The Effects of Vitamin D Receptor Polymorphism on Secondary Hyperparathyroidism and Bone Density After Renal Transplantation

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ABSTRACT

Immunosuppressive treatment and secondary hyperparathyroidism (SHPT) are considered among the most important pathogenetic factors for postrenal transplant bone disease. The aim of this study was to investigate the relationships among vitamin D receptor (VDR) gene polymorphism, parathyroid hormone (PTH) levels, and bone density in renal transplant recipients. We enrolled 69 patients (47 men and 22 women; mean age, 47 ± 11 years) who had undergone kidney transplantation 51 ± 5 months before. All patients underwent an evaluation of the main biochemical parameters of bone metabolism as well as bone densitometry. VDR alleles were typed by a polymerase chain reaction (PCR) assay based on a polymorphic BsmI restriction site. When the patients were categorized according to the VDR genotype (BB, Bb, and bb), serum creatinine, and the cumulative doses of immunosuppressive drugs were similar across the groups. PTH levels higher than 80 pg/ml were found in 53.6% of the patients, with the highest values being detected in the bb VDR genotype (p < 0.05). PTH was significantly correlated to urinary type I collagen cross-linked N-telopeptide (NTx) values. Bone density was low in the whole population; however, spinal bone density was lower in the bb subgroup (p < 0.02). In the whole population, only PTH (p < 0.05) and body mass index (BMI; p < 0.01) were independent predictors of spinal bone density. When grouping the patients by the VDR gene polymorphism, only PTH continued to be an independent predictor of spinal bone density in the bb allele subgroup (R² adj. = 0.17). We can conclude that the VDR genotype polymorphism affects bone density of renal transplant recipients via its effects on the severity of SHPT. (J Bone Miner Res 2002;17:1768–1773)

Key words: bone density, bone turnover, renal transplantation, secondary hyperparathyroidism, vitamin D receptor

INTRODUCTION

RENAL TRANSPLANTATION is effective in correcting most of the abnormalities induced by renal insufficiency. However, because of a number of factors, the alterations in bone metabolism may persist after transplant in many cases. The negative effect on bone of immunosuppressive therapy, which is related to the use of corticosteroids(2) and perhaps of cyclosporin A (CsA),(3) is considered the major risk factor. Another important risk factor for bone morbidity is represented by secondary hyperparathyroidism (SHPT),(4–6) This is almost invariably present in patients with end-stage

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1768
renal failure, but it may persist in up to 50% of the patients after successful transplantation.\(^{4,6}\)

Because of the restoration of normal renal function, parathyroid hormone (PTH) levels decrease markedly soon after transplant.\(^{5,7}\) However, a long-term increase of PTH levels after renal transplant has been found even in patients with good renal function,\(^{10}\) thus indicating that the pathogenesis of SHPT after transplant is not exclusively dependent on the reduction in glomerular filtration rate. Although the mechanisms responsible for this phenomenon currently are not fully understood, several factors have been identified. They include the long-term persistence of the parathyroid gland enlargement, the long time required for the involution of the parathyroid gland hyperplasia, and an alteration in calcium set point.\(^{5}\)

Genetic factors are believed to be important determinants of bone mass. Among such heritable factors, a major role has been proposed for the vitamin D receptor (VDR) gene.\(^{8-10}\) The same gene polymorphism has also been related to a higher incidence of sporadic primary hyperparathyroidism as well as to SHPT in patients with chronic renal insufficiency and kidney transplant.\(^{5,11,12}\) However, this point is still a matter of controversy. For example, Torres et al.\(^{13}\) reported PTH levels after kidney transplant, in which distribution based on the VDR genotype was completely different from most of the data obtained in patients with renal insufficiency or after transplantation.\(^{5,12,14}\) In addition, the same authors\(^{13}\) found that the VDR polymorphism affects bone density after renal transplant in the same way as it does in the general population.

Prompted by these uncertainties, we wanted to evaluate the relationships among VDR gene polymorphism, PTH levels, and bone density in renal transplant recipients.

**MATERIALS AND METHODS**

**Patients**

We studied 69 patients (47 men and 22 women; mean age, 47 ± 11 years) who had undergone kidney transplantation 51 ± 5 months before. The cause of end-stage renal failure was chronic glomerulonephritis in 33 patients, polycystic kidney disease in 7 patients, hypertensive nephropathy in 5 patients, obstructive nephropathy in 4 patients, toxic nephropathy in 5 patients, reflux nephropathy in 4 patients, and nephrolithiasis in 2 patients. The cause of end-stage renal failure remained unknown in 9 subjects. Before kidney graft, all the patients had undergone maintenance dialysis (mean duration, 37 ± 33 months).

Patients were excluded from the study if they had a history of diabetes mellitus; serum creatinine higher than 250 mM; or had been treated with calcium supplements, vitamin D, estrogens, or antiresorptive drugs after the graft.

The immunosuppressive treatment was as follows:

1. Methylprednisolone (MP): 0 postoperative day (pod) 500 mg; first pod, 250 mg; second pod, 80 mg; third pod, 64 mg; fourth–sixth pod, 40 mg; seventh–14th pod, 32 mg; and 15th–21st pod, 24 mg. From the 22nd pod, MP was reduced by 2 mg every 14 days, until the maintenance dose of 8 mg/day was reached.
2. CsA was administered as soon as possible and in any case within 8 h from transplant. The initial daily dose was 12 mg/kg body weight administered in two doses. Subsequent doses of cyclosporine were adjusted based on the clinical evidence of the efficacy and of the occurrence of adverse effects. Blood levels ranging from 200 to 350 ng/ml were maintained.
3. From the 1st pod, 100 mg of oral azathioprine was given once a day together with the evening cyclosporine.

Acute episodes of rejection were treated with MP, 500 mg iv for 3 days. If no improvement of the clinical signs and symptoms was noted, the treatment with a mono/polyclonal antibody preparation was begun.

The cumulative intake of the immunosuppressive drugs was calculated for each patient. The mean daily intake of MP and CsA was calculated as the cumulative intake divided by the days elapsed since transplant. The cumulative intakes of MP and CsA in the whole population were 10.3 ± 8.5 g and 342 ± 314 g, respectively.

All patients gave their consent to the study, which was approved by the Institute’s Ethical Committee.

**Biochemical assay**

Fasting blood and 24-h urine samples were obtained from all patients. Serum calcium, phosphate, and creatinine were analyzed by Automatic Analyzer (Technicon Instruments Corp., Tarrytown, NY, USA). CsA concentration on whole blood was determined by the radioimmunoassay (RIA) method (Incstar Corp., DiaSorin, Saluggia, Vercelli, Italy). Bone ALP (b-ALP) isoenzyme in catalytic activity was determined by lectin from wheat germ activation (Iso-ALP; Boehringer Mannheim, Milano, Italy). After the total ALP activity has been determined (according to IFCC; Roche Diagnostics, Milano, Italy), b-ALP is precipitated using lectin from wheat germ as precipitant and the remaining ALP activity in the supernatant is measured. This procedure has a good correlation with an immunoradiometric assay, measuring bone ALP mass concentration.\(^{15}\)

Intact parathyroid hormone (PTH) was evaluated by a commercial immunoradiometric assay (Bio-Rad Laboratories, Milano, Italy), with intra- and interassay CVs of 6% and 8%, respectively. According to Messa et al.,\(^{15}\) PTH values above 80 pg/ml were considered consistent with hyperparathyroidism in this specific population. Calcitriol \{1,25-dihydroxyvitamin D \(1,25(OH)_{2}D\) \} was assayed using a nonequilibrium competitive protein-binding assay (Nichols Institute, San Juan Capistrano, CA, USA) on plasma samples previously extracted with acetone/toluene and then purified on C\(_{18}\)-OH columns. The intra- and interassay variations were 7.9% and 10.3%, respectively. Urine samples were evaluated for urinary type I collagen cross-linked N-telopeptide (NTx). This was measured by competitive-inhibition ELISA (Osteomark; Ostex International, Inc., Seattle, WA, USA). Assay values were corrected for urinary
creatinine (Automatic Analyzer) and expressed in nanomoles of bone collagen equivalents (BCE; nM) per liter per millimole creatinine. The intra- and interassay CVs for this method were 7.6% and 14%, respectively.

Because of the heterogeneity of the population included in the study, the results from bone turnover marker measurements were expressed both as absolute values and as number of SDs with respect to the predicted levels for sex and menopausal status-matched normal controls (Z score). For this reason, as previously described elsewhere,\(^\text{(16)}\) fasting blood and 24-h urine samples for the evaluation of b-ALP and NTx were obtained from 87 normal subjects (60 men, mean age, 45.2 ± 2.1 years; 15 premenopausal women, mean age, 37.6 ± 2.8 years; and 12 postmenopausal women, mean age, 55.2 ± 3.1 years).

**Polymerase chain reaction analysis of the VDR-restriction fragment length polymorphism genotype**

The genomic DNA was extracted from leukocytes by using standard methods. The VDR gene was amplified through a polymerase chain reaction (PCR). The detection of the *BsmI* site was achieved by amplifying (35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 minute) a region spanning the site, with a primer originating in exon 7 (primer 1, 5'-CAACCAAGACTACAAATGGCCT-GTACGTGA-3') and the other in intron 8 (primer 2, 5'-AACCAGCGGAAGGCTCAAGGG-3') producing a 822-bp fragment. Twenty microliters of the PCR product were subsequently incubated at 65°C for 3 h with 5 U of the enzyme *BsmI* (Amersham Pharmacia Biotech Italia, Milan, Italy). The *BsmI* recognizes the sequence

\[
5'\ldots GAATG\ldots 3' \\
3'\ldots CT\text{TACG}\ldots 5'
\]

Digestion products were resolved by electrophoresis in 1% agarose gel containing 0.5 \(\mu\)g/ml of ethidium bromide. The restriction fragment length polymorphisms (RFLPs) were coded as B/b, where the uppercase letter means absence and lowercase letter means presence of the restriction site.

The genotype was determined by observing the number of bands in the PCR-RFLP digest: homozygotes lacking the *BsmI* site (BB, one 822-bp band was present); homozygotes containing the *BsmI* site (bb, one 646-bp band and one 176-bp band were present); heterozygotes (Bb, all three size bands were present).

**Bone densitometry**

DXA evaluation of the lumbar spine (L2–L4) was performed by Hologic, Inc. QDR 4500 A (Hologic, Inc., Waltham, MA, USA). The results were expressed as bone mineral density (BMD; g/cm²), T score (number of SDs of difference between the patient’s BMD value and the BMD level of normal young adults) and, when appropriate, as Z score (number of SDs of difference between the patient’s BMD value and the BMD level of normal subjects matched for sex and age). According to the World Health Organization (WHO) recommendations, osteoporosis is defined as a T score value < −2.5. The in vivo CV, calculated as described in detail elsewhere,\(^\text{(17)}\) was 1.06% for the spine, 1.16% for the total femur, and 1.63% for the femoral neck.

**Statistical analysis**

The results were expressed as mean ± SD. Multiple group comparisons were made by one-way ANOVA or MANOVA. Group-group differences were then assessed by the post hoc test of Bonferroni. Univariate and stepwise multiple regression analysis was used to evaluate the relationships between the variables considered. The \(\chi^2\) test was used to compare frequencies.

**RESULTS**

The main clinical variables of the patients classified according to the VDR alleles are reported in Table 1. The time elapsed since transplantation tended to be longer, although not significantly, in the bb genotype.

Mean levels of serum creatinine were higher than normal and similar among the three groups (Table 2). PTH levels were higher than normal (87 ± 85 pg/ml) in the whole population. PTH levels higher than 80 pg/ml were found in
TABLE 2. BIOCHEMICAL PARAMETERS IN PATIENTS GROUPED ACCORDING TO THE VDR GENOTYPE

<table>
<thead>
<tr>
<th></th>
<th>BB (n = 11)</th>
<th>Bb (n = 28)</th>
<th>bb (n = 30)</th>
<th>Normal value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>s-Creatinine (µmol/L)</td>
<td>116 ± 25</td>
<td>128 ± 38</td>
<td>138 ± 43</td>
<td>53–115</td>
<td>NS</td>
</tr>
<tr>
<td>s-Calcium (mmol/L)</td>
<td>2.33 ± 0.19</td>
<td>2.37 ± 0.21</td>
<td>2.32 ± 0.21</td>
<td>2.10–2.60</td>
<td>NS</td>
</tr>
<tr>
<td>s-Phosphate (mmol/L)</td>
<td>0.99 ± 0.27</td>
<td>1.00 ± 0.30</td>
<td>1.01 ± 0.40</td>
<td>0.87–1.45</td>
<td>NS</td>
</tr>
<tr>
<td>PTH (pg/ml)</td>
<td>48 ± 30</td>
<td>72 ± 42</td>
<td>118 ± 118</td>
<td>10–60</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>PTH &gt; 80 pg/ml (n)</td>
<td>97 ± 23 (2)</td>
<td>128 ± 12 (19)</td>
<td>229 ± 112 (16)</td>
<td>ns</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>1,25(OH)2D (pg/ml)</td>
<td>24 ± 9</td>
<td>32 ± 17</td>
<td>30 ± 15</td>
<td>20–60</td>
<td>NS</td>
</tr>
<tr>
<td>b-ALP (U/I)</td>
<td>22</td>
<td>35 ± 29</td>
<td>43 ± 39</td>
<td>5–56</td>
<td>NS</td>
</tr>
<tr>
<td>s-Phosphate (mmol/L)</td>
<td>0.9 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>1.5 ± 2.7</td>
<td>—</td>
<td>NS</td>
</tr>
<tr>
<td>Z score b-ALP (SD)</td>
<td>3.0 ± 2.7</td>
<td>2.6 ± 4.6</td>
<td>3.6 ± 4.4</td>
<td>—</td>
<td>NS</td>
</tr>
</tbody>
</table>

TABLE 3. BONE MINERAL DENSITY IN PATIENTS GROUPED ACCORDING TO THE VDR GENOTYPE

<table>
<thead>
<tr>
<th></th>
<th>BB (n = 11)</th>
<th>Bb (n = 28)</th>
<th>bb (n = 30)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>T score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spine</td>
<td>-1.6 ± 1.5</td>
<td>-1.6 ± 1.4</td>
<td>-2.6 ± 1.0</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Total femur</td>
<td>-2.0 ± 0.8</td>
<td>-2.0 ± 1.0</td>
<td>-2.2 ± 1.4</td>
<td>NS</td>
</tr>
<tr>
<td>Femoral neck</td>
<td>-2.4 ± 0.7</td>
<td>-2.3 ± 1.3</td>
<td>-2.8 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Z score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spine</td>
<td>-1.1 ± 1.6</td>
<td>-1.1 ± 1.5</td>
<td>-2.0 ± 1.1</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Total femur</td>
<td>-1.5 ± 0.8</td>
<td>-1.5 ± 0.9</td>
<td>-1.9 ± 1.0</td>
<td>NS</td>
</tr>
<tr>
<td>Femoral neck</td>
<td>-1.5 ± 0.8</td>
<td>-1.4 ± 1.2</td>
<td>-1.8 ± 0.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

53.6% of the patients. The highest levels of PTH were detected in the bb VDR genotype (p < 0.05; Table 2). This difference was still present after adjusting for creatinine, time on dialysis and 1,25(OH)2D levels (p = 0.049). When only PTH levels above 80 pg/ml were considered, PTH values were still higher in the group of patients carrying the bb allele (p < 0.05; Table 2).

Mean levels of b-ALP and urinary NTx were within the normal range and similar across the groups. Nevertheless, when expressed as Z score (see Materials and Methods section), these values were significantly higher than expected in the whole population (b-ALP, 1.0 ± 2.2 SD and p < 0.05; urinary NTx, 3.1 ± 4.2 SD and p < 0.001) and tended to be higher, although not significantly, in the bb VDR allele group (Table 2). PTH levels positively correlated with the Z score of urinary NTx (r = 0.32; p = 0.011).

Bone densitometry results are summarized in Table 3. Bone density was low in the whole population, both at the spine (T score, -2.0 ± 1.4) and total hip (T score, -2.1 ± 1.2). The proportion of patients with osteoporosis of the spine or femur was of 36% and 55%, respectively. Spinal bone density was significantly lower in the bb subgroup (p < 0.02). This result was still evident when patients were sorted by gender. Spinal Z score was significantly lower in the bb subgroup, even after adjusting for body mass index (BMI), cumulative intake of MP and CsA, proportion of postmenopausal women, time since menopause and transplant, serum calcium, and creatinine (p < 0.05). A trend toward lower femoral neck bone density was also observed in this group. The proportion of patients with spinal osteoporosis was 27% in the BB VDR genotype, 32% in the Bb VDR genotype, and 46% in the bb VDR genotype (p, NS). Osteoporosis of the femoral neck was observed in 36% of the patients with the BB allele, 57% of subjects with the Bb allele, and 64% of patients with the bb allele (p, NS).

To adjust correlation analysis for possible confounders, the following predictive variables were identified and then put into a stepwise multiple regression model. These were BMI, dialysis duration, time since transplant, cumulative intake of both CsA and MP, serum creatinine, PTH, and 1,25(OH)2D. Bone density was considered as a dependent variable. In the whole population, only PTH (p < 0.05) and BMI (p < 0.01) were independent predictors of spinal bone mass Z score (R2 adj. [adjusted] = 0.22), while only PTH (p < 0.02) was negatively related to the Z score of the total femur (R2 adj. = 0.11). When patients were grouped according to the VDR genotype, PTH (p < 0.03) continued to be an independent negative predictor of spinal bone density (R2 adj. = 0.17) only in the bb allele subgroup. In the same patients, only PTH (p < 0.02) was correlated to the Z score of the total femur (R2 adj. = 0.23). Finally, calcitriol levels (p < 0.01) were correlated positively with spinal Z score in the BB subgroup (R2 adj. = 0.59), while mean daily intake of MP (p < 0.03) and BMI (p < 0.003) were associated with spinal bone density in the Bb patients.

DISCUSSION

Even a successful kidney transplant frequently is complicated by metabolic bone diseases. Osteoporosis is one of the most frequent among them. Low bone density may be found
in the long term after kidney transplant as well. Accordingly, the fracture rate does not seem to decrease over time since surgery. In keeping with this, in this study, which was carried out on patients transplanted since a mean of 36 months, osteoporosis was present in up to 50% of the patients.

The pathogenesis of this condition is certainly multifactorial, but the deleterious effect of immunosuppressive therapy on bone and the persistence of SHPT are certainly the major factors. Persisting SHPT is a very common feature in kidney transplant recipients and high levels of PTH may be found even in the long term after renal transplantation. Accordingly, SHPT persisted in 54% of our patients after transplant. Although the mechanisms responsible for this phenomenon are not fully understood, several factors have been identified. They include the long-term persistence of parathyroid gland enlargement, the long time required for the involution of parathyroid gland hyperplasia, and some degrees of renal insufficiency after the graft. However, a particular pattern of the VDR gene polymorphism has been involved as well. The VDR allele has been found in 60% of patients with sporadic primary hyperparathyroidism as compared with 33% in controls. In addition, Fernandez and coworkers observed lower degrees of SHPT in patients with chronic renal failure carrying the BB haplotype of the gene. Although we found that mean PTH levels were higher than normal in our transplant recipients, serum concentrations of this hormone were considerably elevated only in the bb VDR polymorphism, with these patients showing the highest levels of PTH among those with persisting SHPT. In contrast, the BB VDR allele was associated with almost normal PTH levels and with the lowest proportion of patients with persisting SHPT. These results are definitely comparable with those from Messa and coworkers, who found that the BB VDR allele was associated with lower PTH and calcium PTH set point levels as compared with the other two genotype patterns. Nevertheless, Torres et al. found a lower degree of SHPT in the bb genotype as compared with the Bb/bb patients pooled together. This discrepancy probably is because of the lower number of patients studied by Torres et al. in comparison with the series investigated by Fernandez, Messa, and our study group. In addition, in the Torres’ study the patients with the BB genotype were grouped together with the Bb genotype and this may have introduced a further confounding factor.

The VDR polymorphism has also been recognized as an influencing factor on BMD. Morrison et al. first correlated the presence of the B allele to lower BMD and the b allele to higher BMD in the general population. However, these results have not been confirmed in several other studies published in the following years. Even considering these uncertainties, the association we found between the b allele and significantly lower bone density in kidney transplant recipients may be somehow surprising. Indeed, an association between the presence of VDR allele and low bone density, if any, could be expected even in this specific population. This has been the case with Torres’ study, in which the BB/Bb allele patients did not recover so much spinal bone density as subjects with the bb haplotype. However, as previously mentioned, Torres et al. also found higher PTH levels by grouping together patients carrying the BB/Bb alleles, which is in contrast with our study as well as with the large majority of the investigations on the relationships between VDR polymorphisms and parathyroid function in patients with primary and SHPT. We also observed that PTH was the only factor affecting both spinal and femoral bone density in the whole study population. When grouping patients according to the VDR genotype, this correlation continued to be present only in the bb patients. This suggests that the very high levels of PTH found in these patients are the most important determinants of low bone density. This hypothesis is also supported by the correlation between PTH levels and a bone resorption marker such as N-telopeptide, in which its values were high in our cohort of patients. This effect of PTH on bone turnover in kidney transplant recipients has been reported already by other investigators and by our group as well.

Although the mechanism by which the VDR gene polymorphism affects parathyroid gland function currently is not defined completely, a decreased transcriptional activity or stability of the VDR mRNA in patients with the bb haplotype, which may in turn decrease calcitriol effects on parathyroid glands, have been suggested. This could explain the reason why in Messa’s study the absence of the b allele was associated with a lower calcium PTH set point, perhaps suggesting increased sensitivity of parathyroids to the calcitriol activity. According to this hypothesis, we found that calcitriol had a protective role on bone density specifically in the BB group.

In conclusion, the effects of VDR gene polymorphism on bone density in renal transplant recipients are widely mediated by the effects on PTH levels and the severity of SHPT. SHPT is in turn one of the major factors conditioning the status of the skeleton after kidney transplantation.

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