Original paper

Identification of a molecular defect in a stillborn fetus with perinatal lethal hypophosphatasia using a disease-associated genome sequencing approach

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Lethal skeletal disorders represent a heterogeneous and clinically variable group of genetic conditions, usually difficult to diagnose without post-mortem radiological assessment. Here we report on a stillborn patient delivered at 22 weeks of gestation who presented with severe skeletal symptoms comprising limb shortening and intrauterine fractures detected upon prenatal ultrasound and autopsy examination. Since post-mortem X-ray was refused and no phenotypic diagnosis could be attempted, we performed next-generation sequencing (NGS) of 2741 genes associated with all known Mendelian disorders. With this strategy, we were able to demonstrate the diagnosis at a molecular level, which turned out to be perinatal lethal hypophosphatasia (HPP). This severe form of HPP represents an inborn defect of ossification often resulting in stillbirth or postnatal death. The NGS panel revealed compound heterozygous *ALPL* missense mutations: c.1283G>C(p.Arg428Pro) and c.1363G>A(p.Gly455Ser). Mutations detected in our case, although previously described in other patients, have not been reported to co-occur in a single individual. The diagnosis established in our index using the NGS-based approach could have been successfully reached by standard radiography. Thus, our report points to the importance of X-ray examination in stillborn cases and highlights the emerging role of NGS strategies in the diagnostic process of prenatally manifesting skeletal disorders.

Key words: hypophosphatasia, ALPL, skeletal dysplasia, prenatal diagnosis, NGS.

Introduction

Lethal skeletal disorders are an extremely heterogeneous group of genetic conditions, which pose a significant diagnostic challenge to the clinical geneticist, especially when resulting in early prenatal death of the fetus [1]. One example of such disease is hypophosphatasia (HPP), an inborn error of bone

metabolism, historically classified as skeletal dysplasia. HPP is a rare genetic disorder characterized by decreased bone density and reduced activity of alkaline phosphatase, resulting from mutations in the liver/bone/kidney alkaline phosphatase gene – *ALPL* (MIM171760) – also referred to as tissue non-specific alkaline phosphatase (*TNAP*) [2, 3]. The gene is located on chromosome 1p36.1, composed of 12 exons, and encodes for a protein hydrolyzing phosphomonoesters with generation of inorganic phosphate [4, 5]. TNAP protein binds to the cell surface of osteoblasts, chondrocytes, and their matrix vesicles, playing an essential role in bone mineralization and formation of primary teeth [6, 7]. Loss of protein function leads to the accumulation of three substrates: inorganic pyrophosphate (PPi), which is a known inhibitor of calcification, pyridoxal 5'-phosphate (PLP), and phosphoethanolamine (PEA) [8].

Six clinical forms of HPP have been traditionally described in the literature, depending on the severity of symptoms and age of onset, i.e. perinatal lethal, prenatal benign, infantile (OMIM #241500), childhood (OMIM #241510), adult, and odontohypophosphatasia (OMIM #14630) [9]. All forms of HPP present with low serum alkaline phosphatase activity and variable bone involvement. The latter feature is however not observed in odontohypophosphatasia, which is characterized by dental abnormalities with no overt skeletal disease. HPP exhibits extreme clinical heterogeneity, ranging from lethal perinatal forms resulting in intrauterine or postnatal death to less severe phenotypes not influencing the lifespan. The broad clinical spectrum of HPP includes poor bone mineralization, thoracic and cranial deformities, long bone shortening and/or bowing, micromelia, and pathologic fractures [10, 11]. In the infantile form, apart from bone hypomineralization and fractures, premature loss of teeth, craniosynostosis, failure to thrive, hypotonia, and pulmonary insufficiency have been described. The milder types (childhood and adult) include delayed gross motor development, myopathy, low bone density, chronic pain of muscles and/or bones, premature loss of teeth, abnormal dentition prone to caries, osteomalacia or pseudogout [10, 11, 12, 13]. The most severe forms of HPP are transmitted in an autosomal recessive manner, whereas the milder types usually result from autosomal dominant mutations or less frequently recessive alterations. Genotype-phenotype correlations suggest that the most severe mutations are located in the active site and its vicinity, the active site valley, the homodimer interface, the crown domain, and the metal binding site of TNAP [7, 14, 15, 16].

The introduction of next generation sequencing (NGS) technologies into medical practice has led to unravelling many genes involved in the pathogenesis of human genetic disorders. Most of the NGS-based studies have used whole-exome sequencing (WES) or whole-genome sequencing (WGS) approaches, which are known to generate massive amounts of uninterpretable sequence variants with unclear clinical significance. In the diagnostic setting, gene panels, although providing less data, seem to be the optimal solution, as they usually allow for reliable interpretation of the detected variants. One interesting example of a comprehensive gene panel strategy was proposed in 2014 by Zemojtel *et al*. [17] and comprised selective targeting of the "disease-associated genome" (DAG), encompassing a total of 2741 known Mendelian genes (all known at that time).

In this report we applied NGS-based DAG panel sequencing in order to establish a diagnosis in a stillborn child affected by severe lethal skeletal dysplasia of unknown origin. With this approach, we detected compound heterozygous *ALPL* mutations demonstrating that the fetus presented with the perinatal type of HPP.

Clinical description

The index fetus affected by a prenatally unrecognizable skeletal disorder was stillborn at 22 weeks of a second gestation. The proband was conceived by a healthy, non-consanguineous couple (31-year-old mother and father), who already had a healthy daughter born from the first pregnancy. The family history revealed that the maternal grandmother of the index case had a single spontaneous abortion, while the sister of the maternal grandfather presented with short stature and rickets. Prenatal ultrasound scans performed in the index fetus at 18 weeks of gestation showed a 4-week delay in femoral length (FL) and humeral length (HL) in comparison with biparietal diameter (BPD), occipitofrontal diameter (OFD), and head circumference (HC). Amniocentesis performed at the same time allowed for conventional cytogenetic GTG banding and showed normal male karyotype (46,XY). Repeated ultrasound examination at 21+2 weeks of gestation revealed abnormal shape and significant shortening of the lower limbs (FL of 24.8 mm; Fig. 1A, B) and moderate shortening of the upper limbs (HL of 33.0 mm) in reference to BPD, OFD, and HC. In addition, the FL to AC ratio was recorded as 14%. Five days later (at 22 weeks of gestation), ultrasound examination revealed absence of the fetal heart rate, and the mother spontaneously delivered a stillborn male fetus, with a birth weight of 530 g. Autopsy examination confirmed that the fetus had severely shortened lower extremities and spontaneous intrauterine fracture of the right lower leg bones, with signs of bone healing. In addition, generalized ischaemia of the internal organs was noted.

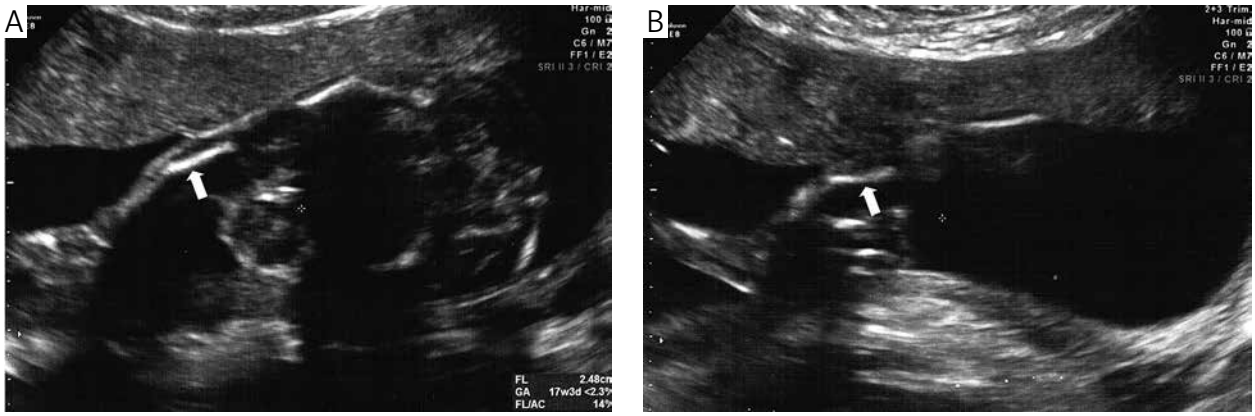

Fig. 1. Ultrasound images of the index patient at 21 + 2 weeks of gestation. Shortening and abnormal shape of the right (A) and left (B) femoral bones associated with a severe manifestation of hypophosphatasia observed in our proband

The Institutional Review Board at the Poznan University of Medical Sciences approved the study, and written informed consent was obtained from all subjects or their legal guardians. Postnatal fetal photographs were unavailable for publication.

Methods

Next generation sequencing panel

Since no clinical diagnosis could be established in our proband, we decided to perform an NGS panel targeting all 2741 genes known to be associated with Mendelian disorders (the disease-associated genome – DAG) [17]. A chorionic sample for genetic testing was taken after placental delivery of the stillborn fetus. Next, genomic DNA was extracted according to standard procedures and subjected to next generation sequencing as described elsewhere [17]. The sample was sequenced using an Illumina HiSeq 1500 sequencer with a mean coverage of 360 reads, with 98% of the target region covered by at least 20 reads. In order to extract potentially causative variants from NGS data, we applied a computational algorithm termed Phenotypic Interpretation of eXomes (PhenIX). The software allows one to filter and rank the variants on the basis of population frequency, predicted pathogenicity as well as clinical relevance by calculating the match between observed symptoms, entered in the form of human phenotype ontology (HPO) terms, and detected gene alterations [17, 18]. In order to calculate the score for clinical relevance we entered the following HPO terms: lethal skeletal dysplasia (HP:0005716), rhizomelia (HP:0008905), and increased susceptibility to fractures (HP:0002659).

Sanger sequencing analysis

Genomic DNA of the parents was extracted from peripheral blood leukocytes using standard protocols. In order to validate the NGS results and test the parents we performed targeted Sanger sequencing. The reactions were performed with dye terminator chemistry (ABI Prism DigDye v3.1) and run on an automated sequencer: Applied Biosystems Prism 3700 DNA Analyzer. The sequencing results were visualized using Bioedit software.

Results

Because the fetal phenotype was strongly suggestive for skeletal disorder (probably skeletal dysplasia) and a postnatal baby-gram was not performed, we decided to apply NGS technology to establish the diagnosis at a molecular level. The NGS DAG panel revealed the compound heterozygous *ALPL* missense mutations c.1283G>C (p.Arg428Pro) in exon 10 and c.1363G>A (p.Gly455Ser) in exon 11 (NM_000478, NP_000469; Fig. 2A, B), which seemed to be the only variants relevant to the clinical phenotype. PhenIX used to interpret the NGS results ranked the two variants in 3rd place with a gene relevance score of 0.781 and a variant score of 1.00 (a total score of 0.891). Both mutations were further confirmed by Sanger sequencing in the index patient (Fig. 2C, D). Next, parental studies demonstrated that c.1283G>C (p.Arg428Pro) mutation was maternal (Fig. 2C), whereas c.1363G>A (p.Gly455Ser) was inherited from the father (Fig. 2D), corroborating "in trans" orientation of both alterations in our proband. To further assess the impact of both *ALPL* mutations on the functionality of alkaline phosphatase, we tested its activity in the serum of both parents. The mother, who was the carrier of the p.Arg428Pro variant, was shown to have enzyme activity of 16 U/l, while the father – a carrier of the p.Gly455Ser variant – had activity of 50 U/l (reference range: 40-150 U/l).

Discussion

Lethal skeletal disorders are an extremely heterogeneous group of genetic conditions, usually

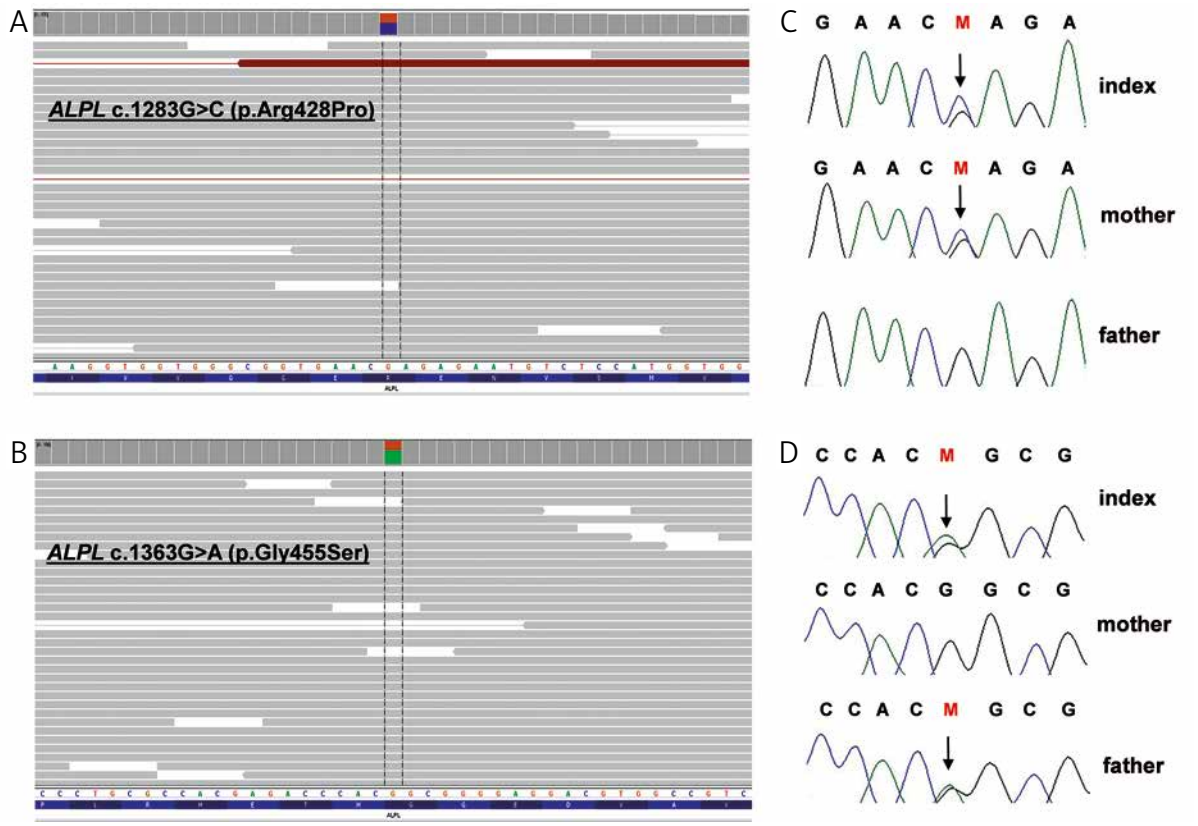

Fig. 2. Representation of the compound heterozygous *ALPL* mutations detected in the proband by means of NGS-based disease-associated genome sequencing. The proband was demonstrated to carry two missense substitutions: c.1283G>C (p.Arg428Pro) in exon 10 (A) and c.1363G>A (p.Gly455Ser) in exon 11 (B). Targeted validation studies of the *ALPL* in the proband and parental testing performed using Sanger sequencing. Mutation c.1283G>C (p.Arg428Pro) in exon 10 was observed in heterozygous state in the index patient and his unaffected mother (C), whereas mutation c.1363G>A (p.Gly455Ser) in exon 11 was detected in the heterozygous state in the index patient and his healthy father (D). Mutation in figures c & d was depicted with "M" and indicated by an arrow

recognizable on the basis of prenatal ultrasound, autopsy examination, and post mortem radiography (baby-gram). Several studies have shown that a specific diagnosis can be achieved in 77% or 84% of prenatally suspected skeletal dysplasia cases, provided availability of detailed and accurate phenotypic data [19, 20]. In this report, we demonstrate an alternative approach that allowed us to identify a specific genetic diagnosis in a stillborn fetus with lethal skeletal disease, in whom correct post mortem examination (including X-ray) was not performed. Using NGS-based DAG panel sequencing, we were able to demonstrate a molecular diagnosis, which turned out to be perinatal lethal hypophosphatasia (pl-HPP). This severe form of HPP represents an inborn defect of ossification resulting in either stillbirth or early postnatal death. Clinical features comprise shortened lower limbs often with femoral and tibial bowing, multiple fractures, short ribs, narrow thorax, poorly ossified epiphyses, large fontanelles, osteochondral spurs, apnoea, and hypercalcaemia [20, 21, 23]. The fetal demise results mainly from generalized bone hypomineralization and respiratory failure due to thorax deformities and lung hypoplasia [9]. In general, the diagnosis of HPP is based on the prenatal ultrasound scan, postnatal X-ray imaging, and evaluation of serum ALP activity. Other laboratory findings of HPP include elevated plasma PLP, elevated serum PPi, and elevated serum or urinary PEA [10].

Upon repeated prenatal ultrasound scans, our patient did not show osteochondral spurs that are pathognomonic for pl-HPP, but presented with shortening of all limbs, lower limb deformities, and an FL to AC ratio of 14%, which falls within the range of lethal skeletal dysplasias [24]. Additionally, autopsy examination showed fractures of the right lower leg. Since stillbirth occurred at 22 weeks of gestation, laboratory blood testing could not be performed. Moreover, postnatal X-ray imaging (a baby-gram) was declined by the physician, so the clinical data were very limited and insufficient to attempt the diagnosis, which could involve at least several genetic conditions, including osteogenesis imperfecta type II, achondrogenesis, campomelic dysplasia, and others (Table I). Using NGS-based DAG panel sequencing, the index was demonstrated to harbour two patho-

Table I. Lethal skeletal dysplasias that clinically overlap with hypophosphatasia along with their clinical and molecular characteristics [1, 3]

Condition	Hypomineralisation of the skull	Hypomineralisation of the vertebrae	Shortened long bones	Bent/bowed bones	Club foot	Fractures of long bones	MIM number	Gene defect
Hypophosphatasia	+	+	+	+	+	+	241500	ALPL
Osteogenesis imperfecta type II	+		+		+	+	166210	COL1A1
							166210	COL1A2
Campomelic dysplasia			+	+	+	+	114290	SOX9
Achondrogenesis IA		+	+	+			200600	TRIP11
Achondrogenesis IB		+	+	+			600972	SLC26A2
Achondrogenesis II		+	+				200610	COL2A1

ALPL – alkaline phosphatase, liver/bone/kidney; COL1A1 – collagen type I, alpha-1 chain gene; COL1A2 – collagen type I, alpha-2 chain gene; SOX9 – sex determining region Y-box 9 gene; TRIP11 – thyroid hormone receptor interactor 11 gene; SLC26A2 – solute carrier family 26 (anion exchanger), member 2; COL2A1 – collagen type II, alpha-1 chain gene

genic missense alterations in the *ALPL* gene, i.e. maternally inherited c.1283G>C(p.Arg428Pro) and paternally inherited c.1363G>A(p.Gly455Ser) mutations. Both variants have been previously described in the literature to be associated with hypophosphatasia [25, 26]. The mutation p.Arg428Pro was described by Spentchian *et al*. [26] in combination with c.997+2T>A (intron 9) splice site mutation in a male newborn presenting with pl-HPP, who died at day 14 after birth due to acute respiratory failure. Radiographic examination showed slender ribs, a small bell-shaped thorax, generalized poor bone mineralization, and erosive changes of the epiphyses of all long bones, while the serum ALP level was very low – 0.04 μmol/s*l (reference range: 0.4-4.17 μmol/s*l). The second pathogenic alteration (p.Gly455Ser) detected in our proband was previously described by Draguet *et al*. [25] in association with the c.341C>G+c.348_349insACCGTC (p.A114G+p.A116_Y117insTV) mutation in a 4-year-old girl affected by the childhood form of HPP. This proband presented with relatively mild symptoms of HPP comprising growth retardation, rickets, premature loss of teeth, bone deformities, and a reduced serum ALP level to 67 and 86 mU/ml (reference range: 100-720 mU/ml). Unlike the patient described by Draguet *et al*., our index manifested perinatal lethal HPP phenotype, suggesting possible severity of the p.Gly455Ser mutation in case of its association with the p.Arg428Pro allele. Alternatively, carrier state of the very severe p.Arg428Pro mutation in the mother may be of great clinical significance due to the deleterious in utero effect of maternal *ALPL* mutations on the baby [27]. We also tested ALPL in the serum of both carrier parents, demonstrating that heterozygosity for p.Arg428Pro and p.Gly455Ser variants was associated with enzymatic activity of 16 U/l and 50 U/l, respectively (reference range: 40-150 U/l), which further confirmed pathogenicity of the mutations. Finally, location of both mutations in the vicinity of the catalytically active site (position 455) or in the crown domain (position 428) supports their damaging effect on ALPL function [28, 29].

In conclusion, our paper describes a stillborn patient delivered at 22 weeks of gestation, presenting with pl-HPP. Mutations detected in our case, although previously demonstrated in other patients, have not been previously reported to co-occur in a single individual. Therefore, our paper may represent a foundation for the prognosis and genetic counselling of other individuals carrying identical mutations. Since the parents were declined access to an X-ray, the diagnosis in our index had to be established by means of NGS-based DAG panel sequencing, although it was apparently achievable with standard radiography. Thus, in our report we strongly emphasize the importance of routine X-ray examination, which should still be a first tier study in similar cases. Finally, we also highlight the emerging role of NGS strategies in the diagnostics of prenatally manifesting skeletal disorders, which should however be reserved only for cases in which clinical data are too sparse to allow for accurate clinical diagnosis and targeted gene analysis.

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The authors declare no conflicts of interest.
Accession numbers: NM_000478, NP_000469.

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