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A homozygous intronic branch-point deletion in the *ALPL* gene causes infantile hypophosphatasia

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ABSTRACT

Hypophosphatasia (HPP) is a multi-systemic inborn disease with an extraordinary spectrum of severity, ranging from the absence of mineralization to high lethality and it involves different organs including bone, muscle, kidney, lung, gastrointestinal tract and the nervous system. The disease is characterized by low levels of serum alkaline phosphatase, caused by loss-of-function mutations within the *ALPL* gene that encodes the tissue-nonspecific alkaline phosphatase TNAP. Here we present the functional characterization of a gene mutation, detected in intron 7 of the *ALPL* gene of a boy with infantile HPP in whom routine sequencing of the coding region failed to detect any mutation. The homozygous c.793del-14_33 mutation results in the loss of the branch-point motif, relevant for correct *ALPL* pre-mRNA splicing. The main transcript skips exon 8 and codes for a C-terminally truncated TNAP protein of 275 amino acids, which was detected in peripheral blood mononuclear cells and serum from the patient. The functional characterization of recombinant $TNAP_{275}$ revealed no enzymatic activity nor any dominant-negative effect, relevant for the heterozygous parents. Nevertheless correct pre-mRNA splicing can take place without the branch-point sequence to a limited extend, as concluded from the *ALPL* cDNA, obtained from patient's PBMC, and from the low serum AP activity.
These data reaffirm that in clear cut clinical cases, where conventional sequencing including the coding sequence and direct exon-intron-boundaries fails to detect mutations, deeper analyses of regulatory important motifs like branch-point sequences are required to establish a genetic diagnosis.

1. Introduction

Hypophosphatasia (HPP) is an inborn error of bone and mineral metabolism characterized by low levels of serum alkaline phosphatase reflecting loss-of-function mutations within the *ALPL* gene (NCBI gene ID: 249; OMIM *171760) that encodes the tissue-nonspecific alkaline phosphatase (TNAP). TNAP is an ubiquitous membrane bound homodimeric enzyme linked to the cell membrane via a GPI (glycosylphosphatidylinositol) anchor and richly expressed in the skeleton, liver, kidney and developing teeth [1,2]. In HPP decreased levels of TNAP result in an extracellular accumulation of its natural substrates, including pyridoxal-5′-phosphate (PLP), phosphoethanolamine (PEA) and inorganic pyrophosphate (PPi), a potent inhibitor of mineralization. Thus, HPP features defective mineralization of the dentation causing tooth loss and of the skeleton causing rickets-like deformities or osteomalacia. Recent studies also suggest an accumulation of other substrates in the microenvironment, such as phosphorylated adenosine-derived compounds, di-phosphoryl lipopolysaccharide (LPS) and phosphorylated osteopontin (p-OPN) which influence purinergic signalling in multiple tissues. In bone purinergic signalling via ATP, its dephosphorylated metabolites and finally adenosine influences bone formation and resorption as well as mineralization via a series of ligand driven channels and transmembrane receptors [3,4].

Due to its ubiquitous expression and its multiple different substrates TNAP deficiency often results in a multi-systemic disease with involvement of different organs, including bone, muscle, kidney, lung, gastrointestinal tract and the nervous system. The disease has an extraordinary spectrum of severity, ranging from the absence of mineralization, severe lung hypoplasia and high lethality in early onset (<6 months of age) HPP to mild late onset syndromes, including manageable dental problems or recurrent fractures and bone marrow edemas in adulthood [5–7]. Further clinical symptoms include defective bone mineralization with bone deformities, rickets-like changes, chronic non-bacterial osteomyelitis, short stature, failure to thrive, craniosynostosis, neonatal seizures, nephrocalcinosis, muscle weakness, motor developmental delay and dental abnormalities with premature exfoliation of teeth and caries [5,6,8–10].

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Severe HPP occurs in approximately 1 in 1–300,000 live births; the calculated incidence of oligo- and asymptomatic carriers of HPP is 1 in 6–7000 [11,12].

The diagnosis of HPP is made on the basis of clinical, biochemical, and radiologic features [5,6]. Low serum alkaline phosphatase activity for age and sex is the biochemical hallmark accompanied by an elevation of PLP [6]. *ALPL* gene mutation analysis is necessary to document the pattern of inheritance and to better understand the recurrence risk. It is also helpful for prenatal diagnosis [6,13].

HPP management so far has been only supportive. Recently, asfotase alfa, a first-in-class bone-targeted human TNAP enzyme replacement therapy (ERT) has been approved for long-term treatment of bone manifestations in pediatric onset HPP. In non-comparative clinical trials, this compound treatment was associated with skeletal, respiratory and functional improvement in perinatal, infantile and childhood onset HPP [7,14,15].

The molecular causes for this mineralization disorder of bone and teeth are in most cases missense mutations in the *ALPL* gene. Almost 75% of the currently listed 317 different mutations (http://www.sesep.uvsq.fr/03_hypo_mutations.php) lead to an amino acid exchange presumably with impact on the three-dimensional structure of the protein. This can result in an impairment of dimerization capability and/or reduction or even complete loss of enzymatic activity [16,17]. Sequence analyses of the coding regions quickly provide evidence for underlying mutations, nonetheless promoter regions [18,19] and intronic sequences include important regulatory motifs for expression and splicing of the gene, which is finally mandatory for an efficient transcription/translation of TNAP mRNA and protein. So far there is almost no documented information available about mutations in regulatory regions and only little about mutations, which might affect the splicing mechanism. According to the *ALPL* gene mutation database (http://www.sesep.uvsq.fr) the estimated frequency of these mutations is 0.3% and 6%, respectively.

In this report we present the functional characterization of a gene mutation, which was detected in intron 7 of the *ALPL* gene of a boy with infantile HPP in whom routine sequencing of the coding region failed to detect any mutation.

2. Materials and methods

Chemicals of molecular biology grade were obtained from Sigma-Aldrich (Schnelldorf, Germany) if not stated otherwise. All primers used in this study were purchased from Eurofins MWG Operon (Ebersberg, Germany).

2.1. Ethics statement

A written informed consent for genetic analyses was provided by the parents. Blood samples were obtained during routine diagnostic blood draws.

2.2. Subjects and samples

The *parents* were of Turkish origin, consanguineous (first cousins) and healthy. At the time of the assessment (age father 33 years, age mother 29 years), the parents had no clinical symptoms of HPP (dental or musculoskeletal). Both had slightly reduced alkaline phosphatase (AP) activity (father: 24 U/l [nr 40–130], mother: 28 U/l [nr 35–105]), normal serum calcium (father 2,31 mmol/l, mother 2,34 mmol/l [nr 2,10–2,60]), normal serum phosphate (father 1,42 mmol/l, mother 1,30 mmol/l [nr 0,6–1,8]), normal PTH (father 38,0 pg/ml, mother 26,2 pg/ml [nr 14,9–56,9]) and low 25(OH)-vitamin D3 (father 19 μg/l, mother 4 μg/l, [nr 30–70]) .

The *patient* was delivered spontaneously at term after an uneventful pregnancy. The first symptoms occurred in the first 5 months of life including a wide open and bulging fontanelle, recurrent vomiting, failure to thrive and muscular hypotonia. He was diagnosed of complex craniosynostosis with premature fusion of the coronal and sagittal sutures and signs of increased intracranial pressure. At the age of 18 months the clinical diagnosis of HPP was suspected and prompted further diagnostic procedures (laboratory analysis, etc.). Skull remodelling was performed at the age of 19 months.

Starting at the age of 18 months premature loss of deciduous teeth occurred. At the age of 3 years he developed a pathologic non-dislocated, distal humerus fracture and at the age of 4 years a pathologic non-dislocated, spiral fracture of the left femur occurred. Physical examination revealed rickets-like changes with metaphyseal widening of the wrists, genua valga, flexion contractures of both hips and knees and a bell shaped thorax. During infancy he developed recurrent infections of the upper and lower respiratory tract (bronchitis, pneumonia and chronic sinusitis) but never needed any ventilatory support.

He showed a delayed motor but normal mental development. He started to walk at the age of 30 months displaying a waddling gait due to his muscular hypotonia. Later on he often complained about pain in the lower extremities and reduced physical capacity with tiredness and exhaustion after a walking distance of below 500 m and he was not able to climb stairs. MRI revealed inflammatory metaphyseal bone lesions and marrow edema of the lower extremities. Antiinflammatory treatment starting at the age of 5 years resulted in a marked improvement of walking distance and overall activity.

Laboratory results at the age of 18 months showed low serum AP activity (15–23 U/l, [nr 110–550]), elevated PLP in the plasma (150 ng/ml, [nr 5–30]). Serum calcium (2,42 mmol/l, [nr 2,19–2,64]) and phosphate (1,87 mmol/l, [nr 1,00–1,95]) were high but in the normal range; creatinine (0,2 mg/dl, [nr 0–0,35]) was normal; and 25(OH)-vitamin D3 (30,5 μg/l, [nr 30–70]) and parathyroid hormone (16 ng/ml, [nr 15–65]) were low but in the normal range. Unfortunately urinary parameters were not available at this time. X-rays demonstrated defects of mineralization especially in the long bones. Kidney ultrasound showed bilateral nephrocalcinosis. Cardiac (electrocardiogram, echocardiography) and neuronal (cerebral ultrasound / magnetic resonance tomography, standard electroencephalogram) diagnostic procedures did not reveal any pathological findings.

Clinical features, laboratory results and radiological findings were consistent with infantile HPP. Nevertheless routine genetic analyses of the *ALPL*-gene by PCR and Sanger sequencing did not detect any mutation. According to Mornet et al. these analyses provide evidence for mutations in the coding sequence and direct exon-intronic boundaries with an analytic sensitivity of 95% [13], but fail to detect for example deeper intronic mutations.

Shortly before his 6th birthday (weight 16.7 kg [3rd percentile], length 106.5 cm [2 cm below 3rd percentile]), he was enrolled in a clinical phase 2 ERT study with recombinant TNAP (Asfotase alfa, Alexion Pharma, NCT01176266). Now, at the age of 9 years (weight 31 kg [50–75th percentile], length 132 cm [25th percentile]) he is going to primary school, able to participate in sports lessons and almost never complains about pain in the lower extremities still treated with Strensiq® (Asfotase alfa).

2.3. Genetic examination of the ALPL gene

Genomic DNA was isolated from blood samples with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), RNA was isolated from peripheral blood mononuclear cells (PBMC) with the NucleoSpin RNA Kit (Macherey-Nagel, Düren, Germany), according to manufacturer's instruction. For amplification of extremely GC-rich sequences within the promoter region a modified 'slowdown PCR protocol' [20] was applied. Briefly, 100 ng gDNA were incubated with 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Triton X-100, 0.1 μM of each primer, 200 μM dATP, 200 μM dCTP, 200 μM dTTP, 50 mM dGTP, 150 μM dc7GTP (Roche Diagnostics, Mannheim, Germany) and 1 unit Taq-DNA-Polymerase (Peqlab, Erlangen, Germany). For heating a ramp