

## Identification of a Frameshift Mutation in *Osterix* in a Patient with Recessive Osteogenesis Imperfecta

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Osteogenesis imperfecta, or “brittle bone disease,” is a type I collagen-related condition associated with osteoporosis and increased risk of bone fractures. Using a combination of homozygosity mapping and candidate gene approach, we have identified a homozygous single base pair deletion (c.1052delA) in *SP7/Osterix* (*OSX*) in an Egyptian child with recessive osteogenesis imperfecta. The clinical findings from this patient include recurrent fractures, mild bone deformities, delayed tooth eruption, normal hearing, and white sclera. *OSX* encodes a transcription factor containing three Cys2-His2 zinc-finger DNA-binding domains at its C terminus, which, in mice, has been shown to be essential for bone formation. The frameshift caused by the c.1052delA deletion removes the last 81 amino acids of the protein, including the third zinc-finger motif. This finding adds another locus to the spectrum of genes associated with osteogenesis imperfecta and reveals that *SP7/OSX* also plays a key role in human bone development.

Osteogenesis imperfecta (OI, MIM 166200, MIM 166210, MIM 259420, MIM 166220) is a bone-related genetic disorder characterized by low bone mass and bone fragility that is clinically and genetically heterogeneous. Patients with OI have clinical features that may range from mild symptoms with a scant number of fractures to severe bone deformities and neonatal lethality.<sup>1</sup> Most cases of OI are caused by mutations in the type I procollagen genes, *COL1A1* (MIM 120150) and *COL1A2* (MIM 120160), and follow an autosomal-dominant pattern of inheritance.<sup>2</sup> Type I collagen is a structural component of the connective tissue that is initially synthesized as a procollagen precursor in the endoplasmic reticulum (ER) by the combination of two procollagen a1 (I) and one procollagen a2 (I) peptide chains paired together into a triple helix, a process that is assisted by a number of molecular chaperons and ER enzymes. Type I procollagen trimers are then exported into the extracellular matrix and transformed into functionally competent type I collagen molecules by the proteolytic removal of the N-terminal and C-terminal propeptides.<sup>3</sup> In a minority of cases with severe or lethal autosomal-recessive OI, there have been mutations identified in the three components of the ER collagen 3-hydroxylation complex, comprising prolyl 3-hydroxylase 1 (LEPRE1, MIM 610339), cartilage-associated protein (CRTAP, MIM 605497), and peptidylprolyl isomerase B (PIIB, MIM 123841).<sup>4–7</sup> These three proteins are responsible for the 3-hydroxylation of a specific residue, Pro986, in the collagen a1 (I) chains.<sup>8</sup> Although the function of this modification is not clear, it appears to be important for

the correct folding or stability of the collagen triple helix.<sup>9</sup> Recently, also in a family with severe OI and recessive inheritance, a homozygous missense mutation (c.233T>C, p.Leu78Pro) was found in *SERPINH1* (MIM 600943; Serpin peptidase inhibitor, clade H), the gene encoding the ER chaperone-like protein HSP47 (heat shock protein 47).<sup>10</sup> *SERPINH1* was first linked to OI by homozygosity mapping in a canine pedigree of Dachshunds.<sup>11</sup> Biochemical analysis of patient-derived primary fibroblasts demonstrated that the p.Leu78Pro HSP47 mutant variants were subjected to proteasome-mediated degradation, whereas there was accumulation of type I procollagen in the Golgi and abnormal folding of a proportion of the secreted type I collagen. Because Pro986 3-hydroxylation was found to be normal in the HSP47 mutant cells, it was concluded that this protein is required downstream of the CRTAP/LEPRE1/PIIB protein complex.<sup>10</sup> Finally, two mutations have been reported in *FKBP10* (MIM 607063) in patients with moderately severe OI and recessive disease transmission.<sup>12</sup> *FKBP10* codes for FKBP65, which is a protein with known type I procollagen chaperone function. Investigation of the FKBP65 mutations in dermal fibroblasts from affected individuals revealed normal 3-hydroxylation of the proline 986 but delayed secretion of type I procollagen.<sup>12</sup> Hence, so far all the genes associated with OI are involved in the synthesis, posttranslational modification, trafficking, processing, or secretion of type I collagen. Herein we report the genetic analysis of a family that has led us to the identification of an additional gene mutated in this disease.

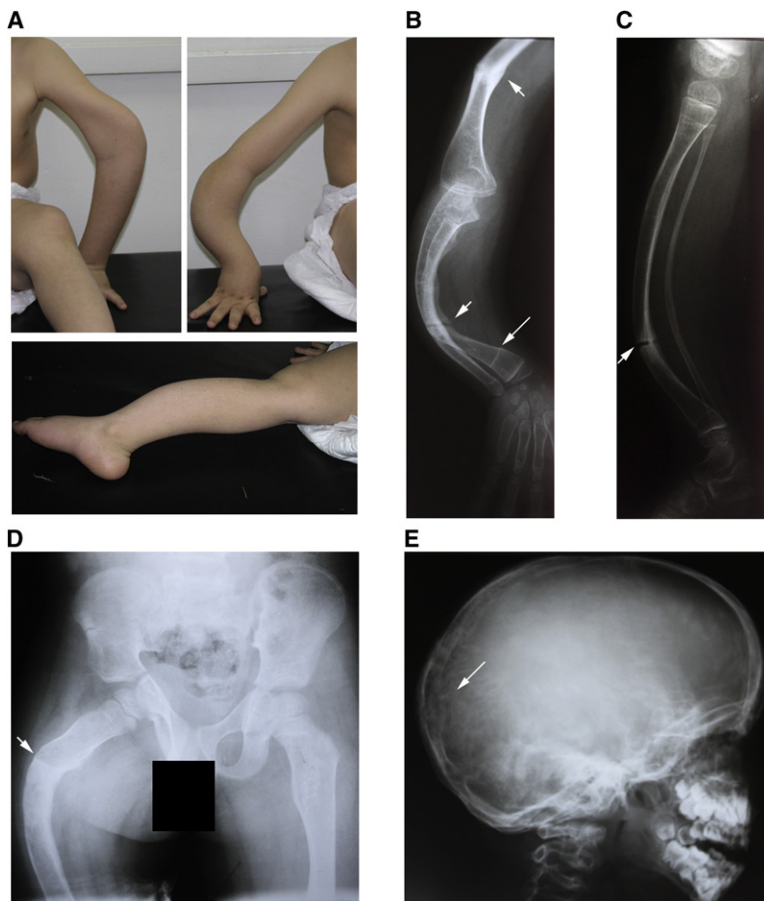
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**Figure 1. Clinical Findings in the Patient with an OSX Mutation**

(A) Photographs of the reported patient showing bowing of the long bones of the arms (top) and sabre tibia (bottom).

(B–E) Radiographical findings in the proband at the age of 8 years.

(B) X-ray of the right upper limb (lateral view) demonstrating anterior bowing, deformed irregular humerus, radius, and ulna, and generalized decreased bone density. An old, improperly healed fracture at the lower third of the humerus and transverse fractures of the radius and ulna are indicated by small arrows. The long arrow designates a transverse line of arrested bone growth at the lower end of the radius.

(C) X-ray of the right leg (lateral view) showing decreased bone density with anterior bowing of the tibia and fibula and transverse fracture at the lower third of the tibia (arrow).

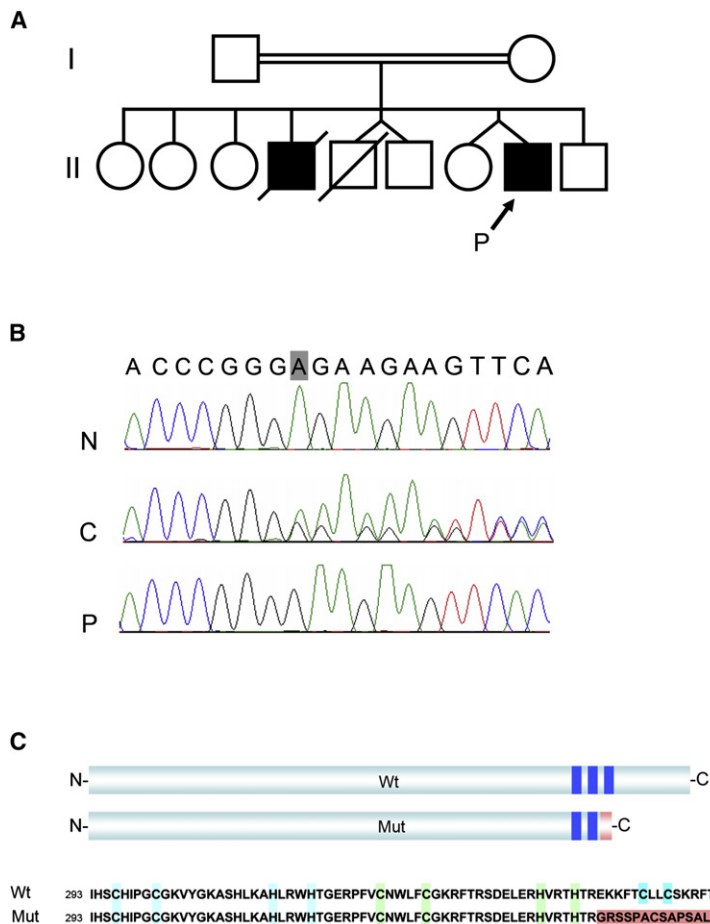
(D) X-ray of the pelvis and upper ends of femora (anterior-posterior view) demonstrating a deformed asymmetric pelvis with thin laterally displaced neck of the femur, bilaterally, mostly on the right side. There is an old, improperly healed fracture at the upper end of right femur with callus formation (arrow).

(E) X-ray of the skull (lateral view) showing wormian bones at the occipital region (arrow).

We have studied the case of an Egyptian 8-year-old boy with OI born to consanguineous parents related as second cousins (Figure 1; Figure 2A). The family had a history of a similarly affected sibling who, in addition to OI with repeated bone fractures, was diagnosed with a congenital heart condition and died at the age of 4 years as a result of pneumonia. We did not see the deceased child, and thus no DNA from this individual was available for molecular studies. Fractures in the proband started at 3 months of age, and since then they occurred frequently, mostly following minor trauma, with an average of 6–7 fractures per year. The number of fractures decreased after the patient began biphosphonate treatment (see Table S1 available online). The child has had delayed motor milestones: he sat at the age of 1 year, was not able to stand unsupported until the age of 6 years, and he is not able to walk independently at the age of 8 years. His hearing and sclerae were normal. Craniofacial features included a skull with wormian bones, mild asymmetry of the face, high prominent forehead with prominent supraorbital ridges more obvious on the right side, midface hypoplasia, depressed nasal bridge, microstomia, micrognathia, and high arched palate. Delayed teeth eruption was noted, but he did not have dentinogenesis imperfecta. Radiographic examination of the skeletal system revealed bowing of the upper and lower limbs; his right leg was shorter than his left leg because of bone deformities, and he also presented hyper-

extensibility of the interphalangeal joints, mild scoliosis, and mild pectus carinatum (Figure 1). Anthropometric measurements for length were below normal ( $-3.6$  standard deviation [SD] at the age of 8 years), and head circumference and weight were within normal range for his age ( $+0.9$  SD and  $-2.1$  SD, respectively, at the age of 8 years). Bone densitometry performed by dual energy X-ray absorptiometry (DEXA) revealed generalized osteoporosis (Z score of  $-3.4$  at the lumbar spine and  $-1.9$  at the femur at the age of 4 years, before the start of biphosphonate treatment). Anthropometric and DEXA data related to the follow-up of this patient are listed in Table S1. Based on the phenotype, a diagnosis of OI type IV was considered following the Sillence classification guidelines.<sup>1</sup> All studies and investigations involving the proband and other members of his family were performed in accordance with the ethical standards of the Medical Research Ethics Committee of the National Research Centre (Egypt), and all DNA samples were obtained with appropriate informed consent.

Recurrence in a sibling and parental consanguinity suggested a recessive pattern of inheritance, and on this basis we embarked on the genetic analysis of this family. Microsatellite analysis in the proband, his father, and two of the unaffected siblings excluded linkage to *COL1A1*, *COL1A2*, *LEPRE1*, and *CRTAP* under a recessive disease model, because the patient was heterozygous for markers



**Figure 2. Identification of a Frameshift Mutation in *Osterix***

(A) Pedigree of the OI family analyzed in this study; the proband (P) is designated with an arrow.

(B) Alignment of genomic sequence chromatograms corresponding to an area encompassing the c.1052delA mutation from a normal individual (N), a heterozygous carrier (C), and the proband (P). The adenine deleted in the patient is squared in gray. Numbering of the mutation correlates to the reference sequence NM\_152860.1, taking the A of the translation start site as nucleotide + 1.

(C) Protein effect of the c.1052delA mutation. The picture on the top shows the position of the three zinc fingers represented by blue rectangles in the normal (Wt) and mutant (Mut) SP7 proteins. The mutant protein is missing the third zinc finger, and the new residues incorporated after the frameshift are indicated in red. The lower panel shows the sequence of amino acids corresponding to the DNA binding region of normal and mutant OSX. Conserved cysteine and histidine residues involved in zinc ion coordination corresponding to each individual zinc finger are highlighted in different colors. The amino acids inserted after the mutation are in red. Protein numbering is related to the reference sequence NP\_690599.1.

surrounding these loci. We also sequenced the five coding exons of *PPIB*, the five exons of *SERPINH1*, and all of the coding exons of *COL1A1* and *COL1A2*, including the intronic boundaries, but no pathogenic sequence variations were found in the patient DNA. To search for the causative gene, we performed homozygosity mapping in the proband and one of his unaffected sisters by using genome-wide high-density SNP arrays (Illumina Human610-Quad BeadChip). The two SNP genotypes generated by this process were then analyzed with Illumina software to look for blocks of homozygosity. Taking an arbitrary cutoff of 300 consecutive homozygous SNPs, we found five large regions of homozygosity in the patient that were not shared by his normal sibling. These regions were located on chromosome 2 (two DNA segments of 3.5 and 3.1 Mb), chromosome 4 (8.1 Mb), chromosome 11 (14.5 Mb), and chromosome 12 (19.3 Mb) and contained 29, 22, 92, 410, and 332 genes, respectively (Genome Reference Consortium Human Build 37.1). Evaluation of the transcripts comprised in each region revealed the presence of a candidate gene, *SP7/OSX* (MIM 606633), in the homozygosity block of chromosome 12 (12q13.13). We considered *OSX* to be a good candidate for the disease because it encodes an osteoblast-specific transcription factor that, in mice, has been shown to be indispensable for bone formation. *Osx* null mice are deficient in osteoblast differentiation and have reduced expression of

osteoblast markers, including *Col1a1*, *Bone sialoprotein*, and *Osteocalcin*.<sup>13</sup> In addition, these mice display bone-bending deformities similar to those seen in patients with OI.<sup>13</sup> We screened the proband for the presence of *OSX* mutations by genomic amplification followed by direct sequencing of the two *SP7* coding exons and at least 100 bp of the adjacent intronic sequences, and we identified a homozygous single nucleotide deletion, c.1052delA, in the last exon of this gene (Figure 2B). The frameshift caused by this mutation introduces 18 novel residues at codon 351 before running into a premature termination codon (p.E351GfsX19). Segregation analysis of the mutation within the family showed that the two parents were heterozygous for the nucleotide deletion and that out of the five normal siblings from whom we could obtain DNA, three carried the mutation in the heterozygous state and two had normal sequence. We checked normal controls for the presence of the c.1052delA change by direct sequencing, but it was not found in 122 control chromosomes of the same ethnic origin or in 284 normal chromosomes from controls of mixed ethnicity.

Because the c.1052delA mutation introduces a premature stop codon within the final exon of the gene, the mutant transcript is predicted to escape the nonsense-mediated mRNA decay machinery.<sup>14</sup> *OSX* is a 431 amino acid protein that belongs to the Specificity protein (Sp)

subgroup of the Krüppel-like family of transcription factors characterized by the invariable presence of three tandem Cys2-His2 zinc-finger DNA-binding domains at their carboxy terminus.<sup>15–17</sup> Based on crystallography and nuclear magnetic resonance studies performed in some members of the family, it has been established that each protein finger recognizes a consecutive trinucleotide sequence, with the three zinc fingers contributing to the strength and specificity of the DNA-protein interaction.<sup>18–20</sup> Sp1, which is the best-characterized member of the Sp group and is closely related to SP7, binds GC-rich DNA boxes of 9 base pairs. Previous experimental studies, including DNA-binding assays, that used Sp1 peptide fragments in which either the N-terminal (1) or the C-terminal (3) zinc fingers were deleted demonstrated a greater contribution of finger 3 to the DNA-binding affinity than the other two remaining fingers.<sup>21,22</sup> The p.E351GfsX19 change leads to a truncated SP7 protein that lacks the last 81 amino acids, including the third Zn-finger domain, and carries instead 18 new residues downstream of codon 351 (Figure 2C). Thus, in the case in which the OSX mutant variant is not degraded by the proteasome, its DNA-binding properties are expected to be altered, and, consequently, the SP7/OSX-mediated transcription regulation will be impaired. Mutations in the third Zn finger of EGR2 (MIM 129010), a very similar Cys2-His2 transcription factor, have been described in patients with autosomal-recessive congenital hypomyelinating neuropathy (MIM 605253), proving that mutations in the third zinc finger can lead to disease.<sup>23</sup> A limitation of this work is that in vivo studies to assess the effect of the OSX mutation on transcript and protein stability and on type 1 collagen production in patient cells could not be performed because of the unavailability of additional biological material from the patient.

*Sp7/Osx* is specifically expressed in cortical and trabecular osteoblasts and at a lower level in the prehypertrophic chondrocytes of the growth plate.<sup>13</sup> This restricted expression pattern is consistent with the patient having normal white sclera. Analysis of *Osx* mouse models has revealed that *Osx* plays an essential role in regulating the differentiation of preosteoblasts to osteoblasts in a step downstream of *Runx2* (MIM 600211),<sup>13</sup> another transcription factor that is a master regulator for osteoblast differentiation and that, in humans, is responsible for cleidocranial dysplasia (MIM 119600).<sup>24</sup> Although *Runx2* null mice do not express *Osx*, *Runx2* expression was shown to be unaffected in the developing bones of *Osx*<sup>-/-</sup> mutants.<sup>13</sup> In agreement with the craniofacial features identified in our patient, both endochondral and intramembranous bone formations were found to be disrupted in the *Osx* null mice. Because constitutive ablation of *Osx* is perinatal lethal, the function of *Osx* in adult bone has been studied by using conditional models. Postnatal inactivation of *Osx* with tamoxifen inducible *Col1a1-CreERT2* mice resulted in impaired bone formation as a result of reduced expression of osteoblast-specific markers.<sup>25</sup> OSX function has not

been explored in detail in humans, but the DNA region around OSX has been identified as one of the osteoporosis susceptibility loci. Several genome-wide association studies have shown significant association of common variants in the region of OSX with bone mineral density (BMD) and osteoporosis, although this association could not be assigned unequivocally to this gene.<sup>26–28</sup> To assess whether the c.1052delA OSX mutation could have an effect on adult BMD in the heterozygous state, we performed DEXA scans in the parents of our patient. The 39-year-old mother had normal bone density at the femur (T score -0.4) and osteopenia at the spine (T score -1.3). The 61-year-old father had borderline osteoporosis of the spine and osteoporosis of the femur (T scores -2.2 and -3.0, respectively). However, the father has been on corticosteroid therapy for the treatment of lung fibrosis since the age of 43 years and had already been identified as having progressive osteoporosis and bilateral glaucoma 2 years after the beginning of this treatment. He also has a history of partial thyroidectomy at the age of 39 years, and he is on L-thyroxine, but thyroid functions are not regulated. Because it is known that prolonged corticosteroid therapy can induce osteoporosis, we cannot conclude from these data that the low BMD of the father is a direct consequence of being a heterozygous carrier for the OSX mutation.

In summary, the data we present here add one more gene to the growing number of causative loci for autosomal-recessive OI and indicate that mutations in transcription factors that regulate the expression of osteoblast-specific genes can also be accountable for OI. Further testing is required to ascertain what proportion of autosomal-recessive OI cases arise from mutations in OSX.

### Supplemental Data

Supplemental Data include one table and can be found with this article online at <http://www.ajhg.org>.

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### Web Resources

The URLs for data presented herein are as follows:

Ensembl Human Genome Browser, <http://www.ensembl.org/index.html>

Online Mendelian Inheritance in Man, <http://www.ncbi.nlm.nih.gov/Omim/>

Primer3 design tool, <http://frodo.wi.mit.edu/primer3/>

UCSC Genome Browser, <http://genome.ucsc.edu/>

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