Lack of a High Prevalence of the BB Vitamin D Receptor Genotype in Severely Osteoporotic Women*

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ABSTRACT

Studies of twins strongly suggest that more than 50% of the peak spinal bone density is determined by genetics. It was reported recently that this genetic effect is primarily determined by vitamin D receptor (VDR) alleles; specifically, a VDR genotype termed BB has been highly associated with low peak bone density. Homozygotes for the second VDR allele, bb, are associated with high peak bone density. If peak bone density is an important determinant of osteoporosis and if the VDR genotype is an important determinant of peak bone density, then patients with severe osteoporosis should have a high prevalence of the BB VDR genotype compared with that of control subjects. To test this hypothesis, we used Southern blot analysis to determine the VDR genotype of 41 Caucasian patients (72 ± 14 yr) with severe osteoporosis (27 women with spinal bone densities below 50 mg/cm² as determined by quantitative computed tomography; 14 women with spinal bone densities below 0.75 g/cm² as determined by dual energy x-ray absorptiometry) and 23 Caucasian control subjects (68 ± 7 yr) without osteoporosis (quantitative computed tomography values at or above the fracture threshold of 100 mg/cm²). Only 6 of the 41 individuals in the group with severe osteoporosis had the BB genotype, whereas 16 had the bb genotype. In the control group comprising 23 individuals, 7 had the BB genotype and only 6 had the bb genotype. We conclude that the BB VDR genotype is not a good predictor of risk for developing severe osteoporosis in our population. (J Clin Endocrinol Metab 80: 2158–2162, 1995)

OSTEOPOROSIS, a disorder characterized by excessive bone loss, increased risk of fractures, and other debilitating conditions, is increasing in incidence in most developed countries (1, 2). Although much of this increase can be attributed to aging, many genetic and nongenetic factors are also known to influence the inherent risk of developing osteoporosis (3). Understanding the causes of osteoporosis and identifying the subjects who have the greatest risk for developing osteoporosis are therefore primary objectives in devising effective therapies for (and delivering effective therapies to) affected individuals.

Peak bone mass is believed to be a major determinant of an individual's risk of developing osteoporosis. Although many factors such as diet, estrogen levels, and growth factors influence bone mass (4, 5), it is well established that genetic factors contribute significantly to the determination of peak bone density (6–9).

Recently, inheritance of a specific vitamin D receptor (VDR) allele was shown to be a major genetic determinant of peak bone density (10, 11). Applying restriction fragment length polymorphism (RFLP) analysis to Caucasian women in Australia, Morrison et al. (10) found a highly significant association between the serum level of osteocalcin (a protein of skeletal origin that is regulated by vitamin D) and a VDR gene allele termed B, which is defined by the absence of a BsmI restriction enzyme site in the intron between exons 8 and 9 of the VDR gene. Equally significant correlations were found using Apal and EcoRV, two other restriction enzymes that can also be used to detect polymorphisms in the VDR gene (10). A subsequent Australian study of twins and nonrelated older women (all Caucasian) revealed a highly significant correlation between low bone density and the B allele in the VDR gene (11), and from this the authors hypothesized that this genetic marker for the VDR gene could perhaps be used to identify subjects with an increased risk for developing osteoporosis.

If peak bone density is a major determinant of osteoporosis and if the VDR genotype is an important determinant of peak bone density, it follows that a population of severe osteoporotic subjects should have a high prevalence of the BB VDR genotype. To test this hypothesis, we used Southern blot analysis to determine the VDR genotype of 41 severe osteoporotic women and 23 age-matched controls. The severe osteoporotic group included 27 women with lumbar spine bone densities below 50 mg/cm² as determined by quantitative computed tomography (QCT) (12), which is 50% of the fracture threshold (13), and 14 women with spinal bone densities below 0.75 g/cm², as measured by dual-energy x-ray absorptiometry (DEXA). Twenty-three age-matched women with QCT spinal bone density measurements above the fracture threshold of 100 mg/cm² were used as controls.

Materials and Methods

Subjects and bone density measurements

Caucasian subjects were selected by a retrospective review of bone density data that had been collected from women evaluated at the Loma Linda University Osteoporosis Research Center. Trabecular bone
density in the spine was measured by either single-energy QCT, as previously described (14), or by DEXA, using a Hologic 1000 (Hologic, Waltham MA). Women having QCT measurements above the fracture threshold of 100 mg/cm³ (13) were selected for inclusion in the control group, and women with QCT values below 50 mg/cm³ or DEXA measurements below 0.75 g/cm² were considered to have severe osteoporosis and were selected for inclusion in the osteoporotic group. Women having intermediate bone density measurements and those with metabolic bone disease such as hyperparathyroidism and Paget's disease, a history of glucocorticoid or anticonvulsant therapy, malabsorption syndrome, or early menopause (i.e.<35 yr) were excluded from this study. Because ethnicity may be an independent determinant of the VDR genotype, non-Caucasian women (e.g. Asian, Hispanic, and African-American) were also excluded from this study. Informed consent was obtained from all subjects.

Southern blot analysis

DNA was isolated from whole blood using the QIAGEN DNA isolation kit (Bio101, La Jolla, CA). For Southern blot analysis, 10 μg DNA was digested with the restriction enzyme BsmAI or Apal (Promega Corporation, Madison, WI) or BsmI (New England Biolabs, Beverly, MA) in the buffer recommended by the manufacturer (e.g. 6 mmol/L Tris-Cl, pH 7.2, containing 150 mmol/L NaCl, 6 mmol/L MgCl₂, 0.1 mg/mL BSA, and 1 mmol/L dithiothreitol, for the BsmAI digestions). The digested fragments were then fractionated by electrophoresis through a 0.8% agarose gel and transferred to Magnagraph nylon membrane (MG1, Westbrook, MA) by standard techniques (15). The 21-kilobase (kb) EcoRI fragment containing the coding sequence of the human vitamin D receptor complementary DNA (cDNA) (16) was obtained from the American Type Culture Collection (ATCC Number 61256; Rockville, MD), purified on low melting point agarose (FMC BioProducts, Rockland, ME), and labeled with [³²P]-deoxycytidine triphosphate (ICN Biomedicals, Irvine, CA) using a random priming labeling kit (Boehringer Mannheim, Indianapolis, IN). Hybridizations were in 5 X SSC (1 X SSC = 0.15 mol/L NaCl, 0.015 mol/L sodium citrate), 2 X Denhardt's solution (1 X Denhardt's = 0.02% each Ficoll 400, polyvinyl pyrrolidine and BSA), 1% sodium deoxycholate, 100 μg/mL salmon sperm DNA, and 50% (vol/vol) formamide at 42 C. Final washes were in 0.5 X SSC, 0.5% sodium deoxycholate at 65 C.

We initially used the restriction enzyme BsmI for our RFLP analysis, but we encountered some problems digesting certain genomic DNA samples. In some cases, high molecular weight DNA did not digest at all or gave incomplete digestions as evidenced by the presence of additional hybridizing bands, indicating incomplete digestion. Although phenol and chloroform extractions of the DNA samples sometimes resolved these problems, we found that digestions with BsmAI, an iso- schizomer of BsmI that recognizes the same DNA sequence as BsmI, reproducibly gave hybridizing fragments of the expected size. The results reported here were therefore determined using the second enzyme BsmAI.

Statistical analysis

Statistical analysis of the proportions of the genotypes was carried out using the computer program Microstat (Ecosoft, Indianapolis, IN). Standard errors of the proportions were calculated as σ = [P(1-P)/N]¹/², and 95% confidence limits were defined by the relationship 1.96[P(1-P)/N]¹/², where P is the decimal proportion and N is the total population. Southern blot analysis and genotype determinations were performed blinded.

Results

We determined the VDR genotype of 64 Caucasian women who were identified in a retrospective analysis of patients from the Loma Linda University Osteoporosis Research Center. Women with QCT lumbar spine bone density measurements above 100 mg/cm³ were selected as control subjects. Women with low bone density measurements (QCT values below 50 mg/cm³ or DEXA values below 0.75 g/cm²) were placed in the severe osteoporotic group. Age and bone density values for control and severely osteoporotic subjects are given in Table 1.

DNA was isolated from whole blood, digested with restriction enzymes, and analyzed by Southern blot analysis using the VDR cDNA as a hybridization probe. Results of a typical Southern blot are shown in Fig. 1 along with the VDR genotype determination. The B allele is classified by the presence of a 7.2-kb band and the b allele by hybridizing bands at 4.6 and 2.6 kb. Thus, a BB homozygote contains two genomic copies of the 7.2-kb fragment and lacks the 4.6- and 2.6-kb fragments (lane 4). The Bb heterozygote has single copy fragments at 7.2, 4.6, and 2.6 kb (lanes 1 and 3), whereas bb lacks the 7.2-kb fragment but has two copies each of the 4.6 and 2.6 band (lanes 2 and 5). Both B and b alleles have invariant bands at 16, 11, and 2.9 kb.

Genotype determinations for both control and osteoporotic groups are summarized in Table 2. Contrary to our hypothesis that the proportion of the BB genotype should be increased in the severely osteoporotic group, the proportion of the BB genotype was actually lower in the osteoporotic group (14.6%) than in the control group (30.4%), although this difference did not reach statistical significance (P = 0.064). However, we did find the proportion of BB in our control group (30.4%) to be significantly higher (P = 0.004) than the proportion seen in the Australian women with the BB genotype having bone densities above the fracture threshold (approximately 3/47 or 6.4%; data taken from Fig. 3, A-C, in reference 11). Surprisingly, the proportion of the bb genotype was not lower in the osteoporotic group compared with that of the controls (i.e. 39.0% and 26.1%, respectively; P = 0.149). As shown in Table 3, differences in genotype within the osteoporotic and controls groups were not associated with differences in trabecular spinal bone density. From these data, we conclude that the BB genotype is not a good predictor of severe osteoporosis in our patient population.

We considered the possibility that our subjects had a different genetic background than did the Australian subjects studied by Morrison et al. (11) and that an RFLP detected by another enzyme might associate more closely with the clinical phenotype than does the BsmI/BsaMI RFLP. We consequently used the restriction enzyme Apal to determine the

<p>| TABLE 1. Age and bone density values for control and severely osteoporotic subjects |
|----------------------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Subjects (n)</th>
<th>Age (yr)</th>
<th>QCT (mg/cm³)</th>
<th>DEXA (g/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (23)</td>
<td>69.3 ± 7.6</td>
<td>132 ± 25</td>
<td></td>
</tr>
<tr>
<td>Osteoporotic (27)</td>
<td>73.1 ± 8.5</td>
<td>30 ± 11b</td>
<td></td>
</tr>
<tr>
<td>Osteoporotic (14)</td>
<td>69.3 ± 7.0</td>
<td>0.676 ± 0.065b</td>
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</tbody>
</table>

All data are shown as mean ± SD. Spinal bone density was measured by QCT in one group of osteoporotic subjects and a group of age-matched controls and by DEXA in a second group of osteoporotic subjects.

b Indicates that all of the osteoporotic subjects in this group had DEXA values that were at least 2 SD below the age-adjusted mean; the average T-score was -3.76 ± 0.58 for this entire group.
FIG. 1. Southern blot analysis of BsaMI digests of genomic DNA using the VDR cDNA as a hybridization probe. The VDR B/b genotype determinations for each sample are given at the top. The arrows at the right indicate the polymorphic BsaMI restriction fragments associated with each allele. Molecular weight size markers are shown to the left.

TABLE 2. Frequencies of VDR genotypes in control and severely osteoporotic subjects using BsaMI

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency with BsaMI number and (%) of total</th>
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<tbody>
<tr>
<td></td>
<td>Control subjects</td>
</tr>
<tr>
<td>Homozygote (BB)</td>
<td>7 (30.4 ± 9.6%)</td>
</tr>
<tr>
<td>Homozygote (bb)</td>
<td>6 (26.1 ± 9.2%)</td>
</tr>
<tr>
<td>Heterozygote (Bb)</td>
<td>10 (43.5 ± 10.3%)</td>
</tr>
</tbody>
</table>

VDR genotypes were determined for a total of 23 control subjects and 41 osteoporotic subjects, using BsaMI, as indicated in Fig. 1. The frequency of each genotype is shown as an absolute number and in parentheses as a percentage of the subjects in each group (mean ± SE). There were no significant differences between the control and osteoporotic subjects with respect to any of the VDR genotypes identified by BsaMI.

VDR genotype of all but three of the subjects who were initially genotyped with BsaMI. In the initial studies of the VDR gene alleles, the A allele was closely associated with the B allele (10). We therefore tested the alternative hypothesis that our severe osteoporotic subjects would have a high prevalence of the AA genotype.

Results of a Southern blot of ApaI digests are shown in Fig. 2 along with the genotype determinations for each sample. The A allele is classified by the presence of a 7.3-kb band, whereas the a allele is characterized by two hybridizing bands 4.3 and 3.0 kb in size. Because an invariant band comigrates with the 4.3-kb band, only the 3.0-kb band was used to determine the a allele.

Genotype determinations using ApaI are summarized in Table 4. As we found with the BB genotype, the AA genotype was not a good predictor for severe osteoporosis. In this case we found approximately equal proportions of AA individuals in the control group (30.4%) and in the osteoporotic group (26.3%). In fact, we found no significant difference between control and osteoporotic subjects for any of the three genotypes classified by ApaI polymorphisms.

A case-by-case comparison of the results of our genotypic analyses using BsaMI and ApaI is summarized in Table 5. A total of 64 subjects were analyzed with BsaMI and a total of 61 subjects (including all 23 of the same controls) were analyzed with ApaI. The same genotype was identified in 77% of the subjects (i.e. 76% for the osteoporotics and 78% for the controls).

TABLE 3. Trabecular spinal bone densities in allelic subgroups of osteoporotic subjects and controls

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of subjects</th>
<th>Genotype</th>
<th>QCT density (mg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoporotic</td>
<td>13</td>
<td>bb</td>
<td>30 ± 7</td>
</tr>
<tr>
<td>Osteoporotic</td>
<td>6</td>
<td>BB</td>
<td>28 ± 8</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>bb</td>
<td>123 ± 15</td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>BB</td>
<td>129 ± 26</td>
</tr>
</tbody>
</table>

Trabecular spinal bone densities (determined by QCT) are shown as group mean values (± SD) for the subgroups of osteoporotic subjects and controls with the bb and BB VDR genotypes. The genotypes were determined using BsaMI, as indicated in Fig. 1. Data for all subjects for whom QCT values were known were included in this analysis. There were no significant differences between the genotypic subgroups of osteoporotic subjects or controls.

TABLE 4. Frequencies of VDR genotypes in control and severely osteoporotic subjects using ApaI

<table>
<thead>
<tr>
<th>Genotype</th>
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Genotype analysis using the ApaI enzyme, which identifies a second polymorphism in the VDR gene, did not alter our conclusions. Because previous studies (11) suggested that the subgroups of (Australian) Caucasian subjects more than 60 yr of age with high and low bone densities also differed with respect to the prevalence of the BB genotype by 21% (i.e. the BB genotype was 21% more frequent in the subjects with low density), our analyses predicted that our study had a power of 0.7 to detect a significant difference. In fact, we expected that the difference in the prevalence of the BB genotype in our selected subgroups of severely osteoporotic and control subjects would be even

Discussion

The results of these studies indicate that the polymorphism in the VDR gene that defines the BB genotype is not a good predictor of severe osteoporosis in our Caucasian patient population. The BB VDR genotype was observed in only 6 of 41 subjects with severe osteoporosis and was not associated with variations in spinal trabecular bone density within the osteoporotic subjects or the controls. Furthermore, 2 of the osteoporotic subjects with the very lowest bone densities (< 20 mg/cm²) had the BB genotype, whereas 2 of the controls with the very highest bone densities (>140 mg/cm²) had the BB genotype. Genotype analysis using the ApaI enzyme, which identifies a second polymorphism in the VDR gene, did not alter our conclusions. Because previous studies (11) suggested that the subgroups of (Australian) Caucasian subjects more than 60 yr of age with high and low bone densities also differed with respect to the prevalence of the BB genotype by 21% (i.e. the BB genotype was 21% more frequent in the subjects with low density), our analyses predicted that our study had a power of 0.7 to detect a significant difference. In fact, we expected that the difference in the prevalence of the BB genotype in our selected subgroups of severely osteoporotic and control subjects would be even
BB VDR GENOTYPE IN OSTEOPOROTIC WOMEN

1. **Fig. 2.** Southern blot analysis of Apol digests of genomic DNA using the VDR cDNA as a hybridization probe. The VDR A/a genotype determinations for each sample are given at the top. The arrows at the right indicate the polymorphic Apol restriction fragments associated with each allele. The 4.3-kb polymorphic fragment associated with the a allele comigrates with an invariant 4.3-kb band and is not highlighted. Molecular weight size markers are shown to the left.

2. Our study had a power of 0.9 to detect a significant difference if the prevalence of BB genotype had differed by 30%.

3. The studies by Morrison *et al.* (11) reporting the association between the BB VDR genotype and low peak bone density comprised twins and unrelated women in Australia. Because our subjects were drawn from areas in and around Loma Linda, California, it is possible that our inability to use the BB genotype as a predictor of severe osteoporosis is a result of differences in the genetic background between our subjects and the Australian group. Consistent with this possibility, Hustmyer *et al.* (17) recently completed a similar genetic analysis investigating the association between VDR alleles and bone mineral density in adult female twins and found no relationship between bone density and polymorphisms in the VDR alleles in the American Caucasian population that they studied. Although our observation of a 20.3% prevalence of the BB genotype in our total population is consistent with previous values of 13.0% (10), 14.5% (11), 16% (18), and 14% (19) in Australian Caucasian subjects and with estimates of prevalence of 14% for American Caucasian twins (17) and 20% and 21% for American Caucasian osteoporotic women and age-matched controls, respectively (20), such comparisons may not be justified, inasmuch as we selected our subjects to represent the extremes of the distribution of spinal bone density among Caucasian women in Southern California (i.e., severely osteoporotic subjects and controls with densities above the fracture threshold).

4. The BsaMI and Apol polymorphisms in the VDR gene represent only 2 nucleotide differences out of at least 15 that have been detected in this gene (10, 11, 17). Besides an additional polymorphism detected by EcoRV, there are several other differences seen in the 3' untranslated region (3' UTR) of the VDR cDNA as well as a synonymous codon change in exon 9, which creates a TaqI polymorphism (11, 17). It may be that in our population neither BsaMI nor Apol polymorphism could correctly identify the allelic change that is associated with the functional change (i.e., a lower bone density).

5. Only one functional study has been reported to analyze these allelic differences. When the 3' UTRs of each allele are spliced to reporter gene constructs and transfected into cells, constructs containing the 3' UTR of the B allele lead to higher reporter activity compared with that of constructs containing...
the 3' UTR of the b allele. Presumably the 3' UTR of the B allele elevates the steady state VDR mRNA levels, but it is not evident how this increase in steady state VDR mRNA leads to a lower bone density.

It is worth noting that the VDR gene is relatively large, spanning approximately 40 kb, and the coding sequence is contained within 10 exons (11, 21, 22). The promoter and other regulatory elements (e.g. upstream and downstream enhancer elements) have yet to be characterized. Therefore, it seems likely that additional differences between the B and b allele that might alter the functional regulation of VDR gene expression would be found if the whole gene were thoroughly analyzed.

We can suggest several possible interpretations to explain the discrepancy between the results of our study and our expectation of a strong association between the BB genotype, a lower peak bone density, and the development of severe osteoporosis. First, it may be that the population of severely osteoporotic patients in Southern California is genetically different from the population of Australian patients who were previously studied. A second possibility is that the peak bone density is not an important determinant of severe osteoporosis. A third explanation could be that the bone resorption rate is a much more important determinant of the development of severe osteoporosis than the peak bone density. We favor a fourth explanation: peak bone density and bone resorption are both important determinants of severe osteoporosis, but the VDR alleles do not make a strong genetic contribution to the peak spinal bone density in our population. Clearly, additional studies are needed in order to resolve this issue. In conclusion, the results of these studies indicate that severe osteoporotic patients in Southern California do not have an overrepresentation of the BB genotype.

Acknowledgments

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References