Molecular diagnosis of hypophosphatasia and differential diagnosis by targeted Next Generation Sequencing

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Highlights

- We developed a NGS array for hypophosphatasia (HPP) and differential diagnosis genes.
- Patients referred for HPP diagnosis may have mutations in *COL1A1* or *COL1A2* genes.
- Testing together *ALPL*, *COL1A1* and *COL1A2* genes was proven efficient and useful.

Abstract

Hypophosphatasia (HPP) is a rare inherited skeletal dysplasia due to loss of function mutations in the *ALPL* gene. The disease is subject to an extremely high clinical heterogeneity ranging from a perinatal lethal form to odontohypophosphatasia affecting only teeth. Up to now genetic diagnosis of HPP is performed by sequencing the *ALPL* gene by Sanger methodology. Osteogenesis imperfecta (OI) and campomelic dysplasia (CD) are the main differential diagnoses of severe HPP, so that in case of negative result for *ALPL* mutations, OI and CD genes had often to be analyzed, lengthening the time before diagnosis. We report here our 18-month experience in testing 46 patients for HPP and differential diagnosis by targeted NGS and show that this strategy is efficient and useful. We used an array including *ALPL* gene, genes of differential diagnosis *COL1A1* and *COL1A2* that represent 90% of OI cases, *SOX9*, responsible for CD, and 8 potentially modifier genes of HPP. Seventeen patients were found to carry a mutation in one of these genes. Among them, only 10 out of 15 cases referred for HPP carried a mutation in *ALPL* and 5 carried a mutation in *COL1A1* or *COL1A2*. Interestingly, three of these patients were adults with fractures and/or low BMD. Our results indicate that HPP and OI may be easily misdiagnosed in the prenatal stage but also in adults with mild symptoms for these diseases.

Keywords

- Hypophosphatasia;
- Differential diagnosis;
- Osteogenesis imperfecta

1. Introduction

Hypophosphatasia (HPP) is a rare inherited skeletal dysplasia due to loss-of-function mutations in the *ALPL* gene encoding the Tissue Nonspecific Alkaline Phosphatase (TNSALP) [1]. Although the clinical spectrum is a continuum, HPP has been divided into 6 clinical subtypes that may however significantly overlap [2]. The perinatal form is the most severe one and is almost always lethal. The patients die a few days after birth due to respiratory distress and seizures. They present with hypoplasic lungs, extensive

hypomineralization, deformities of bone, severe hypercalcemia and hyperphosphatemia likely due to the lack of mineral deposition on the bones. In the prenatal benign form, despite prenatal symptoms, spontaneous improvement of the skeletal defects might occur completely or partially, resulting in nonlethal HPP [3], [4] and [5]. Clinical signs of the infantile form appear during the first 6 months of life and include rickets, premature craniosynostosis, respiratory issues, irritability, seizures and nephrocalcinosis due to hypercalciuria. This form is lethal in approximately 50% of the cases. Childhood HPP mostly occurs after the first year of life and is characterized by rickets causing a short stature, delayed walking and a waddling gait due to bone deformities and pain of the lower extremities. Premature loss of teeth often leads to the diagnosis. Adult HPP presents with osteomalacia, chondrocalcinosis, osteoarthropathy and stress fractures during middle age in patients who had a history of mild rickets in childhood. Many patients present premature loss of permanent teeth. Odontohypophosphatasia (odontoHPP) is characterized by dental manifestations of HPP not associated with abnormalities of the skeletal system.

This high clinical heterogeneity is mainly due to the great number of *ALPL* missense mutations [6], more rarely to other factors as suggested by clinical heterogeneity observed between patients with the same *ALPL* genotype [7], [8], [9] and [10] and even between affected siblings [11]. Interestingly, a recent report showed that screening for low serum alkaline phosphatase (ALP) in patients with low bone mineral density (BMD) evidenced heterozygous *ALPL* sequence variations in 33.8% of these patients vs 1.4% in controls with normal BMD [12]. Thus the role of *ALPL* mutations in various conditions such as osteoporosis is emerging.

There are various differential diagnoses of HPP [13]. They depend on the age at which the diagnosis is considered. *In utero*, osteogenesis imperfecta (OI) type II and campomelic dysplasia (CD) are the most common differential diagnoses of HPP. Rare conditions such as Stuve–Wiedemann syndrome may also be involved. At birth, radiographs may distinguish OI type II, CD, and chondrodysplasias with bone mineralization defect, from HPP, but outwardly the distinction is difficult. In infancy and childhood, OI (typically type III in infancy or type IV later on) is the most common differential diagnosis, but also more rare disorders such as cleidocranial dysostosis, Cole–Carpenter syndrome, idiopathic juvenile osteoporosis, and renal osteodystrophy. In adult osteopenia/osteoporosis and more rarely osteoarthritis and pseudogout may be due to HPP.

We perform genetic diagnosis of HPP and maintain the TNSALP gene mutation database [6] where over 300 mutations are reported. In our experience sequencing the 12 exons and intron/exons borders in the *ALPL* gene by Sanger methodology allows to detect more than 95% of the HPP mutations [14] and [15]. Undetected mutations probably affect intronic or regulatory sequences, or correspond to large deletions partly detected by qPCR or semi-quantitative methodologies like Quantitative Multiplex PCR of Short Fluorescent fragments QMPSF [16]. However the Sanger methodology is expensive and time-consuming, which prevents a strategy of one-time diagnosis including genes of differential diagnosis such as *COL1A1* and *COL1A2* responsible for most cases of OI [17], each harboring over 50 exons. This means that in case of negative result for *ALPL*, other genes had to be analyzed, lengthening the time before diagnosis. The emergence of Next Generation Sequencing (NGS) technologies allows one-time sequencing of several genes (targeted NGS) or even all the coding sequences of genes (exome sequencing) or the full genome, and diagnosis applications have been shown in endocrine disorders as well as in all domains of medical genetics [18],

[19] and [20]. We report here our 18-month experience in testing 46 patients for HPP and differential diagnosis by targeted NGS and show that this strategy is efficient and useful.

2. Materials and methods

2.1. Patients

During the period Dec. 2013–Jul.2015 we tested for diagnosis purpose 46 patients by NGS including 13 fetuses. The patients were referred to us by obstetricians, rheumatologists, endocrinologists, dentists or geneticists. Forty-two were referred to us for suspicion of HPP on the basis of some clinical and/or biological symptoms. Two patients were referred for CD and 2 for skeletal dysplasia without clear suggestion of diagnosis. An informed consent was obtained in each case.

2.2. Genes tested

The array included genes for HPP diagnosis and differential diagnosis: *ALPL*, *COL1A1*, *COL1A2* and *SOX9*. In addition we included genes dedicated to the identification of modifier genes of HPP (*ANKH*, *ENPP1*, *FGFR3*, *PHOSPHO1*, *PTH1R*, *PTH2R*, *SPP1* and *TNFRSF11A*), and the study of variants in the regulatory region of *ALPL* (5000 bp upstream exon 1). We present here the results of patients tested for HPP diagnosis and differential diagnosis. Two multiplex PCR primer pools were obtained from Thermo Fisher Scientific (Ion Ampliseq™). They included primers for the amplification of the coding sequence and 150 bp of exon/intron borders of these genes. The 380 amplicons covered more than 96% of the target coding sequences. Ninety-eight percent of the *ALPL* gene was covered corresponding to all the coding sequence and intron/exon borders except a small part of exon 4. This led us to routinely perform sequencing of exon 4 by standard Sanger methodology.

2.3. Sequencing procedure

Sequencing was performed from 10 ng of genomic DNA by using the Ion Torrent Personal Genome Machine (PGM) according to the recommendations of the manufacturer (Thermofisher Scientific). All the mutations identified by NGS and reported here were confirmed by sequencing with Sanger methodology.

2.4. Analysis

2.4.1. Damaging effect of mutations

The sequence variations were compared to human genome hg19. Identification of variants was performed with Variant Caller and Ion Reporter softwares (Thermo Fisher Scientific) and *in silico* prediction of their pathogenicity was performed by using the free access web servers POLYPHEN2 (http://genetics.bwh.harvard.edu/pph2/) [21], SIFT (http://sift.jcvi.org/) [22] and MUTATION TASTER (http://www.mutationtaster.org/) [23]. The effect of mutations on splicing was tested by using Human Splicing Finder web server (http://www.umd.be/HSF3/) [24].

2.4.2. Dominant effect of mutations

The prediction of dominant negative effect of mutations was performed by using 3D modeling of TNSALP [25] and [26]. Mutations affecting residues located in particular regions, especially the active site, the crown domain and the homodimer interface, were predicted to have a possible dominant effect [15] and [26].

2.4.3. Mutagenesis and expression of PHOSPHO1 (patient P12)

Briefly the PHOSPHO1 c.95_97CCT deletion was introduced into a pCMV expression vector containing the human PHOSPHO1 coding sequence. The resulting construct was transiently transfected into COS-1 cells. The total activity of WT and c.95_97CCT mutant extracts was measured at 405 nm, using *p*-nitrophenyl phosphate (pNPP, 20 mM) as a substrate, at pH 9.8, and using 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (P-Etn) because PHOSPHO1 shows high phosphohydrolase activity towards P-Etn.

3. Results

We present here the sequence variations detected in the genes tested for HPP or differential diagnosis, filtered with MAF (Minimum Allele Frequency) < 0.02. This allows the exclusion of common polymorphisms.

Among the 46 tested patients 29 (63%) were negative for any mutation in the tested genes. Eleven of them were adults (43–91 yo) with stress or atypical fractures, low BMD and musculoskeletal pain and referred by rheumatologists for HPP. Among the patients tested for serum ALP, 5 presented with values below the normal range. Thirteen cases were fetuses with clinical manifestations compatible with HPP and referred by geneticists or obstetricians. The other cases were referred for odontoHPP (3 cases) and for suspicion of infantile HPP (1 case) or childhood HPP (1 case).

In 17 cases (37%) a mutation was detected in *ALPL*, *COL1A1*, *COL1A2 or SOX9*. Except patient P12 all these patients were negative for the other genes included in the array. The results are summarized in Table 1 and Table 2. Only 67% (10/15) of patients referred for HPP were found to carry mutations in the *ALPL* gene and were therefore confirmed to have HPP (Table 1). The others (5 cases) were found to harbor mutations in *COL1A1* or *COL1A2* confirming the interest of testing these genes in routine diagnosis of HPP.

Table 1.

Distribution of positive genes versus referral.

Referral	Number of cases	Genes with detected mutations		
		ALPL	***COL1A1/COL1A2***	***SOX9***
HPP	13	9	4	0
HPP or OI	2	1	1	0
CD	2	1	0	1
Total	17	11	5	1

Table 2.

Mutations detected in 17 patients tested with targeted NGS and estimation of their pathogenicity.

Sequencing results correspond to sequence variations detected in *ALPL* or HPP differential diagnosis genes (see Material and methods), filtered with MAF (minor allele frequency) < 0.02. *In silico* prediction shows the predictions of the three free access web servers POLYPHEN2 (PP2), SIFT and MUTATION TASTER (MT). For PP2 and MT the scores range from 0 (benign) to 1 (damaging), while for SIFT they range from 1 (benign) to 0 (damaging). The threshold benign/damaging is 0.05 for SIFT and 0.90 for PP2. The score indicated by MT is a confidence score for the prediction that the mutation is disease-causing.

"Previously reported mutation" indicates that the damaging effect of the mutation is established because it was previously described in affected patients.

Case	Referral	Sequencing results	*In silico* prediction	MAF	Suggested diagnosis
P1	32 weeks fetus with short and curved long bones. Suspicion of HPP or OI	*ALPL* c.550C > T (p.Arg184Trp) heterozygote	Previously reported mutation	0	Prenatal benign HPP
		ALPL c.984_986delCTT (p.Phe328del) heterozygote	Previously reported mutation	0	
P2	Interrupted fetus with angulation of long bones, low mineralization. Suspicion of HPP	*ALPL* c.1460C > T (p.Ala487Val) heterozygote	PP2: 1 SIFT: 0.01 MT: 0.999 3D: dimer interface	0	Perinatal HPP
P3	Interrupted fetus (20 weeks) with osteochondrosysplasia: short long bones, deformation of long bones, mineralization deficiency: suspicion of HPP	*ALPL* c.299C > T (p.Thr100Met)	Previously reported mutation	0	Perinatal HPP
		ALPL c.1258G > A (p.Gly420Ser)	Previously reported mutation	0	
P4	18 mo female with loss of teeth and low ALP, her	*ALPL*	Previously	0	OdontoHPP

Case	Referral	Sequencing results	*In silico* prediction	MAF	Suggested diagnosis
	twin is affected: suspicion of odontoHPP	c.346G > A (p.Ala116Thr) heterozygote	reported mutation		
P5	59 yo female with spontaneous fractures and low ALP: 30 UI/L (35–120) Suspicion of HPP	*ALPL* c.599G > A (p.Gly200Asp) heterozygote	PP2: 0.994 SIFT: 0.07 MT: 0.999 3D: nonspecific domain	0	Adult HPP
P6	46 yo man with femoral rift and low ALP: 26 U/L (41–120). Suspicion of HPP	*ALPL* c.302_310delACAACACCA (p.Asn102_Asn104del) heterozygote	Previously reported mutation	0	Adult HPP
P7	28 yo female with low ALP and low mineralization (faire confirmer l'indication): suspicion of HPP	*ALPL* c.1328C > T (p.Ala443Val) heterozygote	Previously reported mutation	0	Adult HPP
P8	73 yo female with fractures and low AP: 17 UI/L (35–105) Suspicion of HPP	*ALPL* c.203C > T (p.Thr68Met) heterozygote	Previously reported mutation	0	Adult HPP
P9	41 yo female with pain, tendon calcifications and low AP: 32 U/L (35–105). Suspicion of HPP	*ALPL* c.1381G > A (p.Val461Ile) heterozygote	PP2: 0.014 SIFT: 0.68 MT: 0.989 3D: dimer interface	0.006	?
P10	2 yo female with loss of teeth and low AP: 51 U/L (130–300). Suspicion of odontoHPP	*ALPL* c.1292 T > C (p.Val431Ala) heterozygote	PP2: 0.951 SIFT: 0.04 MT: 0.999 3D: crown domain	0	OdontoHPP
P11	Interrupted fetus with sexual reversion and short	*ALPL*	PP2: 0.984	$5 \cdot 10^{-4}$	CD

Case	Referral	Sequencing results	*In silico* prediction	MAF	Suggested diagnosis
	stature: suspicion of CD	c.436G > A (p.Glu146Lys) heterozygote	SIFT: 0.02 MT: 0.999 3D: nonspecific domain		
P12	Newborn with severe osteochondrodysplasia detected at 22 weeks, hypercalcemia, nephrocalcinosis, many fractures and repeatedly low AP: 95 U/L and 80 U/L (139–449). Suspicion of HPP	*COL1A2* c.1072G > A (p.Gly358Ser) heterozygote	PP2: 1 SIFT: 0 MT: 0.999 Previously reported mutation	0	OI type III
		PHOSPHO1 c.95_97delCCT (p.Ser32del) heterozygote	Na	0.012	
P13	Interrupted fetus (22 w) with fractures of long bones and metaphyseal features: suspicion of HPP or OI	*COL1A1* c.1678G > A (p.Gly560Ser) heterozygote	PP2: 1 SIFT: 0 MT: 0.999	0	OI
P14	88 yo female with atypical femoral fractures: suspicion of HPP	*COL1A2* c.2123G > A (p.Arg708Gln) heterozygote	PP2: 1 SIFT: 0.01 MT: 0.999	0.0008	Heritable disorder of connective tissue?
P15	49 yo female with join pain, chondrocalcinosis and low AP: 25 UI/L (40–100) Suspicion of HPP	*COL1A1* c.517G > A (p.Gly173Arg) heterozygote	PP2: 0.958 SIFT: 0.02 MT: 0.999	0	OI
P16	16 yo female with low BMD (Zscore = − 2SD), diffuse demineralization and low AP: 91 UI/L (120–390) Suspicion of HPP	*COL1A2* c.3712-32A > C heterozygote	MT: polymorphism HSF: Probably damaging (splicing)	0	?
P17	Fetus with short and incurved long bones:	*SOX9*	na	0	CD

Case	Referral	Sequencing results	*In silico* prediction	MAF	Suggested diagnosis
	suspicion of CD	c.813_814insGACTCCGT (p.Val273fs) heterozygote			

3.1. *ALPL* mutations

The fetus P1 was suspected to have OI or HPP on the basis of short and incurved long bones (< 3th percentile, femur: − 2.2 Zscore; humerus − 2.4 Zscore) observed at 32 weeks without fractures and with apparently normal mineralization. The heterozygous mutation c.550C > T (p.Arg184Trp) was detected. No other mutation was found. Screening for large deletions by qPCR was negative. The mutation p.Arg184Trp was previously reported in severe HPP when combined with another mutation [27], but also in moderate HPP (odonto and adult) at heterozygous state [15] and [28]. The dominant effect of the mutation was previously demonstrated by site-directed mutagenesis and transient expression in eukaryotic cells [28]. In addition we previously observed this heterozygous mutation in a case of prenatal benign HPP (our unpublished data). We therefore suggested that the fetus was affected with prenatal benign HPP. In this rare and particular form, the disease may be recessively or dominantly inherited [5], and when it is dominantly inherited the mutation is most often from maternal origin [3] and [5]. Here the mutation p.Arg184Trp was from maternal origin. Genetic counseling provided explanations about this form of HPP and was therefore reassuring. Examination of the baby at birth evidenced only a discreet bowing of the right femur. No other deformation was seen and X-ray showed normal mineralization.

The fetus P2 was tested for *ALPL* gene mutations because of angulation of long bones and abnormal mineralization. The absence of wormian bones and callus did not suggested OI. We found the two heterozygous mutations c.984_986delCTT (p.Phe328del) from maternal origin and c.1460C > T (p.Ala487Val) from paternal origin. The mutation p.Ala487Val was not previously reported. *In silico* tools suggest a damaging effect of this mutation (Table 2) and 3D modeling does not suggest a dominant negative effect. These results confirmed the diagnosis of severe HPP.

The pregnancy of P3 was terminated at 20 weeks because of short and deformed long bones (below the 5th centile), associated with radial deviation of both hands. Post-natal X-rays revealed diffuse hypomineralization, short and bowed long-bones. The forearm bones were severely malformed. Autopsy confirmed the shortening of long bones and radial deviation of hands with presence of 5 rays. Bone histology showed a thickening of the hypertrophic cartilage, persisting cartilage islets in the diaphyseal bone and a defect of membranous ossification. Confirming

the diagnosis of severe HPP two heterozygous mutations were found in *ALPL*, c.299C > T (p.Thr100Met) from paternal origin and c.1258G > A (p.Gly420Ser) from maternal origin. We and others [26] and [29] previously reported these 2 mutations in severe HPP.

Patients P4 and P10 were two young girls (18 mo and 2 yo, respectively) presenting with early loss of teeth. OdontoHPP was suspected. Serum ALP was found low (P4: 94 UI/L, normal range > 120; P10: 51 U/L normal range 130–300). The heterozygous mutations c.346G > A (p.Ala116Thr) in P4 and c.1292 T > C (p.Val431Ala) in P10 confirmed the diagnosis. The mutation p.A116T was previously reported to be responsible for dominant odontoHPP [28] and [30] while the mutation p.Val431Ala was not previously reported. *In silico* a damaging effect is predicted. In addition 3D modeling predicts a possible dominant negative effect due to the localization of Val431 in the crown domain.

Five patients (P5-P9) presented with clinical features of adult HPP, mostly low mineralization, bone fractures without trauma, and when tested, low serum ALP (see Table 2). In each of these patients we found one *ALPL* heterozygous mutation which is coherent with the previous observations that moderate forms of HPP, especially adult and odontoHPP, are mostly due to heterozygous mutations [10] and [15]. The mutation p.Ala443Val was previously observed in a patient with adult HPP [15], the mutations p.Asn102_Asn104del, Thr68Met, and p.Val461Ile were previously observed in severe HPP, associated with others mutations, but the dominant negative effect of these mutations was not tested. However, 3D modeling suggests a possible dominant negative effect of p.Thr68Met and p.Val461Ile. The novel mutation found in P5, c.599G > A (p.Gly200Asp), was predicted to be damaging by *in silico* tools although the SIFT score (0.07) was slightly over the threshold (0.05). 3D modeling did not suggest a dominant negative effect of this mutation.

P11 was a fetus with typical signs of CD, including bone bowing and sexual reversion (46,XY karyotype, female phenotype). For this fetus we did not find any mutation in *SOX9* but the fetal DNA was degraded, probably due to fixation of cells in methanol/acetic acid, and a part of the gene (200 nucleotides in exon 3) could not be analyzed either by NGS or by Sanger methodology. Thus it was not possible to confirm CD. Interestingly we found in this patient the novel heterozygous *ALPL* mutation c.436G > A (p.Glu146Lys) from paternal origin. *In silico* this mutation is predicted damaging by the three prediction softwares, and 3D modeling suggests that this mutation does not have a dominant negative effect. So, for this patient clinically affected with CD, it is not clear whether the CD diagnosis may be confirmed neither there is a role of the ALPL mutation in the phenotype.

3.2. *COL1A1* and *COL1A2* mutations

P12 was a fetus diagnosed at 22 weeks with severe osteochondrodysplasia, very low mineralization and multiple fractures, suggesting HPP or OI. The pregnancy was pursued and among the features observed in the baby, nephrocalcinosis, repeatedly low serum ALP (80 and 95 U/L, normal range 139–449), high Pyridoxal 5′-Phosphate (PLP) (213 nM, normal range < 60), hypercalcemia, hypercalciuria, absence of wormian bones and a large fontanelle were suggestive for HPP diagnosis. We did not find any mutation in *ALPL* but found the heterozygous *COL1A2* mutation c.1072G > A (p.Gly358Ser) previously reported as responsible for OI. We also found the heterozygous sequence variation c.95_97delCCT (p.Ser32del) in *PHOSPHO1*. We hypothesized that this mutation, from paternal origin, could at least in part explain the atypical phenotype suggestive for HPP. However, in addition to its relatively high MAF (0.012), no differences in activity were detected by site-directed

mutagenesis for either pNPP or phosphoethanolamine substrates between wild type and mutant constructs ($p = 0.29$ and $p = 0.23$, respectively). This excludes a role of this variant in the phenotype although these *in vitro* tests do not perfectly reflect *in vivo* function conditions.

P13 was a terminated fetus presenting with fractures of long bones and metaphyseal abnormalities, suggesting HPP or OI. The heterozygous mutation c.1678G > A (p.Gly560Ser) in *COL1A1* was found, indicating that the fetus was affected with OI. This mutation was previously reported (ref [31] and R Dalgleish OI Variant Database (https://oi.gene.le.ac.uk/api/feed.php?select_db=COL1A1) in severe OI and predicted damaging by *in silico* tools (Table 2). The substitution affects a glycine amino acid of the repeated motif (Gly-X-Y), which always results in severe damages because of space constraint in the collagen helix.

Patient P14 was an 88 yo female presenting with atypical femoral fractures. Clinical and biological features of the patient will be extensively described by Funck-Brentano et al. (manuscript in preparation). We found the mutation c.2123G > A (p.Arg708Gln) in *COL1A2*. This mutation was previously reported in an atypical case of Marfan syndrome [32], another heritable disorders of connective tissue with OI and Ehlers–Danlos syndrome (EDS). The mutation affects a highly conserved arginine of a Gly-X-Y motif and *in silico* tools predict a damaging effect of the mutation. However no particular clinical sign of EDS or Marfan syndrome was found in the patient.

Patient P15 was a 49 yo female patient suspected to have HPP because of low serum ALP (25 U/L, normal range 40–100), musculoskeletal pain and tendon calcifications. No *ALPL* mutation were detected but a heterozygous mutation c.517G > A (p.Gly173Arg) in *COL1A1* was found. This unreported mutation, that substitutes a glycine residue, is predicted damaging.

Patient P16 was a 16 yo female presenting with low ALP and diffuse demineralization. A context of mental anorexia could explain these features. The patient is heterozygote for the *COL1A2* mutation c.3712-32A > C predicted to affect splicing by Human Splicing Finder web server (http://www.umd.be/HSF3/). However it was described as a polymorphism by Mutation Taster with a probability of 0.999. The variant was observed once in Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org/) data resulting in an allele frequency of $8.3 \cdot 10^{-6}$.

3.3. SOX9 mutations

The fetus P17 was referred for CD testing because of short and incurved long bones. He carried the novel frameshift mutation c.813_814insGACTCCGT in *SOX9*, allowing us to confirm CD diagnosis.

4. Discussion

On the basis of clinical examination, imaging (X-Ray, ultrasound) and biological data (ALP, PLP, PEA), the distinction between HPP and its main differential diagnoses, CD and OI, is usually assumed to be possible without the need of genetic analysis, the latter simply confirming the diagnosis. However, patient records are often not exhaustive so that the distinction is not always possible, especially in prenatal context where unavailable tools (X-ray, biology) make difficult the correct diagnosis. This is illustrated by our series where 2

fetuses suspected to have HPP and finally found to carry a COL1A1/A2 mutation, and another fetus carrying an *ALPL* mutation and suspected to have OI or HPP while no definitive argument could be made in favor of one of these diagnoses. Interestingly three of our adult patients were suspected to have HPP and were also found to carry COL1A1/A2 mutations, indicating that HPP and OI may be easily mixed up in prenatal context and in adults as well. Moreover, we noted that three of the patients with COL1A1/2 mutations were reported with low serum ALP, yet a marker of hypophosphatasia, and that in two of them none of the conditions that could explain these low levels (hypothyroidism, nutrition problem, bisphosphonates treatment…) were identified. Unfortunately, except patient P12, PLP and PEA levels were not measured in these patients. Given these results it was therefore of interest to design a test including *ALPL*, *SOX9*, *COL1A1* and *COL1A2* genes in order to improve diagnosis rate, turn-around time and costs. Up to date a total of 17 genetic causes of OI have been described [33] but mutations in *COL1A1* and *COL1A2* represent approximately 90% of the cases in European populations [17]. The inclusion in the array of other OI genes as well as other conditions like cleidocranial dysostosis or Stuve–Wiedemann syndrome could still increase the detection rate of mutations.

Excepted for *ALPL* mutations previously reported in HPP patients, assumed to be damaging, the pathogenicity of the mutations detected in this study was estimated by using the prediction softwares POLYPHEN2, SIFT and MUTATION TASTER. According to these tools, most of the mutations described here were predicted damaging, the three softwares giving the same prediction. Together with MAF results, these tools seem efficient to discriminate pathogenic mutations vs polymorphisms. However 2 mutations were not clearly predicted damaging or benign, c.1381G > A (p.Val461Ile) in *ALPL* and c.3712-32A > C in *COL1A2*. The mutation c.1381G > A (p.Val461Ile) found in patient P9 was previously reported by Nielson et al. [12] in a patient with low BMD and low ALP. The three softwares do not suggest a damaging effect while 3D modeling indicates that Val461 is close to the homodimer interface. The variant was not found in 148 controls [12] but the minor allele frequency (MAF) was 0.6% (ExAC data), which may correspond to a rare polymorphism or a mutation. Alignment of 58 mammal TNSALPs shows that position 461 tolerate conservative changes [34], which is the case of Val → Ile change. So although this variant was observed twice in patients with low BMD and low AP ([12] and this study) it remains possible that it is a benign polymorphism. This is also the case of the *COL1A2* intronic mutation c.3712-32A > C found in patient P16. According to Human Splicing Finder the mutation could be damaging but MUTATION TASTER predicted a polymorphism, due to its intronic position and ExAC data. Because of specific HPP features, especially repeatedly low serum ALP, and to the uncertain significance of the *COL1A2* mutation, it remains possible that the patient carried an undetected mutation of the *ALPL* gene.

In conclusion our results confirm the overlap of OI and HPP features in prenatal context, but also in adults, so that a testing strategy including at least *COL1A1*, *COL1A2* and *ALPL* genes is proven useful and efficient.

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