

OSTEOPROTEGERIN DEFICIENCY AND JUVENILE PAGET'S DISEASE

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ABSTRACT

Background Juvenile Paget's disease, an autosomal recessive osteopathy, is characterized by rapidly remodeling woven bone, osteopenia, fractures, and progressive skeletal deformity. The molecular basis is not known. Osteoprotegerin deficiency could explain juvenile Paget's disease because osteoprotegerin suppresses bone turnover by functioning as a decoy receptor for osteoclast differentiation factor (also called RANK ligand).

Methods We evaluated two apparently unrelated Navajo patients with juvenile Paget's disease for defects in the gene encoding osteoprotegerin (*TNFRSF11B*) using polymerase-chain-reaction (PCR) amplification followed by direct sequencing and Southern blotting of genomic DNA. Genetic markers near *TNFRSF11B* were evaluated by both a PCR method that involved sequence-tagged site-content mapping of a deletion of *TNFRSF11B* and PCR spanning the DNA break points.

Results Both patients had a homozygous deletion of *TNFRSF11B*, with identical break points, on chromosome 8q24.2. The defect spans approximately 100 kb, but neighboring genes are intact. We found that serum levels of osteoprotegerin and soluble osteoclast differentiation factor were undetectable and markedly increased, respectively.

Conclusions Juvenile Paget's disease can result from osteoprotegerin deficiency caused by homozygous deletion of *TNFRSF11B*. (N Engl J Med 2002; 347:175-84.)

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JUVENILE Paget's disease, also called hyperostosis corticalis deformans juvenilis or hereditary hyperphosphatasia (number 239000 in Mendelian Inheritance in Man [MIM]),¹ is a rare, autosomal recessive osteopathy of unknown cause that presents in infancy or early childhood with pain from debilitating fractures and deformities due to a markedly accelerated rate of bone remodeling throughout the skeleton.²⁻⁴ The disorder is distinct from the more common condition that sometimes clusters in families, Paget's disease of bone (MIM number 167250), which is typically manifested by focal increases in the rate of bone turnover in middle-aged or elderly people.^{5,6} In patients with juvenile Paget's disease, the continual rapid formation and degradation of osseous tissue impair growth, modeling, and remodeling of the entire skeleton.²⁻⁴ Patients have el-

evated biochemical markers of bone turnover as well as histopathological evidence of weak and disorganized woven bone.²⁻⁴ Approximately 40 cases of juvenile Paget's disease have been reported worldwide.³ Unless it is treated with drugs that block osteoclast-mediated skeletal resorption, the disease can be fatal.⁷⁻¹⁰

In healthy persons, osteoprotegerin (MIM number 602643) suppresses the coupled process of skeletal turnover, functioning as a decoy receptor for osteoclast differentiation factor (MIM number 602642), or receptor activator of nuclear factor- κ B (RANK) ligand.¹¹⁻¹⁴ Osteoclast differentiation factor promotes bone resorption by enhancing the formation and activation of osteoclasts when it binds to RANK (MIM number 603499) on hematopoietic osteoclast progenitor cells as well as on mature osteoclasts.¹³⁻¹⁵

We describe homozygous deletion of the gene on chromosome 8q24.2 that encodes osteoprotegerin, member 11B of the superfamily of tumor necrosis factor receptors (*TNFRSF11B*), in two Navajo patients with juvenile Paget's disease.

METHODS

We used a candidate-gene approach to explore the genetic basis of juvenile Paget's disease. Recently, excessive RANK activity due to tandem duplications of distinctive lengths within exon 1 of *TNFRSF11A*, encoding the signal peptide of RANK, was identified as the cause of three rare autosomal dominant disorders involving rapid skeletal remodeling: familial expansile osteolysis (MIM number 174810), early-onset Paget's disease of bone in Japan (MIM number 602080), and a newly characterized condition called expansile skeletal hyperphosphatasia.¹⁶⁻¹⁸ However, because we found no mutation of *TNFRSF11A* in the proband, osteoprotegerin deficiency as a result of the impaired expression of *TNFRSF11B* became a candidate pathogenesis for juvenile Paget's disease. The fact that osteoprotegerin-knockout mice reportedly have osteoporosis as a result of accelerated rates of remodeling^{19,20} increased our interest in *TNFRSF11B* as the potential cause of juvenile Paget's disease.

Patients**Patient 1**

Patient 1, the Navajo proband (Family 1) who was referred to us at one year of age because of bone deformities and failure to thrive due to juvenile Paget's disease, appeared well until the age of five months, when he was found to have a misshapen skull and thorax.

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Chest radiography for pneumonia at the age of seven months revealed skeletal abnormalities interpreted as juvenile Paget's disease. The child had been treated for streptococcal pneumonia with meningitis at the age of 10 months. He was small (below the 3rd percentile for length and in the 5th percentile for weight), deaf, tachypneic, and weak and had a disproportionately large head (75th percentile for circumference), short humeri, laterally bowed femora, anteriorly curved tibiae, markedly delayed gross motor skills, and poor muscle tone (Fig. 1A). Radiographic findings typical of juvenile Paget's disease included widened, osteopenic long bones with coarse trabeculae and indistinct corticomedullary junctions (Fig. 1B).²⁻⁴ Measurements of biochemical variables of mineral homeostasis were remarkable only for hypercalciuria (401 mg of calcium per gram of creatinine). Markers of skeletal turnover included a striking

elevation in serum alkaline phosphatase activity (2716 U per liter; normal range, 133 to 347), resulting from increased amounts of the alkaline phosphatase isoform emanating from bone, which reflected increased formation of osseous tissue, and excessive urinary excretion of deoxypyridinoline (173 nmol per millimole of creatinine; normal range, 2 to 41), which reflected enhanced resorption of osseous tissue. Substantial clinical and radiographic improvement followed daily subcutaneous injections of synthetic salmon calcitonin given to inhibit bone resorption. However, therapy was not administered consistently in the ensuing years. The child was still below the 3rd percentile for height and weight and had active bone disease when he was seen at the age of seven years in the spring of 2002. His healthy-appearing parents had normal serum alkaline phosphatase activity and unremarkable findings on skeletal radiogra-

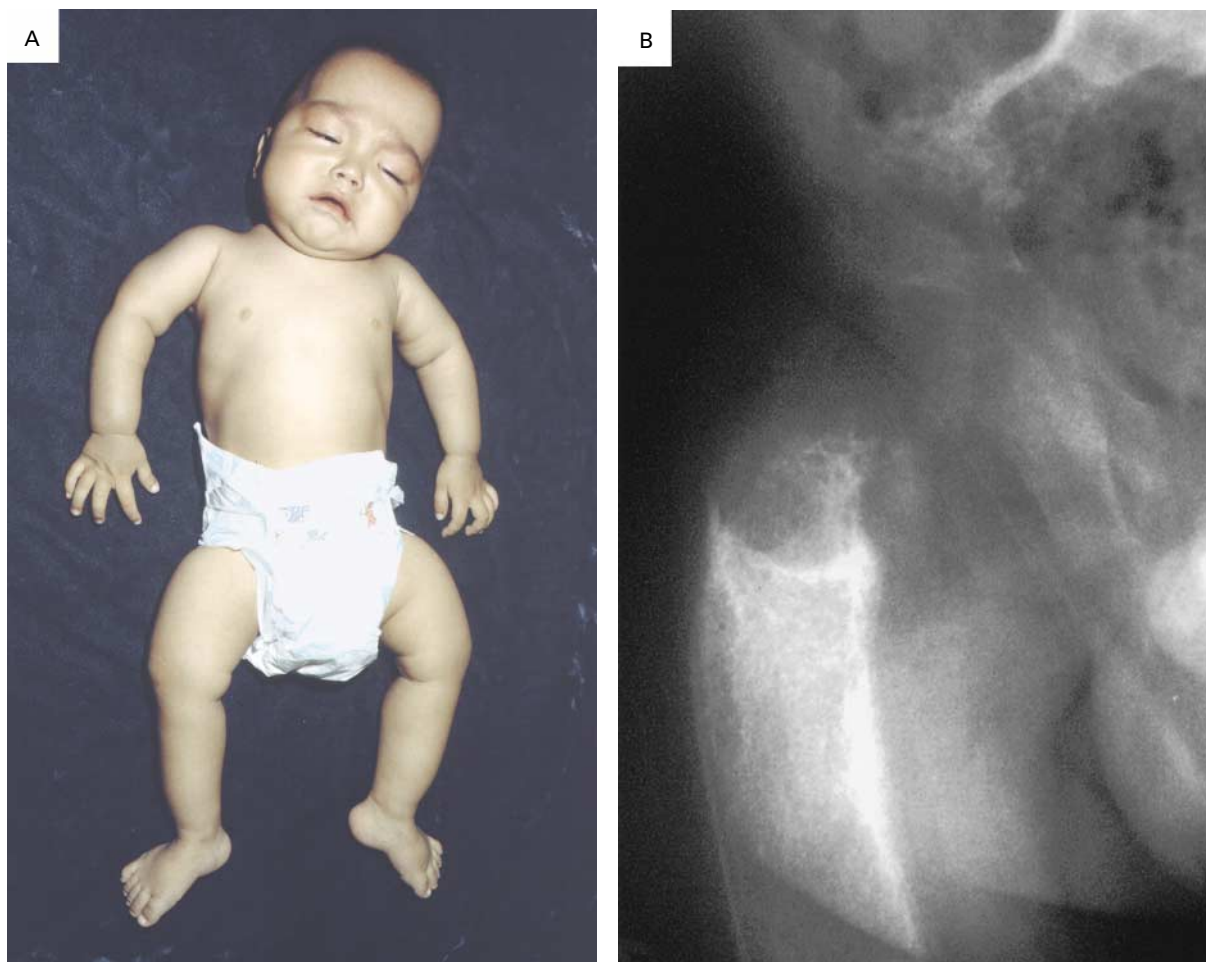
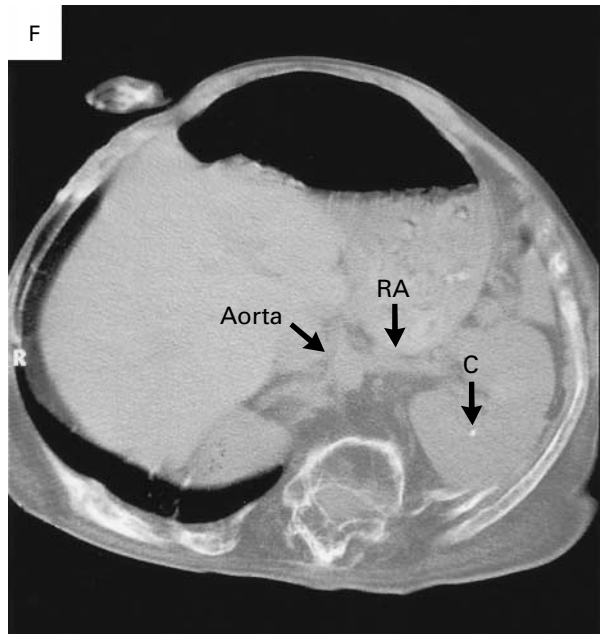
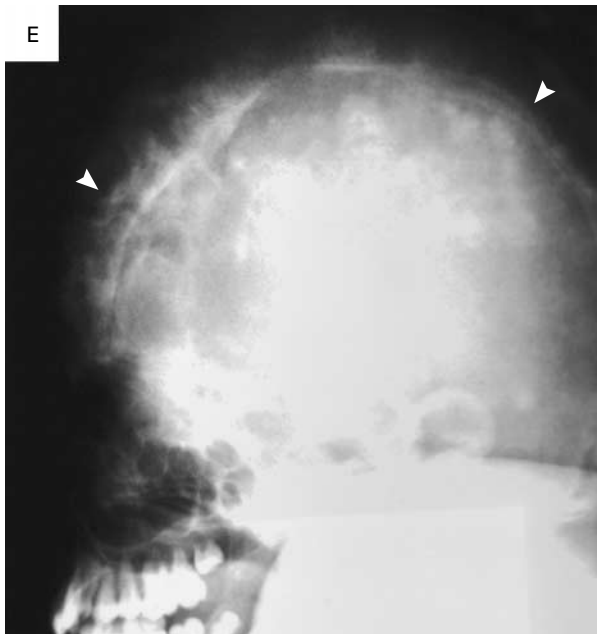


Figure 1. Features of Two Navajo Patients with Juvenile Paget's Disease.

Patient 1, the one-year-old proband, has a head that seems large, a deformed chest, curvature of the limbs, and marked weakness (Panel A). An anteroposterior radiograph of his right proximal femur shows a markedly widened, poorly modeled (shaped), and osteopenic bone, with cortical thinning and a coarse trabecular pattern (Panel B). Panel C shows a radiograph obtained before calcitonin therapy, and Panel D shows a radiograph obtained at 27 months, after the initiation of calcitonin therapy, demonstrating improvement of bone structure. At 15 years of age, Patient 2 had a markedly thickened (surface delineated by arrowheads) and cotton-wool calvarium reminiscent of Paget's disease of bone (Panel E). Computed tomography of the abdomen of Patient 2 at 23 years of age (Panel F) reveals a single calculus (C) in her left kidney, but no ectopic mineralization elsewhere, including skin, renal artery (RA), and aorta.



phy. His family history was negative for consanguinity, which is proscribed by Navajo tradition.²¹

Patient 2

In July 2001, we were contacted by a 26-year-old Navajo woman with juvenile Paget's disease (Family 2) who was seemingly unrelated to Patient 1 and who was deaf, severely deformed, and incapacitated (Fig. 1C and 1D). In 1979, she was described as Case 1 by Dunn and colleagues²² after she and another unrelated Navajo child with juvenile Paget's disease benefited from injections of synthetic human calcitonin. However, antiresorptive therapy had not been given for two decades. Her serum alkaline phosphatase activity was 838 IU per liter (normal range, 36 to 136). Sequential radiographic studies documented worsening osteopenia and profound skeletal deformities that caused chronic, severe pain. She died from pneumonia and heart failure in October 2001. Her parents were healthy and appeared well.

Genetic Studies

DNA was extracted from blood leukocytes after informed written consent had been obtained from the parents of Patient 1 and from Patient 2. To rule out a defect involving the gene that encodes RANK in the proband, we used the polymerase-chain-reaction (PCR) method to amplify *TNFRSF11A* and then sequenced exon 1 using published methods.¹⁶ When this analysis proved unrevealing (data not shown), we analyzed DNA from Patient 1 and his parents and sequenced *TNFRSF11B*, which encodes osteoprotegerin. PCR primers were designed with use of complementary DNA (GenBank accession number NM 002546) and the genomic DNA sequences (GenBank accession numbers AB008821 and AB008822) to amplify each of the five coding exons and adjacent splice sites of *TNFRSF11B*.¹¹

Exon 1 was amplified with use of the primers 5'TGATCAAA-GGCAGCGATAC3' and 5'TGGGAGGTTGGGAGACCAGG3', exon 2 with use of 5'TCATGCTAAGATGATGCCACT3' and 5'CTATCTGACTTTGCATGATCC3', exon 3 with use of 5'CTGCTGGAAACGATTTGAGG3' and 5'CTACAAAATCGTACA-AAGACG3', exon 4 with use of 5'GACTCTCAGAAATCCAA-TTG3' and 5'GGTGCCTTTGATTTCTGATTG3', and exon 5 with use of 5'GCTTGTTTTATGATGGCATTGG3' and 5'GATATC-ACTGAAAGCCTCAAG3'. The *TNFRSF11B* exons were amplified with the use of approximately 12 ng of DNA in a 39- μ l reaction. Sequential PCR conditions included denaturation at 95°C for 2 minutes, followed by 35 cycles at 95°C for 30 seconds, annealing at 55°C for 30 seconds and 72°C for 1 minute, and extension at 72°C for 5 minutes.

To support the observations obtained from study of the PCR amplifications and DNA-product sequencing, we performed Southern blotting of genomic DNA using DNA from the proband, his parents, a healthy sister, and an unrelated control subject (Clontech). The DNA was digested with *Eco*RI restriction enzyme and hybridized with a *TNFRSF11B* probe¹¹ devised by PCR amplification of exon 2 with use of primers described above; normal human genomic DNA (Clontech) was used as a template, tagged with phosphorus-32 by random-primer labeling, and subjected to standard Southern blotting with ExpressHyb solution (Clontech). Before the probe was applied, the gels were stained with ethidium bromide to ensure that there were approximately equal amounts of DNA from each family member. The same procedures were then applied to DNA samples from Family 2.

To characterize the deletion of *TNFRSF11B* in both Navajo patients, we used a high-resolution integrated physical map of 8q22–q24 that was derived from sequence-tagged site-content mapping involving yeast artificial chromosomes and the complete genomic sequence of *TNFRSF11B* (GenBank accession number NT_023811) according to both published²³ and newly designed PCR primers.

We used long-range PCR to amplify, and thereby define, the *TNFRSF11B*-deletion break points. We used the +7.6-kb forward

primer and the –66.7-kb reverse primer (see below). The 39- μ l reaction used the *Taq*Plus Long PCR System (Stratagene) and contained approximately 12 ng of DNA from each patient. The DNA was subjected to denaturation at 95°C for 1 minute, followed by 35 cycles at 95°C for 1 minute, annealing at 55°C for 30 seconds and 72°C for 14 minutes, and extension at 72°C for 10 minutes. The PCR products were isolated and sequenced.

Serum Levels of Osteoprotegerin and Osteoclast Differentiation Factor

Enzyme immunoassay kits (BioNet) were used to quantitate osteoprotegerin and free osteoclast differentiation factor in serum from Patient 1 and his parents. In two separate dedicated assay runs, performed as specified by the manufacturer (Biomedica Gruppe), we compared the osteoprotegerin and osteoclast differentiation factor values with those of six healthy children and six healthy adults chosen randomly as non-Navajo, age-matched controls.

Genetic Heterogeneity in Juvenile Paget's Disease

Finally, we examined *TNFRSF11B* using leukocyte DNA and assayed levels of osteoprotegerin and soluble osteoclast differentiation factor in serum from two young women, one from a nonconsanguineous American family (Patient 3)³ and one from an unrelated Albanian family (Patient 4). Both were referred to us for the treatment of relatively mild juvenile Paget's disease.

RESULTS

Genetic Studies

No PCR products (amplicons) representing any of the five exons of *TNFRSF11B* were formed from DNA from Patients 1 and 2 (Fig. 2A). Conversely, *TNFRSF11B* amplicons were formed from the DNA from an unrelated healthy control subject and from both patients' parents (data not shown). Exon 2 of the gene for transforming growth factor β 1, used as a control, amplified in all subjects (data not shown). The findings indicated that both patients had a homozygous deletion of *TNFRSF11B*, which spans approximately 30 kb.¹¹ The observations were consistent with the mothers' and fathers' being heterozygotes, with one intact *TNFRSF11B* allele.

Southern blots with a probe for exon 2 of *TNFRSF11B* showed no hybridization signal for the two patients (Fig. 2B). However, hybridization occurred in samples from all other family members and non-Navajo controls. These observations evidenced homozygous deletion of *TNFRSF11B* in the patients.

To define the extent of the *TNFRSF11B* deletion, we initially analyzed 30 sequence-tagged site markers spanning approximately 1 megabase — 1 million bp — of genomic DNA encompassing *TNFRSF11B*.²³ On PCR assay, all of these sequence-tagged sites were amplified. However, the markers closest to *TNFRSF11B* were approximately 100 kb upstream (D8S47) and 200 kb downstream (D8S48). Hence, additional sequence-tagged sites closer to *TNFRSF11B* were devised. Using these new markers, we mapped the deletion break points of both patients between 50 and 70 kb upstream and between exon 5 and 10 kb downstream of *TNFRSF11B* (Fig. 3A).

Subsequently, to identify sequence-tagged site markers closer to the *TNFRSF11B* break points, we screened the DNA sequence surrounding each break point to exclude any repetitive elements with use of RepeatMasker.²⁴ Avoiding all repetitive elements, we made 11 additional sequence-tagged sites (Fig. 3A). They identified the deletion break points 56.7 to 66.7 kb upstream and 3.2 to 7.6 kb downstream of *TNFRSF11B* (Fig. 3B). The following were the key primer pairs for mapping the deletion break points:

+7.6 kb, CCTGTCCAATTTTCAGTTGG forward primer and CTCTTGCCTTTTGGTATGCC reverse primer; +3.2 kb, CCAAAGGAGCAGCCAGGGAG-ACCAATC forward primer and GAGGCTCTA-ACCTGGGTAAC reverse primer; -56.7 kb, GATGGCTTAACTATAAGCTGG forward primer and CCCTATCCGTCTATCCAATC reverse primer; and -66.7 kb, CTGCCTGTATTTTCTGCAAAC forward primer and CCAAGCTGTCTGATGGGAAC reverse primer. However, these regions contain sub-

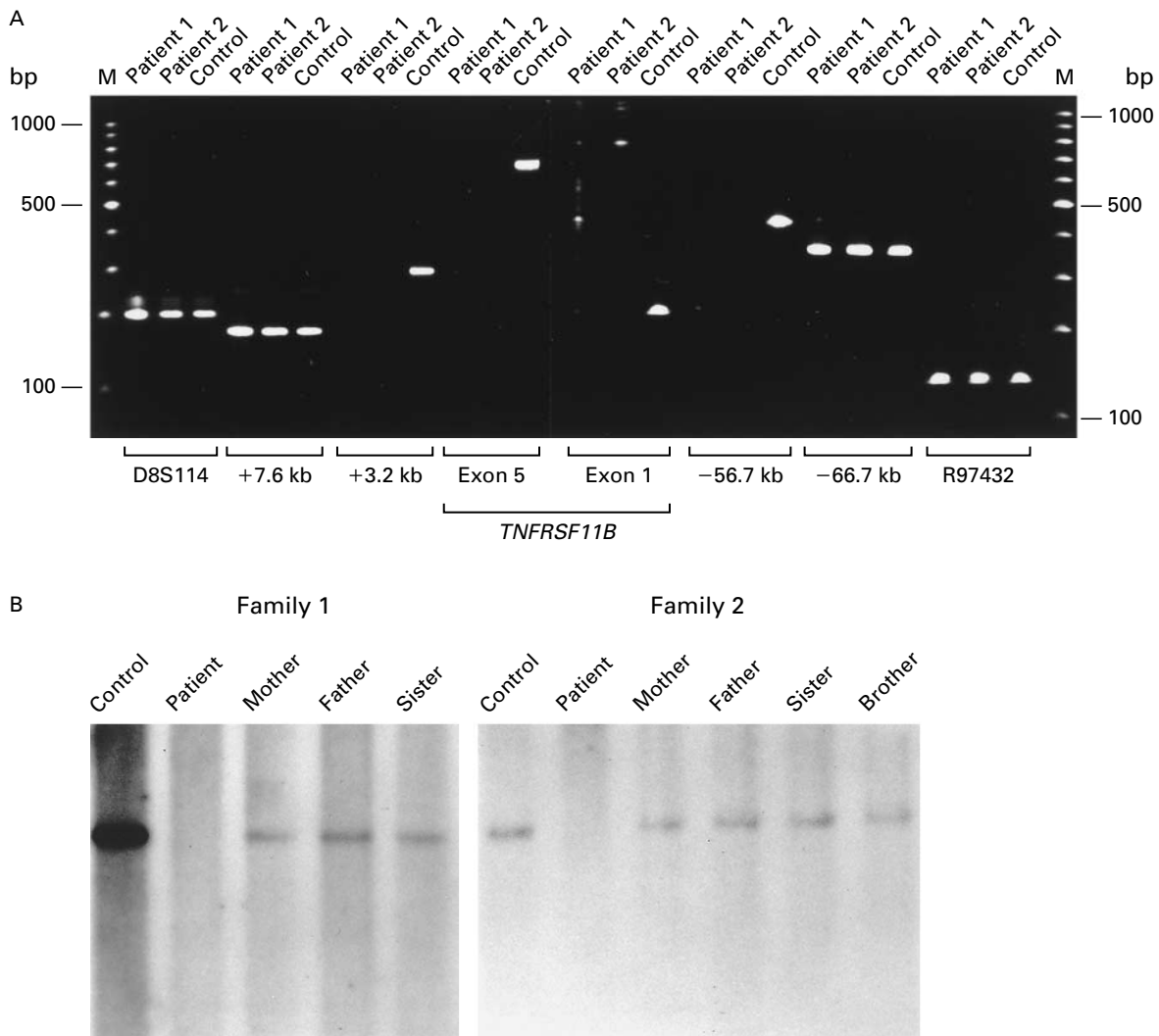


Figure 2. Genetic Studies of Juvenile Paget's Disease in Patient 1, Patient 2, and a Control Subject. Polymerase-chain-reaction analysis of the gene for osteoprotegerin (*TNFRSF11B*) and neighboring markers shows the absence of bands representing *TNFRSF11B* in both patients (Panel A). The plus and minus signs indicate whether the marker is downstream or upstream, respectively, from *TNFRSF11B*. Southern blotting for *TNFRSF11B* in Family 1 and Family 2 shows the absence of hybridization of DNA from Patient 1 and Patient 2 (Panel B). Approximately 20 μ g of genomic DNA from each member of Family 1 and Family 2 and, from a control, approximately 30 μ g (in the case of Family 1) and 15 μ g (in the case of Family 2) of human genomic DNA (Clontech), was digested with *EcoRI* and hybridized with a radiolabeled probe for exon 2 of *TNFRSF11B*.

was absent or had portions deleted. GenomeScan analysis of the deleted genomic DNA (GenBank accession number NT_023811) did indicate the existence of another potential gene in this region composed of four exons containing open-reading frames and appropriate dinucleotide splice signals bracketing the exons. However, analysis involving the Basic Local Alignment Search Tool (BLAST)²⁵ failed to show any substantial similarities, suggesting that this hypothetical gene is not expressed.

Serum Levels of Osteoprotegerin and Osteoclast Differentiation Factor

As anticipated from the molecular studies, osteoprotegerin was undetectable in serum samples from Patient 1 (Fig. 4). His heterozygous parents had osteoprotegerin levels that were approximately half the normal level (Fig. 4). Conversely, his serum level of soluble osteoclast differentiation factor was markedly elevated, whereas the levels were essentially undetectable (below the concentration of the lowest standard used in the assay) in his parents and in the controls (Fig. 4B).

Genetic Heterogeneity of Juvenile Paget’s Disease

No defect in *TNFRSF11B* was found in either Patient 3 or Patient 4 (Fig. 5), both of whom had a rel-

atively mild form of juvenile Paget’s disease (data not shown). Furthermore, their serum levels of osteoprotegerin and soluble osteoclast differentiation factor were unremarkable (data not shown).

DISCUSSION

Our finding of homozygous deletion of *TNFRSF11B*, the gene encoding osteoprotegerin, in two Native Americans with juvenile Paget’s disease provides both a cause and a mechanism for this remarkable osteopathy. Osteoprotegerin, a soluble member of the superfamily of tumor necrosis factor receptors, is normally secreted into marrow spaces by cells derived from mesenchyme. Osteoprotegerin acts as a decoy for osteoclast differentiation factor,¹³⁻¹⁵ which is “both necessary and sufficient for osteoclast development.”²⁶ The serum levels of osteoprotegerin and soluble osteoclast differentiation factor in Patient 1 were consistent with the DNA-based findings. Homozygous deletion of *TNFRSF11B* precludes the biosynthesis of osteoprotegerin, thereby negating its action as a decoy receptor and causing high circulating (and presumably marrow space) levels of biologically active osteoclast differentiation factor, which in turn markedly accelerates the rate of bone turnover.^{19,20}

Our observations demonstrate that osteoprotegerin is a critical regulator of postnatal skeletal develop-

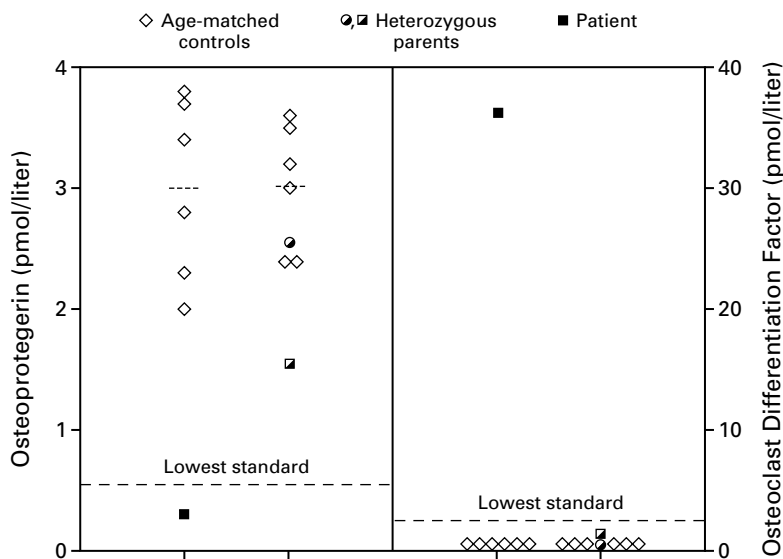


Figure 4. Serum Levels of Osteoprotegerin and Soluble Osteoclast Differentiation Factor in Patient 1, His Parents, and Age-Matched Controls.

In Patient 1, serum levels of osteoprotegerin and osteoclast differentiation factor are lower and higher, respectively, than those of age-matched controls. His parents have similar, but more mild, changes in the osteoprotegerin levels, a finding consistent with their heterozygous carrier status. Short dashed lines depict the mean of the control values. The long dashed lines show the lowest concentration of the standard used in the assays.

ment and homeostasis in humans. Mice that lack osteoprotegerin owing to the knockout of *Tnfrsf11b* reportedly have “osteoporosis,”^{19,20} but they actually have numerous osteoclasts and rapidly remodeling woven bone rather than a paucity of lamellar bone. Accordingly, these animals manifest juvenile Paget’s disease. Although heterozygous mice with osteoprotegerin deficiency can be osteopenic,²⁰ the findings on skeletal radiographs of the proband’s heterozygous (carrier) parents, whose serum osteoprotegerin levels were approximately 50 percent of control values, were unremarkable.

Our findings complement studies of the role of osteoprotegerin among humoral and cellular factors related to atherosclerosis.^{27,28} Studies of *Tnfrsf11b*-knockout mice implicate osteoprotegerin deficiency in atherogenesis, because of the presence of renal-artery and aortic calcification in histopathological studies.¹⁹ However, no mineralization was seen in the media of the aorta or renal arteries of Patient 2 on computed tomographic examination of her abdomen at the age of 23 years (with the use of bone windows sensitive for calcification) or on a postmortem radiographic skeletal survey at the age of 26 years (Fig. 2D). The proband had microscopic hematuria, nephrocalcinosis, and sev-

eral tiny echogenic foci in his kidneys representing small calculi; these calculi probably arose from his hypercalciuria as a result of impaired skeletal growth²⁹ and, perhaps, the calciuric effects of calcitonin therapy. Hence, osteoprotegerin deficiency does not appear to cause macroscopic ectopic mineralization, at least in childhood or early adulthood. Nevertheless, the literature on juvenile Paget’s disease includes the report of “calcifying arteriopathy,” detected by renal sonography and confirmed by histopathological analysis of the internal elastic membrane of a temporal artery, in a six-year-old boy.³⁰ Striking changes consistent with the presence of pseudoxanthoma elasticum (MIM numbers 177850 and 264800), including granular and coarse deposits of calcium in the membranes and intima of the muscular arteries and arterioles, were found at autopsy in all tissues from a 26-year-old man who had severe hypertension and juvenile Paget’s disease.¹⁰ In addition, a girl who seemed to have a dominantly inherited form of juvenile Paget’s disease had transient calcium deposits near collagen and elastin fibers in the mid-dermis basophilic matrix.³¹ However, in order to advance this hypothesis, we must first understand the molecular basis of disease in such patients.

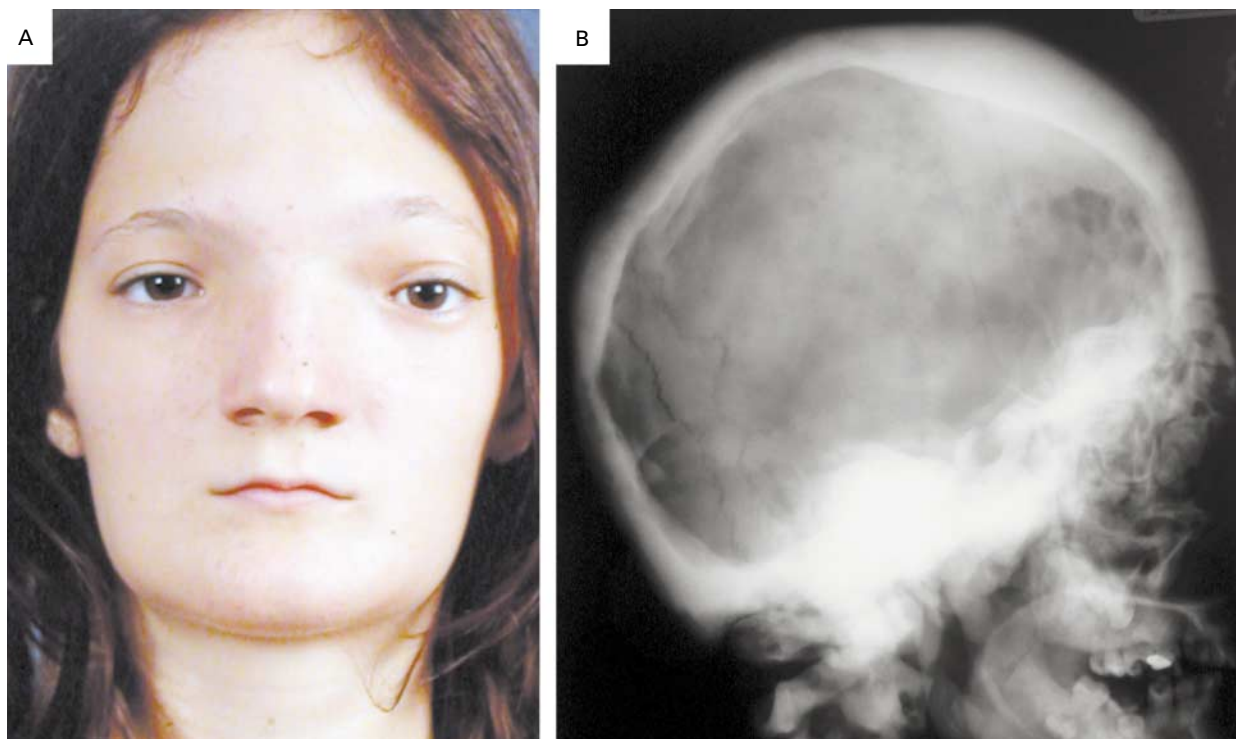


Figure 5. Patient 4, a 15-Year-Old Girl with Mild Juvenile Paget’s Disease.

The patient had hypertelorism and a broad forehead (Panel A) and characteristic, generalized thickening of cortical bone and a disorganized pattern of trabecular bone (Panel B).

We detected no splice-site or exon mutations in *TNFRSF11B* in two unrelated women (Patients 3 and 4) with relatively mild juvenile Paget's disease. Furthermore, their skeletal disease did not seem to result from the diminished expression of *TNFRSF11B*, because they had unremarkable serum levels of osteoprotegerin and soluble osteoclast differentiation factor. Hence, genes other than *TNFRSF11B* seem to be defective in these patients, reflecting genetic heterogeneity for juvenile Paget's disease.

Juvenile Paget's disease belongs among the disorders characterized by excessive signaling along the pathway involving osteoclast differentiation factor, RANK, and nuclear factor- κ B and leading to increased osteoclast

action and an accelerated rate of osseous-tissue turnover (Fig. 6).^{13,32} Nevertheless, autosomal recessive juvenile Paget's disease has clinical and radiographic findings that are distinct from those of autosomal dominant familial expansile osteolysis, early-onset Paget's disease of bone in Japan, and expansile skeletal hyperphosphatasia, showing that additional factors modify the effects of this pathway when it is activated.¹⁶⁻¹⁸

We found that homozygous deletion of *TNFRSF11B* can lead to juvenile Paget's disease. Chromosome 8q24.2 contains *TNFRSF11B*, but it has not been reported as one of the several susceptibility loci for the adult form of Paget's disease of bone.^{1,33} In fact, the coding sequence of *TNFRSF11B* is not

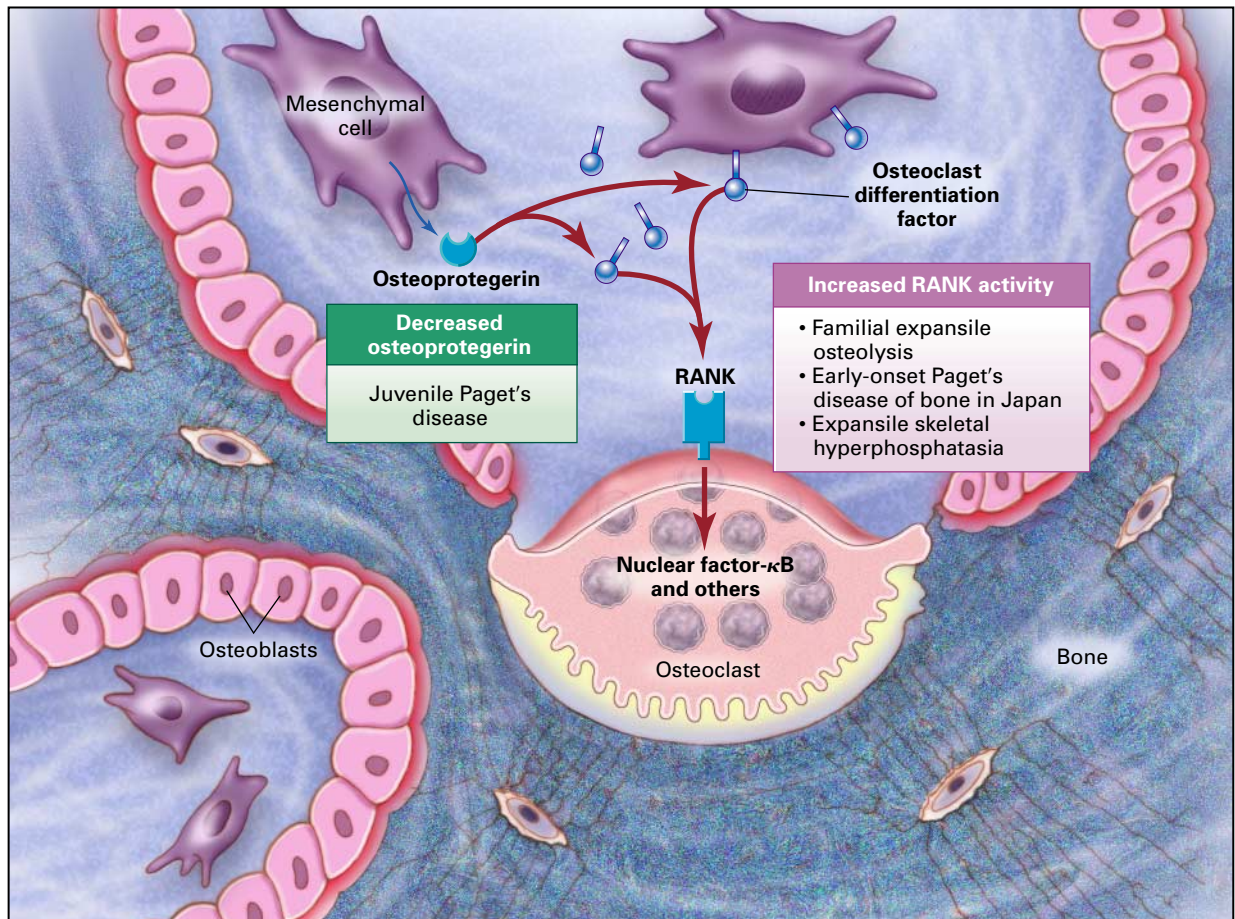


Figure 6. Osteopathies Reflecting Enhanced Signaling along the Pathway Involving Osteoclast Differentiation Factor and Receptor Activator of Nuclear Factor- κ B (RANK).

An excessive effect of RANK on osteoclasts results from three distinctive tandem duplications — 18-bp, 27-bp, and 15-bp — in exon 1 of *TNFRSF11A*, encoding the signal peptide of RANK, that cause familial expansile osteolysis, early-onset Paget's disease of bone, and expansile skeletal hyperphosphatasia, respectively. Homozygous deletion of the gene for osteoprotegerin (*TNFRSF11B*) causes juvenile Paget's disease by abrogating the synthesis of osteoprotegerin by mesenchymal cells. Consequently, the decoy action of osteoprotegerin is abolished, and mesenchymal-cell presentation and (potentially) levels of biologically active osteoclast differentiation factor are increased, enhancing signaling mediated by nuclear factor- κ B and other pathways.

mutated in “garden variety” Paget’s disease of bone.³⁴ Hence, molecular studies distinguish Paget’s disease of bone from juvenile Paget’s disease.

The homozygous deletion of *TNFRSF11B* causing juvenile Paget’s disease in our two Navajo patients most likely reflects a founder effect emerging in this “bottleneck” population, which had decreased to approximately 6000 people in 1868³⁵ and subsequently expanded to approximately 225,000 by 1990.³⁶ Three patients with juvenile Paget’s disease from apparently separate families have been identified since the 1960s. Although the prevalence of the deletion among Navajos is not known, it can now be assessed. Detection of carriers and prenatal diagnosis of juvenile Paget’s disease in this population are also possible. Our observations bolster the rationale for the use of antiresorptive treatment for juvenile Paget’s disease. Furthermore, if osteoprotegerin is not rejected as a foreign protein because of the complete deletion of *TNFRSF11B*, replacement therapy might be especially effective for patients who have juvenile Paget’s disease as a result of the *TNFRSF11B* deletion.

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