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CRTAP AND *LEPRE1* MUTATIONS IN RECESSIVE OSTEOGENESIS IMPERFECTA

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Abstract

Autosomal dominant osteogenesis imperfecta (OI) is caused by mutations in the genes (*COL1A1* or *COL1A2*) encoding the chains of type I collagen. Recently, dysregulation of hydroxylation of a single proline residue at position 986 of both the triple-helical domains of type I collagen α1(I) and type II collagen α1(II) chains has been implicated in the pathogenesis of recessive forms of OI. Two proteins, CRTAP, or cartilage-associated protein, and prolyl-3-hydroxylase-1 (P3H1, encoded by the *LEPRE1* gene) form a complex that performs the hydroxylation and brings the prolyl *cis-trans* isomerase cyclophilin-B (CYPB) to the unfolded collagen. In our screen of 78 subjects diagnosed with OI type II or III, we identified three probands with mutations in *CRTAP* and sixteen with mutations in *LEPRE1*. The latter group includes a mutation in patients from the Irish Traveller population, a genetically isolated community with increased incidence of OI. The clinical features resulting from *CRTAP* or *LEPRE1* loss of function mutations were difficult to distinguish at birth. Infants in both groups had multiple fractures, decreased bone modeling (affecting especially the femurs), and extremely low bone mineral density. Interestingly, "popcorn" epiphyses may reflect underlying cartilaginous and bone dysplasia in this form of OI. These results expand the range of *CRTAP/LEPRE1* mutations that result in recessive OI and emphasize the importance of distinguishing recurrence of severe OI of recessive inheritance from those that result from parental germline mosaicism for *COL1A1* or *COL1A2* mutations.

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Keywords

Osteogenesis Imperfecta; Prolyl 3-Hydroxylation; *CRTAP*; *LEPRE1*

INTRODUCTION

Individuals with osteogenesis imperfecta (OI) have fragile bones and increased susceptibility to fracture (Byers and Cole, 2002; Sillence and Rimoin, 1978). OI is usually inherited in an autosomal dominant pattern and caused by heterozygosity for mutations in the *COL1A1* or *COL1A2* genes (MIM #120150 and #120160) (Byers and Cole, 2002; Pihlajaniemi, et al., 1984; Rauch and Glorieux, 2004). Mutations that lead to a quantitative decrease in production of structurally normal type I collagen result in the mildest OI phenotype (OI type I) (Wenstrup, et al., 1990). Mutations that cause structural changes in either of the chains of type I procollagen can affect chain association, triple helix formation, secretion, and/or fibril formation, and generally result in more severe phenotypes, including the perinatal lethal OI phenotype. Biochemically, these structural abnormalities are reflected by increased post-translational modification of type I procollagen chains (Byers and Cole, 2002; Rauch and Glorieux, 2004; Wenstrup, et al., 1990).

Alteration of type I procollagen post-translational processing, in the absence of mutations in type I collagen genes, can also cause OI (Barnes, et al., 2006; Morello, et al., 2006). In those instances, mutations in *CRTAP* (MIM #605497) and *LEPRE1* (MIM #610339, which encodes the protein prolyl 3-hydroxylase-1, abbreviated P3H1, and the protein leprecan) can cause recessively inherited OI. CRTAP, or cartilage associated protein, forms a molecular complex in the rough endoplasmic reticulum (RER) with P3H1 and cyclophilin B (MIM #123841, CYPB or peptidylprolyl isomerase B, PPIB) in a 1:1:1 ratio (Vranka, et al., 2004). In both mice and humans with mutations that lead to loss of function mutations in *CRTAP*, there is absent 3-hydroxylation of the single proline target residue at position 986 in the triple helical domain or residue 1164 measured from the initiator methionine in the proα (1)I chain (Morello, et al., 2006). Prolyl 3-hydroxylation is one of several modifications of proα chains that contribute to the proper folding, stability, and secretion of procollagen. Prolyl 4-hydroxylation is important for thermal stability of the triple helix while lysyl hydroxylation and hydroxylysyl glycosylation contribute to extracellular crosslink stability between molecules (Lamande and Bateman, 1999; Myllyharju and Kivirikko, 2004). In human forms of OI, homozygosity or compound heterozygosity for *CRTAP* and *LEPRE1* mutations are associated with post-translational overmodification of the chains of type I collagen synthesized by cultured fibroblasts *in vitro*, causing retarded mobility on electrophoresis. Although all molecules are overmodified in the recessive disorders, and either half or three quarters are affected in the dominant disorders, depending on the gene in which the mutation occurs, it can be difficult to distinguish collagen overmodification due to structural mutations of type I collagen genes from mutations in the prolyl 3-hydroxylation complex.

To identify mutations that disrupt prolyl 3-hydroxylation and, at the same time, to assess the contribution of mutations in *CRTAP*, *LEPRE1*, and *PPIB* (which encodes CYPB) to the pathogenesis of OI, we screened DNA from 78 affected individuals, identified either *in utero* or at birth, for mutations in these genes. In our cohort, we found three additional instances in which OI was caused by novel mutations in *CRTAP*, including the first reported missense mutation. We also identified sixteen instances in which OI was caused by mutations in *LEPRE1*. Nine of the eleven mutations we identified in *LEPRE1* are novel. We found no mutations in the *PPIB* gene.

MATERIALS AND METHODS

Human Subjects

We selected 78 subjects for screening based on reported consanguinity, origin from communities with a known increased rate of consanguinity, multiple affected siblings from the same family, or failure to identify mutations in the *COL1A1* or *COL1A2* genes even when cultured cells made abnormal proteins; some subjects were chosen for screening due to clinical and radiographic phenotypic similarity to previously reported mutation-positive cases. With institutional review board approvals, blood, fibroblasts or tissue were collected from affected individuals and DNA was prepared by standard protocols.

PCR and Sequence Analysis

The seven exons of *CRTAP*, fifteen exons of *LEPRE1*, and five exons of *PPIB*, as well as surrounding intronic regions, were amplified from genomic DNA by PCR and analyzed by fluorescent dye-terminator sequencing (Seqwright DNA Technology Services, Houston, TX). Results were analyzed using Sequencher 4.6 software (Gene Codes Corporation, Ann Arbor, MI) or Mutation Surveyor (Softgenetics, Hershey, PA). Subject sequences were compared to the Ensembl gene sequences ENSG00000117385, ENSG00000170275, and ENSG00000166794 for *LEPRE1*, *CRTAP* and *PPIB*, respectively. Previously known SNPs were identified by using GeneCards, www.genecards.org (Weizmann Institute of Science, Rehovot, Israel). For both *CRTAP* and *LEPRE1* we have used the conventional coding sequence numbering in which the A of the initiator methionine codon is the first nucleotide (+1), and the initiator methionine is amino acid +1. This makes the reference point for the *LEPRE1* coding sequence different from that used by Cabral et al.(Cabral, et al., 2007b) where the first nucleotide of the cDNA sequence is numbered as +1, which is at position −41 with respect to the A of the initiator methionine codon. Both this analysis and that by Cabral et al. (11) used the same convention for amino acid numbering.

RT-PCR

Total RNA was extracted from fibroblasts using Trizol (Invitrogen, Carlsbad, CA), and first-strand cDNA synthesis was performed with the SuperScript III reverse transcriptase kit (Invitrogen). Quantitative real-time quantitative PCR was performed on the LightCycler version 1.1 instrument with Roche Applied Science (Indianapolis, IN) reagents according to manufacturer recommendations. cDNA template concentrations were determined using Ribogreen (Molecular Probes) and equivalent concentrations were used for each q-PCR. Fluorescence was captured at the end of each extension cycle, over a total of 45 cycles. Crossing points were determined by a second derivative algorithm intrinsic to the Lightcycler software and normalized to a constitutively expressed gene (β2-microglobulin). Primer sequences are available upon request.

Mass Spectrometry

3-hydroxylation of proline at position 986 of the triple helix in the α1(I) chains of type I collagen was determined on collagen prepared from bone tissue or cell cultures of skin fibroblasts or bone osteoblasts. Either CNBR-derived peptides (CB peptides) or pepsinized collagens were isolated by SDS-PAGE and tryptic digests prepared for MS/MS. Electrospray MS was performed on an LCQ Deca XP ion-trap mass spectrometer with in-line liquid chromatography (LC) (ThermoFinnigan) using a C8 capillary column (300mm × 150mm; Grace Vydac 208MS5.315) eluted at 4.5ml/min. The mobile gradient was made from buffer A (0.1% formic acid plus 2% buffer B in MilliQ water) and buffer B (0.1% formic acid in 3:1 acetonitrile:n-propanol v/v). An electrospray ionization source (ESI) under atmospheric pressure was used

to introduce the sample stream into the mass spectrometer. The spray voltage was 3 kV and the inlet capillary temperature 160°C.

RESULTS

From our screen of 78 affected individuals, we found three probands with mutations in the gene *CRTAP* and sixteen probands with mutations in the gene *LEPRE1*. Two of the probands were compound heterozygous and the remaining subjects were homozygous for their respective mutations (Supplementary Table S1). This finding reflects known consanguinity of the parents in at least eight of the nineteen probands (Supplementary Table S1). All of the probands had severe forms of OI, either type II (perinatal lethal OI) or type III (severely deforming OI): seven died within the first year after birth; five were diagnosed with severe OI *in utero* and the pregnancies were terminated; an additional five probands survived the first year after birth with the oldest being 16 years of age; and one proband is presently alive at 5 months of age (Supplementary Table S1).

Representative radiographic findings of more severely affected probands with *CRTAP* and *LEPRE1* mutations are shown in figures 1 and 2, respectively. At the time the radiographs were taken, Proband 2 was a 12 month old Caucasian female who was compound heterozygous for *CRTAP* mutations (c.278_293dup and c.822_826delAATACinsT). Both mutations predict frameshifts and downstream premature termination codons. At birth she had multiple fractures of the ribs, clavicle, humerus, radius, mandible, and ulna, with sclerosis, poor definition and widened long bone metaphyses (Fig. 1). At age 12 months, she was alive without need for respiratory support. Proband 7 was found to be homozygous for a *LEPRE1* nonsense codon, c.392C>A, p.Ser131X. At birth she had multiple fractures of long bones and ribs (Fig. 2A–D), short limbs, decreased tone, and respiratory distress. Radiographically, there was osteopenia, rhizomelia, brachycephaly, decreased calvarial thickness, and thin ribs with multiple fractures.

Representative radiographic features of longer-lived patients with *CRTAP* and *LEPRE1* mutations are shown in figures 3 and 4, respectively. Proband 3 was homozygous for the first reported missense mutation in *CRTAP*, c.200T>C (p.Leu67Pro). She was the product of an Iranian, first cousin mating. At the time of this study, she was 9 years-old. She had multiple fractures at birth and exhibited growth delay soon after birth. She has had at least 16 documented fractures from birth to age 7.5 years (Fig. 3), but none in the last 18 months. At age 7.5 years her length was 71 cm ($z = -12.87$, 50% for age 10.5 months) and her weight was 11 kg ($z = -7.91$, 50% for age 18 months; $z = 2.46$ in weight-for-height). Her lung function (FV1, FVC, PEF) was normal for a child her height, and close to 100% of what is expected for a child her size. She had significant scoliosis, and marked bowing of her long bones. Radiographically, these bones were gracile and demonstrated large, bulbous “popcorn” epiphyses (Fig. 5A). She does not stand or walk, uses a wheel bed for mobility, and is intellectually normal. A second pregnancy from this family was terminated at 19 weeks gestation when ultrasound findings also showed OI. Testing of this pregnancy revealed no mutations in type I collagen genes, but our study confirmed homozygosity for the same missense mutation in *CRTAP* seen in Proband 3 (c.200T>C; p.Leu67Pro). Both parents were heterozygous for the mutation and this variant was not found in 100 unaffected control samples. The level of *CRTAP* mRNA was normal in patient fibroblasts (Table 1). This amino acid residue in *CRTAP* is evolutionarily conserved among vertebrate sequences, with the exception of some fish species.

Proband 10 was another of the longer lived probands in this cohort. This case was previously reported by Cabral et al. (2007) as their Proband 5, and the mutation noted by Cabral et al. (c. 1656C>A; p.Tyr552X) was confirmed in our study (Cabral, et al., 2007b). The parents are half-first cousins and share a common grandfather. During their first pregnancy, *in utero* fractures

were noted and the infant was confirmed to have fractures at birth. When evaluated at age 6 years (the radiographs published in Cabral et al., 2007), he was small, depended on a wheel chair for mobility, and had marked deformity of all limbs and significant scoliosis (Cabral, et al., 2007b). By age 11 years, his clinical findings were similar but the radiographs demonstrated large, bulbous epiphyses of the long bones, with very irregular regions ("popcorn epiphyses") (Fig. 4A–C, 5B).

Both of the longer-lived cases show striking bulbous "popcorn" epiphyses (Fig. 5) which may be a distinguishing feature of recessive versus dominant OI. However, from the cases described above, the phenotypes of probands with mutations in *CRTAP* and *LEPRE1* are difficult to distinguish. This conclusion is supported by the additional radiographs available in Supplementary Figures 1–4, which show the clinical phenotypes of Proband 1 with a mutation in *CRTAP* and Probands 4, 5, 14, 16 and 17 with mutations in *LEPRE1*.

Among *CRTAP* or *LEPRE1* mutations identified in this study (Fig. 6), seven probands had frameshift mutations, all of which lead to downstream termination codons and nonsense mediated decay (NMD). Seven additional probands had mutations that disrupt splice donor sites and are predicted to lead to abnormal splicing. In all but two of these cases (Probands 18 and 19), missplicing should lead to premature termination and NMD. These include Probands 11, 12, 13, and 14, who were all homozygous for the West African *LEPRE1* mutation previously identified (Cabral, et al., 2007b), and Proband 15 who was a compound heterozygote for this West African mutation and a nonsense mutation. Four additional probands had nonsense mutations. Four of these nonsense mutations are predicted to lead to NMD, while the mutation in Proband 9 (c.2041C>T) is within the last 50 nucleotides of the penultimate exon, which can allow for escape from NMD. Proband 3, discussed above, is the only one found with a missense mutation in either *LEPRE1* or *CRTAP* to date. Hence, it appears that for the majority of severe recessive OI cases, loss of mRNA stability represents the primary consequence of mutation.

To confirm the effects of these genomic mutations on transcript stability, quantitative real-time PCR was performed when RNA from cultured cells was available. In all instances in which frameshift, nonsense, or splice site mutations created premature termination codons, there was significant reduction in the quantity of *LEPRE1* or *CRTAP* transcripts relative to controls, consistent with the predicted effect of NMD (Table 1). In cells from Proband 3, who was homozygous for a missense mutation in *CRTAP*, there was no reduction in transcript levels. Cells from Proband 9, who was homozygous for a nonsense mutation within the last 50 base pairs of the penultimate exon of *LEPRE1*, contained about half the normal level of the transcript, suggesting that a significant portion of transcripts escaped NMD (Khajavi, et al., 2006). Probands 18 and 19 were each homozygous for unique single base pair substitutions at the splice donor site at the start of intron 6 of *LEPRE1* (c.1170+2T>A and c.1170+5G>C, respectively). Both of these mutations resulted in normal quantities of mRNA transcript (Table 1 and data not shown), because the only apparent splice outcome from cells from both probands was in-frame skipping of exon 6 resulting in deletion of 90 nucleotides.

To evaluate the biochemical effects of the mutations, levels of 3-hydroxyproline were analyzed by mass spectrometry of type I collagen from fibroblast-conditioned media, and bone when available. In all samples that we tested, there were significantly decreased levels of 3-hydroxylation of the proline at position 986 of the triple helix in the α(I) chain of type I collagen from cultured fibroblasts as shown in table 1. Fibroblast collagen from Proband 3, who was homozygous for a missense mutation in *CRTAP*, and who seemed to have a milder phenotype than those in which the amount of mRNA was predicted to be close to absent, hydroxylated 79% of the target proline residues. Cells from Proband 11, who had a splice site mutation in *LEPRE1* that results in several products (Cabral, et al., 2007b), had 4% of 3-hydroxyproline

in fibroblast collagen but 55% of 3-hydroxyproline in type I collagen from bone derived cells (Fig. 7). In contrast, Proband 1, who was homozygous for a frameshift mutation in *CRTAP*, had 4% of 3-hydroxyproline in collagen from fibroblasts and 2% in bone compared to controls (Fig. 7).

DISCUSSION

Based on the phenotypic findings in our cohort, complete or nearly complete loss-of-function of either *CRTAP* or *LEPRE1* gives rise to the severe forms of OI, specifically types II and III. We found three newly identified alleles of *CRTAP* and nine newly identified alleles of *LEPRE1*, thereby expanding the spectrum of mutant alleles discovered to date (Fig. 6). Probands 11, 12, 13, and 14 were homozygous for, and Proband 15 was heterozygous for a mutation at the consensus splice donor site in intron 5 of *LEPRE1* that was reported as a "West-African allele" (Cabral, et al., 2007b). Proband 2 was a compound heterozygote for *CRTAP* mutations and shared the 16 base pair duplication allele with infant 3 described by Barnes et al.(Barnes, et al., 2006).

The *CRTAP* missense mutation, c.200T>C (p.Leu67Pro), in Proband 3 demonstrates that disruption of the amino acid sequence of CRTAP can lead to OI, and in this instance to a similar albeit milder phenotype than seen with complete loss of the protein. Additional studies are needed to determine how this amino acid substitution disrupts CRTAP function.

The presence of a unique loss-of-function *LEPRE1* allele (c.232delC) in three members of the Irish Traveller population whose families had not considered themselves related has important clinical and epidemiological implications for this community. This population has significant consanguinity and an increased prevalence of genetic and metabolic conditions with autosomal recessive inheritance, including osteogenesis imperfecta (Barry and Kirke, 1997; Van Cleemput, 2000). Previous work showed that OI in the Irish Traveller population is not linked to the two type I collagen genes, *COL1A1* and *COL1A2* (Williams, et al., 1989). The increased prevalence of OI in this population probably reflects a high carrier frequency for this *LEPRE1* mutation so that carrier testing could be effectively implemented. We are uncertain if the extended survival of Proband 4, one of the Irish Traveller subjects who is now 5 years old, intellectually normal, and uses a wheelchair for mobility, results from treatment since birth with bisphosphonates (IV pamidronate, 12 mg/kg/year divided into every three month doses) that are frequently used in treatment of children with severe OI (Glorieux, 2007), or reflects a different genetic background on which the homozygous *LEPRE1* mutations are expressed. We speculate that early treatment with these drugs might reduce the extent to which the bulbous epiphyseal structures (often referred to as "popcorn epiphyses") form, considering their absence in this treated child at age 5 years and presence in a child previously described who has abnormal epiphyses by age 6 years (Fig. 5B) (Cabral, et al., 2007b).

Mutations in the *CRTAP* and *LEPRE1* genes can each have a relatively broad range of clinical presentation, from a very severe and often lethal phenotype in OI type II/III to milder OI type VII seen with mutations in *CRTAP* that lead to production of some normal protein (Morello, et al., 2006). Parental consanguinity is one feature that helps to separate those with recessive mutations in *CRTAP* or *LEPRE1* from those with mutations in type I collagen genes in whom parental mosaicism can lead to recurrence that mimics recessive inheritance. Among those infants with *CRTAP* or *LEPRE1* mutations that leave little functional mRNA, the clinical presentations are difficult to distinguish. These infants often are identified *in utero* with features similar to lethal forms of OI that result from heterozygosity for mutations in type I collagen genes (*COL1A1* and *COL1A2*) and may have multiple fractures of the long bones and the ribs at birth, diminished calvarial mineralization, and decreased bone modeling. Cabral et al. (2007) suggested that the severely affected infants with *LEPRE1* mutations differ from those

with mutations in type I collagen genes in that they have white rather than grey or blue sclerae, round rather than a triangular face, and barrel-shaped rather than narrow thorax. Based on our findings, it is not clear that these features are consistently found in all cases with *LEPRE1* mutations. More individuals with mutations in these genes must be identified and better characterized at the clinical level to permit definitive conclusions. The "popcorn epiphyses" in the surviving patient who harbors a homozygous missense mutation in CRTAP (Proband 3, Fig. 5A) and the patient who has a homozygous null mutation in P3H1 (Proband 10, Fig. 5B) are striking. These abnormal epiphyses may reflect a cartilaginous dysplasia at the developing growth plate in addition to the defects in bone formation in patients with OI caused by mutations in the prolyl 3-hydroxylation complex. This speculation is consistent with type II collagen in cartilage also being modified by prolyl 3-hydroxylation, and cartilage from CRTAP knock-out mice showing absent prolyl 3-hydroxylation (Morello, et al., 2006).

Null mutations in *CRTAP* and in *LEPRE1* may not have entirely comparable results. For example, while Proband 1 with a *CRTAP* mutation has 2–4% of the expected amount of 3-hydroxyproline at proline 986 of the triple helix in α1(I) collagen chains from bone and cultured dermal fibroblasts and α1(II) from cartilage (data not shown), another patient, Proband 11, with a *LEPRE1* mutation, has about 50% of the expected amount of 3-hydroxyproline in cultured osteoblast α1(I) chains, but only 4% in cultured dermal fibroblast α1(I) chains. This suggests that there may be compensation for P3H1 loss-of-function in bone cells, perhaps by other prolyl 3-hydroxylases, such as P3H2 or P3H3. We cannot exclude the formal possibilities that the data from fibroblasts may be an artifact of culture or that the *LEPRE1* mutation may lead to different splicing outcomes in bone versus fibroblasts.

The precise mechanisms by which mutations in *CRTAP* and *LEPRE1* result in osteogenesis imperfecta remain undefined. It is clear that a population of the protein product of the *LEPRE1* gene can interact with CRTAP protein to form a complex with cyclophilin B, the prolyl cis-trans isomerase. There are, however, at least two transcripts from the *LEPRE1* gene reported in the Ensembl database: one encodes what is presumably the P3H1 protein and has a KDEL endoplasmic reticulum retention domain at its carboxyl-terminus (accession number ENST00000296388); a second splice product results from alternate splicing at the end of exon 14 and creates a protein that lacks the KDEL sequence and contains 804 residues (accession number ENST00000236040) compared to the 736 in P3H1. This protein may well be the "true" secreted leprecan protein that contains two glycosoglycan addition sites. The function of leprecan and its localization in the extracellular matrix is still being explored. The identification of cells with null mutations will provide tools with which to dissect the functions of the different elements of this complex of proteins. It is likely that the molecular pathogenesis is more complex than simply the lack of prolyl 3-hydroxylation in type I and perhaps other collagens.

Our work demonstrates that mutations in *CRTAP* and *LEPRE1* contribute significantly to the cohort of individuals with severe phenotypes and recessive OI. In populations from West Africa the severe form of OI may result as frequently from mutations in the *LEPRE1* gene as from mutations in the type I collagen genes. We identified one carrier among 91 normal African American males (data not shown) and Cabral et al. identified 5 in 1429 newborns (Cabral, et al., 2007a). This suggests a carrier frequency of between 1/100 and 1/280, with expected affected infant frequency as high as 1/40,000 births. The estimate of lethal OI in about 1/50,000–1/60,000 live births suggests that a significant proportion of those in African Americans could result from this single allele, rather than from parental mosaicism for mutations in type I collagen genes.

These observations come in the face of increasing ease of DNA-based diagnosis and raise the question of how best to approach the diagnosis of OI. In all fourteen individuals in this series from whom cultured skin fibroblasts were available for testing, the cells synthesized abnormal

type I procollagen chains that had altered electrophoretic mobilities (data not shown). It was sometimes difficult to determine if all chains had abnormal mobility, however. Thus, while protein studies will identify abnormalities in cells from infants with severe forms of OI and mutations in *CRTAP*, *LEPRE1*, and the type I collagen genes, testing for those mutations is essential to distinguish the mode of inheritance, prediction of recurrence risk, and facilitate testing for parental mosaicism when mutations occur in the collagen genes. Ultimately, the mutation data will provide the basis for genotype-phenotype studies, including determining whether the observed "popcorn epiphyses" are distinguishing features of surviving patients with mutations in *CRTAP* or *LEPRE1*. Until all splice site mutations have been identified, understanding the outcome of new mutations will have to rest on the clinical picture and study of RNA processing in cells. Moreover, the implementation of high density arrays will facilitate the detection of subtle deletions and duplications, and may become an important complement to sequencing based diagnostic approaches. In addition to blood samples for DNA isolation and diagnostic testing, understanding the molecular pathogenesis of OI will continue to require either cultured cells from the target tissue or sophisticatedly engineered animals that allow the study of the mechanisms by which mutations in all of the OI disease genes lead to reduced bone mineral density.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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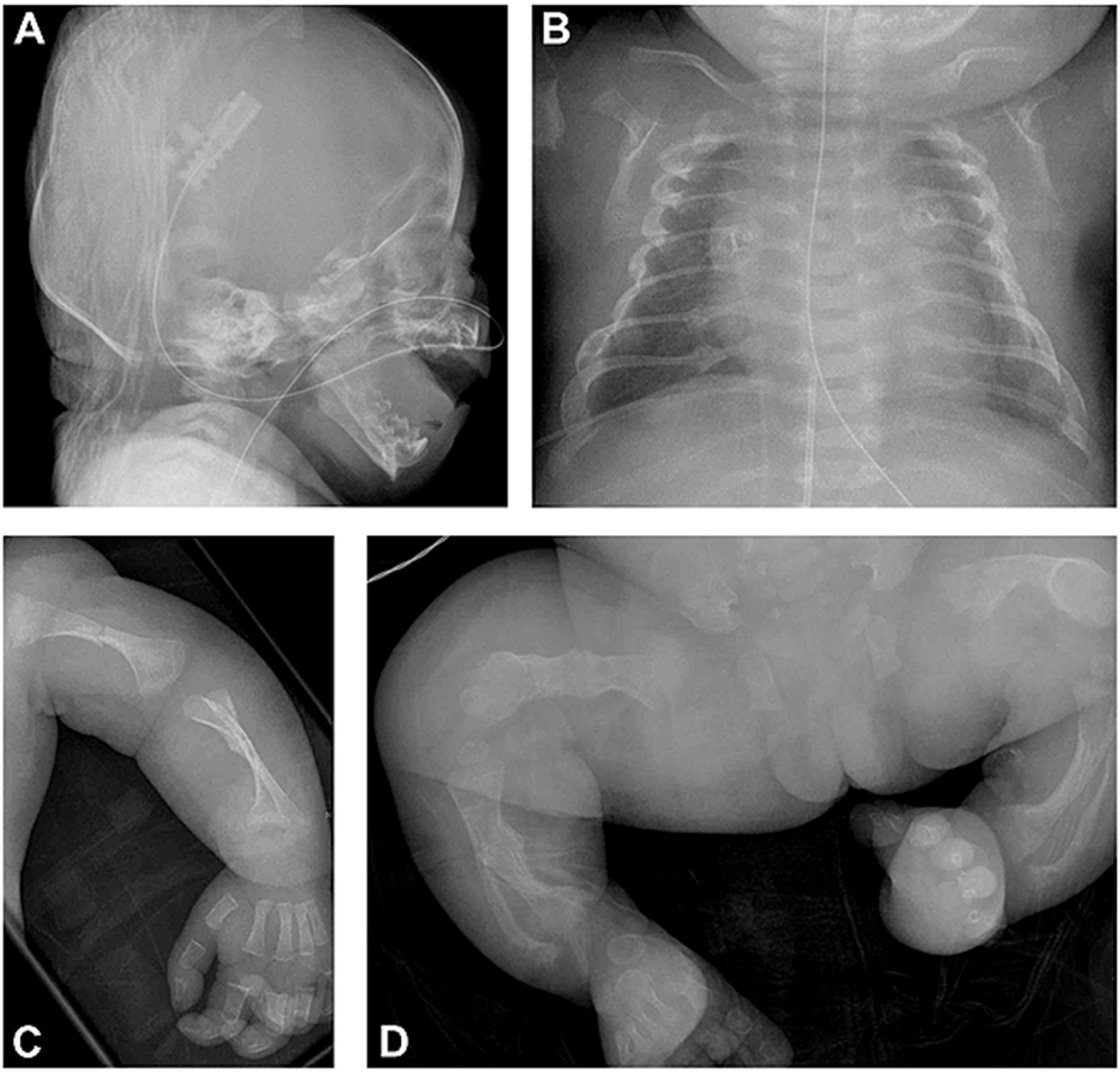

Figure 1.
Early radiographs of Proband 2 with *CRTAP* mutation. *A, B, C,* and *D,* radiographs at age one day, consistent with severe OI. This infant was a compound heterozygote for mutations in *CRTAP* (c.278_293dup; c.822_826delAATACinsT).

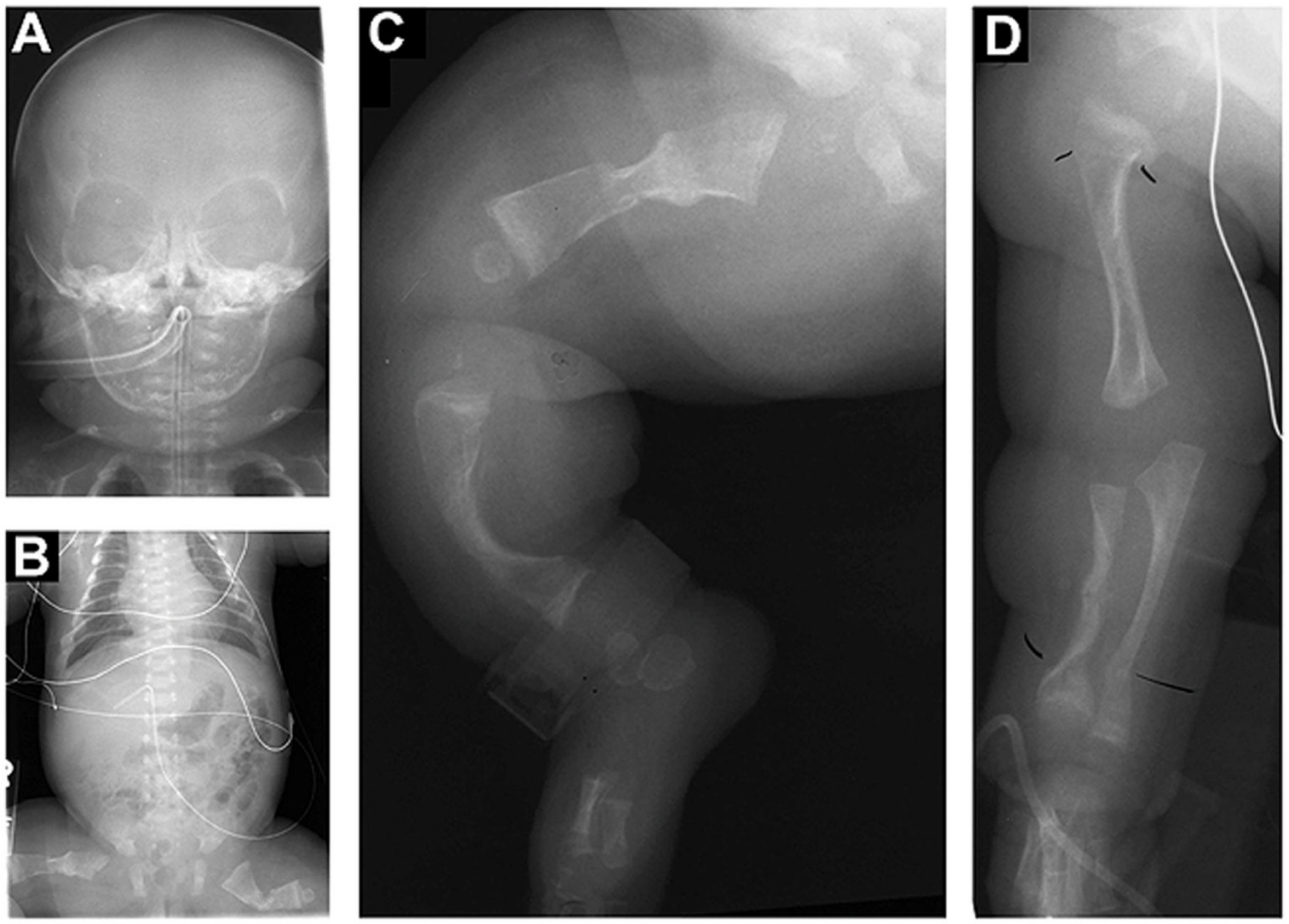

Figure 2.
Early radiographs of Proband 7 with *LEPRE1* nonsense mutation. *A, B, C, D,* radiographs of Proband 7 at birth, consistent with severe OI, found to have a homozygous nonsense mutation in *LEPRE1*, c.392C>A, p.Ser131X.

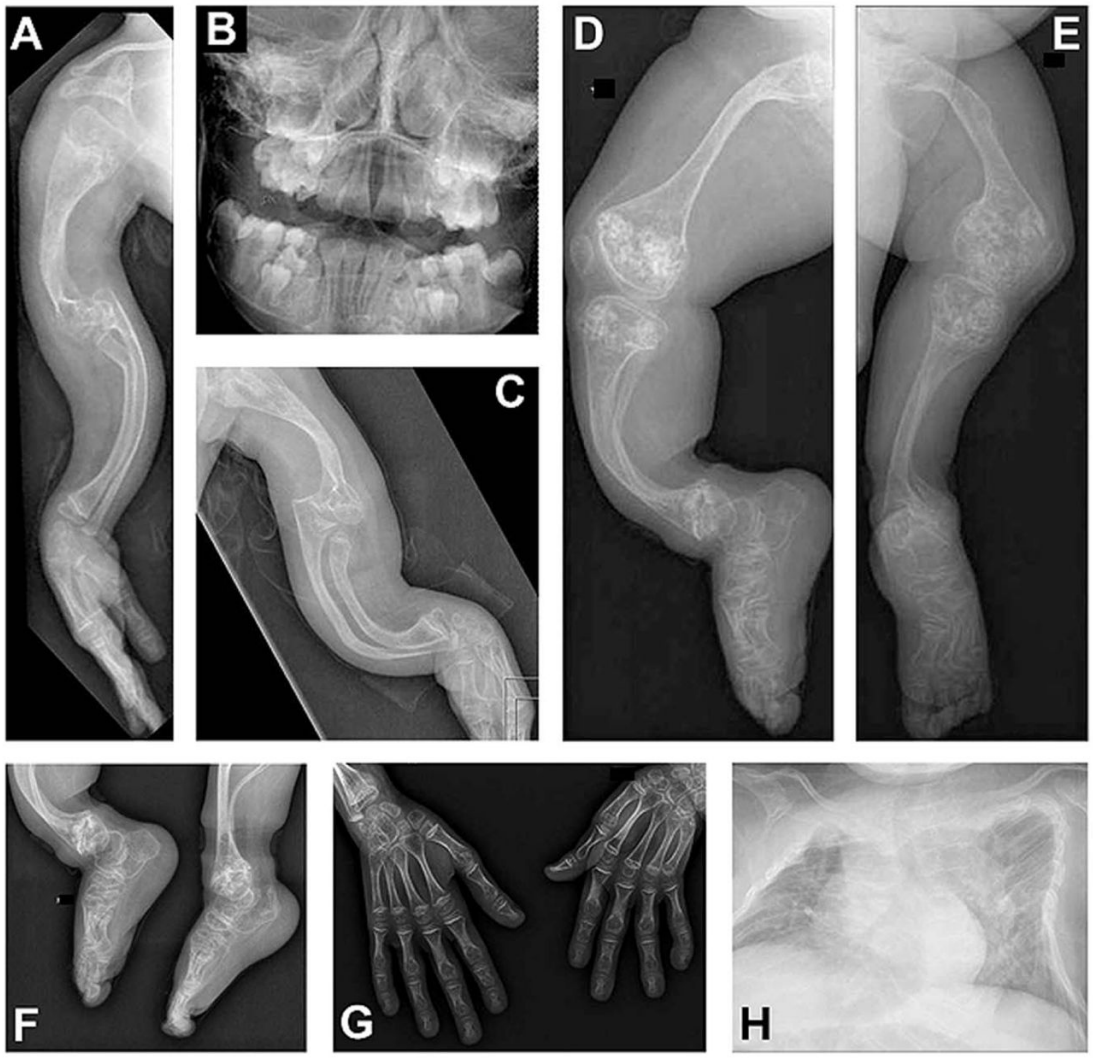

Figure 3.
Late radiographs of Proband 3 with *CRTAP* missense mutation. *A–H*, radiographs at age 8 years, consistent with OI, found to have a homozygous missense mutation in *CRTAP*, c. 200T>C; p.Leu67Pro.

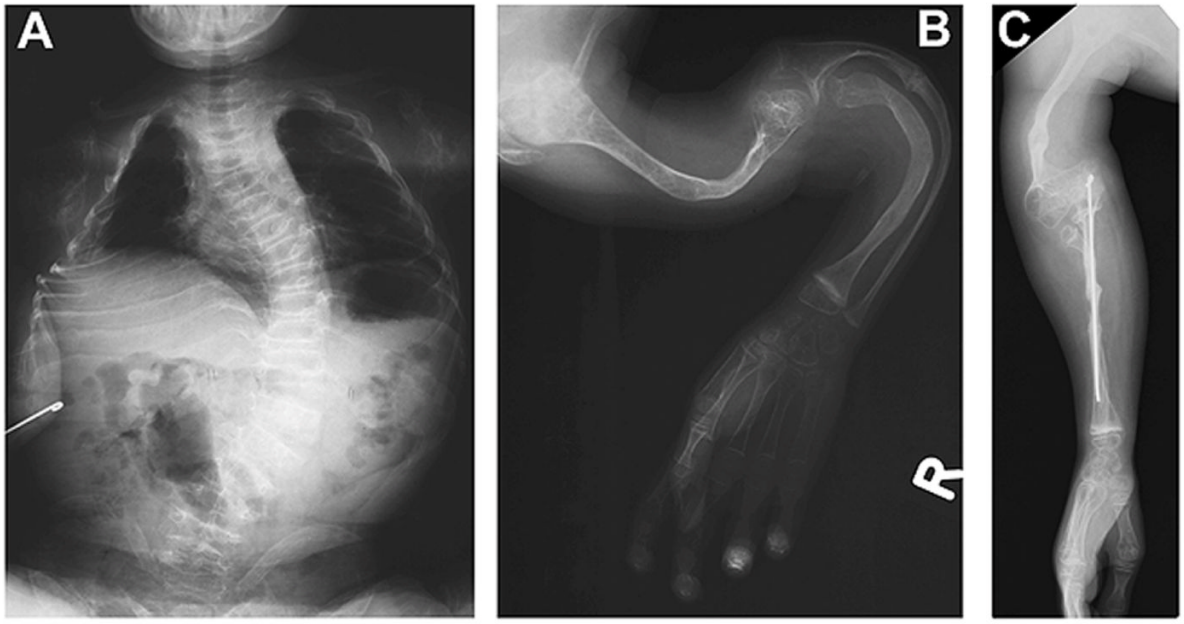

Figure 4.
Late radiographs of Proband 10 with *LEPRE1* nonsense mutation. *A, B, C*, radiographs of Proband 10 at age 11 years, previously described at age 6 years by Cabral et al.(Cabral, et al., 2007b) and confirmed to have a homozygous nonsense mutation in *LEPRE1*, c.1656C>A; p.Tyr552X.

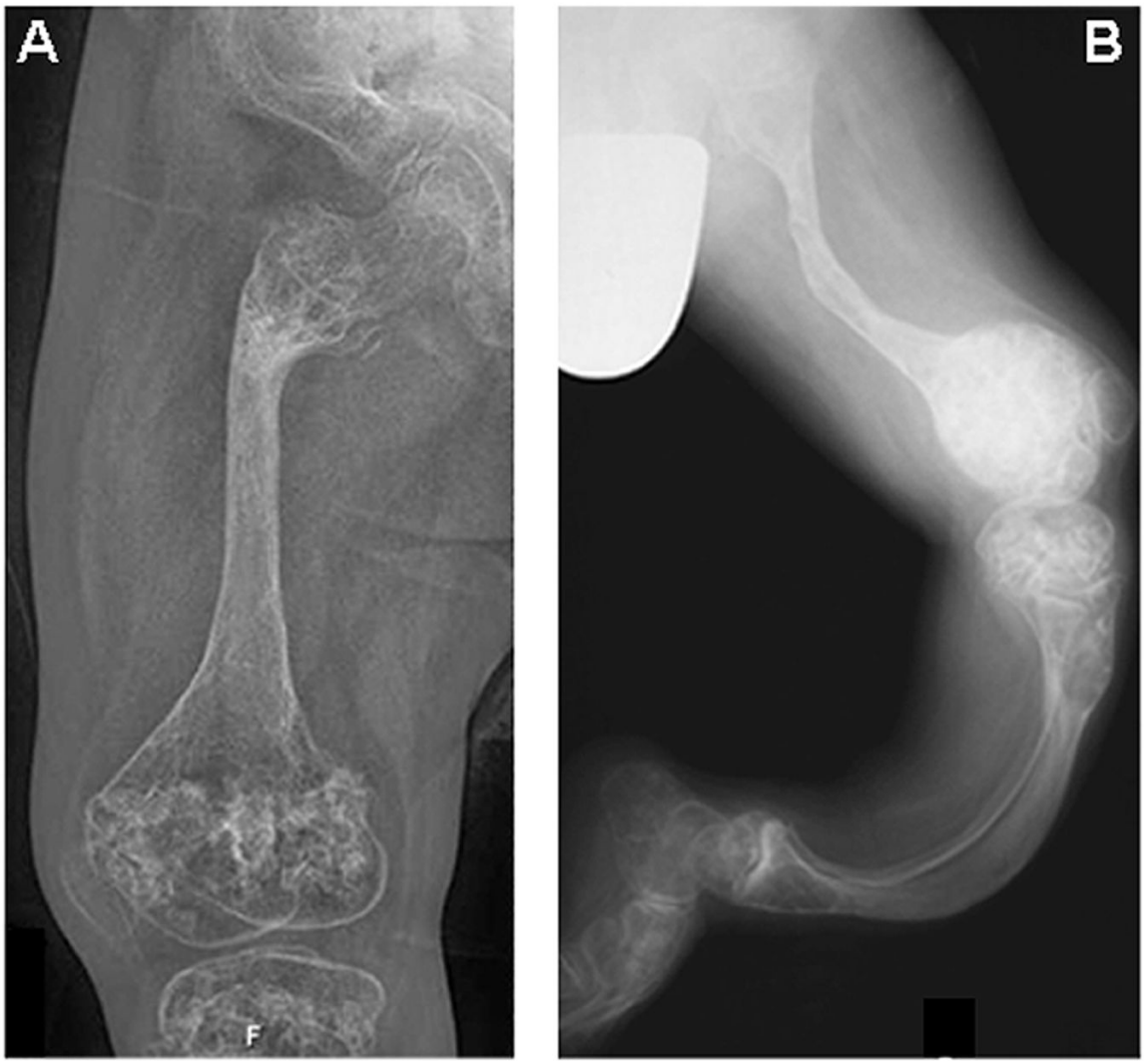

Figure 5.
Bulbous "popcorn epiphyses." *A*, radiograph of femur of Proband 3 with a homozygous missense mutation in *CRTAP*, c.200C>T; p.Leu67Pro. *B*, radiograph of leg of Proband 10 with a homozygous nonsense mutation in *LEPRE1*, c.1656C>A; p.Tyr552X.

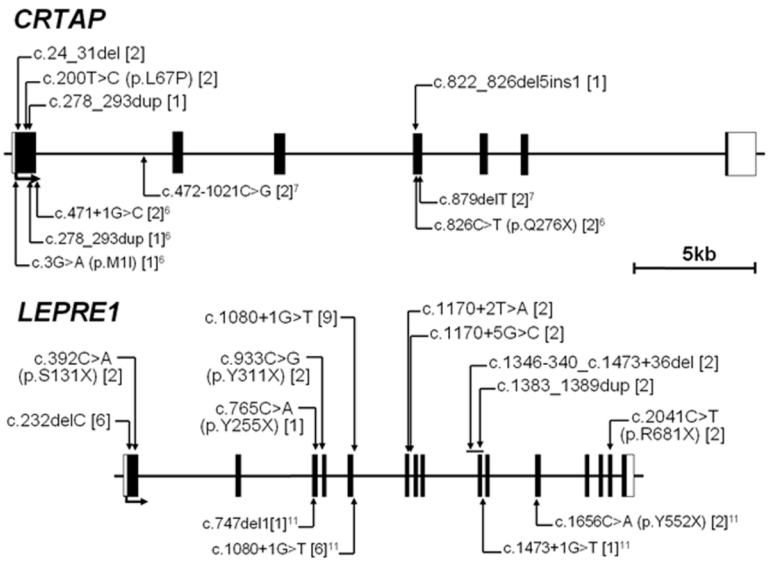

Figure 6.

Schematic diagram of the exons and the locations of mutations found in the *CRTAP* and *LEPRE1* genes. The mutations described in this report are shown above each gene and those previously described are shown below the respective gene. The number of alleles identified are in brackets [] and the references are indicated by superscript. Amino acids are represented by the single letter code: I, isoleucine; M, methionine; Q, glutamine; R, arginine; Y, tyrosine; and X, termination codon.

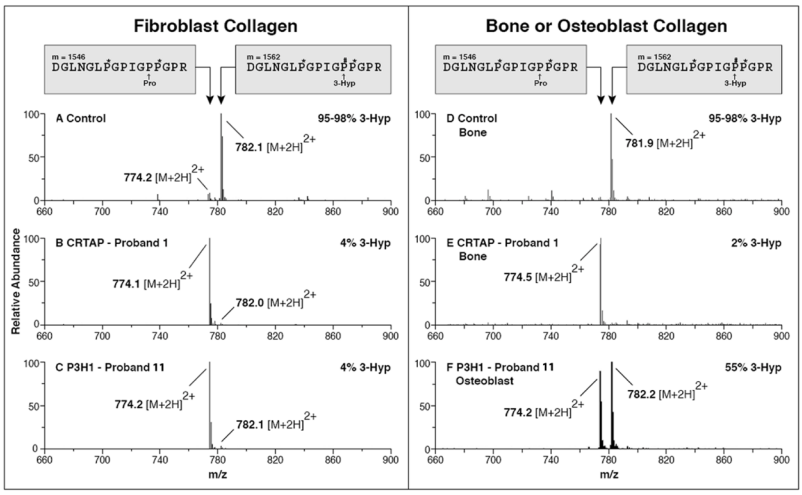

Figure 7.

Tandem mass spectrometric analyses of α1(I) collagen from patient tissues and cultured cells with *CRTAP* (Proband 1) or *LEPRE1* (Proband 11) mutations shows decreased 3-hydroxyproline relative to controls. *A*, *B*, and *C*, analyses from patient and control fibroblast-conditioned media. *D*, *E*, and *F*, analyses from patient and control bone and cultured osteoblast collagen.

Table 1

Mutations in *CRTAP* and *LEPRE1* with mRNA and 3-Hyp levels.

Proband	Mutation [Allele 1] [Allele 2]	mRNA expression (%)	3-Hyp fibroblasts (%)	3-Hyp bone (%)
CRTAP				
1	[c.24_31del] [c.24_31del]	19	4	2[a]
2	[c.278_293dup] [c.822_826delAATACinsT]	4	5	NA
3	[c.200T>C] [c.200T>C] (p.Leu67Pro)	128	79	NA
LEPRE1				
4	[c.232delC] [c.232delC]	0.8	15	NA
8	[c.933C>G] [c.933C>G] (p.Tyr311X)	6	5	NA
9	[c.2041C>T] [c.2041C>T] (p.Arg681X)	57	4	NA
11	[c.1080+1G>T] [c.1080+1G>T]	Skin 5 Bone 0.4	4	55[b]
16	[c.1383_1389dup] [c.1383_1389dup]	8	6	NA
18	[c.1170+2T>A] [c.1170+2T>A]	87[c]	7	NA

NOTE.—mRNA and 3-hydroxyproline (3-Hyp) levels are given as percentages relative to normal controls. Reference sequences used are CRTAP: Ensembl ENST00000320954, and LEPRE1: Ensembl ENST00000296388.

[a] Bone tissue.

[b] Cultured osteoblasts.

[c] This mRNA is abnormally spliced such that exon 6 (90 base pairs) is skipped, resulting in a stable product. NA = Not available.