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CASE REPORT

Novel heterozygous *tissue-nonspecific alkaline phosphatase* (*TNAP*) gene mutations causing lethal perinatal hypophosphatasia

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Abstract Hypophosphatasia is a rare inherited disorder characterized by poor bone mineralization and deficiency of alkaline phosphatase activity. It is caused by mutations in the liver/bone/kidney alkaline phosphatase gene encoding the tissue-nonspecific isoenzyme of alkaline phosphatase (TNAP), which displays many allelic heterogeneities, leading to different clinical phenotypes. This study reports the case of a patient diagnosed with lethal perinatal hypophosphatasia. His gene analysis showed compound heterozygocity of two novel mutations: c.650delTinsCTAA and c.984_986delCTT, which led to p.217delVinsAK and p.328delF, respectively. The two mutations originated separately from his parents, consistent with autosomal recessive perinatal hypophosphatasia. For these two novel mutations, we analyzed their functions through three-dimensional structural analysis. This revealed that V217 is located in the β-sheet area, V217 is deleted, and insertion of alanine and lysine alter the secondary structure, causing instability in the hydrophobic region, which may influence the metal-binding vicinity. This mutant structure loses its catalytic activity. Deletion of 328F also results in protein structural alteration and affects TNAP functions. These results may provide an explanation of the two novel mutated alleles correlating with the lethal phenotype of our patient. In conclusion, we demonstrated the case of a patient with lethal perinatal hypophosphatasia caused by two novel heterozygous mutations.

Keywords Hypophosphatasia · *Tissue-nonspecific isoenzyme of alkaline phosphatase* · Mutation · Structure modeling

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Introduction

Hypophosphatasia (OMIM no. 171760) is a rare inherited disorder characterized by poor bone mineralization and deficiency of alkaline phosphatase activity [1–4]. It is caused by mutations in the liver/bone/kidney alkaline phosphatase gene encoding the tissue-nonspecific isoenzymes of alkaline phosphatase (TNAP) [1, 2]. Although the exact role of TNAP in bone mineralization is not clear, it is involved in hydrolyzing inorganic pyrophosphate [5]. Deleterious mutation of TNAP protein leads to extracellular accumulation of inorganic pyrophosphate, pyridoxal-5′-phosphate and phosphoethanolamine (PEA) [3, 4]. Inorganic pyrophosphate impairs tooth and bone mineralization. An increased level of urine PEA supports the diagnosis of hypophosphatasia [1, 2].

Clinical manifestations of hypophosphatasia are divided into six classes: perinatal lethal, perinatal benign, infantile, childhood, adult and odontohypophosphatasia [1, 2]. The

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perinatal lethal and infantile forms are autosomal recessive, while the other milder forms are either autosomal dominant or recessive [1, 2]. Traditionally the diagnosis is usually made on the basis of clinical features, bone imaging, low serum alkaline phosphatase activity and elevated PEA. Genetic testing was used for making the current diagnosis of hypophosphatasia [6]. We here present a patient who was affected with lethal perinatal hypophosphatasia because of a novel combination of heterozygous TNAP mutations. We also used three-dimensional (3D) structural analysis to predict functional impairment of the mutant TNAP protein, which correlated with patient's lethal condition.

Clinical report

This patient was a 12-h-old male infant referred to our hospital for acute respiratory distress. Nasal continuous positive airflow pressure was required to support his respiratory insufficiency. He was a full-term infant of a G3P2SA1 mother who delivered by Cesarean section.

On physical examination, his weight was 2796 g (10–25th percentile), length was 45 cm (<10th percentile) and head circumference was 31.5 cm (<10th percentile). He had a plump face, rachitic chest wall, soft calvarium and skull bone, and short limbs. Radiographs demonstrated thin ribs, thoracic cage deformities, poor ossification of the skull, and epiphysis of the bilateral humerii, radii and ulnas (Fig. 1). Biochemistry revealed undetectable serum alkaline phosphatase (ALP; normal range 60–220 U/l), elevated serum phosphate of 8.6 mg/dl (range 2.5–4.5 mg/dl) and normal serum calcium of 2.2 mmol/l (range 2.02–2.6 mmol/l), while parathyroid hormone levels were normal (33.4 pg/ml; range 12–72 pg/ml). Urine excretion of PEA was markedly elevated at 927.1 mmol/molCRE (normal range 0–70 mmol/molCRE). Considering the clinical features and laboratory results, the male infant was diagnosed as having hypophosphatasia.

Tracing the family history, both his parents are ethnic Han Chinese. They are asymptomatic, married and non-consanguineous (Fig. 2a). The mother previously had a spontaneous abortion at 20 weeks' gestation, without known etiology. Both father and mother had normal serum ALP levels at 89 and 62 U/l, respectively. After genetic counseling, the parents agreed to undergo an ALPL gene study and signed the informed consent. Leukocyte DNA was obtained from the patient and his parents for polymerase chain reaction and bi-directional sequencing. Two novel mutations were found in both alleles of this patient; they were separately from his parents. The mother's mutation was c.650delTinsCTAA (p.217delVinsAK) (Fig. 2b), and the father's mutation was c.984_986delCTT (p.328delF) (Fig. 2c). Genetic testing confirmed the diagnosis of autosomal recessive hypophosphatasia of perinatal type. The patient developed progressive respiratory failure and repeated pneumonia. He died at the age of 28 days because his parents refused to allow resuscitation because of his poor prognosis.

Discussion

We have identified two novel mutations in the TNAP gene that result in the phenotype of lethal perinatal hypophosphatasia. Our patient presented severe clinical manifestations such as defective bone mineralization, abnormal serum ALP and elevated urine PEA levels. Without active treatment, he eventually succumbed to respiratory failure. Sequencing analysis of the *TNAP* gene revealed compound heterozygous mutations. The two alleles were c.650delTinsCTAA and c.984_986delCTT, which resulted in deletion of valine and insertion of alanine and lysine at position 217, and deletion of phenylalanine at position 328, respectively. To our knowledge, these two mutations have not been reported yet.

To investigate the correlation of phenotype and genotype, we analyzed protein functions using 3D structural analysis [7]. For comparative structural modeling, we simulated these two novel mutants using the MODELLER program based on the template of the crystal structure of human placental alkaline phosphatase (PDB code: 1ew2), which is an isoform of TNAP and shares the same active site at zinc and magnesium areas [8–10]. The structural basis for active homodimer of TNAP and physically hydrolyzing inorganic pyrophosphate is now well understood [8, 9]. Mutations altering the structure of the active site cleft, including itself, the roof and floor, may cause the various degrees of functional impairment, resulting in a variety of disease presentations [8–10]. The 3D structure of the two novel mutations was generated using the UCSF Chimera program.

In the 3D structure (Fig. 3a), V217 is located in a β-sheet, and its residue is buried in the TNAP hydrophobic region, which contributes the floor of the active site cleft [8]. Deletion of V217 and insertion of alanine and lysine, which do not belong in β-sheet principle amino acids and have a large polar side chain, cause steric hindrance and disrupt the β-sheet structure, affecting the proper folding of the TNAP protein (Fig. 3b) [11]. The structural alteration may indirectly affect the active site, resulting in diminished catalytic activity [10, 12]. This mechanism is similar to the one found in a prior study of missense mutations in the same β-sheet, p.I218T, which affect the hydrophobicity of the same region, causing functional impairment and clinically perinatal lethal hypophosphatasia [12, 13]. The

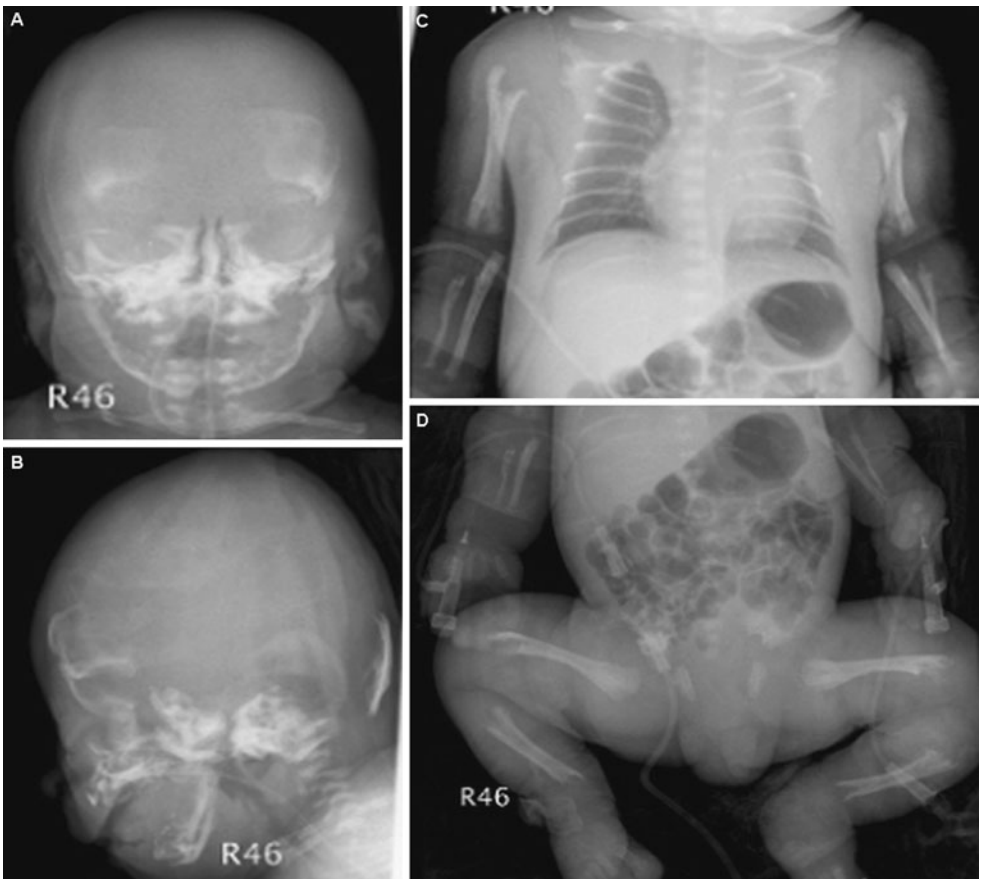

Fig. 1 Patient radiography. **a**, **b** Anterior-posterior and lateral views of the skull reveal diffuse demineralization and globular cranial shaping, with calcification scattered in the frontal and occipital bones. **c** Chest radiography shows generally thin and widened ribs, lacking ossification. **d** Diffuse irregular bilateral lower metaphyses for distal femurs

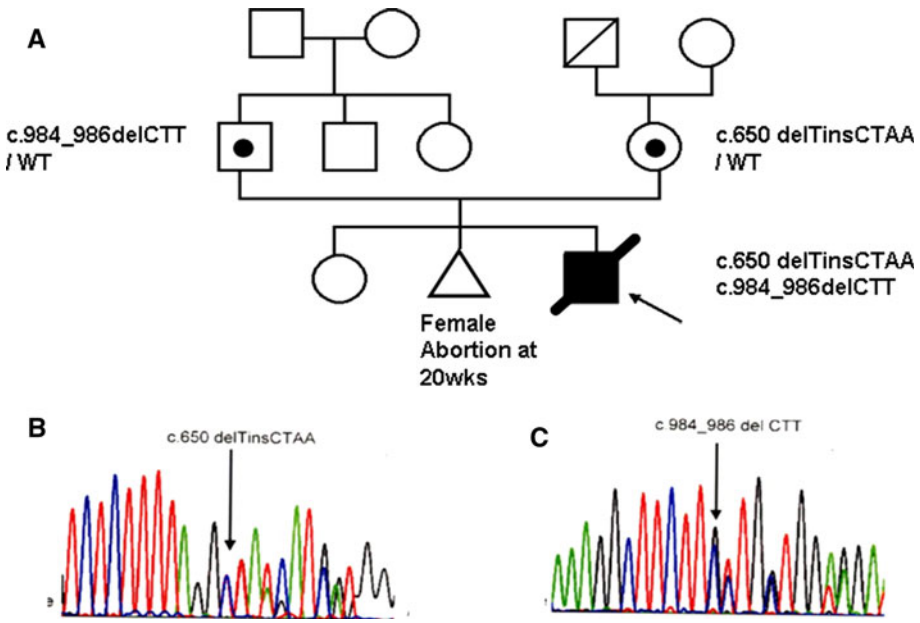

Fig. 2 Pedigree of this family with sequencing data for the two novel mutations in the TNAP gene

p.I218T missense mutation in the COS-7 cell yielded 3.7% of enzymatic activity compared with wild-type TNAP protein [12]. Furthermore, this β-sheet displays a high degree of conservation of amino acid during the evolution between fish and *Homo sapiens* (data not shown). These facts reflect that mutations in this area, like

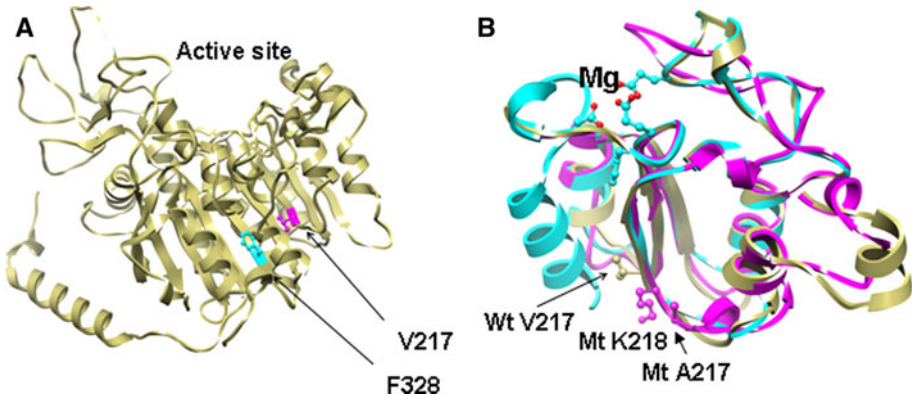

Fig. 3 3D modeling structure of TNAP. **a** Ribbon presentation of the wild-type (wt) TNAP monomer. V217 and F328 are located in the β-sheets, which contribute to the stability of the hydrophobic region. **b** Comparative structural modeling of the p.217delVinsAK mutation (mt) TNAP monomer (*pink color*), superimposed on the wt TNAP (*khaki color*) and placental alkaline phosphatases (PDB code: 1ew2, shown in *blue*). Wt TNAP and placental alkaline phosphatases are aligned closely in the β-sheet area and the metal-binding vicinity. However, changes in A217 and K218 in mt TNAP disrupt the β-sheet secondary structure and may cause steric hindrance because of the large polar side chain of K218. The structure of the metal-binding site was also influenced, which may explain the loss of catalytic functions of mt TNAP

p.217delVinsAK or p.I218T, can lead to severe functional impairment of TNAP, consistent with the clinical lethal phenomenon. For another mutation, the trinucleotide deletion (c.984_986delCTT) leads to deletion of phenylalanine at position 328 (p.328delF). F328 lies at the β-sheet and contributes to the hydrophobic region of TNAP. Deletion of F328 may disrupt the local structural stability and cause regional rearrangement. Notably, the mutation p.327delF of TNAP, which has the same amino acid sequence as p.328delF, shows about 10–20% activity when this mutant protein is expressed in the COS-7 cells; it is measured by the Bessey-Lowry method using *p*-nitrophenylphosphate as a substrate [14]. Taken together, 3D structural analyses show the marked functional defects caused by genetic mutations of c.650delTinsCTAA and c.984_986delCTT, compatible with the clinical phenotype of lethal hypophosphatasia in our patient.

Currently, there are 224 known deleterious mutations in the TNAP gene that contribute to various types of hypophosphatasia (for a review, see the TNSALP gene mutation database at the SESEP website). Their different consequences on enzyme activity account for the clinical heterogeneity of the presentations among patients [15]. Disease severity variably ranges from intrauterine death without mineralized bone to a mild adult form involving pathological fracture or odontohypophosphatasia [1, 2]. Genetic tests are currently used to diagnose hypophosphatasia; however, a great effort is still needed to correlate genotypes and phenotypes as most patients have compound heterozygosity of TNAP genes, making it difficult to determine the respective roles of each mutation. Study of mutation effects of disease genes usually proceeds through epidemiological phenotype-genotype study or functional assays. However, epidemiological phenotype-genotype study is not suitable in rare mutations; functional assays are not widely available in clinical practice, although they provide qualitative evidence to characterize the mutant proteins. Therefore, we used 3D structural modeling to predict whether functional impairment of the two novel mutants exists. Significant structural alteration of the two novel mutants is shown, so impaired phosphatase function can be strongly predicted. This finding correlates with the clinical presentation of our patient.

In conclusion, we presented a case of lethal perinatal hypophosphatasia, caused by novel heterozygous deleterious mutations in the TNAP gene. Using 3D structural modeling, we characterized the two novel mutations, which led to significant structural alteration resulting in the loss of phosphatase activity. This finding will prove relevant for genetic counseling and perinatal gene testing for affected families.

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