A polyadenylation site variant causes transcript-specific BMP1 deficiency and frequent fractures in children

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We had previously published the clinical characteristics of a bone fragility disorder in children that was characterized mainly by lower extremity fractures and a mineralization defect in bone tissue but not on the growth plate level. We have now performed whole-exome sequencing on four unrelated individuals with this phenotype. Three individuals were homozygous for a nucleotide change in BMP1 affecting the polyadenylation signal of the transcript that codes for the short isoform of BMP1 (BMP1-1) (c.241T>C). In skin fibroblasts of these individuals, we found low levels of BMP1-1 transcript and protein. The fourth individual was compound heterozygous for the c.241T>C variant in BMP1-1 and a variant in BMP1 exon 15 (c.2107G>C) that affected splicing in both BMP1-1 and the long isoform of BMP1 (BMP1-3). Both the homozygous 3′UTR variant and the compound heterozygous variants were associated with impaired procollagen type I C-propeptide cleavage, as the amount of free C-propeptide in the supernatant of skin fibroblasts was less than in controls. Peripheral quantitative computed tomography showed that all individuals had elevated volumetric cortical bone mineral density. Assessment of iliac bone samples by histomorphometry and quantitative backscattered electron imaging indicated that the onset of mineralization at bone formation sites was delayed, but that mineralized matrix was hypermineralized. These results show that isolated lack of BMP1-1 causes bone fragility in children.

INTRODUCTION

Osteogenesis imperfecta (OI) is a heritable disorder of the connective tissue that is typically characterized by high fracture rate, low bone mass and variable short stature (1). In most cases, OI is caused by variants in one of the two genes coding for procollagen type I α chains, COL1A1 (MIM 120150) and COL1A2 (MIM 120160). Procollagen type I is produced in osteoblasts and other cell types as a heterotrimer that consists of two α1 chains and one α2 chain (1). Upon secretion, the C- and N-terminal propeptides are cleaved off and the mature type I collagen molecules assemble into collagen fibrils. Variants leading to OI are most often found in the helical domains of the α1 and α2 chains, leading to autosomal dominant OI. Recessive OI is much rarer and is usually caused by variants in genes encoding proteins that are involved in the processing of procollagen type I (2).

Cleavage of the C-propeptide of procollagen type I is performed by four different proteases, which are encoded by three different genes (3). The most active of these C-proteinases are BMP1 isoform 1 (BMP1-1) and BMP1 isoform 3 (BMP1-3; also called mammalian tolloid protein), which arise from alternatively spliced transcripts of BMP1 (MIM 112264) (4,5). BMP1-1 is the shorter isoform and consists of 730 amino acids, whereas BMP1-3 has 986 residues. The common N-terminal portion of BMP1-1 and BMP1-3 (residues 1–702) is encoded by exons

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1–15 of BMP1, but the two proteins differ in their C-terminal ends, which are encoded by exon 16a for BMP1-1 and exons 16b–20 for BMP1-3 (6).

Two reports have described two pairs of siblings from consanguineous families who had recessive OI due to homozygous BMP1 variants (7,8). In one family, a missense variant affecting the signal peptide (p.Gly12Arg) led to decreased secretion of both BMP1-1 and BMP1-3, resulting in bone fragility of intermediate severity (8). This was associated with high lumbar spine areal bone mineral density (BMD), which is a very unusual finding in the context of OI, as almost all children with OI due to COL1A1/COL1A2 variants have low areal BMD at that location (9). Bone tissue examinations from one affected individual revealed an increased amount of unmineralized bone matrix, while the mineralized bone matrix contained a higher than normal amount of mineral and thus was hypermineralized (10). In the second family with a BMP1 variant, a missense variant in the common protease domain of BMP1-1/BMP1-3 (p.Phe249Leu) was associated with very severe bone fragility and extreme short stature (7). Bone tissue was not examined in these individuals, but their areal BMD appeared relatively high when taking the extreme short stature into account.

The variants in BMP1 reported to date are located in exons 1 and 6, respectively, and thus affect both the BMP1-1 and the BMP1-3 protein (7,11). It is therefore unclear at present whether a decrease in the function of only one of these two isoforms is sufficient to cause disease in humans. In the present report, we describe four children of French–Canadian origin with mild-to-moderate bone fragility. Three of these children had a homozygous variant in exon 16a of BMP1, which affected the polyadenylation signal in the 3′UTR of BMP1-1 but did not directly affect BMP1-3. The fourth individual was compound heterozygous for the same 3′UTR variant of BMP1-1 and a missense variant in the terminal nucleotide of BMP1 exon 15. A decade ago, we had published the clinical and bone histological characteristics of two of these individuals and had suggested that they suffered from a novel bone fragility disorder (12). The present report shows that this disorder is caused by BMP1-1 deficiency.

RESULTS
Clinical descriptions

The four individuals with bone fragility were from four non-consanguineous French–Canadian families (Supplementary Material, Fig. S1). Family histories were negative for bone disorders. Birth weight was normal for all individuals. At the time of first presentation at our institution, all four individuals had normal height and weight (Table 1). All had normal dentition and white sclera. Outside of fracture episodes, mobility was not impaired. Fracture healing occurred normally.

Individual P1 sustained her first fracture (left tibia) at the age of 4 years when she fell off a height of 70 cm (Table 1). Until the age of 11 years, she suffered five additional long-bone fractures subsequent to minor trauma (one tibia fracture; four forearm or elbow fractures) as well as five hand or foot fractures. In the following 6 years, only one finger fracture occurred, and a mild curvature of the spine developed (Fig. 1). She did not receive bisphosphonate treatment.

Individual P2 was previously described as Patient 1 in the report by Munns et al. (12). By the time she was first evaluated at our institution at the age of 3.6 years, she had sustained five tibia fractures (Table 1). Until the age of 9 years, she suffered 11 more tibia fractures, but only two small-bone fractures (finger, toe) occurred in the subsequent 19 years. No bisphosphonate therapy was given.

Individual P3 was previously described as Patient 2 by Munns et al. (12). He was first examined at our institution when he was 9.4 years old after he had sustained 12 long-bone fractures and a Grade 1 vertebral compression fracture (Table 1). He was treated with intravenous pamidronate for 12 months, starting at 9.6 years of age. In the following 12 years, he sustained three more upper extremity fractures (two radius and ulna fractures, one carpal fracture), which however all occurred after significant trauma.

Individual P4 was born by spontaneous vaginal delivery at term with a weight of 3460 g. She had a clavicle fracture at birth and suffered the first postnatal fracture (right tibia) at 15 months of age after a fall from standing height. Cognitive development was normal but she was able to walk only at 20 months of age. A muscle biopsy at that age did not reveal any abnormalities.

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Table 1. Clinical characteristics of individuals with homozygous or compound heterozygous BMP1 variants

<table>
<thead>
<tr>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>F</td>
<td>F</td>
<td>M</td>
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<tr>
<td>Age at time of first evaluation</td>
<td>11.5</td>
<td>3.6</td>
<td>9.4</td>
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<tr>
<td>Age at first fracture (years)</td>
<td>4.0</td>
<td>0.8</td>
<td>2.5</td>
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<tr>
<td>Number of long-bone fractures</td>
<td>6</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Vertebral compression fractures</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Joint hyperlaxity</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Height (z-score)</td>
<td>−0.7</td>
<td>−0.9</td>
<td>−0.6</td>
</tr>
<tr>
<td>Weight (z-score)</td>
<td>−1.3</td>
<td>−0.6</td>
<td>−1.9</td>
</tr>
<tr>
<td>Lumbar spine areal BMD (z-score)</td>
<td>−0.4</td>
<td>1.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Serum total calcium (mmol/l)</td>
<td>2.40</td>
<td>2.50</td>
<td>2.32</td>
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<tr>
<td>Serum inorganic phosphorus (mmol/l)</td>
<td>1.83</td>
<td>1.56</td>
<td>1.27</td>
</tr>
<tr>
<td>Serum parathyroid hormone (pmol/l)</td>
<td>1.2b</td>
<td>5.6c</td>
<td>8.0c</td>
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<tr>
<td>Urinary NTX/creatinine (pmol/mmol)</td>
<td>492</td>
<td>n.a.</td>
<td>399</td>
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<tr>
<td>At the time of the last follow-up</td>
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<td></td>
<td></td>
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<tr>
<td>Age (years)</td>
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<td>28.8</td>
<td>22.6</td>
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<tr>
<td>Height (z-score)</td>
<td>−0.2</td>
<td>−1.8</td>
<td>−0.4</td>
</tr>
<tr>
<td>Weight (z-score)</td>
<td>−0.9</td>
<td>1.0</td>
<td>−0.7</td>
</tr>
<tr>
<td>Serum pigment-epithelium-derived factor (mg/l)</td>
<td>22.5</td>
<td>na</td>
<td>9.5</td>
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<tr>
<td>Lumbar spine areal BMD (z-score)</td>
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<td>1.1</td>
</tr>
<tr>
<td>Radius trabecular volumetric BMD (z-score)</td>
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<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>Vertebral compression fractures</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Biochemical and bone density results outside the reference range are shown in bold print.

na, not available.

Reference data (13,14): calcium: 2.20–2.70 mmol/l; inorganic phosphorus: 4–11 years 1.20–1.80 mmol/l, adult 0.90–1.50 mmol/l; alkaline phosphatase: 1–9 years 145–420 U/l, 16–19 years 50–130 U/l; urinary NTX/creatinine: Ref. (13); pigment-epithelium-derived factor: range in 19 healthy controls: 2.0–17.5 mg/l (15). Reference range for all results given as z-scores: −2 to +2.

aAt 5.7 years of age.

bNorm 1.3–5.4 pmol/l.

cNorm 2.6–10.0 pmol/l.

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By the time she was referred to our institution at 3.7 years of age, she had sustained three more tibia and two metatarsal fractures. Lateral spine radiographs in addition demonstrated grades 2–3 vertebral compression fractures in the thoracic spine (Fig. 1). Lumbar spine areal BMD was low (Table 1). Intravenous therapy with zoledronate was started and has been maintained until the most recent follow-up at 8 years of age. This treatment was associated with a rapid normalization of lumbar spine areal BMD and some improvements in the shape of the compressed vertebral bodies (Fig. 1). Two more long-bone fractures...
(one tibia, one fibula fracture, in separate events) and one toe fracture occurred while receiving zoledronate.

Bone analyses

Biochemical parameters of bone and mineral metabolism were within normal limits at baseline, apart from low serum 25-OH vitamin D in individual P3 (Table 1). Lumbar spine areal BMD was normal for individuals P1, P2 and P3, but was low in individual P4. Peripheral quantitative computed tomography of the radius showed elevated cortical volumetric BMD for all individuals (Table 1; Fig. 1).

Histomorphometric analyses of iliac bone samples showed normal or slightly elevated cortical width and a normal amount of trabecular bone in all individuals (Supplementary Material, Table S1). Osteoid thickness and mineralization lag time, which are the histomorphometric indicators of mineralization defects, were elevated in individuals P2, P3 and P4, but these parameters were normal in individual P1. Mineral apposition rate, a marker of osteoblast activity, was low in all samples, but osteoclast surface, an indicator of bone resorption, was normal. Quantitative backscattered electron imaging of mineralized bone in the same samples showed a shift towards increased mineralization both in trabecular and in cortical bone (elevated \( \text{Ca}_{\text{Mean}}, \text{Ca}_{\text{Peak}} \) and \( \text{Ca}_{\text{High}} \); Fig. 1; Supplementary Material, Table S2).

The histomorphometric findings somewhat resembled those of OI type VI, where circulating pigment-epithelium-derived factor is lacking due to \( \text{SERPINF1} \) variants (MIM 172860) (15). We therefore measured serum levels of pigment-epithelium-derived factor in three of the individuals described here, but found normal results (Table 1).

Variant detection

We performed whole-exome sequencing on genomic DNA of individuals P1 to P4. The samples from individuals P2, P3 and P4 were analyzed first, using Illumina TruSeq Exome Kit for exome capture and an Illumina HiSeq 2000 sequencer for sequencing. This did not reveal any common disease-causing variants in these three samples. However, in P4 we observed a heterozygous variant in the terminal nucleotide of \( \text{BMP1} \) (chromosomal location: 8p21.3) exon 15 (c.2107G>C) (Supplementary Material, Fig. S2), which was predicted to either lead to a transcript-specific amino acid substitution (p.Glu703Gln in \( \text{BMP1-1} \) (NP_001190.1); p.Asp703His in \( \text{BMP1-3} \) (NP_006120.1)) or to affect splicing (Fig. 2A). However, \( \text{BMP1} \) variants are expected to be recessive; therefore, this heterozygous variant was initially not considered to be causative of this patient’s condition.

Figure 2. Location of \( \text{BMP1} \) variants. (A) \( \text{BMP1-1} \) and \( \text{BMP1-3} \) transcripts. Variants found in the present study (bold print) and previously reported variants [asterisk, reference (8); number sign, reference (7)] are indicated by arrows. The c.2107G>C variant affects the 3' end of exon 15. The location of alternative splicing leading to \( \text{BMP1-1} \) and \( \text{BMP1-3} \), respectively, is indicated. (B) \( \text{BMP1-1} \) and \( \text{BMP1-3} \) proteins. The domain structure is indicated. S, signal peptide; Pro, prodomain; CUB, CUB domains (complement/Uef/BMP1); E, EGF (epidermal growth factor) domain. The locations of the exons in the transcripts shown in (A) are aligned relative to the regions of protein each exon encodes. (C) Sequence of the \( \text{BMP1-3} \) UTR (NM_001199.3). The stop codon is highlighted. The polyadenylation signal is underlined and highlighted by dark background. The T affected by the c."241T>C variant is printed in white.
The DNA sample of individual P1 became available only 2 years after the analyses in the other three patients had been performed. We analyzed this sample using SureSelect Human All Exon Kit version 4 for exome capture and an Illumina HiSeq 2000 sequencer for sequencing. This identified a homozygous novel point variant in BMP1, affecting the polyadenylation site in the 3′UTR of BMP1-1 exon 16a (c.*241T>C; NM_001199.3; Fig. 2A and B; Supplementary Material, Fig. S2). Given the close phenotypic similarities between the individuals described here, we reviewed the 3′UTR of BMP1-1 exon 16a in the previously obtained exome sequencing results of P2, P3 and P4 (Supplementary Material, Fig. S3). Individual P4 appeared to share one haplotype of the same region with the three other individuals.

Subsequent Sanger sequencing showed that individuals P1, P2 and P3 were homozygous for the c.*241T>C variant in the 3′UTR of BMP1-1, while individual P4 was compound heterozygous for both the c.2107G>C and the c.*241T>C variant (Supplementary Material, Fig. S1). Neither the c.*241T>C nor the c.2107G>C variant was found in the databases of dbSNP, 1000 Genomes or the NHLBI/NGHRI Exome Project. On the transcript level, individual P4 is thus compound heterozygous for the c.2107G>C and c.*241T>C variants in BMP1-1 and is heterozygous for the c.2107G>C variant in BMP1-3 (Supplementary Material, Fig. S4).

In order to elucidate whether the c.2107G>C variant in P4 represented a missense or rather a splicing variant, we performed transcript-specific PCR amplification of cDNA from skin fibroblasts using forward primers in exon 14 and reverse primers in either exon 16a (BMP1-1) or exon 16b (BMP1-3) (Supplementary Material, Fig. S4). Apart from the PCR products that had the expected length, this resulted in additional PCR products that arose from alternative splicing. In BMP1-1, exon 15 was spliced out, which was predicted to lead to a frameshift (p.Val643Lysfs*99; NP_001190.1). Overall, these results thus indicate that in P4, the c.2107G>C variant leads to a splicing defect rather than an amino acid substitution.

Functional studies
Skin fibroblasts for functional studies were available only from individuals P1, P3 and P4. Transcript-specific real-time PCR of cDNA from skin fibroblasts (primer sites indicated in Fig. 2A) showed that the BMP1-1 transcript was less abundant in individuals P1 and P3 than in control fibroblasts, whereas the BMP1-3 transcript was more abundant than in control fibroblasts (Fig. 3). Western blot revealed a decrease of BMP1-1 protein in individuals P1, P3 and P4, whereas BMP1-3 was detectable at apparently normal levels. Collagen type I analysis in skin fibroblasts from the same individuals showed a lower proportion of pNα1(I) (the partially processed procollagen type I) after removal of the N- or C-terminal propeptides (PICP). pCα1(I) and pNα1(I) are intermediate variants following removal of the N- or C-terminal propeptides, respectively.

Results in heterozygous carriers
Among family members of the individuals described above, seven heterozygous carriers of BMP1 variants were assessed clinically (Supplementary Material, Fig. S1). None of these individuals had a history of fractures outside of the context of...
significant accidents. Bone densitometry did not reveal abnormalities apart from slightly low results for bone mineral content and trabecular volumetric BMD at the distal radius in individual II2 of Family 4 (Supplementary Material, Table S3).

**DISCUSSION**

In the present study, we identified a variant in the polyadenylation site of BMP1-1 as the cause of a bone fragility disorder that we had previously described in French–Canadian children. The variant was associated with a decrease in the expression of BMP1-1 and a decrease in procollagen type I C-propeptide cleavage activity. All individuals had elevated volumetric cortical BMD at the radius and increased mineralization of the mineralized bone matrix.

Polyadenylation is an essential step in the processing of most mRNAs (17). A key element in this process is the polyadenylation signal in the 3′ UTR, which is located ~30 nucleotides upstream of the cleavage site. The polyadenylation signal is recognized by the cleavage and polyadenylation specificity factor, which initiates the assembly of a multiprotein complex that performs pre-mRNA cleavage, followed by the addition of a poly(A) tail at the 3′ end (17). These modifications are required for the stability of mature transcripts and for efficient translation.

Only a few disorders are known to be caused by variants in polyadenylation sites, including rare forms of thalassemias, Fabry disease and metachromatic leukodystrophy (17,18). The ‘canonical’ hexameric polyadenylation signal is AATAAA, but the polyadenylation sequence of BMP1-1 is AGTAAA (19), which is observed in only 3.7% of human genes (20). However, the T on the third position of the hexamer, which is affected by the c.*241T>C variant found in the present study, is almost universally conserved in human polyadenylation sites (20). *In vitro* studies have shown that replacing the T by a C at this position decreases the binding of cleavage and polyadenylation specificity factor (21) and markedly decreases mRNA polyadenylation (22). The substitution of a C for a T in a polyadenylation signal has also been implicated in human disease before, as such a variant has been reported in the polyadenylation signal of *HBB*, leading to β-thalassemia (23).

Our results are in accordance with an effect of the BMP1-1 c.*241T>C variant on both the availability of BMP1-1 mRNA and on translation. The variant was associated with low but detectable BMP1-1 mRNA levels in fibroblasts from homozygous patients, which may be explained by mRNA instability. Nevertheless, BMP1-1 protein was not detectable by western blot despite the presence of BMP1-1 mRNA, which indicates an additional effect of the variant on translation. In contrast to BMP1-1, BMP1-3 transcript levels were elevated and BMP1-3 protein was expressed normally in fibroblasts affected by the 3′ UTR variant in BMP1-1.

On the functional level, the specific lack of BMP1-1 protein was associated with a decrease in the cleavage of procollagen type I C-propeptide, in accordance with the notion that BMP1-1 is the most active C-propeptide cleavage enzyme (4,5). Removal of the C-propeptide by BMP1 is a critical step in the assembly of collagen fibrils (24). BMP1 variants may therefore cause bone fragility by interfering with collagen type I fibril assembly, as was demonstrated previously for the p.Gly12Arg signal peptide variant (8). Nevertheless, a recently described mouse model with postnatally induced ablation of both BMP1 and mammalian tolloid-like 1, a functionally related proteinase, had severe bone fragility but did not show a major abnormality in the appearance of collagen fibrils (25).

Similar to that mouse model, our histomorphometric and quantitative backscattered electron imaging data suggest a disturbance in at least two aspects of the mineralization process. First, mineralization lag time and osteoid thickness are increased, indicating a delay in the start of mineralization. Second, there is hypermineralization of the mineralized matrix showing that after the delayed start, mineralization progresses to a higher than normal level. This may be an important aspect of the bone brittleness, as hypermineralization seems to be a constant feature of OI, where the bone fragility is related to abnormalities in collagen type I (10,26,27), in contrast to bone fragility disorders caused by defects in other genes, such as *LRP5*, *WNT1* or *PLS3* (28–30).

Despite hypermineralization of the bone matrix, baseline lumbar spine areal BMD, as measured by dual-energy X-ray absorptiometry, was normal in three of the four individuals with BMP1 variants. This indicates that a decrease in bone volume (e.g. lower thickness of cortices or trabeculae, fewer trabeculae) must have been present in addition to the increase in bone tissue mineralization, resulting in overall normal density on the level of the entire bone. This can make the diagnosis of this condition somewhat difficult, as the typical routine diagnostic methods of X-rays, dual-energy X-ray absorptiometry and serum markers of bone and mineral metabolism may not indicate pathology. However, as cortical bone at the radius is largely ‘solid’ bone, the hypermineralization of the bone matrix becomes detectable by peripheral quantitative computed tomography, as shown by the consistently elevated cortical volumetric BMD that we found in affected individuals.

The children described here had less severe bone fragility than was previously reported for individuals who had BMP1 variants that affected both BMP1-1 and BMP1-3 (7,8). In particular, the variant in the enzymatically active astacin domain of BMP1-1 and BMP1-3 (p.Phe249Leu) was associated with extremely severe bone fragility and marked short stature (7). The milder disease severity in individuals with a homozygous polyadenylation signal variant may be explained by the presence of functional BMP1-3, which will provide residual C-propeptidase activity. This is also suggested by the observation that individual P4 who had a compound heterozygous variant was more severely affected than the individuals with homozygous BMP1-1 3′ UTR variants. The compound heterozygous variant found in this girl affected BMP1-3 in a heterozygous manner, which may have decreased the activity of BMP1-3 in addition to that of BMP1-1, which is expected to lead to a more severe functional deficit of overall BMP1 activity than the lack of BMP1-1 alone.

The disease-causing variant in the 3′ UTR of BMP1-1 was initially missed in individuals P2, P3 and P4 because of low coverage in the region affected by the mutation. This indicates that the exome capture kit used for these three samples had poor capturing efficiency for exon 16a of *BMP1*. The DNA sample of individual P1 was analyzed 2 years later using a different exome capture kit and the variant was easily detected. This highlights the methodological progress in the rapidly evolving field of whole-exome sequencing.
In conclusion, this study presents a rare polyadenylylation site variant that leads to a specific lack of BMP1-1. These observations show that isolated BMP1-1 deficiency is sufficient to cause bone fragility in children.

SUBJECTS AND METHODS

Study participants

Study participants were assessed at the Shriners Hospital for Children in Montreal. Clinical data were extracted by retrospective chart review. The present study population initially comprised three individuals who had previously been described by Munns et al. (12) and two children with a similar phenotype who were referred to us later. After BMP1 variants had been found in four index individuals, we invited their family members to participate in the study. In one of the individuals reported by Munns et al. (referred to as Patient 3 in that publication) we identified a stop variant in COL1A1 (NM_000088.3; c.3421C>T; p.Arg1141*). This variant had not been detected by the heteroduplex screening method for COL1A1 variants that we had used at the time of the previous report (12). Stop variants in COL1A1 are the typical cause of OI type I (31) and therefore no further analyses were undertaken in this sample. The study was approved by the Institutional Review Board of McGill University. Informed consent was provided by study participants, or, in minors, their legal guardians. Study participants aged 7–17 years provided assent.

Anthropometry and serum biochemistry

Height was measured using a Harpenden stadiometer (Holtain, Crymych, UK). Height and weight measurements were converted to age- and sex-specific z-scores on the basis of reference data published by the Centers for Disease Control and Prevention (32).

Blood and urine samples (second morning void) were obtained between 8 a.m. and 9 a.m. after an overnight fast. Serum alkaline phosphatase activity was measured using standard laboratory methods. Serum parathyroid hormone concentrations were determined by the immunoradiometric assay (12) and two children with a similar phenotype referred to us later. After BMP1 variants had been found in four index individuals, we invited their family members to participate in the study. In one of the individuals reported by Munns et al. (referred to as Patient 3 in that publication) we identified a stop variant in COL1A1 (NM_000088.3; c.3421C>T; p.Arg1141*). This variant had not been detected by the heteroduplex screening method for COL1A1 variants that we had used at the time of the previous report (12). Stop variants in COL1A1 are the typical cause of OI type I (31) and therefore no further analyses were undertaken in this sample. The study was approved by the Institutional Review Board of McGill University. Informed consent was provided by study participants, or, in minors, their legal guardians. Study participants aged 7–17 years provided assent.

Radiology

Dual-energy X-ray absorptiometry was performed in the anterior–posterior direction at the lumbar spine (L1–L4) using a Hologic QDR Discovery device (Hologic Inc., Waltham, MA, USA). Lumbar spine areal BMD (LS-aBMD) results were transformed to age- and gender-specific z-scores using reference data provided by the densitometer manufacturer (35,36). Peripheral quantitative computed tomography (pQCT; XCT-2000, Stratec Inc., Pforzheim, Germany) was performed at the metaphysis (4% site) and at the diaphysis (65% site) of the radius as described, and z-scores were calculated based on reference data established by one of the authors (37–40). Vertebral compression fractures were graded using the semiquantitative Genant score (41).

Iliac bone analyses

Iliac bone samples were obtained at a site 2 cm posterior of the superior anterior iliac spine. Tetracycline double labeling was performed prior to biopsy. Sample preparation and histomorphometric analyses were performed using previously described procedures (42). Results were compared with the average value of the age- and gender-specific reference range using reference data established in our laboratory (42). The bone mineralization density distribution in trabecular and cortical bone from this sample was analyzed by quantitative backscattered electron imaging, as described (43). We have previously validated this method and applied it to analyze bone samples representing a wide variety of clinical and experimental situations (44). Results were compared with a pediatric reference database that we have established previously (16).

Sequencing

Whole-exome sequencing (library preparation, capturing, sequencing and bioinformatics) was performed at the Genome Quebec Innovation Center in Montreal, as described (29). Briefly, the exome of individual P1 was captured by the SureSelect Human All Exon Kit version 4 (Agilent Technologies, Inc., Santa Clara, CA, USA). For the other individuals, TruSeq Exome Enrichment Kit (Illumina, San Diego, CA, USA) was used. Subsequently, 100 bp paired-end reads were generated on an Illumina HiSeq 2000 sequencer. Alignment, variant calling and annotation were performed using Burrows-Wheeler Aligner (v.0.5.9) (45), Samtools (46) and Annovar (47), respectively. Mean coverage was 131X for P1 and between 51X and 88X for the other individuals after aligning high-quality reads against human reference genome hg19 for all consensus coding sequence exons. To find novel causative variant(s), we focused on those located in coding regions (i.e. nonsense, frameshift and missense), splicing sites and untranslated regions and having low allele frequency in public (the 1000 genome <0.05) or Exome Variant Server and our in-house (<0.005) exome databases. Finally, we used Integrative Genomics Viewer (48) and Mutalyzer v2 (49) to manually examine all of potential candidate variant positions and descriptions, respectively.

Sanger sequencing was used to confirm the presence of the BMP1 variants in the index individuals of each family and to test available other family members for the presence of the variant. The relevant exons of BMP1 (exon 15 and/or 16) were amplified by PCR and directly sequenced using an Applied Biosystems 3730xl sequencer (Applied Biosystems, Foster City, CA, USA). PCR primers for the site in BMP1 exon 15 were: TGCAAGTACGACTTCGTGGA (forward), GGCTTGATGG- CATCAGTGTG (reverse). PCR primers for the locus in BMP1 exon 16a were: CCGGACAGAATCTGCTGAT (forward), CT GTTGGAGAGAGAGT (reverse). Sequence traces were aligned with GenBank reference sequences NM_001199.3

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(BMP1-1) and NM_006129.4 (BMP1-3). The same primers were used for the sequencing reactions.

**Fibroblast analyses**

Skin fibroblasts were seeded at an initial density of 25 000 cells/cm² in 6-well plates and maintained in DMEM-Dulbecco’s modified Eagle medium (Life Technologies). To initiate procollagen biosynthesis and secretion, cells were treated with ascorbic acid 24 h after plating. One day before the experimental end point, cells were washed two times with serum-free medium, and maintained in serum-free medium for 24 h. The conditioned media was collected, and RNA was harvested using the Trizol reagent (Invitrogen). Two micrograms of RNA were used for cDNA transcription.

To assess the effect of the BMP1 c.2107G>C variant on the transcript level, cDNA was subjected to transcript-specific PCR amplification using the following primer pairs: CGGA TCTCTACAAAGCTCAAC (forward; site in exon 14), GAAGCCCCCATCCATTCGG (reverse; site in exon 16 of BMP1-1) (NM_001199.3). TTCTCACAAGCTCAACGG (forward; site in exon 14), GCAGCTGACTCATAAC (reverse; site in exon 16 of BMP1-3) (NM_006129.4). PCR products were directly Sanger sequenced.

Real-time PCR assessment of gene expression was performed on an ABI 7500 Real-Time PCR Machine using the TaqMan Universal PCR Master Mix and Taqman primers specific for BMP1-1 (Hs00986781_m1), BMP1-3 (Hs00196183_m1) and GAPDH (Hs02758991_g1) (Applied Biosystems). The relative expression levels for BMP1-1 and BMP1-3 were normalized to those of GAPDH using the threshold cycle method and values were expressed as the 2^(-ΔΔCt). Results were expressed as fold difference relative to control fibroblasts.

For protein analysis, samples were separated on 6% SDS–polyacrylamide gels. Primary antibodies were applied against BMP1 (goat anti-human BMP1, R&D Systems) and the triple helical domain of collagen type I (polyclonal anti-collagen type I antibody, Millipore). We also applied LF42 antibody, a generous gift from Dr Larry Fisher, NIH, Bethesda, MD, USA). The membranes were incubated with the corresponding anti-horseradish peroxidase secondary antibody. The separated protein bands were visualized by using Amersham ECL prime western blotting detection reagent (GE Healthcare Life Sciences).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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