Evaluation of the Role of RANK and OPG Genes in Paget’s Disease of Bone

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Paget’s disease of bone (PDB) is one of the most common bone disorders in the western world. PDB is characterized by focal areas of increased osteoclastic bone resorption and bone formation, which leads to the formation of poorly structured bone. These abnormalities of bone turnover and structure predispose affected individuals to various complications including bone pain, deformity, pathological fracture, and an increased risk of osteosarcoma. One of the main mechanisms of osteoclast formation and activation involves the receptor activator of nuclear factor -κB (RANK) ligand (RANKL)/osteoprotegerin (OPG) pathway, where binding of RANKL to RANK results in the differentiation of osteoclast precursors. OPG, on the other hand, acts as an inhibitor of osteoclastogenesis by serving as a decoy receptor for RANKL. Recently, mutations in the RANK gene have been shown to cause familial expansile osteolysis, a rare bone disorder showing great similarity to PDB. We performed mutation analysis in the RANK and OPG genes in 28 PDB patients to investigate whether mutations in these genes could be responsible for PDB. Our data suggest that RANK is not directly involved in PDB in our set of patients, as no mutations in the RANK coding region could be identified and allele frequencies of RANK polymorphisms did not differ in PDB patients as compared with the random population. Also, in the OPG gene, we could not detect PDB-causing mutations. However, of the several polymorphisms identified, one (400 + 4 C/T in intron 2), showed a statistically significant increased frequency for the C allele in PDB patients, suggesting that individuals harboring this allele may be more susceptible for developing PDB. (Bone 28:104–107; 2001) © 2001 by Elsevier Science Inc. All rights reserved.

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Introduction

Paget’s disease of bone (PDB) has an estimated prevalence up to 5% in the population over the age of 55 years,2,11 and is thereby the second leading skeletal disorder affecting the older population of the western world, exceeded only by osteoporosis. The disease involves expedited remodeling, resulting in the formation of structurally abnormal bone, causing an increased risk of fracture, pain, deformity, and osteosarcoma. Both familial and sporadic PDB cases have been described, but it is well recognized that genetic predisposition plays an important role in the development of this disease.9 Recent studies have revealed that PDB is a genetically heterogeneous disorder, with a suggested association between PDB and the HLA complex5 on chromosome 6 (PDB1), a second locus (PDB2) determined by linkage analysis on chromosome 18q,3,5 and at least one other locus elsewhere in the genome.5 Interestingly, the PDB2 locus has been mapped on chromosome 18q,3,5 in a candidate interval containing the gene for receptor activator of nuclear factor κ B (RANK). This gene was initially identified as an important regulator of interactions between T cells and dendritic cells.1 Since its isolation, however, it has been shown that RANK (also known as TNFRSF11A) and its ligand, RANKL,1 also called ODF,17 OPGL,8 or TRANCE,16 are key proteins in bone development and, more specifically, in osteoclastogenesis. Osteotropic factors such as parathyroid hormone (PTH), interleukin-11 (IL-11), and 1,25(OH)2D3 upregulate RANKL in osteoblasts, which is then recognized by RANK expressed on the surface of osteoclast precursors. Binding of RANKL to RANK results in the activation of the NF-κB and c-Jun N-terminal kinase signaling pathways leading to differentiation of osteoclast precursor cells into preosteoclasts, which then further mature into active osteoclasts. The RANK/RANKL interaction is negatively controlled by osteoprotegerin (OPG)13 (also known as OCIF15 or TR115), a known inhibitor of osteoclastogenesis, which acts as a soluble decoy receptor for RANKL.14
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Table 1. Primers amplifying RANK coding exons

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sequence (5'-3')</th>
<th>Tm (°C)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>aagggcaggagccgacagatgcg</td>
<td>63</td>
</tr>
<tr>
<td>2</td>
<td>ttggtgctcggcaagcaggag</td>
<td>58</td>
</tr>
<tr>
<td>3</td>
<td>ctcttccttggtatccttgcg</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
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<td>57</td>
</tr>
<tr>
<td>5</td>
<td>aatggaatcttaccattgggccg</td>
<td>60</td>
</tr>
<tr>
<td>6</td>
<td>gggtgctcaatatgtgactagg</td>
<td>58</td>
</tr>
<tr>
<td>7</td>
<td>attctgaggtttgatttaccgg</td>
<td>57</td>
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<tr>
<td>8</td>
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<td>58</td>
</tr>
<tr>
<td>9</td>
<td>gattcgcacggattaaaataggg</td>
<td>57</td>
</tr>
<tr>
<td>10</td>
<td>aatggaatcttaccattgggccg</td>
<td>60</td>
</tr>
</tbody>
</table>

Recently, mutations affecting the RANK signal peptide were shown to cause familial expansile osteolysis (FEO), a rare autosomal-dominant bone disorder.7 FEO is characterized by the presence of focal lesions of expanding bone, causing serious deformities and increased fracture risk.12 There are many similarities between FEO and Paget’s disease of Bone, both clinically and histologically, and some investigators have suggested FEO to be a severe form of PDB. In keeping with this hypothesis, one family described by Hughes et al.7 showed clinical features of early-onset PDB and FEO, raising the possibility that both disorders are indeed allelic variants caused by different mutations of the same gene. To investigate whether the RANK and OPG genes might be directly involved in Paget’s disease of bone, we analyzed both genes for the presence of disease-causing mutations in PDB patients.

Materials and Methods

Patients

Twenty-eight PDB patients, 24 sporadic and 4 familial cases, and 50 control individuals of the same ethnic origin were included in this study. The familial PDB cases originated from Spain (two families), the USA (one family), and Barbados (one family). All sporadic PDB patients were of Belgian origin. Diagnoses of all PDB patients were based on radiographs. DNA was isolated from peripheral blood according to standard procedures.

Single-stranded Conformation Polymorphism (SSCP)

SSCP was performed with intronic primers amplifying all coding exons of the RANK and OPG genes.10 All primers are listed in Tables 1 and 2.

The PCR reaction amplification mixture (20 μL) contained dNTPs (10 mmol/L each), 10 pmol of two specific primers, 1 × polymerase chain reaction (PCR) buffer, (32P)-odCTP, and Taq-DNA polymerase. PCR conditions were 5 min at 96°C, 35 cycles of 45 sec at 94°C, 45 sec at Tm and 45 sec at 72°C, and 10 min at 72°C. Tm values for each combination are listed in Tables 1 and 2. For the amplification of exon 1 of the RANK gene, PCR enhancer solution (Gibco) was added to a final concentration of 1× to improve amplification. Electrophoresis was performed at 500 V for 16 h at room temperature.

Sequencing

PCR amplification products were purified directly from the PCR reaction using a PCR-purification system (Concert Rapid, Clontech) according to the manufacturer’s recommendations. Sequencing was performed using Big-Dye Terminator (Perkin-Elmer) chemistry and sequences were analyzed on an ABI 377 automated sequencer.

Results

RANK Mutation Analysis

Previously, the RANK coding region was shown to be comprised within 10 exons7 and primer sets amplifying all exons, including intron/exon boundaries, were developed (Table 1). SSCP analyses in 4 familial and 24 sporadic PDB cases showed similar migration patterns for all samples in all exons except for exons 4 and 6.

For further analysis of exon 4, two samples associated with each SSCP pattern were reamplified and sequenced. This revealed that the difference in migration between the various samples was caused by a C421T polymorphism, which results in a H141Y substitution in the RANK protein. As presence of the 421T allele generates an Rsal restriction site, we determined the allele frequencies for this polymorphism in our set of PDB patients and a random population of 50 individuals by reamplification of exon 4 followed by Rsal restriction digest. No statistically significant difference in distribution was observed in our PDB population compared to the control group with the C allele present in 47% of the PDB alleles and in 40% of the control chromosomes.

Sequence analysis of exon 6 showed a C575T variation resulting in a V192A substitution in the RANK protein. A modified PCR primer was developed to create a CfoI restriction site in the presence of the C allele to determine the allele frequencies for the C575T variation in our PDB population and 50 control individuals. The C allele was detected in 53% of the alleles of PDB patients, and in 51% of the control alleles.

OPG Mutation Analysis

The genomic organization of the OPG gene has been described previously10 and primers amplifying all five coding exons were designed based on sequences available in Genbank AC AB008821 and AC AB008822 (Table 2). SSCP analysis showed a uniform pattern for exon 5 in all patients analyzed, whereas, for
the remaining four exons, different patterns were observed between the patients. Reamplification and sequence analysis identified several polymorphisms in these four exons.

In exon 1, a G9C polymorphism was identified causing a K3N substitution in the OPG protein. The sequence difference did not generate or remove a restriction site, nor could a suitable modified primer be developed. As specific SSCP bands could be assigned to each allele, we performed SSCP analysis on the control population and the results were extrapolated to calculate the allele frequencies. No difference in allele distribution was found between the PDB patients and the control individuals with a frequency of 53% for the G allele in the PDB population and 52% in the controls.

A 400 + 4 C/T polymorphism was detected in intron 2. Both alleles could be distinguished after amplification of exon 2 and digestion with RsI I for which a restriction site is present in the presence of the C allele. The C allele was found to be present on 75% of the control chromosomes, a frequency that was statistically increased (p = 0.028) to 89% in the PDB patients.

Intron 2 contains another polymorphism five bases before the first base of exon 3 where a C/T variant was detected. An additional primer set containing a modified primer creating a restriction site in the presence of the T allele was used to determine the allele frequencies. In the control population the T allele was present on 3.6% of the chromosomes, whereas PDB patients showed a frequency of 5.5% for this allele, a difference that did not reach statistical significance (p = 0.520).

Exon 4 harbors an A768G polymorphism, which corresponds to the third codon position of Leucine 256, and therefore does not change the amino acid sequence. By analysis of the SSCP pattern of our control and PDB populations, the A allele was shown to be present in 92% of the PDB chromosomes and in 85% of the control chromosomes, a difference that did not reach statistical significance (p = 0.188).

Discussion

Paget’s disease of bone is initiated by increased osteoclast mediated resorption characterized by the presence of numerous, multinucleated abnormal osteoclasts. Based on its function in osteoclastogenesis and its genetic localization in the PDB2 candidate region, the RANK gene could be considered a serious candidate for Paget’s disease of bone. Moreover, the recent identification of RANK mutations in FEO again raised the question whether both FEO and PDB could be allelic variants caused by different mutations in the same gene. From this perspective it is noteworthy that one of the families harboring a RANK 75dup27 mutation showed a pagetic phenotype with involvement of the axial skeleton, including lesions in the spine, pelvis, and mandible, sites that are normally unaffected in FEO.7 Therefore, we performed RANK mutation analysis in both sporadic and familial cases of PDB. However, in our set of patients, no RANK mutations could be detected in the coding region. In addition, no association of the C421T (H121Y) polymorphism in exon 4 and the C575T (V192A) variation in exon 6 with PDB was detected by comparison of our set of PDB patients with a control group. These data suggest that mutations in the RANK gene are not responsible for the majority of PDB cases. This is not totally surprising, as linkage analysis has also shown that linkage to the locus on 1q is relatively uncommon in PDB and that PDB2 thus probably represents only a minor locus.

The genetic heterogeneity implies that mutations in genes other than the RANK gene must be responsible for the majority of PDB cases. Based on its function as a negative regulator of RANK-RANKL binding, OPG represents a serious candidate gene for PDB. However, we could not detect any disease-causing mutation in the OPG gene in our set of PDB patients. This suggests that mutations in the OPG coding region are not the major cause of PDB. Interestingly however, of the several polymorphisms identified in our study, the 400 + 4 C/T variation showed a different distribution in PDB patients compared to the general population. It appears that the presence of a cytosine at this position was seen more frequently in PDB patients, suggesting that individuals harboring this allele may be more susceptible to develop PDB. From this perspective, it is of interest to note that, although this sequence variation represents an intronic polymorphism, it is close to the splice junction of exon 3. Although highly speculative, one could hypothesize that the presence of either allele might have some effect on the splicing efficiency of this exon. Further analysis must reveal whether this is indeed the case and whether the association of the 400 + 4 C allele with PDB is of any clinical significance. Therefore, further evaluation of the allele frequencies in a larger set of PDB patients is under way. Alternatively, the observed results could reflect the presence of another, proximal polymorphism associated with predisposition to PDB. In this regard, it must be kept in mind that our analysis does not exclude the presence of sequence variations in the promoter region or possible regulatory sequences upstream or downstream from the analyzed OPG and RANK genes, which may affect the expression of these genes. Therefore, a search for polymorphisms in these regions may be worthwhile.

In conclusion, we have demonstrated that the RANK and OPG genes, coding for key players in osteoclastogenesis, do not harbor mutations in their coding sequences in the large set of PDB patients analyzed, but a possible positive association of the 400 + 4 C allele in intron 2 of the OPG gene with PDB warrants further investigation.

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References


