Vitamin d-receptor gene polymorphisms and vertebral bone density in men with idiopathic hypogonadotrophic hypogonadism

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SUMMARY

Background: Optimal peak bone mass is closely related to sufficient and appropriately timed androgen release. However, attainment of peak bone mass in men, as in women, is under genetic control, as well as being subject to hormonal and mutational effects. With increasing recognition of osteoporosis and related fractures in men, it is of interest to consider whether there is relationship between bone density and vitamin D receptor (VDR) polymorphisms, as described in women.

Material and methods: To assess the influence of allelic variation in the VDR gene on vertebral bone density in men with idiopathic hypogonadrotrophic hypogonadism (IHH), 27 patients (mean age 21.4±0.4 yrs) and 25 age-and-BMI matched healthy males (mean age 21.2±0.3) were genotyped using three restriction enzymes (Apa I, Bsm I, and Taq I). Vertebral bone mineral density was measured using dual energy X-ray absorptiometry (DEXA).

Results: As expected, vertebral bone density was reduced significantly in patients with IHH (p<0.001). Despite weak evidence for an association between Apa I polymorphism and vertebral bone density in the IHH group (r=0.454, p=0.017 and r²=0.20), VDR genotype was not associated with vertebral bone density in either group. When analyzing homozygous haplotypes, the probability of carrying the favorable BAt haplotype was greater in the control group (OR=2.000 vs. 0.500).

Conclusion: We conclude that VDR genotype has no influence on vertebral bone density in men with IHH. Thus, allelic variation in the VDR cannot help define those at increased risk for osteoporosis and related fractures among such patients.

BACKGROUND

Albright and Reifenstein first described the pathological association of androgen deficiency and osteoporosis in the late 1940s [1]. Since then, it has required decades to demonstrate the presence of androgen receptors, androgen metabolism, and androgenic effects in bone cells [2–8]. Despite the hypothesis suggesting a relationship between peak bone mass (bone mass achieved in early adulthood) and sufficient and appropriately timed androgen release [9], attainment of peak bone mass in men is largely under genetic control [10].

Bone density is low in hypogonadal men [11–16]. If the hypogonadal state is present before achievement of peak bone mass both cortical and trabecular bone density are low [17]. However, patients with Klinefel-
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ter’s syndrome present with cortical bone loss, which may be attributable to the variable level of hypogonadism, or to the chromosomal abnormality existing in such patients [16]. Restoring cortical bone mass in the postpubertal period with androgen replacement is often unsuccessful in patients with Klinefelter’s syndrome or in males with IHH [16,17]. This supports the view that puberty is crucial for determining peak cortical bone mass. In the case of hypogonadism experienced after attainment of peak bone mass (e.g. elderly men), bone turnover is increased and trabecular bone loss ensues [12,15]; the effects on cortical bones, however, remain unclear.

The vitamin D receptor (VDR) is currently a major focus of research in the field of bone and calcium metabolism. This receptor mediates vitamin D action by binding the active metabolite of vitamin D, 1,25-dihydroxyvitamin D3, and subsequently increasing or decreasing the transcription of target genes. The VDR plays a critical role in the maintenance of serum calcium levels, modulating the levels of calcium binding proteins in the intestine and kidney, and playing a role in the regulation of bone. Allelic variations in the VDR gene have been suggested to account for up to three quarters of the genetic effect on bone density in healthy women [18]. How might these polymorphisms exert such a profound effect on bone density remains to be elucidated, but current data raise the question of whether such a genetic defect might also exist in healthy and osteoporotic men.

The present study aims to gain further insight into the genetic contribution of the VDR gene to low bone mass in men with IHH.

**MATERIAL AND METHODS**

**Subjects**

Twenty-seven untreated men with IHH (mean age: 21.4±0.43 (mean±SEM) years, age range: 19–24 yrs, BMI: 21.7±0.6 Kg/m²) and sex- and age-matched 25 healthy male individuals (mean age: 21.2±0.3 yrs, age range: 19–23 yrs BMI: 22.4±0.30 Kg/m²) were enrolled in the study. The diagnosis of IHH was based on absent or severely retarded sexual maturation and failure to undergo spontaneous puberty before 18 yr of age, and was confirmed by a decreased serum testosterone concentration below normal range of adults, FSH and LH levels within or below the normal range, absence of a pituitary or hypothalamic mass lesion on computerized tomography or magnetic resonance imaging, the presence of a gonadotropin response to repetitive doses of GnRH, normal smell test and normal karyotypes (46,XY). None of the patients had hyposmia or anosmia, or a family history of IHH. There were no other hypothalamic or pituitary functional abnormalities in these patients. All patients are sporadic and there was no relativity with each other. A history of chronic illness, use of medications that may influence bone metabolism within the previous six months, and smoking were the exclusion criteria. Informed consent was obtained from each subject and the study protocol was approved by the local ethical committee.

**Genotyping**

The genotypes for three restriction-fragment-length polymorphisms of the VDR gene were determined by polymerase chain reaction (PCR) amplification and enzymatic digestion of the products with Apa I, Bsm I, and Taq I. The polymorphic sites for Bsm I and Apa I were in the region of the gene between exon 7 and the 3’ UTR [19]. No polymorphic sites were in the coding region, except for a T-for-C synonymous coding change in exon 9, changing ATT to ATC (isoleucine), creating a Taq I polymorphism. The forward and reverse primers for the DNA sequence flanking the Bsm I site were 5’-CAACCAAGACTACAAGTACCGCGTCAGTGAA-3’ and 5’-AACCAGCGGGAAGAGGTCAAGGGGCT CCAGTGA-3’. The forward primer for the Apa I and Taq I polymorphisms was the same as that used for Bsm I site. The reverse primer for the Apa I and Taq I polymorphisms was 5’-CACTTCCGAGCAACAGGGGT GAAAGGTCAAGGGG3’. The forward primer for the Apa I and Taq I polymorphisms was the same as that used for Bsm I site. The reverse primer for the Apa I and Taq I polymorphisms was 5’-CACCTCGAGACCAAGGGCTCCAGTGA-3’. PCR was performed with a thermal cycler (Perkin-Elmer, USA) under standard conditions for 35 cycles, using 65°C as annealing temperature. The PCR product for Bsm I polymorphism was 825 base pairs (bp) long, and the restriction fragments were 650 bp and 175 bp long. The PCR products for the Apa I and Taq I polymorphisms were 2000 bp long; the length of the fragments after digestion with Apa I were 1700 and 300 bp, and the lengths of the fragments after digestion with Taq I were 1800 and 200 bp.

With the enzymes Apa I, Bsm I, and Taq I, the respective genotypes were defined as A, B, T (indicating the absence of the restriction site) or a, b, t (indicating the presence of restriction site) [20].

**Hormonal analysis**

After an overnight fast, blood was collected for the measurements of serum FSH, LH, and free testo-
sterone. FSH and LH were measured using a chemiluminescence kit (Chiron/Diagnostics, Halsted, Essex, UK). Free testosterone was also measured using a chemiluminescence kit (Diagnostic Systems Laboratories Inc, Webster, TX, USA). Normal values for FSH, LH, and free testosterone were <15mIU/mL, <15mIU/mL, and 8–55 pg/mL, respectively.

Bone density measurement

Vertebral bone mineral density (BMD) was measured using DEXA (Norland XR 36-WBL, Norland Inc, Fort Atkinson, WI, USA) at the level of L2–L4.

Statistical analysis

Mann-Whitney-U and Chi Square Fisher’s exact tests were used to assess differences between the groups. Spearman’s Rho test was used to assess significant correlation between the variables. The values were expressed as mean ± SEM. A p value less than 0.05 was accepted as significant.

RESULTS

Clinical and laboratory characteristics of both groups are shown in Table 1. The mean serum FSH, LH, and free testosterone levels were significantly low in the IHH group. The mean BMD was also significantly reduced in the patient group. Percentage distributions of genotypes were similar in both groups. The most frequent genotype was Aa in both groups, whereas aa was the least frequent genotype (Table 2).

Comparison of the homozygous haplotypes (BAT and baT) between both groups, using Fisher’s exact test, showed no significant variation (p = 0.315) (Table 2). However, the probability to carry the favorable BAT haplotype was four-fold greater in the control group as compared to the IHH group (OR = 2.00 vs. 0.50; 95% CI: 0.589–6.790 and 0.169–2.237, respectively) (Table 2).

When analyzing whether any of the three polymorphisms (Apa I, Bsm I, and Taq I) were correlated to BMD in either group, there was weak evidence for an association between the Apa I polymorphism and BMD in the patient group (r = 0.454, p = 0.017). However, the r² value was 0.216, which indicates that the observed correlation was true only for 21% of the patients. In other words, the correlation was rather weak. This was further investigated with Kruskal-Wallis test and no significant association between Apa I genotypes and BMD was found (χ²=5.517, p=0.63).

<table>
<thead>
<tr>
<th>Variable</th>
<th>IHH group (mean±SEM)</th>
<th>Controls (mean±SEM)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>21.4±0.4</td>
<td>21.2±0.3</td>
<td>0.940 (NS)</td>
</tr>
<tr>
<td>Bone age (year)</td>
<td>17.5±0.32</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.7±0.6</td>
<td>22.4±0.3</td>
<td>0.634 (NS)</td>
</tr>
<tr>
<td>Free Testosterone (pg/mL)</td>
<td>2.33±0.25</td>
<td>23.94±1.85</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FSH (mIU/mL)</td>
<td>1.38±0.36</td>
<td>2.44±0.30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LH (mIU/mL)</td>
<td>1.18±0.25</td>
<td>2.41±0.25</td>
<td>0.001</td>
</tr>
<tr>
<td>BMD (g/cm²)</td>
<td>0.77±0.029</td>
<td>1.190±0.013</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Mann-Whitney U test; NS: not significant

Table 2. Percentage distribution of genotypes and frequencies of BAT and baT haplotypes in IHH and control groups.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (%)</th>
<th>Controls (%)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apa I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>40.7</td>
<td>40.0</td>
<td></td>
</tr>
<tr>
<td>Aa</td>
<td>44.4</td>
<td>52.0</td>
<td>0.712 (NS)</td>
</tr>
<tr>
<td>aa</td>
<td>14.9</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>Bsm I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BB</td>
<td>29.6</td>
<td>24.0</td>
<td></td>
</tr>
<tr>
<td>Bb</td>
<td>29.6</td>
<td>40.0</td>
<td>0.729 (NS)</td>
</tr>
<tr>
<td>bb</td>
<td>40.8</td>
<td>36.0</td>
<td></td>
</tr>
<tr>
<td>Taq I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>37.0</td>
<td>32.0</td>
<td></td>
</tr>
<tr>
<td>Tt</td>
<td>29.6</td>
<td>40.0</td>
<td>0.734 (NS)</td>
</tr>
<tr>
<td>tt</td>
<td>33.4</td>
<td>28.0</td>
<td></td>
</tr>
<tr>
<td>BAT</td>
<td>11.1</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>BaT</td>
<td>14.8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>OR</td>
<td>0.50</td>
<td>2.00</td>
<td></td>
</tr>
<tr>
<td>95% CI</td>
<td>0.169 – 1.477</td>
<td>0.589 – 6.790</td>
<td></td>
</tr>
</tbody>
</table>

*Chi square test; NS: not significant
The relationship between VDR polymorphisms and vertebral bone density are shown in figure 1.

**DISCUSSION**

Bone density, as with other complex traits, is likely to be determined by multiple genetic and environmental factors. Recent molecular studies have shed considerable light on the genetic component. However, heritable determinants of peak bone mass are still poorly understood, particularly in males. Common allelic variations in the gene encoding the VDR have been shown to be determinants of circulating osteocalcin levels and bone density [18,19]. Thus, genotyping of this locus might be used to predict an individual’s risk of osteoporosis. However, there are substantial racial differences in genotype frequencies of the VDR polymorphism, and not all studies have confirmed the relation between VDR polymorphism and bone remodelling [21,22].

Male hypogonadism is known to be associated with low bone mass. A few studies have investigated the relation between BMD and polymorphisms at the VDR locus in males with varying age [23–25], but there are no studies investigating this relationship in men with IHH. Gunnes et al. found no such association in a group of healthy boys [23]. However, work by Ferrari et al. suggested that healthy young men with the BB genotype had significantly low BMD at the lumbar spine and femoral neck [24]. Moreover, serum PTH levels were reported to be significantly greater in the BB genotype at baseline and remained so after a low or high calcium – phosphorus diet.

It is of interest that Francis et al. also found no association between VDR gene polymorphism and BMD or fractional calcium absorption in a group of males comprising healthy adults and men with vertebral crush fractures [25]. In the present study, we did not find an association between VDR gene polymorphism and BMD at the lumbar spine in either healthy males or men with IHH. However, it is of interest that Francis et al. also found no association between VDR gene polymorphism and BMD or fractional calcium absorption in a group of males comprising healthy adults and men with vertebral crush fractures [25]. In the present study, we did not find an association between VDR gene polymorphism and BMD at the lumbar spine in either healthy males or men with IHH. In contrast to previous studies, the most frequent genotype was Aa, and the least frequent genotype was aa in both groups of men. However, Sainz et al. have also reported that Aa was the most common genotype girls of Mexican descent [20]. These differences in genotype frequency may represent differences in genetic background. This study is the first to analyse the frequency of the VDR genotypes in Turkish population.

The molecular mechanisms by which bone density might be affected by polymorphisms in the VDR gene are not certain, although allelic variations in the 3’ untranslated region may alter mRNA levels [18]. Morrison et al. have demonstrated that the homozygous VDR haplotype, Bat, is associated with increased VDR gene transcription and/or stability of its mRNA in vitro [18]. Gennari et al. have reported that the Bat haplotype is more prevalent in osteoporotic postmenopausal women [26]. In conjunction with Morrison et al. [19], another in vitro study revealed that the Ca++ concentration needed for half maximal inhibition of PTH release was lower in parathyroid adenoma cells homozygous for Bat compared to those exhibiting the bat haplotype [27]. In our study, the probability of carrying the favorable Bat haplotype was greater in the control group (OR=2.000 vs. 0.500). Although, we realize a power analysis is important, we can only study the patients we can collect because IHH is rare as observed in other countries. Our results reveal no association between the VDR polymorphisms and vertebral bone mineral density.

**CONCLUSIONS**

We conclude that VDR genotype has no influence on vertebral bone density in men with IHH. Thus, allelic variation in the VDR cannot help define those at increased risk for osteoporosis and related fractures among such patients.

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