osteomalacia, and hypercalcaemia have been reported in Turkish population[33-36]. The association between VDR gene polymorphism and urolithiasis in a Turkish population has been shown and the genotype frequencies in the studied control group for *Bsm*I, *Apa*I, *Taq*I were similar to the frequencies seen in our healthy controls[37].

In conclusion, within the limitations of the sample selection and number, findings of the present study indicated that the *Bsm*I, *Apa*I, and *Taq*I polymorphisms of the VDR gene were not associated with severe generalized CP. The present study showed that composite haplotypes GAGGTC and GAGTTC were associated with CP in the studied population. The increasing interest in finding genetic markers for periodontitis is essential, because in the future, diagnostic periodontal risk assessments may be useful in identifying individuals susceptible for periodontitis.

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