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Phenotypic and Molecular Characterization of Bruck Syndrome (Osteogenesis Imperfecta With Contractures of the Large Joints) Caused by a Recessive Mutation in *PLOD2*

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Bruck syndrome (BS) is a recessively-inherited phenotypic disorder featuring the unusual combination of skeletal changes resembling osteogenesis imperfecta (OI) with congenital contractures of the large joints. Clinical heterogeneity is apparent in cases reported thus far. While the genes coding for collagen 1 chains are unaffected in BS, there is biochemical evidence for a defect in the hydroxylation of lysine residues in collagen 1 telopeptides. One BS locus has been mapped at 17p12, but more recently, two mutations in the lysyl hydroxylase 2 gene (*PLOD2*, 3q23-q24) have been identified in BS, showing genetic heterogeneity. The proportion of BS cases linked to 17p22 (BS type 1) or caused by mutations in *PLOD2* (BS type 2) is still uncertain, and phenotypic correlations are lacking. We report on a boy who had congenital contractures with pterygia at birth and severe OI-like osteopenia and multiple fractures. His urine contained high amounts of hydroxyproline but low amounts of collagen crosslinks degradation products; and he was shown to be homozygous for a novel mutation leading to an Arg598His substitution in PLOD2. The mutation is adjacent to the two mutations previously reported (Gly601Val and Thr608Ile), suggesting a functionally important hotspot in *PLOD2*. The combination of pterygia with bone fragility, as illustrated by this case, is difficult to explain; it suggests that telopeptide lysyl hydroxylation must be involved in prenatal joint formation and morphogenesis. Collagen degradation products in urine and mutation analysis of *PLOD2* may be used to diagnose BS and differentiate it from OI. © 2004 Wiley-Liss, Inc.

KEY WORDS: lysyl hydroxylase; Bruck syndrome; osteogenesis imperfecta; recessive mutation; collagen telopeptide

INTRODUCTION

The majority of cases of osteogenesis imperfecta (OI) are caused by mutations in one of the two genes, *COL1A1* and *COL1A2* that code for the two chains that trimerize to form the procollagen 1 molecule. In rare instances, a phenotype of bone fragility resembling OI is inherited as a recessive trait, like in the osteoporosis-pseudoglioma syndrome (OPS; "ocular" OI) caused by mutations in a *LRP5*, a receptor in the *wnt* signaling pathway [Gong et al., 2001], and in other, as yet molecularly undefined, severe OI variants that are not linked to *COL1A1* or *COL1A2* [Aitchison et al., 1988; Williams et al., 1989; Wallis et al., 1993; Labuda et al., 2002]. A further recessively-inherited OI-like phenotype is Bruck syndrome (BS; OMIM no. 259450). The name of "Bruck syndrome" proposed in 1989 [Viljoen et al., 1989] is actually a misnomer, since the patient reported by Bruck in 1897 had OI but developed contractures only later in life. However, at least 21 children with "Bruck syndrome" from 13 different families have been reported [Sharma and Anand, 1964; Viljoen et al., 1989; Brenner et al., 1993; Brady and Patton, 1997; McPherson and Clemens, 1997; Blacksin et al., 1998; Breslau-Siderius et al., 1998; Leroy et al., 1998; Bank et al., 1999; van der Slot et al., 2003]. In BS, bone fragility is associated with the unusual finding of pterygia and contractures of the large joints. Length is usually normal at birth but joint contractures/deformities are responsible for short stature. Most reported patients have fragile bones, normal sclerae, normal dentinogenesis and no hearing loss, and their intelligence is normal [Bruck, 1897; Sharma and Anand, 1964; Viljoen et al., 1989; Brenner et al., 1993; Brady and Patton, 1997; McPherson and Clemens, 1997; Blacksin et al., 1998; Breslau-Siderius et al., 1998; Leroy et al., 1998; Bank et al., 1999; van der Slot et al., 2003]. Typical radiographic findings are Wormian bones in the skull, generalized osteopenia, bowing of long bones and multiple fractures [Blacksin et al., 1998]. Other features observed are pterygia of the elbows and knees, bowing of long bones, clubfeet, Wormian bones, and kyphoscoliosis [Blacksin et al., 1998; Leroy et al., 1998; McPherson and Clemens, 1997]. Some patients present with blue sclerae [Sharma and Anand, 1964]. BS can be distinguished from OI by the absence of hearing loss and dentinogenesis imperfecta, and by the presence of clubfoot and congenital joint limitations. Notably, several studies have failed to show any abnormality in the biosynthesis of collagen 1 as is found in fibroblasts from OI individuals [McPherson and Clemens, 1997; Breslau-Siderius et al., 1998; Leroy et al., 1998] (and unpublished observations). There seems to be considerable variability among the reported cases of BS concerning the age of onset of contractures, and the presence of pterygia, and the correlation between the extent of articular involvement and the degree of osteopenia is poor.

Recently, evidence was obtained for a specific defect of procollagen telopeptide lysine hydroxylation in BS [Bank et al.,

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1999]; one large family showed evidence of linkage to chromosome 17 (designated BS type 1); while two BS families that did not link to chromosome 17, had mutations in the gene *PLOD2* (on chromosome 3), that codes for one isoform of lysyl hydroxylase, (BS type 2) [van der Slot et al., 2003]. Clinical and radiographic features of PLOD2-associated, BS type 2 have not been described in detail. We report on the clinical, radiographic, and biochemical findings of a child with BS in whom we identified a novel *PLOD2* mutation, in order to facilitate recognition and diagnosis of this syndrome that can now be screened for by biochemical analysis on a urine sample and diagnosed by molecular means.

CLINICAL REPORT

The proband was the first child of healthy, consanguineous Turkish parents. Pregnancy and delivery were uneventful. He was born at term with a weight of 3,000 g (10–50th centile). Length and head circumference at birth are not known. Pterygia were present at the left elbow and at both knees, and extension of these joints was limited. Contractures were present at the wrists, and there were bilateral clubfeet. A fracture of the left arm diagnosed immediately after birth was attributed to trauma during delivery. Bilateral inguinal hernias were also diagnosed. In the first 3 months the boy had two more fractures, one in the left arm, the other in the right arm. At the age of 3 months OI was diagnosed because of recurrent fractures and evidence of marked osteopenia and bowing of the long bones. Blood calcium and phosphate were normal. Treatment with intermittent intravenous pamidronate was started. At age 6 months, weight was 5,740 g (below the 3rd centile), length 56 cm (3rd centile), and head circumference 41 cm (below the 3rd centile); blue-grey sclera and pectus carinatum were noticed. Radiographs documented bilateral femoral bowing, periosteal reaction on tubular bones, a pathological fracture of the right humerus and diffuse osteoporosis. Surgical correction of bilateral clubfoot was performed at the age of 11 months. An operation to release the knee contractures was performed at the age of 18 months. His weight and head circumference always remained below the 3rd centile and his length was at the 3rd centile (measured with his legs only partially stretched) and between the 10th–25th centile if measured going over the deformities with the measuring tape. Mental development was normal (Fig. 1).

At age 2 years, the diagnosis of BS was considered by one of us (Y.A.). A skeletal survey was performed, and previous available radiographs were reviewed. It must be noted that obtaining standard projections was difficult because of the joint contractures. The radiographic changes can be summarized as follows (Fig. 2): marked osteopenia was present from the first months of life in all skeletal elements. Several diaphyseal fractures of the long bones (femurs, tibiae, ulnae, and fibulae) were documented. Callus formation was not exuberant, and healing appeared to proceed normally. A large number of Wormian bones were present in the occipital region of the skull (not shown). There was no platyspondyly in the lumbar region, and only moderate platyspondyly in the thoracic region. There was a vertebra plana at T5, indicating a previous crush fracture. There was a nodular thickening of ribs 4–9 on the left side, adjacent to the costovertebral joint; the lesions looked like healing fractures, but their paravertebral position was unusual and reminiscent of fractures caused by child abuse; it cannot be excluded that those fractures occurred during handling of the child, although there was no other evidence of abuse. There was a healing fracture of rib 9 on the left side, positioned more laterally. The long bones showed multiple striations at more or less regular intervals as well as a mild impairment of metaphyseal modeling, characteristic for cyclic intravenous bisphosphonate treatment.

When last examined at age 25 months (Fig. 1), his weight was 8,740 g (well below 3rd centile), length 84.5 cm (25th centile) measured going over the deformities, head circumference 46 cm (below 3rd centile). There were persisting pterygia at the left elbow and knee. He had had no fracture in the last 7 months. His fine motor, language and social development were normal. He could sit without support when feet were descended from the side of the examination table but standing and walking were impossible because of the joint contractures. He was still being treated with cyclic intravenous pamidronate. The family was informed of the presumptive diagnosis of BS, and blood and urine samples were taken with appropriate consent for further studies.

METHODS

Biochemical Studies

Analysis of collagen crosslinks in urine of patient and controls was performed with the method described by one of us [Bank et al., 1997a], whereas hydroxyproline levels in urine were determined by means of OPA/FMOC derivatization [Bank et al., 1997b]. Results were compared with those obtained in urine samples from healthy children and those of a group of individuals with OI.

cDNA Analysis

For cDNA analysis, total RNA was extracted from white blood cells of our patient and of a healthy control subject

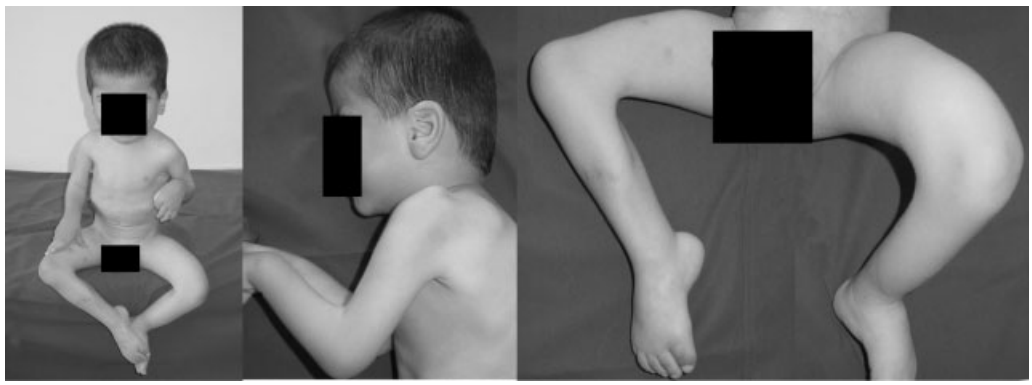

Fig. 1. Clinical photographs of the proband at age 2 years. The boy can sit when his legs are in a dependent position. The thorax is moderately short and the sternum is pushed anteriorly. There are pterygia at the left elbow and at both knees. Residual clubfoot can be seen at the right foot. Surgery had been performed at age 18 months at both knees and both feet.

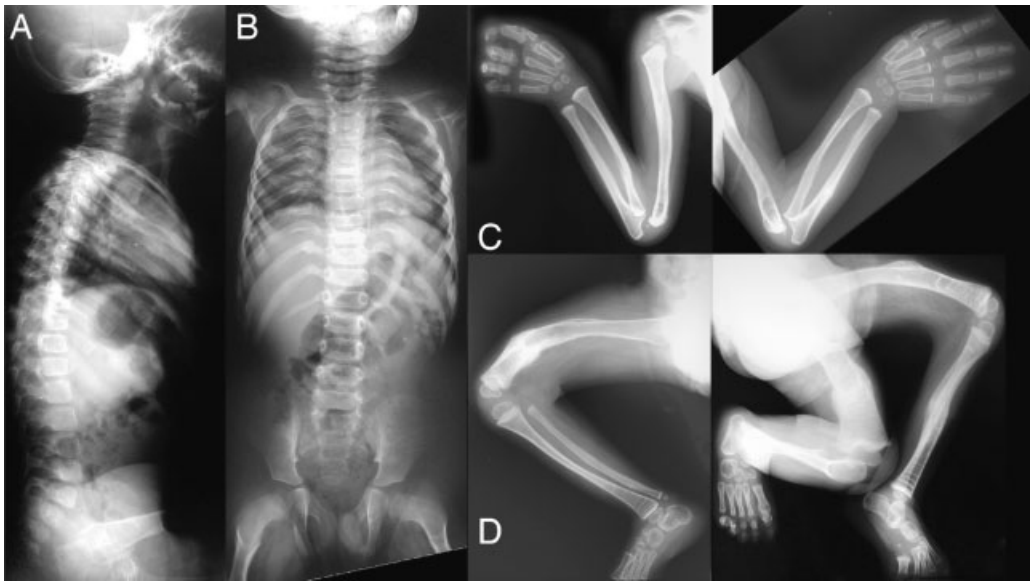

Fig. 2. Radiographic changes at age 2. **A**: The thoracic vertebrae are mildly flattened, the lumbar vertebrae are normal. **B**: Vertebral body T5 is flat; there are paravertebral bone swellings at ribs 4–10 on the right side; and a healing fracture of rib 9 on the left side. **C**: There is generalized osteopenia with lines of sclerosis at the metaphyses (secondary to bisphosphonate therapy). Fractures at different stages of healing and remodelling can be recognized at the right humerus and radius and at the left humerus, radius, and ulna. **D**: Osteopenia, bisphosphonate striations, and mild metaphyseal modelling defect in the long bones of the legs. Fractures at different healing stages at both humeri, the left tibia, and the right ulna.

(RNeasy Mini Kit, Qiagen, Hilden, Germany). On the basis of the published *PLOD2* mRNA sequences (LH2a and LH2b), four fragments of different sizes were amplified by one-step reverse-transcription PCR (One step RT-PCR, Qiagen). The following primers were designed on the GenBank sequence (accession number NM_000935): 5′-CCGGGTCTCTGCGTT-F, 5′-GATAGCGTTTCCCAATGTGC-R (amplimer length 571 bp); 5′-ATTCCAAAAGGCAAACCAC-F, 5′-GTGCTTCTTTTGGGTAATCC-R (amplimer length 599 bp) 5′-GTCCATCCAAACGTATCAA-F, 5′-CATTCATCTCTGATCGGAG-R (amplimer length 553 bp) 5′-GAAGGCTATTATCCACTGC-F, 5′-ATTCCTCTCATCTTCTCAGC-R (amplimer length 769 bp). All nucleotide primers were custom synthesized by Microsynth (Balgach, Switzerland).

The amplified cDNA fragments were purified (Qiaquick PCR purification kit, Qiagen) and directly sequenced using fluorescence-labeled terminator reagents (Big Dye v.1.1) and an automated sequencer (ABI prism 3100, Applied Biosystems, Foster City, CA). The same primers used for amplification were also used for sequence analysis. A short region of cDNA between c.1357 and c.1624/1668, which comprise the region of exon 13a, was amplified using primers 5′-GTAGGAGTATGGAATGTCCC-F and 5′-CCTTCCAGTCCACAGGATTT-R (amplimer length 267/330 bp). Leucocyte RNA yielded two transcripts as found in other organs. The products were separated by electrophoresis on a 3% agarose gel, and each band was extracted from the gel and directly sequenced as described above. The primers used for amplification were also used for sequencing.

Genomic DNA Analysis

Genomic DNA was obtained from blood of our patient and his parents and analyzed by PCR amplification and direct sequencing of exon 17 of *PLOD2* gene. Amplification was carried out at an annealing temperature of 52°C and 39 cycles. The following primer pair, PD2ex17F (5′-AATGGGAATTGCACGTGGAG-3′) and PD2ex19R (5′-GCCACAACTTCAAAGACGTGT-3′), was used to amplify an 834 bp fragment containing exon 17. To exclude polymorphism in the exon 17 of *PLOD2* gene, genomic DNA from 60 anonymous unrelated controls was also studied by PCR amplification and sequencing. The amplimers from genomic DNA were purified and directly sequenced as described above for cDNA analysis.

RESULTS

Biochemical Analysis

The results of the analysis of urinary collagen degradation products, hydroxylysylpyridinoline (HP), lysylpyridinoline (LP), and hydroxyproline (Hyp), are presented in Table I. In the proband's urine, the excretion of Hyp, derived from bone turnover, is high; the HP/LP ratio is normal (unlike in EDS VI, where this ratio is markedly reduced); and the sum of excreted crosslink products is also within the normal range, but is low

TABLE I. Urinary Levels of Collagen Metabolites in the BS Patient and in Controls

	Proband with Bruck syndrome	Control values, mean (±SD) (n = 76; age 0.6–18 years)
HP (nM/mM creatinine)	204	440 (±140)
LP (μM/mM creatinine)	54	106 (±91)
HP/LP	3.9	4.2 (±0.98)
Hyp (μM/mM creatinine)	535	185 (±156)
(HP + LP)/Hyp	0.48	2.8 (±0.50; range: 1.83–4.31)

when related to the excretion of hydroxyproline: the HP + LP/Hyp ratio is 0.48 in the proband's urine, whereas it is on average 2.80 (range: 1.83–4.31) in healthy children. The latter ratio appears to be a good diagnostic marker for BS, as it has found to be below 1 in other BS patients tested so far (Bank et al., unpublished data). In 25 children with non-lethal OI, the average HP + LP/Hyp ratio was 2.67 (range: 1.31–5.10) [Bank et al., unpublished data]; thus, the ratio appears to discriminate also between BS and OI.

Molecular Analysis

PLOD2 is known to have splicing variants in different tissues. Two *PLOD2* mRNA variants were found: the first one was isolated from human fetal kidney and human pancreas [Valtavaara et al., 1997] and encodes for a 737 amino acid protein. Yeowell and Walker [1999] have isolated a cDNA from human skin fibroblasts that encodes for a protein of 758 amino acids of which 21 amino acids are encoded by an additional exon designated 13A. The two transcripts were found in frontal lobe, spleen, kidney, liver, and cartilage, whereas dura, lung, aorta, and skin only express mRNA containing exon 13A. We amplified both splice variants LH2b (+exon13A, 758aa) and LH2a (737aa) from patient's and control's RNA extracted from leucocytes, showing for the first time that leucocytes express both transcripts. In the proband's cDNA, we found a homozygous G > A transition at cDNA position 1793/1856 (LH2a/LH2b). The mutation leads to an R > H substitution in position 598/619 (LH2a/LH2b) of the protein (Fig. 3.). The mutation was confirmed in the patient's genomic DNA, and each parent was found to be heterozygote for the mutation. None of 60 control individuals of mixed European ancestry showed the same mutation. To exclude a possible polymorphism specific to the Turkish population, we analyzed a further sample of 24 untelated Turkish individuals, and none was found to carry the mutation. The mutation is located in close proximity to the two mutations previously identified (G > V 601/622, and T > I 608/629), and all three are in a region that is highly conserved throughout the species [van der Slot et al., 2003] (Fig. 3).

DISCUSSION

Collagen fibrils are stabilized by intermolecular covalent bonds called cross-links. Multiple different cross-links are known, and their structure, number, and location are often tissue specific [Robins, 1982; Eyre et al., 1984b; Eyre, 1987; Reiser et al., 1992]. Lysine (Lys) is the main amino acid residue involved in collagen cross-links. Lysyl hydroxylases (LH) are responsible for the hydroxylation of lysine residues both within the triple helical sequence and in the amino- and carboxy-terminal telopeptide. Three human lysyl hydroxylase genes, coding for at least three isoforms of lysyl hydroxylase, are known (*PLOD1*, MIM no. 153454, 1p36.1-p36.2*; PLOD2*, MIM no. 601865, 3q23-q24; *PLOD3*, MIM no. 603066; 7q22). Hydroxylysine (Hyl) residues in the telopeptide are then converted to the aldehyde hydroxyallysine (Hylald), which then reacts with a Lys or Hyl residue of the triple helix to form di-, tri-, or tetra-functional cross-links [Knott and Bailey, 1998; van der Slot et al., 2003]. These mature cross-links, derived either from one Hyl and one Lys residue (lysylpyridinoline crosslinks; LP) or from two hydroxylysine residues (hydroxylysylpyridinoline crosslinks; HP) are found in a variety of connective tissues including bone and cartilage [Eyre et al., 1984a]. Among the three PLOD isoforms, *PLOD2* has been found to be expressed preferentially in cells with osteoblastic activity [Uzawa et al., 1999].

The first human disease to be related to a deficit in lysine hydroxylation, and, therefore, in collagen cross-linking, was the Ehlers–Danlos syndrome type VI (EDS VI) [Pinnell et al., 1972; Hautala et al., 1992, 1993; Heikkinen et al., 1994; Yeowell and Walker, 2000; Steinmann et al., 2002]. EDS VI combines severe joint laxity, skin hyperelasticity, muscular hypotonia, osteoporosis, scoliosis, and vascular and ocular fragility [Steinmann et al., 2002]. EDS VI is the result of recessive mutations in the *PLOD1* gene that codes for lysyl hydroxylase 1 (LH1) [Hautala et al., 1992, 1993; Heikkinen et al., 1994]. LH1 specifically hydroxylates lysine residues in X-Lys-Gly triplets in the triple helical region of collagen [Pasquali et al., 1997; Bank et al., 1999].

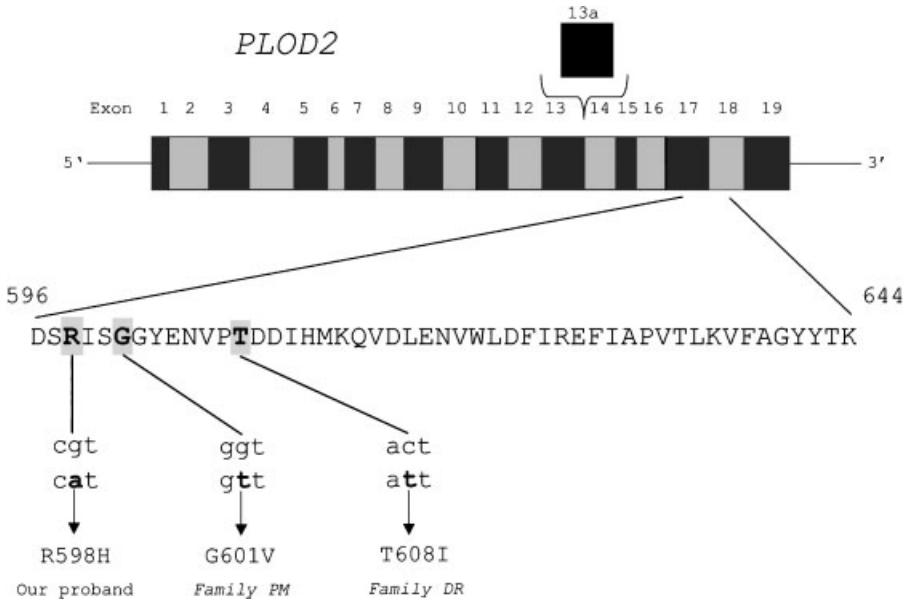

Fig. 3. Scheme of the exon composition of *PLOD2* mRNA (**upper part**), and blow-up of exon 17 (**lower part**) showing the three mutations known so far (families PM and DR are from van der Slot et al., 2003). All three mutations have been found at the homozygous state in affected individuals. Amino acid numbering shown is for the shorter mRNA variant, LH2a, lacking exon 13a (see text).

Studies on bone samples obtained from individuals with BS showed reduced hydroxylation of lysine residues specifically derived from collagen telopeptides. This anomaly was detectable in bone tissue, but not in cartilage or ligamentous tissue [Bank et al., 1999]. A genome-wide homozygosity scan in one consanguineous family identified a locus at 17p12 [Bank et al., 1999] that was initially supposed to contain the gene for the telopeptide-specific lysyl hydroxylase, but no known gene at that map location seemed to be a good candidate to explain the biochemical findings; specifically, none of the procollagen lysyl hydroxylase isoenzymes map there. The study of two further families yielded evidence against linkage to the locus on chromosome 17; in these two families, a candidate gene approach resulted in the identification of two different missense mutations in exon 17 of *PLOD2*. *PLOD2* and *PLOD3* are two genes coding for two isoforms of lysyl hydroxylase (LH2 and LH3, respectively) [Hautala et al., 1992; Valtavaara et al., 1997, 1998; Yeowell and Walker, 1999; Rautavuoma et al., 2000]. *PLOD2* was thereby identified as coding for the putative telopeptide lysyl hydroxylase and as the gene responsible for BS type 2. No *PLOD2* mutation was found in the family that showed linkage to chromosome 17, suggesting genetic heterogeneity of this condition [van der Slot et al., 2003]. Mutation analysis of our patient with BS revealed homozygosity for a missense mutation in *PLOD2*. Interestingly, the mutation is located in exon 17 as were the two mutations previously identified, and affects an amino acid residue highly conserved throughout the species and showing high homology between the three different lysyl hydroxylase isoforms [van der Slot et al., 2003]. This points to a functionally important region of the PLOD2 molecule. Unfortunately, functional studies of mutant *PLOD2* proteins are still lacking, as producing enough substrate to test for this particular enzymatic activity is a formidable task. Thus, while it is true that formal proof of pathogenicity is still lacking, absence of the mutation in controls, correct segregation, localization in the same domain as the two others known, and—most significantly—correlation with a reproducible pattern of changes in hydroxylated metabolites in the urine leaves little doubt as to its causative role.

In EDS VI, hydroxylysine is deficient all along the triple helix of collagen in several tissues; the urinary metabolite pattern shows a marked deficiency of HP and an elevation of LP, and this can be used as a reliable diagnostic marker [Eyre et al., 1984a, 2002; Steinmann et al., 1995, 2002]. We were interested in testing whether urinary excretion of collagen metabolites in BS would reflect the biochemical anomalies and thus be a further diagnostic handle, as it is in EDS VI. In BS, hydroxylysine deficiency affects the telopeptide only, and appears to be restricted to bone; changes would be expected to be less marked than in EDS VI. The urinary excretion of collagen metabolites in our patient reflects a condition with a normal ratio of HP to LP (unlike EDS VI, where hydroxylation of lysyl residues in the whole helical region is affected, the PLOD2 mutation seems to be much more specific for hydroxylation of telopeptides in bone), a high bone turnover (consistent with the findings of osteopenia and fractures), and a reduction of the absolute number of crosslinks in bone collagen (low HP + LP/Hyp ratio). Excretion of crosslink products in BS is particularly low in relation to the high bone turnover; thus, the HP + LP/Hyp ratio appears to be the most useful parameter, as it may differentiate between BS and controls but also between BS and OI. The number of BS individuals tested so far is small, and more studies must be done to validate urine metabolite testing as diagnostic help.

The combination of bone fragility with contractures is unusual and apparently contradictory. In OI and in several types of EDS (notably in EDS VI), moderate osteopenia is accompanied by joint laxity as a consequence of a disturbance in collagen synthesis or metabolism. How can a collagen crosslink defect lead to joint contractures? The contractures seen in our proband and in some other individuals with BS are different from the progressive contractures seen in other connective tissue diseases like e.g., the mucopolysaccharidoses; they are associated with pterygia (from pteros, greek for wing) present over certain large joints. Pterygia are usually present at birth and only rarely acquired postnatally. It is not known whether they reflect a primary morphogenetic defect (with secondary restriction of joint movements), or whether they are merely a consequence of restricted movements of the joints before birth. Although the mechanism remains unclear, the findings of contractures and pterygia in BS seems to indicate that telopeptide lysyl hydroxylation must be involved in prenatal joint formation and morphogenesis.

The radiographic changes in our proband are superficially similar to those of OI; they would be classified as severe type IV or mild type III. The susceptibility to fractures in our proband appears to be higher than expected based on the severity of radiographic changes. In particular, rib fractures and vertebral crush fractures are unusual in infants with OI. Fracture rate may be influenced by the joint contractures that make handling even more difficult than in OI.

Given the apparent phenotypic heterogeneity and the evidence of at least two loci in the etiology of BS, accurate phenotype-genotype correlations may become important to ascertain whether there are differences in presentation and clinical course that are determined by the nature of the underlying defect. No phenotypic description is available yet for those BS patients that have been characterized at biochemical and molecular level so far [van der Slot et al., 2003]. The combination of OI-like bone changes with congenital contractures and pterygia, as illustrated by our patient, is distinctive enough to be useful for a diagnostic orientation. The biochemical and laboratory findings confirm that mutations in *PLOD2* may be a common etiology of BS, identify exon 17 as a potential hotspot, and point to the value of urinary crosslink analysis as an additional diagnostic tool. Future study should show the phenotypic spectrum of *PLOD2* mutations, in particular whether BS is the sole *PLOD2*-associated phenotype or whether some individuals may lack contractures; *PLOD2* and even *PLOD3* may be candidate loci for recessive forms of OI that are not linked to the loci coding for type 1 collagen chains.

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