Polymorphisms of the Vitamin D Receptor Gene and Stress Fractures

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Abstract

Our aim was to evaluate the association between *VDR* polymorphisms and calcaneal Stiffness Index (SI) with stress fractures in a case control study including male military personnel. Thirty-two patients with stress fractures were matched with 32 uninjured healthy volunteers (controls), by gender, age, height, body weight, and level of physical activity. The two groups were genotyped for the FokI, BsmI, ApaI, and TaqI polymorphisms of the *VDR* gene with PCR-RFLP method. In addition, calcaneal SI was measured by heel quantitative ultrasound in both groups. Data were analyzed by chi-squared test and logistic regression analysis. The f allele was significantly more frequent in patients than in controls (p=0.013), while the B allele showed such a tendency without reaching statistical significance (p=0.052). Among the entire cohort, a 2.7-fold and a 2.0-fold increase in risk of stress fractures was associated with the f and B alleles (OR, 2.7, 95% CI, 1.2–5.9; p=0.014 and OR, 2.0, 95% CI, 1.0–4.1; p=0.053, respectively). No statistically significant association was found between the incidence of stress fractures and t or a alleles. Decreased T-scores were also associated with the presence of f and B alleles. Mean values of T-scores of SI were statistically significantly lower in patients than in controls (p=0.018). These results suggest that the FokI and BsmI polymorphisms of the *VDR* gene could be associated with increased risk of stress fractures among military personnel. Moreover, a low calcaneal SI could represent a measurable index of this increased risk.

Introduction

Stress fractures occur with no history of specific injury to account for the fracture and are common injuries frequently seen in athletes and military recruits. Several intrinsic and extrinsic risk factors have been implicated although there is no general agreement as far as the relationship between these factors and the risk for stress fractures is concerned[1,2]. Vitamin D plays an important role in skeletal metabolism, as well as in other metabolic pathways, such as those involved in the immune response and cancer[3]. The vitamin D endocrine system includes vitamin D, its active form 1,25-dihydroxyvitamin D [1,25-$(OH)_2D_3$ - calcitriol], the metabolizing enzymes involved in the formation of the biologically active form of the hormone, as well as a specific receptor - Vitamin D Receptor (*VDR*) - to mediate its genomics actions[4,5].

Polymorphisms sequence variations in the *VDR* gene, have been reported. These changes can occur in coding and noncoding parts of the gene and lead to changes in the protein sequence or affect the degree of expression of the gene, and thus the levels of the protein respectively[4,5]. The relationship between the BsmI and FokI polymorphisms and bone mass

density (BMD) has been extensively studied, but whether these two genetic changes affect *VDR* function and/or predisposition to various skeletal conditions or bone disease still remains a matter of controversy[5]. A positive association between the b allele and BMD has been found and the haplotypes Bat and BAt have been strongly associated with osteoporosis[6]. Conflicting results have been reported for the FokI polymorphism and its association with BMD[5].

It was recently shown that adequate levels of vitamin D play an important role in the prevention of stress fractures in female navy recruits[7]. Earlier observations imply that a genetic component for stress fractures must exist (for example, monozygotic twins with multiple stress fractures in identical anatomical sites[8]). Candidate genes for stress fractures must logically be involved in bone formation, remodeling, or bone matrix formation. Thus, *VDR* alleles could be considered attractive candidates as the genetic determinants of stress fractures.

In the present study, we hypothesized that *VDR* polymorphisms may affect skeletal strength and thus constitute intrinsic risk factors for stress fractures. We thus determined the prevalence of *VDR* genotypes - the FokI, BsmI, ApaI, TaqI polymorphisms in particular- and their association with stress fractures and calcaneal Stiffness Index (SI) in military personnel.

Materials and Methods

Patients and clinical protocol

In this case-control study, 32 patients with stress fractures and 32 healthy volunteers (controls) were included. The two groups were matched on an individual basis, for gender, age, height, body weight and level of physical performance such that a control had to be within±10% of a patient, during a period of two years. All patients and healthy volunteers were genotyped for the FokI, BsmI, ApaI, TaqI polymorphisms by standard methods[4,5,6], and see below).

In both groups of patients and controls, only male military personnel beyond basic training were included. Controls were recruited among healthy volunteers with no history of stress fracture or other bone disease, who visited the hospital for routine health checkup. After institutional review board approval and appropriate informed consent, calcaneal quantitative ultrasound (CQUS) was measured by heel ultrasound and an appropriate questionnaire concerning the alcohol consumption (doses/week), calcium intake (mg/day) and smoking habits (packs/week) was completed for each patient and healthy volunteer.

Stress fractures were diagnosed by the first author of this study, using a predetermined algorithm. Soldiers that were referred for pain in the lower extremity with no history of injury or any specific event prior to the symptoms were examined clinically. In patients with localized bony tenderness a stress fracture was diagnosed with an anterior/posterior and lateral radiograph (n=21), or a technetium-99 bone scanning (n=11). Twenty-three patients sustained a stress fracture of the metatarsals, five patients of the tibia, three patients of the calcaneus, and one patient of the femoral neck.

DNA genotyping

Approximately 10 ml of venous blood was collected with a standard venipuncture technique in ethylenediaminetetraacetic acid (EDTA)-containing tubes. Genomic DNA was isolated from whole blood using standard procedures. All individuals were genotyped for four different *VDR* polymorphisms (FokI in exon 2, BsmI and ApaI in intron 8, and TaqI in exon 9) using PCR amplification with published oligonucleotide primers and conditions[9,10],

followed by restricted endonuclease digestion and electrophoresis on 3% agarose gels. In all cases, genotypes were designated by a lowercase letter for the presence of a restriction site and a capital letter for the absence ("f" and "F" alleles correspond to the presence and absence of a FokI site, respectively).

Calcaneal Quantitative UltraSound (CQUS) measurement

The CQUS was measured on the day of the diagnosis for the subjects and on the day of the first visit for the controls. The dominant limb was chosen for both groups, by checking limb use during a specific physical exercise (kicking a football). The three patients, who sustained a calcaneal stress fracture, were included in the study group because the nondominant limb was affected. The Achilles Express Ultrasonometer (Lunar Corporation, Madison, WI, USA) was used for the measurements. This ultrasonometer measures the ultrasound properties of the heel namely, the broadband ultrasound attenuation (BUA) and speed of sound (SOS) through calcaneus, and automatically calculates the patients Stiffness Index (SI), which is compared to young adult (T-score) references.

The SI is the sum of the scaled and normalized BUA and SOS values. It is a measure of bone strength and is sensitive to bone structure and bone mineral density. It can be calculated either automatically by the ultrasonometer (as in our case), or through an empirically derived formula[11]. According to the manufacturer's operation manual of our equipment, the SI has a precision error in osteoporotic patients comparable to that of X-ray absorptiometry (about 2%).

Statistical analysis

Statistical analysis of the data was performed using the Statistical Package for the Social Sciences (SPSS), version 13.0 (SPSS, Inc., Chicago, IL, USA). The normality of quantitative variables was tested with Kolmogorov-Smirnov test. Normally distributed variables were expressed as the mean±standard deviation, while non-normally distributed variables were expressed as the median and range. Categorical variables were expressed as frequencies (and percentages). The chi-square test was used to evaluate any potential association between categorical variables. It was also used to compare the observed frequency of each genotype with that expected for a population in the Hardy-Weinberg equilibrium. Student's *t*-test or median test was used to assess differences of quantitative variables between two groups. One-way analysis of variance (ANOVA) was used to assess differences of Calcaneal Quantitative UltraSound measurements between three groups according to their genotype; post hoc analysis was performed using Tukey's test. Multivariate stepwise linear and logistic regression models were separately constructed, adjusted for age, BMI, smoking, alcohol and calcium intake, to evaluate the independent effect of *VDR* polymorphisms on stress fractures and Calcaneal Quantitative UltraSound measurements, respectively. Odd ratios (OR) and 95% confidence intervals (CI) were estimated as the measure of association of *VDR* polymorphisms with stress fractures. All tests were two tailed and statistical significance was considered for p-values less than 0.05.

Results

Clinical data

The patients' age ranged from 19 to 30 years, with a mean age of 22.91±2.99 years. Controls' age ranged from 19 to 30 years, with a mean age of 22.91±3.21 years. The characteristics of healthy controls and patients with a stress fracture are compared in (Table 1). There were no statistically significant differences in age (p=1.000), height (p=0.498), weight (p=0.624), alcohol consumption (p=0.562), calcium intake (p=0.284), and smoking (p=0.724) between these two groups. Mean values of T-scores, measured by heel ultrasound, were statistically

significantly lower in patients than in controls (0.21±0.95 vs. 0.33±0.82, p=0.018 for T-scores).

FokI polymorphism

The FF, Ff and ff genotypes of FokI polymorphism were found in 62.5%, 34.4% and 3.1% of healthy controls and in 31.2%, 56.2% and 12.6% of patients, respectively. A statistically significant difference of genotype frequencies (p=0.033) was found between the two groups. In particular, the patients were more likely to have the Ff or ff genotypes and less likely to have the FF genotype, than healthy controls. Statistically significant higher frequencies of the f allele (40.6% vs. 20.3%, p=0.013) and the f-containing genotypes (68.8% vs. 37.5%, p=0.012) were also found in patients compared to controls. Further analysis of genotype frequencies revealed that the genotype distribution in both groups was in Hardy-Weinberg equilibrium (p=1.000 in controls and p=0.871 in patients).

Logistic regression analysis, which was conducted to evaluate the risk of stress fractures according to the FokI polymorphism, revealed that stress fractures were 8.0 (95% CI, 0.8–81.3; p=0.079) and 3.3 (95% CI, 1.1–9.5; p=0.029) times more likely to be found in patients with ff and Ff genotypes, respectively, compared to homozygous FF genotype. The presence of the f-containing genotypes yielded an odds ratio for stress fractures of 3.7 (95% CI, 1.3–10.3; p=0.014) compared to the FF genotype. In multivariate analysis, adjusted for age, BMI, smoking, alcohol and calcium intake, the presence of homozygous ff (aOR=10.2, 95% CI, 1.0–88.7, p=0.047), heterozygous Ff (aOR=3.7, 95% CI, 1.2–11.6, p=0.028), and f-containing (aOR=4.1, 95% CI, 1.3–12.7, p=0.014) genotypes remained independent risk factors of stress fractures.

BsmI polymorphism

The bb, Bb and BB genotypes were found in 28.1%, 59.5% and 12.5% of healthy controls and in 6.2%, 68.8% and 25.0% of patients, respectively. A statistically significant difference of genotype frequencies (p=0.050) was found between the two groups, with patients being more likely to have the BB and Bb genotypes and less likely to have the bb genotype, than healthy controls. Moreover, the B-containing genotypes (93.8% vs. 71.8%, p=0.020) were more prevalent in patients compared to healthy controls, while the B allele itself, was more prevalent but without reaching statistical significance (59.4% vs. 42.2%, p=0.052). The genotype distribution in both groups was in Hardy-Weinberg equilibrium (p=0.585 for Control group and p=0.258 for Patients' group). The presence of BB, Bb, and B-containing genotypes were associated with a 9-, 5.2-, and 5.9-fold increase in risk of stress fractures compared to the bb genotype, respectively (all p<0.05). After adjustment for possible confounders, the presence of two copies of B allele and the presence of B-containing genotypes remained independent risk factors of stress fractures, with adjusted odds ratios of 8.8 (95% CI=1.1–68.6, p=0.039) and 5.3 (95% CI=1.0–29.4, p=0.050), respectively. Heterozygous Bb genotype showed an intermediate risk of 4.6 (95% CI, 0.8–26.4), which did not reach statistical significance (p=0.085).

TaqI and ApaI polymorphisms

Regarding TaqI and ApaI polymorphisms, both allele and genotype frequencies were similar between healthy controls and patients with a stress fracture (all p>0.400; Table 2). The genotype distribution in the control group (p=0.747 and p=0.595) and in the patients' group (p=0.878 and p=0.305) was in Hardy-Weinberg equilibrium for TaqI and ApaI polymorphisms, respectively. There were no statistically significant associations between TaqI or ApaI polymorphisms and stress fracture risk (Table 2).

T-score

We analyzed the association of *VDR* polymorphisms with the Calcaneal Quantitative UltraSound measurements. One-way analysis of variance revealed that the effect of FokI and BsmI polymorphisms was statistically significant on the T-score (p=0.013 and p<0.001, respectively). Post hoc analysis indicated that both scores were significantly lower in homozygous ff genotype compared to heterozygous Ff (T-score: p=0.044) and homozygous FF (T-score: p=0.014) genotypes, and significantly higher in homozygous bb genotype compared to heterozygous Bb (both p<0.001) and homozygous BB (both p<0.001) genotypes. No significant associations of TaqI and ApaI polymorphisms with the Calcaneal Quantitative UltraSound measurements were found (all p>0.10; Table 3). The results of the multivariate regression analyses, which were performed to examine the relationship between *VDR* polymorphisms and the Calcaneal Quantitative UltraSound measurements, controlling for the effect of potential confounders, showed that the presence of BB, Bb, and ff genotypes remained significantly independent determinants of low T-scores (p<0.001, p<0.001 and p=0.027, respectively).

Discussion

The main finding of this study is that the presence of f and, possibly, B alleles is associated with an increased risk for stress fracture as the f allele was significantly more frequent in subjects than in controls, while B allele showed such a tendency without reaching statistical significance. In addition to this, T-scores of SI in Calcaneal Quantitative UltraSound measurement were statistically significantly lower in subjects than in controls.

Stress fractures have been suggested to be a biological process in a susceptible individual during which, increased and prolonged mechanical usage stimulates bone turnover, resulting in focally increased bone remodeling and decreased bone mass with subsequent bone micro-damage and failure[12]. Several observations imply that a genetic component for stress fractures may exist[8,13,14]. In addition, ample evidence suggests that bone mass and size influence stress fracture risk[15,16,17].

Until now, *VDR* polymorphisms have been linked to bone mineral density[18,19] but few investigators have tried to examine the relationship between stress fractures and DNA genotyping in general and the prevalence of *VDR* genotypes in subjects presenting with stress fractures [20]. *VDR* polymorphisms could be involved in stress fractures not only through bone mass density changes, but also through subtle changes in bone structure that would make the bone more susceptible to stress fractures[21]. These effects could be quantitatively measured through ultrasound examination of related bone characteristics [22]. Because the etiology of stress fractures is multifactorial[1,2], the study was conducted in military personnel beyond basic training in an effort to reduce the confounding variables (risk factors). Military personnel beyond basic training represent a more homogenous group compared to military recruits in whom previous physical activity and the way of accommodation in military life - including training and nutrition - may differ significantly. Although there was no significant difference between subjects and controls in terms of age, BMI, calcium intake, smoking and alcohol intake, a multivariate stepwise logistic regression analysis was separately conducted with an adjustment for these factors including SI measurements. Fokl and BsmI polymorphism were found to be independent risk factors for stress fractures.

VDR genotypes and BMD have been extensively studied. Currently it is accepted that Bsml polymorphism is related to the BMD but its effect is relatively small and strongly influenced by some other non-genetic factors like diet[5]. Despite the conflicting results that have been reported in studies[23,24,25,26] that followed the initial reports[27, 28], a recent meta-analysis

found that the B allele was significantly associated with BMD at the spine, with the BB genotype having lower BMD than Bb/bb genotypes at baseline[29].

In our study, the B allele showed a tendency (without reaching statistical significance) to be more frequent in patients, in whom SI (mean values of T-scores) was statistically significantly lower than in controls. Although the ultrasonometer does not directly measure the bone mineral density but rather is designed for fracture risk assessment, this observation could be useful for future studies. Calcaneal ultrasound measurement can be easily performed with a portable machine and a large number of subjects could be assessed with no exposure to radiation.

Conflicting results have been reported for the FokI polymorphism and its association with BMD and bone biology in general. Strong, weak[30,31,32,33,34,35,36], or no association[37,38,39,40] has been found with f allele and low BMD at various sites in the body, in adolescents, pre- or postmenopausal women. In our study the f allele was significantly more frequent in patients in whom SI (mean values of T-scores) was statistically significantly lower than in controls.

Although there are few studies that have found correlation between ApaI and TaqI polymorphisms with BMD and markers of bone biology[23,40,41], we did not manage to detect any association of those polymorphisms with the calcaneal ultrasound measurements in our cohort.

In conclusion, our results suggest that the FokI and BsmI polymorphisms of the *VDR* gene could be associated with increased risk of stress fractures among men of military personnel. Moreover, a low calcaneal SI could represent a measurable index of this increased risk.

Acknowledgments

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Table 1

Characteristics of patients and healthy controls[*]

	Patients	Controls	p-Value
Age (years)	22.91±2.99	22.91±3.21	1.000
Height (cm)	179.27±6.55	178.00±5.77	0.498
Weight (kg)	76.91±7.53	77.86±5.06	0.624
Alcohol consumption (doses/week)	2 (0–6)	2 (0–6)	1.000
Calcium intake (mg/day)	870 (0–1820)	1–090 (0-1–660)	0.284
Smoking (pack/week)	0.50 (0–14)	0.75 (0–14)	0.724

[*] Data were expressed as means±standard deviation for normally distributed quantitative variables and as medians and range for non-normally distributed quantitative variables

Table 2

Distribution of *VDR* polymorphisms among patients with a stress fracture and controls[#]

	Patients[*]	Controls[*]	aOR[†]	95% CI	p-Value
FokI polymorphism					
Genotype					
FF	10 (31.2)	20 (62.5)	1.0		
Ff	18 (56.2)	11 (34.4)	3.7	1.2–11.6	0.028
ff	4 (12.6)	1 (3.1)	10.2	1.0–88.7	0.047
Ff or ff	22 (68.8)	12 (37.5)	4.1	1.3–12.7	0.014
Allele					
F	38 (59.4)	51 (79.7)	1.0		
f	26 (40.6)	13 (20.3)	2.8	1.2–6.3	0.017
BsmI polymorphism					
Genotype					
BB	8 (25.0)	4 (12.5)	8.8	1.1–68.6	0.039
Bb	22 (68.8)	19 (59.5)	4.6	0.8–26.4	0.085
bb	2 (6.2)	9 (28.1)	1.0		
BB or Bb	30 (93.8)	23 (71.9)	5.3	1.0–29.4	0.050
Allele					
B	38 (59.4)	27 (42.2)	2.2	1.0–4.4	0.051
b	26 (40.6)	37 (57.8)	1.0		
TaqI polymorphism					
Genotype					
TT	9 (28.1)	11 (34.4)	1.0		
Tt	18 (56.3)	18 (56.3)	1.1	0.3–3.7	0.846
tt	5 (15.6)	3 (9.4)	4.5	0.6–33.5	0.138
Tt or tt	23 (71.9)	21 (65.6)	1.4	0.4–4.4	0.577
Allele					
T	36 (56.3)	40 (62.5)	1.0		

	Patients*	Controls*	aOR†	95% CI	p-Value
t	28 (43.8)	24 (37.5)	1.7	0.8–3.5	0.199
ApaI polymorphism					
Genotype					
AA	9 (28.1)	8 (25.0)	1.0		
Aa	10 (31.3)	12 (37.5)	0.5	0.1–2.1	0.355
aa	13 (40.6)	12 (37.5)	0.7	0.2–2.7	0.598
Aa or aal	23 (71.9)	24 (75.0)	0.6	0.1–2.1	0.425
Allele					
A	28 (43.8)	28 (43.8)	1.0		
a	36 (56.2)	36 (56.2)	0.9	0.4–1.8	0.747

[#]Statistical significance for differences between patients and controls in genotypes frequencies (p=0.033, p=0.050, p=0.705, and p=0.869), f-, B-, t- and a-containing genotypes frequencies (p=0.012, p=0.020, p=0.590, and p=0.777) and allelic frequencies (p=0.013, p=0.052, p=0.472, and p=1.000) of FokI, BsmI, TaqI, and ApaI polymorphisms, respectively

[*]Data given are number of subjects and percentage (%)

[†]Adjusted for age, BMI, smoking, alcohol, and calcium intake

aOR: adjusted odds ratio; CI: confidence interval

Table 3

Calcaneal Quantitative UltraSound measurements (mean ± SD) in relation to *VDR* polymorphisms

	T-score N	p-Value
FokI polymorphism		0.013
FF	0.47±1.12	
Ff	0.16±0.65	
ff	−0.84±0.53	
BsmI polymorphism		<0.001
BB	−0.33±0.71	
Bb	0.09±0.88	
bb	1.30±0.51	
TaqI polymorphism		0.188
TT	0.40±0.98	
Tt	0.25±0.91	
tt	−0.32±0.98	
ApaI polymorphism		0.517
AA	0.36±0.90	
Aa	0.31±0.83	
aa	0.05±1.08	