ORIGINAL ARTICLE

Homozygous sequence variants in the *FKBP10* gene underlie osteogenesis imperfecta in consanguineous families

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Osteogenesis imperfecta (OI, MIM 610968) is a genetically and clinically heterogeneous disorder characterized by bone fragility. It is one of the rare forms of skeletal deformity caused by sequence variants in at least 14 different genes, including *FKBP10* (MIM 607063) encoding protein FKBP65. Here we present three consanguineous families of Pakistani origin segregating OI in an autosomal-recessive pattern. Genotyping using either single-nucleotide polymorphism markers by Affymetrix GeneChip Human Mapping 250K *Nsp* array or polymorphic microsatellite markers revealed a homozygous region, containing a candidate gene *FKBP10*, among affected members on chromosome 17q21.2. Sequencing the *FKBP10* gene revealed a homozygous novel nonsense variant (c.1490G>A, p.Trp497*) in the family A and two previously reported variants, including a missense (c.344G>A, p.Arg115Gln), in the family B and duplication of a nucleotide C (c.831dupC, p.Gly278ArgfsX295) in the family C. Our findings further extend the body of evidence that supports the importance of *FKBP10* gene in the development of skeletal system.

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INTRODUCTION

Osteogenesis imperfecta (OI) is clinically characterized by fragile bones, increased susceptibility to fractures and low bone mass. Overall incidence of different types of OI is approximately 1 in 15 000–20 000 births. More than 90% of autosomal-dominant forms of OI are caused by sequence variants in type I procollagen chains pro α 1 (*COL1A1*, MIM 120150) and pro α 2 (*COL1A2*, MIM 120160) encoding genes. Such sequence variants result in delayed posttranslational modifications, triple helical folding and disturb primary structure of the collagen type I.^{1,2} Clinically, severity of OI varies widely and patients show variable features, including short stature (from normal to severely compromised height), sclerae that vary from blue to white coloration, bone fragility and fractures (from mild osteopenia to severe osteoporosis) and dentinogenesis imperfect (dental abnormality).³

Autosomal-recessive forms of OI are caused by sequence variants in genes encoding proteins that are involved in folding and posttranslational modifications of type I collagen. Ten genes, involved in causing autosomal-recessive forms of osteogenesis imperfect, include *SERPINF1* (MIM 172860), *CRTAP* (MIM 605497), *LEPRE1* (MIM 610339), *PPIB* (MIM 123841), *SERPINH1* (MIM 600943), *FKBP10* (MIM 607063), *SP7* (MIM 606633), *BMP1* (MIM 112264), *TMEM38B* (MIM 611236) and *WNT1* (MIM 164820) (Table 1).^{3,4}

Autosomal-recessive forms of OI type XI, commonly known as Bruck syndrome type 1 (BRKS1; MIM 259450), result from sequence variants in the *FKBP10* gene. A similar condition Bruck syndrome type 2 (BRKS2; MIM# 609220) is associated with mutations in the *PLOD2* gene (MIM 601865). Both conditions have many common features, such as bone fragility, congenital contractures, fractures starting in infancy, limb deformities, short stature, scoliosis and pterygia.^{5,6}

The FKBP65/FK506-binding protein, encoded by *FKBP10* gene, is a member of protein family carrying peptidyl-prolyl-isomerase (PPIase) activity and are also called as immunophilins. These immunophilins catalyze *cis-trans* isomerization of peptidyl-prolyl bonds that are important for proper folding of proteins.^{7–9} The FKBP65, which localizes in the endoplasmic reticulum, is comprised of four PPIase domains, two Ca²⁺-binding EF hand motifs and an endoplasmic reticulum retention sequence at its carboxyl end (Figure 4c).¹⁰

Deficiency of FKBP65 in autosomal-recessive type of OI was first reported in 2010 in cases of progressive deforming from Turkey and Mexico.⁹ Later, it was reported that phenotype of Bruck syndrome type I (MIM 259450) with congenital joint contractures as well as osteoporosis, short stature and fragile bones overlapped with those caused by sequence variants in the *FKBP10* gene (Figure 4a).^{11–14}

In the study presented here, we have investigated three consanguineous families of Pakistani origin (A, B, C) segregating OI in

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Table 1 Genes involved in causing osteogenesis imperfecta segregating in an autosomal-recessive manner

Sl. No.	Gene	Types	Protein	Inheritance/phenotype	Bone collagen abnormalities
1	<i>SERPINF1</i>	VI	PEDF	Autosomal recessive (AR), bone fragility and low mineral density, osteoid seams	Failed mineralization, no other collagen abnormalities, symptomatically similar to the type III, histological differences, impaired mineralization
2	<i>CRTAP</i>	VII	CRTAP	Multiple fractures at birth, short stature, decreased bone modeling, extreme low bone mineral density and poor prognosis	Symptomatically similar to type II/III
3	<i>LEPRE1</i>	VIII	P3H1	AR, bone fragility	$\alpha 1$ (I) P986 and $\alpha 2$ (I) P707 under prolyl-3-hydroxylation
4	<i>PPIB</i>	IX	CYPB	Reduced mineral density	High HP/LP (CRTAP and LEPRE1), low HP/LP (PPIB)
5	<i>SERPINH1</i>	X	HSP47	AR, bone fragility (type III OI)	High HP/LP, and abnormal arrangement of crosslinking bonds, symptomatically similar to the type III/II
6	<i>FKBP10</i>	XI	FKBP65	AR, Bruck Syndrome: bone	Lack of telopeptide hydroxylysines produces skin-like symptomatically similar to the type III/IV
7	<i>SP7</i>	XII	OSX (Osterix)	AR, recurrent fractures, mild bone deformities, delayed tooth eruption, normal hearing and white sclera moderate	Symptomatically similar to type IV
8	<i>BMP1</i>	XIII	BMP1	AR, severe	Symptomatically similar to type III
9	<i>TMEM38B</i>	XIV	TM38B	AR, variable severity	Multiple fractures and osteopenia, with normal teeth, sclerae and hearing
10	<i>WNT1</i>	XV	WNT1	AR, variable severity, early-onset osteoporosis+	Early-onset recurrent fractures, bone deformity, short stature

Abbreviation: OI, osteogenesis imperfecta.

an autosomal-recessive pattern. Screening of the *FKBP10* gene led to the identification of a novel (p.Trp497*) and two previously reported homozygous sequence variants (p.Arg115Gln, p.Gly278ArgfsX295) in the three families.

MATERIALS AND METHODS

Human subjects and ethics statement

The present study describes clinical and molecular analysis of three consanguineous families (A–C) segregating autosomal-recessive form of OI. These were recruited from remote regions in Khyber Pakhtunkhwa province of Pakistan. The study was approved by the Institutional Review Board of Quaid-i-Azam University Islamabad, Pakistan. Informed written consent of the study, including presentation of photographs for publication, was provided by patients and elders of the families.

Extraction of genomic DNA

Venous blood samples from 22 individuals, including 3 affected (IV-1, IV-2, IV-7) and 7 unaffected (III-3, III-5, III-7, IV-3, IV-4, IV-8, IV-9) in family A, 2 affected (IV-3, IV-7) and 4 unaffected (III-1, III-3, IV-1, IV-6) in family B and 3 affected (V-1, V-2, V-5) and 3 unaffected (IV-3, IV-4, V-6) in family C, were collected in EDTA tubes (BD, Franklin Lakes, NJ, USA). DNA was extracted using GenElute Blood Genomic DNA Kit (Sigma-Aldrich, St Louis, MO, USA). Quantification of genomic DNA was performed using Nanodrop-1000 spectrophotometer (Thermal Scientific, Wilmington, MA, USA).

Genotyping

In order to identify the disease gene, human genome scan using single-nucleotide polymorphism (SNP) markers was performed in two families (A, B). In the third family C, search for the gene was carried out by genotyping microsatellite markers linked in the vicinity of 10 genes (*SERPINF1*, *CRTAP*, *LEPRE1*, *PPIB*, *SERPINH*, *FKBP10*, *SP7*, *BMP1*, *TMEM38B*, *WNT1*) involved in causing autosomal-recessive form of OI. Microsatellite markers were selected from Rutgers combined linkage physical map of the human genome (Build 36.2).¹⁵ Amplification of microsatellite markers was performed according to standard procedure in a total volume of 25 μ l. PCR was carried out in a 0.2-ml PCR tube with 40 ng human genomic DNA, 2.5 ml of 10 $\times$ ammonium sulfate buffer (MBI Fermentas, Pittsburgh, PA, USA), 15–20 pmol of each reverse and forward primer, 1.5 mM MgCl₂, 200 μ M of each deoxynucleoside triphosphate (dNTP), and 1 unit of Taq DNA polymerase (MBI Fermentas, Life Sciences, UK). The standard thermal conditions used were: 95°C for 7 min, 95°C for 1 min, 55–58°C for 1 min 40 cycles, 72°C for 1 min, and 72°C for 10 min.

PCR was performed in T3000 thermocycler (Biomera, Göttingen, Germany), and PCR products were resolved on 8% non-denaturing polyacrylamide gels. Gels were stained with ethidium bromide, and genotypes were assigned by visual inspection using gel documentation system (Kodak Ink, San Gabriel, CA, USA).

In family A, DNA of three affected (IV-1, IV-2, IV-7) and seven unaffected (III-3, III-5, III-7, IV-3, IV-4, IV-8, IV-9) and in family B two affected (IV-3, IV-7) and four unaffected individuals (III-1, III-3, IV-1, IV-6) were subjected to SNP analysis. The genotyping was performed using Affymetrix GeneChip Human Mapping 250K *Nsp* array (Affymetrix, Santa Clara, CA, USA) containing probes for 250 000 SNPs. The genotype of each SNP was determined by the BRLMM algorithm incorporated in Affymetrix Genotyping Console. A call rate (percentage of SNPs genotyped by sample) of >98% was obtained across the entire sample. Mapping order and physical and genetic distances of SNPs were obtained from Affymetrix. Analysis of SNP data was conducted using online tool Homozygosity Mapper (<http://www.homozygositymapper.org/>).

Sequencing *FKBP10*

Genomic sequence of the *FKBP10* gene, including exons, introns, 5' untranslated region and 3' untranslated region, were retrieved from the University of California Santa Cruz genome database browser (<http://genome.ucsc.edu/cgi-bin/hgGateway>). Primer sequences for PCR amplification of the exons and flanking regions of the gene were designed using the primer-3 software (<http://frodo.wi.mit.edu/primer3/>). The entire coding sequence of the *FKBP10* gene was sequenced in all affected and unaffected subjects of each family. Purification of the PCR-amplified DNA was performed with commercially available kits (Marligen Biosciences, Ijamsville, MD, USA). The products were then sequenced using the DTCS Quick Start Sequencing Kit (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's instructions. Sequence variants were identified via BIOEDIT sequence alignment editor version 6.0.7 (Ibis Biosciences Inc., Carlsbad, CA, USA).

In silico analysis

The pathogenicity index for the detected sequence variants were calculated using Mutation Taster (<http://www.mutationtaster.org/>), Polymorphism Phenotyping V2 (PolyPhen-2) (<http://genetics.bwh.harvard.edu/pph2/>) and Sorting Intolerant From Tolerant (SIFT) (<http://sift.bii.a-star.edu.sg/>). The frequency of the variant in the general population was determined using Exome Variant Server (<http://evs.gs.washington.edu/EVS/>) and 1000 genomes.

Figure 1 Pedigree of family A segregating osteogenesis imperfecta in an autosomal-recessive manner. Circles and squares represent females and males, respectively. Clear symbols represent unaffected individuals while filled symbols represent affected individuals. Double lines are indicative of consanguineous unions. The individual numbers labeled with asterisks indicate the samples which were available for this study (a). In affected individuals (IV-1, IV-2), upper and lower limbs show typical features of fixed flexion deformity (FFD) (b). Affected individual (IV-1) showed clubbed foot deformity in supinated form (c). Extension of proximal and distal interphalangeal joints and Cubitus varus form of elbow were observed in both the affected individuals (d, e). X-ray of chest and vertebrae of affected member IV-1. Red arrows indicating kidney stones (f, g). Radiographic features of upper limbs indicating fractures of elbow joints and ulna (h). Irregular bending of lower limbs due to multiple fractures and self-healing in affected member IV-1 (i). DNA sequence analysis of the *FKBP10* gene shows a substitution of G with A at nucleotide position 1490 (c.1490G>A; p.Trp497*), upper panel showing nucleotide sequence in homozygous normal (wild type), middle panel in heterozygous carrier and lower panel in homozygous affected member. Arrows represent position of the mutated nucleotide (j). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

RNA extraction and cDNA synthesis

For isolation of RNA, 3 ml of the peripheral blood was collected in Tempus Blood RNA Tubes (AB, Foster City, CA, USA). Total RNA from whole blood was isolated using Tempus Spin RNA Isolation Kit (AB) according to the manufacturer's protocol. RNA was further purified by treatment with 10× DNase (Fermentas Inc., Hanover, MA, USA) to remove traces of genomic DNA and stored at −20°C. cDNA was prepared from total RNA by reverse

transcriptase-PCR reaction using TaqMan PreAmp Master Mix Kit (AB) according to the manufacturer's protocol. For PCR-amplification of *FKBP10* cDNA, two sets of primers (Set 1: forward 5'-GGGATTTTGTGCGCTACC-3'; reverse 5'-CATCCGGTGGAATGAGC-3' and Set 2: forward 5'-CCACGGGGA GAGTGGAG-3'; reverse 5'-CATGAGGCGTCCTTTGCC-3') were used. As a control, glyceraldehyde-3-phosphatase dehydrogenase was PCR-amplified from cDNA using the two sets of primers.

Figure 2 Pedigree of family B segregating OI in an autosomal-recessive pattern. The individual numbers labeled with asterisks indicate the samples that were available for this study (**a**). Affected members (IV-3, IV-7) in family B had frequent fractures and healing of upper limbs, which resulted in extension of metacarpophalangeal, proximal interpalangeal and distal interpalangeal joints (**b–d**). Lower limbs were weak and unable to ambulate (**e**). Radiographs of an affected member (IV-3) presenting severe progressive kyphoscoliosis. Spine deformity consists of both dorsal (front-to-back) curves (kyphosis) and lateral (side-to-side) curves (scoliosis) (**f, g**). Lower limbs with protrusio acetabula (severe pelvic girdle deformity) and weak tibia and fibula (**h**). Upper limbs showed frequent fractures and healing of radius, ulna in affected member (IV-3) in family B (**i**). Sequence analysis of the *FKBP10* gene shows a substitution of nucleotide G with A at position 344 (c.344G>A; p.R115Q), upper panel showing nucleotide sequence in homozygous normal, middle panel in heterozygous carrier and lower panel in homozygous affected member. Arrows represent position of the mutated nucleotide (**j**). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

RESULTS

Clinical features

Affected individuals in all three families (A–C) shared typical features of OI. Parents of the affected individuals showed no abnormality and were healthy like the other normal members of the family.

Family A

In family A, affected members exhibited several different types of skeletal abnormalities. Two of the affected members IV-1 and IV-2 at

the age of 17 and 10 years, respectively, showed restricted limb movements and were unable to ambulate freely. Clinical analysis revealed reduction in total lung capacity as a result of severe and progressive kyphoscoliosis. Dentinogenesis imperfecta was observed in an affected member (IV-2). Both the affected members (IV-1, IV-2) had fractures of radius, ulna, tibia, fibula and femur. Humerus and mandibles were not affected. They were short statured (58.4 cm) with head circumference of 44.5–50.8 cm. Radiographs of an affected member IV-1 showed grossly scoliotic spine and flattened vertebral

Figure 3 Pedigree of family C segregating osteogenesis imperfecta in an autosomal-recessive pattern. The individual numbers labeled with asterisks indicate the samples that were available for this study. The shaded blue alleles are forming risk haplotype, while the alleles shown in white are not co-segregating with the disease (a). An affected member (V-5) in the family C reported frequent fractures and healing of radius and ulna, growth retardation and weakening of lower limbs that resulted in restriction of the movement to wheel chair (b, c). Sequence analysis of the *FKBP10* gene shows duplication of a nucleotide C at position 831 (c.831dupC; p.Gly278ArgfsX295). Upper panel showing nucleotide sequence in homozygous normal, middle panel in heterozygous carrier and lower panel in homozygous affected member. Arrows represent position of the mutated nucleotide (d). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

body (platyspondyly) (Figures 1b–i). Hearing impairment, intellectual disability and pterygia were not observed in the affected individuals.

Family B

From very early age, affected members were reported facing recurrent long bone fractures, which lead to severe deformity and restricted their movement. Two affected members IV-3 and IV-7 at the age of 15 and 11 years, respectively, had bilateral flexion contracture of knee and

elbow and bilateral extensions contractures of ankles. As a result of underdeveloped maxilla and mandible bones, triangular facial appearance was observed in both the affected members. Radiographic analysis of an affected member (IV-3) demonstrated severe osteopenia, wedging of the vertebrae and kyphoscoliosis, frequent fractures and healing of radius, ulna of both hands and right femur. Head circumference of the affected members IV-3 and IV-7 was 58.45 and 30.48 cm, respectively. Height of IV-3 and IV-7 was 68.57 and

Figure 4 Several proteins are involved in posttranslational modifications of type I collagen and mutation in the *FKBP65* protein causes both osteogenesis imperfecta (OI) and Bruck syndrome (**a**). Red bars indicate mutations identified in exons 2, 5 and 9 of the *FKBP10* gene in the present study. Blue bars indicate previously reported mutations in the gene (**b**). Schematic representation of the *FKBP65* protein exhibiting a signal peptide, four PPlase domains, EF/Hand domain, HEEL and a putative ER-retention sequence. Arrows indicate mutations identified in various domains of the *FKBP65* protein in the current study. The mutations p.Trp497*, p.Arg115Gln and p.Gly278ArgfsX295 reside in the EF/Hand domain, first PPlase domain and third PPlase domain of *FKBP65* protein, respectively. Numbers below represent position of the amino acids in various domains of the protein (**c**). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

55.88 cm, respectively, at the time of the study (Figures 2b–i). The 50th centile for height and head circumference for the affected individuals in families A and B are 58.4 and 47.65 cm, respectively. Mental disability, hearing impairment and pterygia (webbing) were not observed in any affected member.

Family C

In this family, affected members V-1, V-2 and V-6 were 14, 17 and 18 years of age, respectively, and had severe progressive OI. They had multiple bone fractures, which restricted their movement to wheelchair. In addition, they showed kyphoscoliosis and suppressed development of trunk region, which resulted in weak pelvic girdle and short stature. Hearing was normal. Intellectual disability was not observed in the affected members (Figures 3b and c).

Genotyping

In two families A and B, human genome was scanned using 250K *Nsp* array containing probes for 250 000 SNPs. Analysis of SNP array data revealed homozygous region of ~9.2 and ~8.7 Mb among the affected members in families A and B, respectively, on chromosome 17q21.1–q21.31. The region had a candidate gene *FKBP10*, reported

earlier to be involved in causing OI. In the third family C, genotyping using microsatellite markers (D17S946, D17S2189, D17S1299, D17S760, D17S965, D17S930, D17S934, D17S920, D17S1150, D17S2180) and haplotype analysis identified homozygous region containing *FKBP10* gene on chromosome 17q21.1–q21.31 (Figure 3a).

Sequencing *FKBP10*

Screening *FKBP10* gene (NM_021939) identified a novel nonsense variant (c.1490G>A, p.Trp497*) in exon 9 in family A, a missense variant (c.344G>A, p.Arg115Gln) in exon 2 in family B and duplication of a nucleotide C (c.831dupC) in exon 5 in family C. The duplication (c.831dupC) revealed in the later family generated a frameshift (p.Gly278ArgfsX295) predicting a stop codon 17 amino acids downstream in the third PPlase domain of the *FKBP65* protein in all the affected individuals. The three sequence variants were present in the heterozygous state in the obligate carriers of the families (Figures 1j, 2j and 3d). To exclude the nonpathogenic nature of the novel variant (p.Trp497*), identified in family A, exons 9 of the *FKBP10* gene was sequenced in 200 ethnically matched control individuals. The novel variant was not identified outside the family. Reverse transcriptase–PCR analysis failed to amplify *FKBP10* mRNA from blood samples of both affected and control individuals.

DISCUSSION

The study, reported here, characterized OI segregating in an autosomal-recessive manner in three consanguineous families of Pakistani origin. Distinctive clinical features of OI, including severe osteopenia, frequent fractures and healing of long bones accompanied with ambulatory disabilities and progressive kyphoscoliosis with platyspondyly, reported previously^{9,11–13,16} were observed in the affected members in the three families. However, condition was more severe in patients of the present families. Affected individuals in two families (A and B) had bilateral flexion contracture of knee and elbow and bilateral extensions contractures of ankles. Similar features were reported in families with OI by three other groups.^{11–13,16} An additional feature of pterygia (webbing) reported by these groups was not observed in patients of our families. In family A, an affected individual (IV-2) had dentinogenesis imperfecta, a feature reported previously in three brothers affected with OI by Steinlein *et al.*¹⁷

To date, only 27 sequence variants, including 11 missense/nonsense, 1 splice site, 6 small deletions and 9 small duplications in the *FKBP10* gene, causing OI have been reported. The *FKBP10* gene, located on chromosome 17q21.2, is composed of 10 exons encoding 582 amino acids extracellular matrix-binding protein FKBP65.⁸ In the present study, sequence analysis of *FKBP10* gene revealed a novel nonsense variant (p.Trp497*) in family A and two previously reported a missense (p.Arg115Gln) in family B and a duplication (c.831dupC) in family C. The missense variant (p.Arg115Gln) and duplication (c.831dupC) were reported by four different groups.^{9,11,13,16}

The protein FKBP65 is composed of four PPIase domains, EF-hand 1 and EF-hand 2 domains. The nonsense variant (p.Trp497*), identified in family A, is located in EF-hand 1 domain and most likely resulted in loss of function of the FKBP65 protein either through nonsense-mediated mRNA decay (NMD) or in the production of a truncated FKBP65 protein. In case of later event, the variant p.Trp497* potentially reduced size of the EF-hand 1 domain and resulted in loss of function of EF-hand 2, CA-BIND 1 and CA-BIND 2 domains. Subsequently, this effected secretion of the protein from endoplasmic reticulum and thus produced OI phenotypes (Figure 4c).

FKBP65 prevents premature crosslinks between procollagen chains and assists in proper registration, folding into the collagen triple helix and subsequent trafficking.^{18,19} Sequence variants in the *FKBP10* gene resulting in loss of function of FKBP65 leads to effect the secretion of type I procollagen and marked diminution of lysyl hydroxylation.^{20,21} The pathogenesis of OI related to FKBP65 deficiency encoded by *FKBP10* is still unclear. Most of the OI cases are dominantly inherited owing to main defects in the type I procollagen genes that cause abnormal structure and/or lead to reduced synthesis of type I collagen while recessively inherited severe-to-lethal OI are caused by loss of the prolyl-3-hydroxylation complex owing to defect in posttranslational modification of type I procollagen.^{22,23}

In conclusion, we have reported one novel and two previously reported disease-causing sequence variants in the *FKBP10* gene, resulting in an autosomal-recessive form of OI phenotypes. This report is the first case of OI resulting from variants in the *FKBP10* gene in Pakistani population. This study will provide support genetic testing and promote the understanding of genotype–phenotype relationships within OI patients in Pakistan.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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