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A Novel *De Novo* Heterozygous *ALPL* Nonsense Mutation Associated with Adult Hypophosphatasia

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Abstract

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Author contribution

LM contributed to the conception and design of the study, molecular data acquisition, data analysis and interpretation, and manuscript drafting. EJLS, ABA, and KRK contributed to dental data acquisition, analysis and interpretation, patient care, and critically revised the manuscript. AML contributed to clinical data acquisition and interpretation, patient care, and critically revised the manuscript. RAM contributed to molecular data acquisition, data analysis, and interpretation and critically revised the manuscript. BLF, RDC, and FHN made substantial contributions to the study's conception and design, and manuscript review and editing. All authors gave final approval of the submitted version and agree to be accountable for all aspects of the work.

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Conflict of interest

The authors declare that they have no conflict of interest.

Compliance with ethical standards

The study was conducted in compliance with the recommendation of the World Medical Association Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects, and written informed consent or assent was obtained for the individuals enrolled to report their clinical data, retrospective medical histories, and genetic information. The study protocol was approved by the Piracicaba Dental School - State University of Campinas Institutional Review Board (IRB#88910618.0.0000.5418).

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Summary: Hypophosphatasia (HPP) is a rare inherited metabolic disorder caused by genetic alterations of the alkaline phosphatase (*ALPL*) gene, encoding the tissue-nonspecific isozyme of alkaline phosphatase (TNSALP). The purpose of this study was to perform a clinical and molecular investigation in a 36-year-old Caucasian woman suspected to present adult-HPP based on clinical symptoms and low alkaline phosphatase serum activity.

Methods: Full medical and dental histories were obtained for the probanda and family members, including biochemical, radiographic and dental assessments. *ALPL* mutational analysis was performed by the Sanger sequencing method and the functional impact prediction of the identified mutations was assessed by bioinformatic methods.

Results: We identified a novel heterozygous nonsense mutation in the *ALPL* gene (NM_000478.6:c.768G>A; W[TGG] > *[TGA]) associated with spontaneous vertebral fracture, severe back pain, musculoskeletal pain, low bone density, and short-rooted permanent teeth loss. Functional prediction analysis by homology modeling revealed that the Trp256Ter mutation led to a complete loss of TNSALP crown domain and extensive loss of other functional domains (calcium-binding domain, active site vicinity, and zinc-binding site) and over 60% loss of homodimer interface residues, suggesting that the mutant TNSALP molecules are nonfunctional and form unstable homodimers. Genotyping of the *ALPL* in the probanda's parents, sister and niece revealed that in this case, HPP occurred due to a *de novo* mutation.

Conclusion: The present study describes a novel genotype-phenotype and structure-function relationship for HPP, contributing to a better molecular comprehension of HPP etiology and pathophysiology.

Keywords

hypophosphatasia; alkaline phosphatase; nonsense mutation; *de novo* mutation; 3D modeling

Introduction

Hypophosphatasia (HPP; OMIM# 146300, 241500, 241510) is an inborn error-of-metabolism characterized by defective mineralization as a result of loss-of-function mutations in the *ALPL* gene, which encodes a phosphate-monoester phosphohydrolase, known as tissue-nonspecific isozyme of alkaline phosphatase (TNSALP), which is active as a homodimer and especially abundant in skeletal, hepatic and renal tissue [1–2]. Six major clinical forms are recognized according to the age of first symptoms, including lethal perinatal, benign prenatal, infantile, childhood, adult and odonto-HPP forms [2]. Despite an extremely variable clinical spectrum of HPP, all affected individuals exhibit reduced TNSALP activity as a result of impaired function of one or both *ALPL* alleles [1–3]. In adult-HPP, the disease typically manifests in or after middle age with pain due to stress fractures, osteomalacia and chondrocalcinosis [2–5]. In addition, bone mineral density (BMD) has been reported to be affected and fracture healing is problematic and delayed [2–6]. Interestingly, some cases of adult-HPP have milder symptoms [4–7].

The remarkably broad-ranging severity and clinical expressivity of HPP have been attributed to a combination of strong allelic heterogeneity of the *ALPL*, distinct inheritance patterns (dominant or recessive), compound heterozygosity, dominant-negative effects, incomplete

gene penetrance, and/or actions of modifier genes and other factors that, altogether making diagnosis and phenotype-genotype associations quite challenging [7–11]. Severe forms of HPP (perinatal and infantile) are usually inherited as autosomal recessive traits with homozygosity or compound heterozygosity for two defective *ALPL* alleles, whereas mild forms (including childhood, adult and odonto-HPP) are inherited as an autosomal dominant trait [2–3]. Individuals carrying *de novo* mutations of the *ALPL* are rare [1]. Although a number of studies have focused on homology modeling and *in vitro* residual activities of HPP-causing *ALPL* mutations [6–12], many structural and functional aspects related to the heterogeneous range of HPP clinical manifestations still remain to be clarified as new relevant genetic information continues to emerge.

Here, we present an unusual case of adult-HPP caused by a novel *de novo* heterozygous nonsense mutation in the *ALPL*. Genetic analysis, clinical, biochemical and radiographic assessment and bioinformatic approaches were performed in order to predict the structural and functional impact of the mutation on TNSALP and to establish a genotype-phenotype and structure-function association for the current case.

Methods

Clinical assessments:

The proposita, a 36-year-old Caucasian woman with low serum alkaline phosphatase (ALP) activity, was referred to the State University of Campinas - Brazil, for genetic testing to support a diagnostic hypothesis of adult-HPP. The proposita's medical and dental histories were obtained, while other family members, including her mother, father, sister, and niece were also examined and genetically analyzed in order to identify inheritance and genotype-phenotype association patterns. Oral charting and dental radiographs (panoramic and periapical) were obtained for all of the enrolled subjects.

Mutational analysis and homology modeling:

ALPL mutation analysis, homology modeling and multiple sequence alignment were performed as previously described [7]. Then, genetic alterations, identified in the proposita's DNA sequencing by Sanger method were searched in the Single Nucleotide Polymorphism Database (dbSNP; <https://www.ncbi.nlm.nih.gov/snp/>) and the Exome Aggregation Consortium database (ExAC; <http://exac.broadinstitute.org/>). Native and mutant 3D models were built based on the alkaline phosphatase, tissue-nonspecific isozyme canonical sequence (UniprotKB # P05186-1) and the crystal structure of alkaline phosphatase from human placenta (PDB: 1EW2; sequence identity up to 57%) in the SWISS-MODEL program, in order to predict the functional impact of the putative causative mutation. 3D models were visualized in the PyMOL program (PyMOL Molecular Graphics System, v.2.0.4; Schrödinger, LLC), and the interface residues, domain, and functional sites were highlighted in the native and mutant TNSALP structure. Prediction of monomer-monomer interaction sites was performed using distance-based methods and cutoff values of 4.5 Å in PyMOL software (PyMolWiki, <https://pymolwiki.org/index.php/InterfaceResidues>, November 01, 2019). For the normal *ALPL* sequence, the Leu18 to Ile490 residues were represented in the 3D model, whereas only Leu18 to Trp256 residues were represented for the mutant

sequence. An interactive figure was created and displayed in a web interface, using the Three.js JavaScript 3D framework (<https://threejs.org/>).

Results

Clinical findings:

The proposita, weighing 138 pounds (63 kg) and standing at 68.5 inches (1.74 m) tall, presented a medical history of childhood and adolescent fractures in the wrist and ankle as a result of falls, as well as recurrent, long-term episodes of renal lithiasis at the age of 24-25 years old. She reported recently experiencing back and musculoskeletal pain in arms, legs, and joints, excessive tiredness, persistent muscle fatigue, malaise, weakness, and dizziness with a sporadic episode of fainting. She reported ingesting approximately 500 mg of calcium per day, as directed by a physician. Osteopenia was indicated by a spinal BMD below the expected range for individuals matched by age, sex, ethnicity, weight, and height (L1-L4 region; 0.887 g/cm², Z-score = -2.3), whereas BMD measurements in the femoral neck (0.817 g/cm², Z-score = -1.2) and total femur (0.744 g/cm², Z-score = -1.9) were within the normal limits. Radiographs of hands, feet, and knees indicated no relevant or significant findings. Posteroanterior and profile chest X-rays revealed discrete vertebral joint arthrosis associated with anterior vertebral fracture/wedging of the middle dorsal vertebra (Fig. 1A). These findings suggest that the spontaneous fractures of thoracic vertebrae experienced by the proposita may be associated with low bone mass as no other potential cause was found. Blood test showed that the proposita exhibited low ALP activity (15 U/L; normal range for adult: 46 to 116 U/L), whereas other markers were within the normal limits, including: 25-hydroxyvitamin D [25(OH)D] serum levels (34.6 ng/mL; desirable range: 20-50 ng/mL); pyridoxine (30.3 mcg/L; normal range: 5.2-34.1 mcg/L); vitamin B12 (275 pg/mL; normal range: 187-883 pg/mL); calcium (9.1 mg/dL; normal range: 8.3-10.6 mg/dL); phosphorus (4.2 mg/dL; normal range: 2.4-5.1 mg/dL); creatinine (0.85 mg/dL; normal range: 0.57-1.11 mg/dL); creatine phosphokinase (72 U/L; normal range: 34-145 U/L); parathyroid hormone (PTH; 36.1 pg/mL; normal range: 18.5-88.0 pg/mL); thyroid-stimulating hormone (TSH; 2.19 mUI/L, normal range: 0.35-4.94 mUI/L); urine phosphorus (418 mg/24 h; normal range: 400-1300 mg/24 h); urine calcium (185 mg/24 h; normal range: 100-300 mg/24h); and serum CTX (0.350 ng/mL; normal range: 0.016-0.584 ng/mL). Pyridoxal phosphate (PLP) serum levels were not measured. Dental history and clinical assessments revealed that the proposita experienced the premature loss of primary (starting at the age of 4-5 years old) and permanent (with no history of caries or periodontal disease) teeth, and displayed tooth mobility in the remaining dentition. Intraoral examination revealed staining on most of the remaining teeth that was associated with the use of mouthwash containing chlorhexidine (Fig. 1B). Evidence for enamel or dentin mineralization defects was neither radiographically nor clinically detectable. Several teeth displayed gingival recession, but without signs of gingivitis, bleeding on probing, swelling, or any evidence of periodontal pocketing. Radiographs revealed a short-rooted phenotype for the vestibular roots of the maxillary molars and evidence of generalized atrophy or loss of alveolar bone (Fig. 1C-F).

ALPL mutation analysis and segregation pattern:

Mutation analysis revealed a *de novo* heterozygous substitution G>A at the 768-nt position of the *ALPL* (exon 7; NM_000478.6:c.768G>A, rs760649979), resulting in the change of a tryptophan (Trp, W) residue to a stop codon (Ter,*) at protein position 256 (NP_000469.3:p.Trp256Ter) (Supplementary Fig. 1). This nonsense mutation, which results in a shortened TNSALP protein, has been previously reported in dbSNP (rs760649979). However, no study has reported this genetic alteration in association with HPP or any other genetic disorders before (dbSNP, <https://www.ncbi.nlm.nih.gov/snp/rs760649979> accessed on July 9, 2019). Furthermore, no report was found in the ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/>, July 9, 2019), whereas in the GnomAD browser, the p.Trp256Ter heterozygous mutation was reported in a South American man, 50-55 years-old, (https://gnomad.broadinstitute.org/variant/1-21894716-G-A?dataset=gnomad_r2_1, accessed on March 26, 2020). To the best of our knowledge, this is the first case of adult HPP associated with the p.Trp256Ter heterozygous nonsense mutation, as this variant has not been reported among the 405 HPP-causing mutations recorded to date (http://www.sesep.uvsq.fr/03_hypo_mutations.php, December 12, 2019). Genetic analysis further identified a paternally derived heterozygous substitution C>T at the 1565-nt position of *ALPL* (exon 12; NM_000478.6:c.1565T>C; rs34605986), resulting in substitution of the valine (Val, V) residue for alanine (Ala, A) at protein position 522 (NP_000469.3:p.Val522Ala) (Supplementary Fig. 1B). However, this missense substitution has a Global Minor Allele Frequency (GMAF) of 0.07189 and was previously reported as benign in the ClinVar database (VCV000193996.1). Residue conservation analysis additionally confirmed that Val522 is not a conserved residue in *ALPL* orthologs and paralogs (Supplementary Fig. 2), suggesting this variation is a neutral mutation and is not associated with HPP. Medical history and genetic screening in the probanda's relatives revealed that none of them were *ALPL* mutation carriers (p.Trp256Ter), suggesting that the *de novo* nonsense mutation, and not an inherited mutation, contributed to the manifestation of HPP in the probanda.

Structural and functional impact of the p.Trp256Ter ALPL mutation:

Homology modeling and prediction of monomer-monomer interaction sites revealed that the p.Trp256Ter mutation resulted in over 60% loss of protein interface residues predicted for the TNSALP native homodimer (Fig. 2A, B and C). In addition, the computational analysis revealed that the p.Trp256Ter mutant molecule lost all residues from the crown domain and collagen-binding site, and most of the residues from the calcium-binding domain, active site vicinity, and metal-binding sites (Fig. 2 D and E).

Discussion

Hypophosphatasia (HPP) is a disorder with a strong allelic heterogeneity of the *ALPL* gene. Adult-HPP clinical form is typically diagnosed after middle age with many patients exhibiting nonspecific clinical symptoms [2–6]. Genetic analyses have been used to identify *ALPL* mutations, to determine the mode of inheritance, as well as to provide a basis for genotype-phenotype associations [7–13]. In the present study, complete *ALPL* sequencing supported the suspected diagnosis of adult-HPP, revealing a novel *de novo* heterozygous

nonsense mutation. Around 71% of the HPP-associated mutations are missense, which confers a variable degree of residual activity to the protein. Nonsense mutations represent less than 4,2% (17/405) of genetic variation of the *ALPL*, and in most of the cases, nonsense mutations are found as a part of a heterozygous compound genotype. Only in 3 reports involving childhood and adult-HPP clinical forms, the HPP phenotype was in consequence of a dominant nonsense mutation [http://www.sesep.uvsq.fr/03_hypo_mutations.php, December 12, 2019]. Unlike the highly variable effect of missense mutations, nonsense mutations are expected to produce nonfunctional TNSALP and result in a dramatic ALP activity decrease [12]. Despite this, heterozygous nonsense mutation in the *ALPL* (c.558G > A; p.Trp186*) has been reported to result in a non-specific clinical phenotype [12]. Such large complexity to link genotype with phenotype in HPP disorder has also been observed in retrospective studies, where ALP activity and clinical symptoms may vary strongly among patients which are carriers of the same mutation [14], and has been explained by the influence of other genetic, epigenetic or non-genetic factors in this disorder [12]. In the present investigation, the probanda's clinical features, including low BMD, bone fragility, dental abnormalities, and musculoskeletal pain were associated with persistently low ALP activity (15 U/L) and a nonsense autosomal dominant mutation (p.Trp256Ter). Interestingly, a recent study has evidenced that hypophosphatasemia and elevated ALP substrate levels present a good correlation with the occurrence of fractures and other adult-HPP symptoms but, in general, these biochemical features are not always associated with low bone mass [14]. However, some adults with persistent hypophosphatasemia do not fall within the spectrum of the characteristic clinical manifestations of the adult-HPP form, resulting in incorrect or delayed diagnosis [13]. Noteworthy, the majority of adults with persistent hypophosphatasemia and elevated ALP substrate values are *ALPL* mutation carriers [13].

With respect to HPP pathophysiology, studies have consistently shown that skeletal manifestations are due to the bone matrix hypomineralization as a result of inorganic pyrophosphate (PPi) accumulation and inhibition of hydroxyapatite crystals formation [2, 10, 15], while the propensity to increased dental mobility and/or teeth premature loss is due to defects in the dental cementum mineralization [16]. In contrast, the mechanisms underlying other manifestations of HPP are poorly understood. For instance, high extracellular levels of PPi, reduced neurotransmitter synthesis as a consequence of improper vitamin B6 metabolism or reduced hydrolysis of the endogenous pronociceptive nucleosides have been implicated with HPP musculoskeletal symptoms [10, 15], whereas nephrolithiasis may be associated to defective Pi excretion, hypercalcemia and hypercalciuria resulting from blocked mineral entry into the skeletal, or still renal deposition of calcium pyrophosphate dihydrate crystals [1, 2, 15]. Here, the probanda experienced premature tooth loss, bone fractures, musculoskeletal pain, and recurrent renal lithiasis. Short-rooted permanent teeth were revealed by radiographic evaluation. Also, added to the well-known impact of HPP on acellular cementum formation [16], short-rooted permanent teeth have become a more established finding associated with HPP, previously reported in association with cases of childhood [17], adult [7], and odonto-HPP [18].

Noteworthy, the current study is the first to identify an individual with adult-HPP carrying a *de novo* autosomal dominant mutation in *ALPL*. Previous studies have suggested that the variable patterns of inheritance (autosomal dominant or recessive), associated with distinct

HPP forms, may partially explain the HPP phenotype heterogeneity [2–3]. For instance, perinatal and severe infantile HPP are usually inherited as autosomal recessive traits, whereas milder forms, including benign prenatal, infantile, childhood HPP, adult HPP, and odonto-HPP are usually inherited in an autosomal recessive or autosomal dominant manner [2–3]. On the other hand, *de novo* *ALPL* mutations are thought to be a rare transmission mode and have only been reported twice [19–20]. The first was a case of childhood HPP where the subject carried heterozygous compound *ALPL* mutation (p.E174K missense mutation of maternal origin and p.M45I *de novo* mutation) [19]. The second was a case of adult HPP harboring a homozygous splice site mutation (c.298-1G>A) with one maternally derived allele and the other resulting from *de novo* transmission [20].

In the current investigation, homology-modeling analyses suggest that the mutant homodimer or mutant-native dimer formation is quite unstable due to a 60% loss of protein interface residues. Therefore, no dominant-negative effect should be expected for the p.Trp256Ter mutation and a residual activity would only be expected from TNSALP native homodimers (Fig. 2). The present study contributes to improving the efficiency of the molecular diagnosis and clinical interpretation of *ALPL* variants. However, a limitation of our approach was the absence of a model that might reflect the complexity of *in vivo* situations, as computational models do not consider, apart from the effect of *ALPL* variant, the influence of other genetic or non-genetic factors in the heterogeneous clinical expression of the HPP. Also, it is important to emphasize that our findings should not be seen as the only diagnostic tool, but the clinician plays a key role in determining diagnostic and managing therapy.

Conclusions

We report a novel *de novo* heterozygous nonsense mutation in the *ALPL* (NP_000469.3: p.Trp256Ter) leading to an adult-HPP phenotype including short root anomalies in permanent teeth, as the result of impaired TNSALP activity and dimer stability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Clinical findings in an individual diagnosed with adult HPP. **(A)** Profile chest X-ray showing discrete vertebrae joint arthrosis (dorsal spondylosis) associated with anterior vertebral fracture/wedging of the middle dorsal vertebra (T7; Genant grade 2). The reduction in anterior vertebral body height is estimated at 35%. **(B)** Oral photograph from the propoita showing generalized gingival recession and brown staining associated with the use of chlorhexidine mouthwash. Panoramic **(C)** and periapical **(D-F)** dental radiographs illustrating the absence of posterior mandibular teeth, generalized atrophy of alveolar bone, and short-rooted maxillary and mandibular molars.

Fig. 2.

3D models of native and mutant TNSALP dimers. **(A)** The native structure of a TNSALP homodimer highlighting monomers 1 and 2 (salmon and cyan, respectively), interface residues (brick red), and ion binding sites (yellow). **(B)** Putative unstable dimer between two p.Trp246Ter mutant monomers, revealing a dramatic loss of protein structure (detailed in Figure 4). **(C)** Putative unstable dimer between native (monomer 1 on left) and p.Trp246Ter mutant TNSALP (monomer 2, on right). **(D)** Native structure of a TNSALP homodimer highlighting monomers 1 and 2 (salmon and cyan, respectively) and color coding structural

and functional domains, including N-terminal alpha-helix (dark green), active site vicinity (pink), calcium-binding domain (plum), crown domain (royal blue), and the collagen-binding domain (navy blue), as well as locations of Zn^{2+} and Mg^{2+} ion binding. **(E)** The p.Trp256Ter mutant homodimer presents extensive loss of residues from important domain and functional sites compared to native TNSALP homodimer, including the entire crown domain and collagen-binding site, portions of the calcium-binding domain, and residues in the active site vicinity. The interactive figures may be accessed at <http://www.ludolab.com.br/lumazy/?grupo=interface> and <http://www.ludolab.com.br/lumazy/?grupo=domain> and displayed as a web page on a monitor or mobile device.