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

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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.

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1. Introduction

Hypophosphatasia (HPP) is a rare inherited skeletal dysplasia due to loss-of-function mutations in the *ALPL* gene encoding the Tissue Non-specific 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 [3–5]. 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 [7–10] 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 [14,15]. 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 [18–20]. 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 [25,26]. 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 [15,26].

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