Introduction  Pycnodysostosis is a rare autosomal recessive skeletal dysplasia characterized by short stature, osteosclerosis, acro-osteolysis, bone fragility and skull deformities. Recently, mutations in the gene encoding cathepsin K (CK), a lysosomal cysteine protease localized exclusively in osteoclasts, were found to be responsible for this disease. In this study, we analyzed the sequence of the coding region of the cathepsin K gene in four patients from unrelated families and identified three different mutations of the cathepsin K gene. Two of those mutations were altering the amino acid sequence of the mature peptide, while one was an amino acid substitution in the signal peptide region of this protein. We examined the expression of this mutant cathepsin K protein in COS-7 cells using a transient expression assay. The signal peptide mutation detected in our patient was a new type of mutation disrupting the function of cathepsin K protein.

Materials and Methods  Preparation of DNA - Whole blood was obtained from each patient, as well as from the parents and sister of patient 4. Blood samples from 80 normal individuals were used as a control. Genomic DNA was extracted from whole blood samples. Cathepsin K genomic DNA were amplified in six overlapping fragments by genomic DNA PCR. Sequencing - PCR.  Cathepsin K cDNA was amplified in two overlapping fragments by cDNA primers and the Superscript II preamplification system (Gibco BRL). Total RNA was extracted from whole blood samples. Cathepsin K genomic DNA were amplified in two overlapping fragments by genomic DNA PCR. RNA extraction, reverse-transcription PCR, and PCR amplification of cathepsin K c-DNA - RNA was prepared from fracture callus and cultured bone marrow cells of patient 4. Callus was harvested at the time of surgery for a fractured right femur. Total RNA was extracted with guanidinium thiocyanate followed by centrifugation in CsCl. Bone marrow blood samples from each patient, as well as from the parents and sister of patient 4. Blood samples were also obtained from patient 4 and bone marrow cells were cultured for 14 days in a-MEM (Gibco BRL, Gaithersburg, MD) supplemented with 10-8M 1a, 25(OH)2 vitamin D3, 10% FBS, and 10% autologous bone marrow supernatant to induce the osteoclastic phenotype. Total RNA was extracted using Trizol reagent (Gibco BRL). cDNA was synthesized using oligo(dT) primers and the Superscript II preamplification system (Gibco BRL). Cathepsin K cDNA was amplified in two overlapping fragments by cDNA PCR. Sequencing - The amplified products were subjected to direct sequencing or were subcloned into pCR®2.1 vector (Invitrogen Corp., San Diego, CA). Positive clones were selected and the isolated plasmids were used for sequencing.

Transient expression of wild-type and mutant cathepsin K genes tagged with FLAG in COS-7 Cells - The entire cDNA of the nucleotide 131C mutant and wild-type cathepsin K genes tagged at the COOH terminus with a FLAG epitope was constructed as follows. First, a PCR fragment containing the full coding sequence of the nucleotide 131C mutated cathepsin K was amplified from the cDNA of patient 3. A single point mutation (C to T) was introduced in the signal peptide of cathepsin K. The amplified DNA showed that the patient was homozygous for this mutation. This point mutation resulted in the substitution of an Ala277 by a Val (A277V) in the mature cathepsin K polypeptide. Patients 2 and 3 had a same 1 bp deletion of nucleotide 531 in exon 5 (531 del T). Direct genomic sequencing showed that the patient was also homozygous for this mutation, which resulted in frameshift from codon 142 and premature stop at codon 160.

Direct sequencing of genomic DNA indicated that patient 4 was homozygous for a T to C transition of nucleotide 935 in exon 7. Direct sequencing of genomic DNA showed that the patient was homozygous for this mutation. This point mutation resulted in the substitution of an Ala277 by a Val (A277V) in the mature cathepsin K polypeptide. No other mutations were found in the other amplified DNA fragments. The patient's parents were heterozygous for this mutation, and the unaffected sister was homozygous for the normal gene. This base substitution was not detected in any of the 80 normal controls by direct sequencing of this region of the cathepsin K gene, suggesting that it was unlikely to be a neutral polymorphism. Sequencing of the cDNA of patient 4 transcribed from fracture callus and from cultured bone marrow cells showed the same mutation. No other mutations were found in the amplified cDNA fragments.

Expression of wild-type and mutant cathepsin K genes tagged with FLAG in COS-7 cells - wild-type and LP mutant cathepsin K genes were transcribed at an approximately equal level, as confirmed by RT-PCR of the mRNA of each transfectant, consistent with the fact that these two genes were driven by the strong CMV promoter. In the immunohistochemical study, however, cells transfected with FLAG-tagged mutant cathepsin K cDNA did not show any positive staining by the monoclonal FLAG antibody, while cells transfected with the wild-type fusion gene showed positive staining. On Western blotting, positive bands were detected corresponding to the size of procathepsin K, although the density of the mutant fusion gene product was significantly reduced compared to that of the wild-type product.

Discussion  Mutations of cathepsin K gene were detected in all the patients with pycnodysostosis that we studied. While one had a mutation identical to that previously reported, the others had novel mutation sites. Expression of the LP mutant protein was markedly reduced, suggesting decreased mature product in this patient, which may have been due to dysfunction of the signal peptide. These results provide evidence that a structural change to the signal peptide of the CK protein was involved in the pathogenesis of pycnodysostosis.