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ARTICLE

Unexpected high intrafamilial phenotypic variability observed in hypophosphatasia

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Hypophosphatasia (HPP) is a clinically heterogeneous rare, inherited disorder of bone and mineral metabolism with extensive allelic heterogeneity in the *ALPL* gene. In this report, we present a family with heterozygous parents (maternal p.(Glu191Lys), paternal p.(Gly334Asp) mutations in the *ALPL* gene) and four children (one genotypically normal, one heterozygous carrier and two compound heterozygous) showing an unexpected high phenotypic variability. One of the compound heterozygous showed clinical symptoms of the mild childhood form mainly affecting the teeth. The other one was more seriously affected with severe failure to thrive, delayed motor development, need for oxygen supply and profound mineralization deficit compatible with an infantile form of HPP. Functional *in vitro* studies identified p.(Glu191Lys) as mild (68%, no dominant-negative effect) and p.(Gly334Asp) as severely affected allele (1.2%, dominant-negative effect). *In vitro* simulation of the children's genetic status showed a residual AP activity of 29%, while the biochemical AP activity in the serum was comparably reduced in both children (22 and 36 U/l). This family report indicates that mapping *ALPL* mutations within the gene does not necessarily help to predict the clinical severity of the phenotype. Therefore, results of prenatal diagnostics have to be interpreted with caution and prenatal genetic diagnosis and counseling for HPP should be provided within an experienced multidisciplinary team. Research about other confounding factors is urgently needed.
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INTRODUCTION

Hypophosphatasia (HPP) is a rare, inherited disorder of bone and mineral metabolism, caused by mutations in the *ALPL* gene encoding the tissue-nonspecific alkaline phosphatase (TNAP). Mutations result in a decreased level of TNAP activity and increased levels of its substrates: inorganic pyrophosphate (PPi) and pyridoxal-5′-phosphate (PLP). The disease is highly variable in its clinical presentation. Depending on the age when HPP is diagnosed and the severity of disease, five clinical subtypes have been distinguished: perinatal (divided in perinatal lethal and prenatal benign), infantile, childhood, adult and odonto-HPP.[1] Prognosis is very poor in severe forms of HPP with most patients dying from respiratory failure.[2] Recently enzyme replacement therapy (ERT) has been reported to be efficient and improve the outcome in life-threatening forms of HPP.[3]

According to the current experience, there seems to be a good correlation between genotype and phenotype.[4] In families with an index case of recessively inherited, mostly compound heterozygous HPP, the severity of symptoms has been reported to be comparable or to differ only moderately from one child to another.[4] Here, we present a family with heterozygous parents and four children showing an unexpected high intrafamilial phenotypic variability. We describe the functional consequences of the mutations and discuss the problems in genotype–phenotype correlation and genetic counseling in HPP.

Clinical report

The *parents* were Caucasian, non-consanguineous and healthy. Both had slightly reduced alkaline phosphatase (AP) activity (father: 25 U/l, mother: 35 U/l), and, at the time of the assessment, mild to no clinical symptoms of HPP. The father had a history of dental caries but no premature loss of teeth.

Child 1, a 14-year-old asymptomatic girl, showed an AP activity in the normal range, 155 U/l.

Child 2, an 11-year-old girl, had a history of premature loss of deciduous teeth, starting at the age of 12 months and tooth enamel defects. Her body weight increased along the 20th percentile, her length along the 10th percentile. She showed a slow but age-appropriate motor and a normal mental development and she was able to fully participate in sports lessons. Sometimes she complained about muscle soreness after physical activity. Physical examination showed mild genua valga and thorax deformity. Laboratory work-up revealed a low serum AP activity (22 U/l), elevated PLP in the plasma (120 ng/ml, (normal range (nr) 5–30 ng/ml)), slightly elevated serum phosphate (1.96 mmol/l, (nr 0.81–1.94 mmol/l) and a low level of 25(OH)-vitamin D3 (20.9 μg/l (nr 30–70 μg/l))).[5] She was clinically diagnosed of childhood HPP at the age of 1 year.

Child 3, a 3-year-old healthy girl, did not show any clinical symptoms of HPP. Her AP activity was slightly reduced, 100 U/l, and serum phosphate and PLP were in the normal range, 25 ng/ml.

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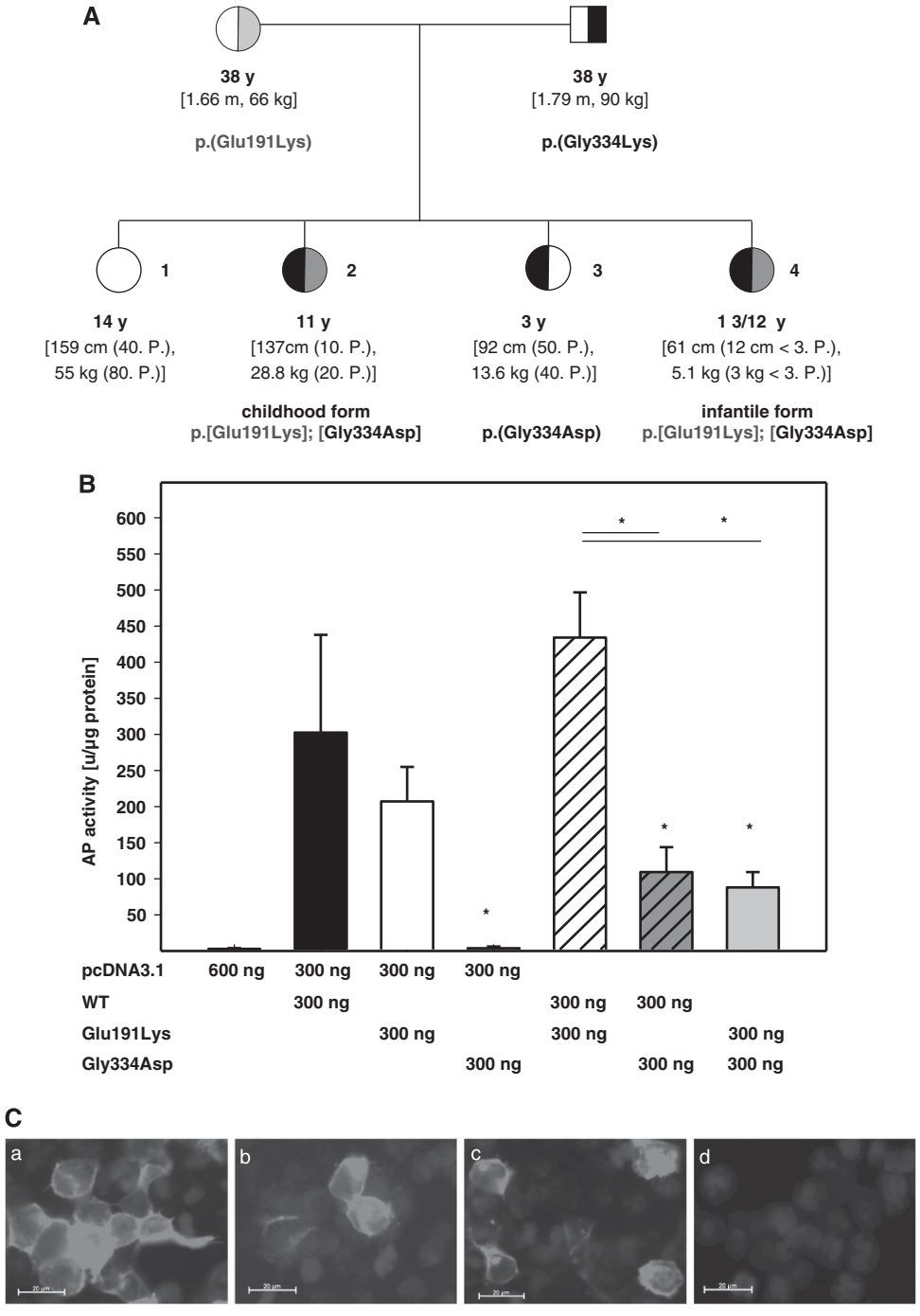

Figure 1 (**A**) Pedigree of the family showing the detected mutations, age, height and weight of each family member. (**B**) AP enzymatic activity in whole cell lysates of HEK 293 cells after transfection with empty expression plasmids pcDNA3.1, ALPL-WT, -Glu191Lys, -Gly334Asp. PcDNA3.1+ transfected HEK-293 cells have no relevant enzymatic AP activity. WT AP activity (after transfection of 300 ng WT and 300 ng pcDNA3.1) was defined 100%. The enzymatic activity for the *ALPL* mutant p.(Glu191Lys) (300 ng Glu191Lys and 300 ng pcDNA3.1) was 68% of WT and for p.(Gly334Asp) (300 ng Gly334Asp and 300 ng pcDNA3.1) 1.2% of WT, respectively. Co-transfection of WT and each mutant at the ratio 1:1 (300 ng mutant + 300 ng WT) were performed to explore whether one of the mutated proteins could affect the WT enzyme in a dominant-negative way resulting in an activity reduction of >50% of the unaffected protein. The mutant p.(Glu191Lys) did not show a dominant-negative effect (143%). The mutant p.(Gly334Asp) exhibits considerable inhibitory impact on ALPL-WT activity, which is reduced to 36%. Co-transfection of both mutants at the ratio of 1:1 (300 ng Glu191Lys + 300 ng Gly334Asp) – representing the genetic situation of child 2 and 4 – resulted in a residual AP activity of 29%. Significances were calculated with Mann–Whitney *U*-test (**P*<0.001). (**C**) Immunocytochemical staining of HEK293 cells transiently transfected with ALPL-expression plasmids for TNAP-WT (a, d), -Gly334Asp (b) and -Glu191Lys (c), respectively. Samples a–c were stained with the ALPL-specific antibody TNAP B4-78, sample d was incubated with mouse serum instead of first antibody. Strong signals can be detected in the region of the cell membrane in all transfected cells (a–c). Scale bar corresponds to 20 μm. The full colour version of this figure is available at *European Journal of Human Genetics* online.

Table 1 Characteristics of the parents and children including detected mutations in the *ALPL* gene (genotype), AP values (normal range, nr) and PLP values (nr: 5–30 ng/ml)

	Father	*Mother*	*Child 1*	*Child 2*	*Child 3*	*Child 4*
Genotype	p.G334D	p.E191K	no mutation	p.E191K, p.G334D	p.G334D	p.E191K, p.G334D
AP values (nr)	25 U/l (20–129)	35 U/l (35–104)	155 U/l (130–700)	22 U/l (130–700)	100 U/l (110–550)	36 U/l (110–550)
PLP values	63 ng/ml	23 ng/ml	n.d.	120 ng/ml	25 ng/ml	100 ng/ml

Abbreviation: n.d., not done.

Child 4, a 15-month-old girl, was diagnosed with HPP in her first year of life. She presented with severe failure to thrive (length 61 cm (12 cm below 3rd percentile), weight 5.1 kg (3 kg bellow 3rd percentile)), dysphagia and recurrent vomiting. She showed profound muscular hypotonia with marked delayed neuromotor skills, including absent head control. She was able to lift her extremities against gravity but could not sit without help, turn or crawl. Physical examination revealed rickets-like changes with thorax deformity, tachydyspnea and mild oxygen demand at night. She was diagnosed of complex craniosynostosis and increased intracranial pressure with the need for neurosurgical intervention. Laboratory results showed low serum AP activity (36 U/l), elevated PLP in the plasma (100 ng/ml (nr 5–30)). Serum calcium, phosphate, creatinine, 25(OH)-vitamin D3 and parathyroid hormone were in the normal range. X-rays demonstrated impaired skeletal ossification of the skull, severe delay of mineralization of, for example, thorax and long bones as well as mild lung hypoplasia. Kidney ultrasound showed bilateral nephrocalcinosis. Clinical features, laboratory results and radiological findings were consistent with the infantile HPP. She is currently enrolled in a clinical phase 2 ERT study with recombinant TNAP. Figure 1A and Table 1 summarize the clinical findings of the presented family.

MATERIALS AND METHODS

Genetic examination of the ALPL gene

Analyses of child 2 and the parents were performed as previously described.[7] Children 1, 3 and 4 were analyzed by direct sequencing of PCR-amplified genomic DNA using primers IntEx6s (5′-gtggatggggagactgagac-3′) and IntEx6as (5′-gagcctcccaggtgtgtg-3′) for exon 6 and IntEx10s (5′-gtggtgctagctc agagtgg-3′) and IntEx10as (5′-tgtcattgagtccccaccat-3′) for exon 10, respectively.

Mutagenesis and transfection studies

Two mutated TNAP proteins were generated by site-directed mutagenesis of a pcDNA3.1-*ALPL* wild-type (WT) expression plasmid as previously described by Mentrup *et al*.[6] Briefly, mutagenic primers g571a (5′-tggtactcagacaacaagatg ccccctgagg-3′) and g1001a (5′-tgctggtggaaggagacagaattgaccacgg-3′) were used to generate the mutants p.(Glu191Lys) and p.(Gly334Asp), respectively. AP activities and dominant-negative effect on WT protein were analyzed with the Konelab Alkaline Phosphatase (IFCC) Plus Kit (Fisher Scientific GmbH, Schwerte, Germany) 48 h after transfection or co-transfection of HEK-293 (ATCC CRL-1573). All experiments were performed three times in triplicates and data were normalized to protein content. Results are presented as mean of all experiments $+/-$ standard error of the mean (s.e.m.). Significances were calculated with the Mann–Whitney *U*-test ($^*P<0.001$).

Immunocytochemistry

The subcellular localization of TNAP-WT, -p.(Glu191Lys) and -p.(Gly334Asp) in transfected HEK-293 cells were analyzed as described in detail in Mentrup *et al*.[6] For staining the TNAP (B4-78) primary antibody (sc-81754; Santa Cruz Biotechnology, Heidelberg, Germany, diluted 1:100 in 1% BSA in PBS for 16 h at 4 °C, for specificity: www.scbt.com/datasheet-81754-tnap-b4-78-antibody.html) and a fluorescein-like secondary antibody (Northern Lights anti-mouse IgG-NL493; R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany, diluted 1:400 for 2 h at room temperature) were used. Finally the coverslips were applied to a drop of Vectashield with DAPI (LINEARIS GmbH, Wertheim, Germany) on slides and cells were observed under a fluorescence microscope (Axioskop 2, Carl Zeiss MicroImaging GmbH, Jena, Germany) using AxioVision Rel 4.6.

RESULTS

Genetic examination of the ALPL gene

Genetic analysis revealed two heterozygous *ALPL* mutations in the family (Figure 1A). All sequence variations have been deposited in the Tissue Nonspecific Alkaline Phosphatase Gene Mutation Database (http:www.sesep.uvsq.fr/Database.html); reference HGNC ID 438; NCBI Reference Sequence NM_000478.4.

The maternally inherited mutation c.571 G>A (p.(Glu191Lys)) is located in the first position of codon 191 (GAG–AAG) in exon 6, and results in an amino-acid exchange of glutamate to lysine. The paternally inherited mutation, p.(Gly334Asp), a c.1001 G>A transition, is localized in the second position of codon 334 (GGC–GAC) in exon 10. The parents were heterozygous carriers for one mutation, child 1 showed a normal genotype, child 2 and 4 a compound heterozygous genotype with both mutations (p.[Glu191Lys]; [Gly334Asp]) and child 3 was a heterozygous carrier for p.(Gly334Asp).

Enzymatic activity of AP mutants

The unimpaired TNAP protein reveals 302.5 ($\pm$102.2) U/μg protein and is defined 100% (Figure 1B). The p.(Glu191Asp) mutant has a reduced enzymatic activity of 207.1 ($\pm$39.3) U/μg protein (68% of WT), whereas the p.(Gly334Asp) mutant has (3.7 ($\pm$1.8) U/μg protein; 1.2% of WT, $P<0.001$)) only marginally exceeds the measurement of the background (pcDNA3.1: 2.9 ($\pm$0.7) U/μg protein; 1%). Co-transfection of WT and mutants showed no effect of p.(Glu191Asp) (434 ($\pm$52.4) U/μg protein; 143%) but a considerable dominant-negative effect of p.(Gly334Asp) on WT activity (109.2 ($\pm$28.6) U/μg protein; 36%, $P<0.001$). Co-transfection of equal amounts of both mutated expression constructs revealed an activity of 88 ($\pm$28.6) U/μg protein (29%, $P<0.001$). All data are presented as means of nine different measure points of three independent experiments $\pm$ s.e.m. and statistics were calculated with Mann–Whitney *U*-test.

Protein expression of AP mutants

HEK293 cells transiently transfected with *ALPL*-expression plasmids TNAP-WT, -p.(Gly334Asp) or -p.(Glu191Asp) and stained with the *ALPL*-specific antibody TNAP B4-78 showed strong signals in the cell membrane for all three approaches indicating a correct dimerization and subcellular localization of the glycosylphosphatidylinositol (GPI)-anchored proteins (Figure 1C).

DISCUSSION

Until now, diversity of the clinical course of HPP has been explained mainly by the extensive allelic heterogeneity in the *ALPL* gene with >270 different mutations (http://www.sesep.uvsq.fr/03_hypo_mutations.php). Zurutuza *et al*[4] were able to show a good correlation between clinical forms of the disease and the genotype characterized with *in vitro* mutagenesis experiments and three-dimensional structure study. *In vitro* testing allows to distinguish between severe and moderate alleles according to their residual AP activity. Protein structure studies indicated that severe mutations were localized in crucial domains such as the active site, the homodimer interface or GPI anchor relevant sites.

In this report, we present a family with two compound heterozygous children p.[Glu191Lys]; [Gly334Asp] showing an unexpected high phenotypic variability. The maternal mutation p.(Glu191Asp) has been described previously in a compound heterozygous patient with an infantile form (p.[Gly334Asp]; [Asp378Val]).[7] It occurs with a high frequency (up to 55%) in HPP patients with European ancestry.[7,8] In our *in vitro* testing, this moderate mutation resulted in 68% WT AP enzyme activity and it has no dominant-negative effect. The paternal mutation p.(Gly334Asp) has been found previously in homozygous Mennonite patients with the perinatal lethal form.[9] It results in a change of a polar glycine to an acidic aspartate located in the highly conserved active site region of the central β sheet flanked by residues involved in metal binding. In accordance to Fauvert *et al*,[9] *in vitro* testing showed very low residual activity of this severe mutation (1.2%). Co-transfection experiments revealed a clear dominant-negative effect (36%). The father and child 3, all heterozygous, showed a slightly reduced serum AP activity and mild/no clinical symptoms. It can also be noticed that the penetrance of dominant HPP is incomplete and very variable so that recessive genotypes including a dominant mutation may also undergo variable expressivity.[8] Nevertheless, carriers especially of a dominant-negative mutation may develop relevant clinical problems in later life and therefore should be followed by an experienced multidisciplinary team.

In order to mimic the genetic situation of child 2 and 4 *in vitro,* we performed co-transfections of both mutants in the ratio of 1:1 resulting in a residual AP activity of 29% of the WT AP enzyme activity. Both children showed comparable reduced AP levels but a remarkable variability of the phenotype: child 2 had typical clinical symptoms of the mild childhood form without current substantial reduction in quality of life whereas child 4 was significantly more severely affected. She had characteristic symptoms of the infantile form and was enrolled in an ERT study with recombinant TNAP for life-threatening forms of HPP. As both children are female one may conclude that gender is not inevitably the cause for the very varied phenotypes.

Of note, in our HPP cohort there are four other patients with exactly the same genetic compound heterozygous phenotype. A 9-year-old girl was diagnosed with a childhood form of HPP at the age of 2 years. Two siblings – a 16-year-old girl and an 11-year-old boy – both diagnosed in their first year of life with failure to thrive, musculoskeletal and dental problems, craniosynostosis/hydrocephalus. The fourth patient is an adult (65-year-old, AP 12 U/l (nr 42–98 U/l)) with a history of musculoskeletal and dental problems since early childhood. She developed severe bone pain mistreated with bisphosphonates, multiple rib fractures and stress and fragility fractures in both hips and a metatarsal bone resulting in arthrodesis of the destroyed ankle and hip joint replacements/plate screw fitting. Interestingly, her affected brother also (unknown genotype) was bound to a wheel-chair and died at the age of 53 years due to skin cancer. This even more illustrates the great clinical variability during lifetime in patients showing the identical genotype with respect to the *ALPL* gene. It is not known whether this heterogeneity reflects a common phenomenon in HPP and it could be noticed here because this genotype is frequent or if it is limited to only some particular genotypes. There may exist AP thresholds during life beyond whose the needs are fulfilled and below whose they are not; it remains possible that the combination p.[Glu191Lys]; [Gly334Asp] corresponds to one of these thresholds at a particular step of development. This might explain why for this particular genotype, the phenotype may easily switch to one or to another form.

The list of potential confounding factors is long, comprising both regulatory components of transcription/translation of AP itself as well as other phosphatases and phosphate-metabolizing enzymes. In addition, TNAP substrates like adenosine and its phosphorylated derivatives ATP, ADP, AMP and the complex downstream pattern of purinergic signalling may be important with respect to the variability of the clinical phenotype.[10,11]

In conclusion, a high variability of phenotypes can be observed in patients with HPP. This seems to predominantly be based on an extensive allelic heterogeneity. According to our clinical observations however, also intrafamilial and interfamilial variability of phenotypes can be observed in patients with identical genotypes. Further genetic, epigenetic or environmental factors may explain the clinical variability. Molecular mechanisms leading to a greater expression of the allele harbouring the severe *ALPL* defect (p.(Gly334Asp)) might be of relevance in the more severely affected child. No microarray analysis has been performed and further intronic mutations or polymorphisms in the *ALPL* or in other possibly relevant genes might also have escaped notice. Finally, this family report indicates that knowing the *ALPL* mutations does not necessarily help for prognostication of the clinical severity of the phenotype. Therefore, results of prenatal diagnostics in the case of a couple with an affected first child (index case of recessive HPP with two mutations identified) have to be interpreted with caution. Prenatal genetic diagnosis and counseling for HPP should be provided in combination of mutation characterization with careful prenatal ultrasonography within an experienced professional team of clinical geneticist, prenatal diagnostician, obstetrician and paediatrician. In the light of this, a prenatal prediction of severe versus mild forms of HPP may be difficult.[1]

CONFLICT OF INTEREST

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