Phenotypic Profiling in Subjects Heterozygous for 1 of 2 Rare Variants in the Hypophosphatasia Gene (*ALPL*)

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Context: Hypophosphatasia (HPP) is a syndrome marked by low serum alkaline phosphatase (AlkP) activity as well as musculoskeletal and/or dental disease. While the majority of subjects with HPP carry a pathogenic variant in the *ALPL* gene or its regulatory regions, individual pathogenic variants are often not tightly correlated with clinical symptomatology. We sought to better understand the genotype/phenotype correlation in HPP by examining the clinical and biochemical data of 37 subjects with 2 rare variants in *ALPL*.

Methods: Through BioVU, a DNA biobank that pairs individuals' genetic information with their de-identified medical records, we identified subjects with 2 rare variants with distinct reported clinical phenotypes (p.D294A and p.T273M). We then performed a manual review of these subjects' de-identified medical records along with computational modeling of protein structure to construct a genetic, biochemical and clinical phenotype for each subject and variant.

Results: Twenty subjects with the p.D294A variant and 17 with the p.T273M variant had sufficient data for analysis. Among subjects in our cohort with the p.D294A variant, 6 (30.0%) had both clinical bone disease and serum AlkP activity below 40 IU/L while 4 subjects (23.5%) with the p.T273M variant met the same criteria despite the distinct clinical phenotypes of these variants.

Conclusions: Given the loose genotype/phenotype correlation in HPP seen in our cohort, clinical context is crucial for the interpretation of genetic test results to guide clinical care in this population. Otherwise, over- or under-diagnosis may occur, resulting in misidentification of those who may benefit from additional screening and perhaps pharmacologic intervention.

Hypophosphatasia (HPP) is a disorder of reduced serum activity of the tissue nonspecific alkaline phosphatase (TNSALP) protein, a product of the *ALPL* gene [1-6]. Subjects with *ALPL* variants have a widely variable clinical phenotype, likely due to variable expressivity and incomplete penetrance, from neonatal seizures and hypomineralization to isolated dental or joint disease in adults [3, 7-11]. Those with mild phenotypes may progress into adulthood with subclinical disease and present only as older adults after years of subclinical disease [1, 9, 12]. If these subjects come to clinical attention, it is often due to mild, nonspecific findings or incidental findings on otherwise routine lab evaluation which prompts further evaluation and ultimately a diagnosis of HPP [13, 14]. This highly variable clinical phenotype leads to significant diagnostic challenges, particularly in those with minor disease manifestations or a phenotype that may have multiple potential etiologies [8, 15-17]. While there are no formal criteria for diagnosis of HPP, factors often considered supportive of a diagnosis include an alkaline phosphatase (AlkP) value below the normal range for age/sex and clinical features such as bone and/or dental disease consistent with the diagnosis [1-3, 16]. However, as more becomes known about the genetic and biochemical underpinnings of the disease, genetic testing is often offered to subjects to help clarify their diagnosis [1, 3, 9, 16, 18].

While significant effort has been expended to catalog and identify pathogenic variants in the *ALPL* gene, the rarity of the diagnosis has complicated these efforts [16, 19]. Furthermore, with more than 400 described variants, knowledge about the effect of a particular variant is usually based upon clinical experience with an extremely small cohort of subjects [9, 20]. The absence of larger cohorts and the lack of clear genotype-phenotype correlations makes diagnosis difficult and can lead to both over- and under-diagnosis [17]. Subjects with genetic testing positive for a pathogenic variant may undergo further testing and monitoring and ultimately never develop symptomatic disease [13]. Conversely, other subjects may have genetic testing results demonstrating no variant or a variant of undetermined significance; this can inappropriately be interpreted by providers to be inconsistent with HPP, resulting in exclusion of subjects who may otherwise require additional testing and treatment [14, 21]. To better understand the relationship between genotype and phenotype, we identified subjects with TNSALP mutations from a general population database and reviewed their medical records for evidence of an HPP phenotype. Through this unbiased approach, we are able to better understand the phenotypic diversity of individuals with 2 variants to aid better understand phenotypic diversity in this disease.

Methods

Subject identification

The cohort studied for this case series was taken from BioVU, Vanderbilt's de-identified DNA biobank that pairs DNA isolated from leftover clinical blood samples from across the medical center with a de-identified and randomly date-shifted image of the electronic health record as previously described [22]. In 2012-2013, approximately 40 000 subjects in BioVU were genotyped using the Illumina Infinium HumanExome BeadChip (http://genome.sph. umich.edu/wiki/Exome_Chip_Design), which includes >240 000 mostly exonic markers, as well as single nucleotide polymorphisms (SNPs) from the genome-wide association study (GWAS) catalog [23]. Genotyping was performed at the Vanderbilt Technologies for Advanced Genomics (VANTAGE) Core, and genomic data were processed by the Vanderbilt Technologies for Advanced Genomics Analysis and Research Design (VANGARD) Core. Genotyping quality was evaluated using SNP call rates and concordance rates with HapMap controls; SNPs with <99.8% call rate or <98% concordance were excluded [24]. Further details about GWAS coverage and quality control protocols have previously been discussed in detail [25].

Ethics statement

This project was determined to be nonhuman subject research by the Vanderbilt Institutional Review Board [VUMC IRB# 120277], since there are no personal health identifiers in the dataset. In accordance with the ethical framework previously described for BioVU studies [26], all investigators must have IRB determination prior to accessing the resource and also must sign a standard data use agreement [22, 26].

Case identification

We identified 13 *ALPL* SNPs which passed quality control as described above. The Illumina chip included 13 variants in the *ALPL* gene on the HumanExome BeadChip. Each variant was initially reviewed for this study. From the 13 identified variants, 2 were selected for further study—*ALPL:* rs121918002 (c.881A>C, p.D294A) and rs148405563 (c.818A>T, p.T273M) as they had sufficient subject numbers (having at least 15 subjects within the BioVU database), were sufficiently rare (minor allele frequency [MAF] <0.002), and had adequate lab data (at least 2 AlkP values during the study time period) [27]. The total number of subjects in the cohort was 37; variant data are summarized in Table 1.

Manual chart review

We then performed a manual record review to better define the clinical phenotype of subjects with these variants. The de-identified chart was accessed in the BioVU interface, which allows review of the entire medical record of each subject. A search function was used to identify key phrases within documents to flag them for further review. For bone disease, chart documents were flagged for review if they included the following terms: *fracture, broken, broken bone, broke, compression, lumbar, long bone, humeral, radial, clavicular, sternal, tibial, fibular, femoral, spinal, cervical, thoracic, metaphyseal, spondylolisthesis, spondylosis, scoliosis, osteonecrosis, CPPD, pseudogout, gout, pyrophosphate, osteoporosis, osteopenia, osteomalacia, rickets, DEXA, bone density, osteogenesis, short stature, stone, calculus*. Charts were similarly flagged for review of dental disease if they included the following terms: *dental, teeth, tooth, dental fractures, extractions, tooth loss, broken tooth, broken teeth, caries, periodontal, tooth extraction, crown, implant, tooth abscess, ONJ, TMJ, osteonecrosis of the jaw, cracking, chipping*. A blinded manual abstraction of the medical records was performed by 2 independent reviewers for the 37 total subjects with identified *ALPL* variants. Records were manually abstracted to document clinical and laboratory findings consistent with the known skeletal and dental phenotype of HPP. On average, electronic health record (EHR) data from 2000 to 2012 were reviewed, which also includes problem lists, lab results, imaging reports, and narrative reports from the EHR. AlkP assays were performed by a single lab as a part of routine clinical care. The assay used during this time period was a photometric assay done at nonphysiologic pH and with an artificial substrate. This assay has the following reference ranges for age and sex: 0 to 14 days, 90-273 U/L; 15 days to <1 year, 134-518 U/L; 1 year to <10 years, 156-369 U/L; 10 to <13 years, 141-460 U/L; 13 years to <15 years, 62-280 U/L (female [F]) and 127-517 U/L (male [M]); 15 years to

Table 1. *ALPL* Variants Identified Among Subjects Included as Cases

SNP	rsID	Previously Reported Phenotype [20]	Molecular consequence	Functional Consequence	Carrier Count	MAF [27]
exm28158	rs121918002	Infantile [3]	c.881A > C p.D294A	35% normal [37]	20	0.00002
exm28148	rs148405563	Asymptomatic, low BMD [14]	c.818C > T p.T273M	Unknown	17	0.00118

Abbreviations: BMD, bone mineral density; MAF, minor allele frequency—the frequency of the second most common allele in a given population to differentiate between common and rare variants; rsID, accession number assigned to refer to specific SNPs; SNP, single nucleotide polymorphism.

<17 years, F 54-128 U/L, M 89-365 U/L; 17 years to <19 years, F 48-95 U/L, M 59-164 U/L. Descriptive statistics were calculated manually as presented in Table 2.

Computational modeling

A model of dimeric TNSALP was generated using RosettaCM to combine elements from 2 template crystal structures [28]. The model was based on 4kjg.pdb (rat intestinal alkaline phosphatase) and 1zef.pdb (human placenta alkaline phosphatase) [29, 30]. In order to compare the location of each variant to the spatial distributions of known benign versus disease-associated variants, the PathProx algorithm was used to map known ExAC and ClinVar missense variants to the structure and calculate a relative proximity score [31, 32]. Thermodynamic destabilization by the variants was predicted using Rosetta's ddG_monomer application, which calculates energy scores for the structure before and after mutation, allowing repacking of sidechains without backbone movement [33]. Because the model was not a high-resolution x-ray crystal structure, the low-resolution protocol was used, corresponding to row 3 of Table 1 from Kellogg et al [33]. Molecular graphics were generated using UCSF Chimera (supported by NIH P41 RR-01081) as distributed by SBGRID [34, 35].

Results

Among the 40 000 patients who had been evaluated at our facility for any reason and for whom SNP data was available, 2 variants were identified for further study. The first variant, p.D294A, has previously been studied in vitro and has 8.9% of native protein function and shows negligible dominant negative effect [36]. In vivo, this variant has been found to be disease-causing with a broad range of ages of onset and disease severity [5, 37, 38]. The MAF of 0.00002 demonstrates that this is a rare variant in the population but was detected within our sample in 20 subjects of approximately 40 000 screened [32]. The second variant, p.T273M, has been reported in the literature as causing low AlkP and low bone mineral density [14, 39]. Recent data show this variant to have near normal in vitro enzymatic activity

Table 2. Aggregate Demographic, Alkaline Phosphatase, and Skeletal Phenotype Data Skeletal Observed Within the Study Populations Defined by Their Genetic Variants

Demographics	p.D294A	p.T273M
Number of subjects with variant	20	17
Age (SD)	60.2 (28.65)	71.8 (19.05)
Percent Female	35.0%	35.3%
Alkaline Phosphatase		
Mean (SD)*	59.6 (42.47)	50.65 (11.95)
Subjects with at least one AlkP under 50	17 (85%)	15 (88.2%)
Subjects with at least one AlkP under 40	16 (80%)	8 (47.1%)
Bone Disease		
Scoliosis	2 (10.0%)	4 (23.5%)
Low bone mineral density	4 (20.0%)	7 (41.2%)
Long bone fractures	2 (10.0%)	6 (35.3%)
Spine fractures	2 (10.0%)	2 (11.8%)
Dental disease	1 (5.0%)	3 (17.6%)
Disease Phenotype		
Both AlkP <40 and disease phenotype	6 (30.0%)	4 (23.5%)

Phenotypic data are the result of a manual chart review of subjects with each of the noted SNPs. No subject had a clinical diagnosis of hypophosphatasia.

Dental disease included dental fractures, extractions, or other tooth loss. Long bone fractures included humeral, radial, clavicular, sternal, tibial, fibular, or femoral fractures. Spinal fractures included cervical, thoracic, lumbar, or sacral fracture.

*reference range 40–150 (IU/L).

Abbreviation: AlkP, alkaline phosphatase.

without a dominant negative effect [36]. No data regarding *the in vivo* enzymatic activity for this variant was identified in our review of the literature. While still rare, this variant is slightly more common in the general population, with an MAF of 0.00118, and it was present in 17 subjects within our sample [27].

Computational modeling of the TNSALP protein was performed to provide additional insight into the impact of the variants in this study. The p.D294A variant is highlighted in Fig. 1 within the dimeric TNSALP enzyme. This mutation, D294A, was analyzed using PathProx, an algorithm which compares the proximity to the 3D distribution of known pathogenic variants from the ClinVar database versus proximity to the putative neutral variants from the ExAC database. The PathProx score of 0.44 indicates strong colocalization with pathogenic variants. This variant also produced a positive value of 2.0 in Rosetta's delta-delta-G calculation, predicting thermodynamic destabilization of the structure as a result of the mutation. In contrast, the p.T273M variant scored only 0.10 by PathProx, indicating weaker colocalization with known disease-associated variants. It also produced a small negative delta-delta-G score of −1.51, suggesting a minor overstabilizing effect, if any.

Detailed chart review of this cohort of subjects demonstrated that regardless of their variant, the majority of subjects had at least one HPP associated finding—a low AlkP activity or clinical history associated with HPP—and a smaller, but significant number had both associated findings (Table 2). Despite this, none of the subjects had a clinical diagnosis of HPP. While those with the p.T273M variant were slightly older than those in the p.D294A cohort (71.8 years vs 60.2 years, respectively), the cohorts were similar in number and sex composition. As would be predicted from previous in vitro work noted above, subjects in the p.D294A cohort were more likely to have at least one abnormally low AlkP value (80% vs 47.1%). Interestingly, while these low values were more common in the p.D294A cohort, the mean serum AlkP was lower in the p.T273M cohort compared with those with the p.D294A variant (53.92 vs. 70.67). In terms of clinical phenotype, bone and dental disease (scoliosis, fractures, low bone mineral density, and dental disease) identified during chart review were each more common among patients with the p.T273M variant. The rate of long bone fractures was 10.0% in the p.D294A cohort and 35.3% in the p.T273M group. The rate of scoliosis was 10.0% in the p.D294A cohort and 23.5% in the p.T273M group. Finally, 30.0% of the p.D294A cohort had both a low AlkP activity and
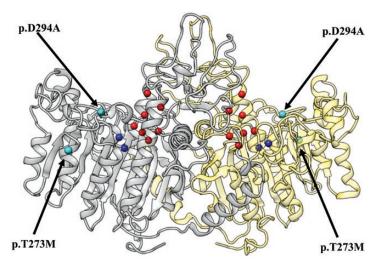

Figure 1. Location of variants of interest, metal ions, and other known pathogenic variants within the dimeric alkaline phosphatase, tissue-nonspecific isozyme (TNSALP) homodimer. Structural model of dimeric TNSALP, showing the location of the p.D294A and p.T273M variants (cyan) in the context of the pathogenic variants collected from ClinVar (red) and bound zinc and magnesium ions (blue). The p.D294A substitution has a higher PathProx score (0.44 vs 0.10) compared to the p.T273M variant, indicating greater spatial colocalization with the pathogenic variants than with the neutral distribution from ExAC.

clinical findings associated with disease while these were observed together in 23.5% of the p.T273M group.

On an individual level, there was no observed correlation between average serum AlkP and clinical manifestations of disease (Figs. 2 and 3). In subjects with the p.D294A variant, all subjects except for subjects 7, 8, 19, and 20 had at least one recorded AlkP below the lower limit of normal, but only subjects 1 through 8 had a finding in their clinical history consistent with disease. This left subjects 1 through 6 with both a low AlkP level and a clinical manifestation associated with HPP. All subjects within this genotype cohort except for subjects 19 and 20 had a least one biochemical or clinical history marker associated with HPP. Among those with the p.T273M variant, subjects 21 to 24 and 32 to 36 had at least one low AlkP value while subjects 21 to 31 each had at least one clinical feature consistent with disease. Subjects 21 to 24 had both a low AlkP and at least one disease-related manifestation associated with HPP. Within this genotype cohort, all subjects except for subject 37 had either a biochemical or clinical history consistent with disease. Interestingly, while many subjects in the p.T273M cohort had multiple bone or dental findings, only subjects 1 and 2 in the p.D294A had multiple co-occurring markers on clinical history.

Discussion

Current knowledge about genotype/phenotype correlations in HPP are limited by the condition being an uncommon diagnosis caused by a multitude of *ALPL* variants. Additional complexity occurs as *ALPL* variants can exhibit a dominant-negative effect as a result of interactions between mutated and wild-type monomers, and it has been suggested that clinical presentation of HPP may be affected by strength of the dominant-negative effect [36]. We evaluated the phenotypes of subjects with 2 distinct variants in the *ALPL* gene who did not have a clinical diagnosis of HPP, and thus, our cohort is likely to include a broad representation of the clinical manifestations of HPP, not driven by presumptive diagnosis. Despite using multiple methods to attempt to predict a phenotype consistent with disease, no single predictor, including in vitro assays, in silico modeling, and in vivo enzyme activity, consistently correlated with disease in our cohort. With respect to the p.D294A variant, our in silico modeling gives a clear hypothesis for the observed decreased functional activity of this enzyme due to the replacement of the negatively charged aspartate residue by a neutral alanine in the active site of the enzyme. This is also reflected in the in vivo results from the chart review of subjects, as they have low-normal average levels of serum AlkP levels and frequently have serum AlkP levels below the lower limit of normal. Despite the correlation between genotype and enzymatic phenotype, only 30% of subjects with this variant have both a low serum AlkP and a musculoskeletal manifestation consistent with the clinical disease. In contrast, the molecular modeling in the p.T273M variant places this away from the enzymatic active site and metal or collagen binding domains, making a clear hypothesis about the biologic consequence of this residue change more difficult to construct. Furthermore, recent evidence suggests that this variant does not result in a significant

Subject Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Sex	F	F	F	M	M	F	M	F	M	M	M	M	M	M	M	F	F	M	M	M
Age at Review	82	98	45	56	59	91	26	72	62	87	76	84	74	93	8	66	72	13	17	23
Scoliosis						X														
Low BMD	X	X	X					X												
Long Bone Fracture	X	X																		
Spine Fracture	X				X															
Dental Disease				X																
≥1 phenotypic marker	X	X	X	X	X	X	X	X												
Mean AlkP	36	24	27	56	44	28	81	110	82	17	41	41	26	28	40	196	49	104	83	79
One AlkP value ≤ 40	X	X	X	X	X	X			X	X	X	X	X	X	X	X	X	X		
Both markers present	X	X	X	X	X	X														

Figure 2. Subject data for those with the p.D294A variant.

Subject Number	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
Sex	F	M	M	M	M	M	M	F	M	F	M	M	M	F	F	M	F
Age	90	94	102	70	90	91	71	61	88	50	49	59	59	47	44	68	88
Scoliosis		X			X						X						
Low BMD	X		X	X	X				X	X							
Long Bone Fracture	X	X			X	X		X	X				X				
Spine Fracture						X		X	X								
Dental Disease						X		X	X	X							
≥1 phenotypic marker	X	X	X	X	X	X	X	X	X	X	X						
Mean AlkP	41	39	47	39	64	52	58	65	81	52	52	50	37	43	42	39	60
One AlkP value ≤ 40	X	X	X	X									X	X	X	X	X
Both markers present	X	X	X	X													

Figure 3. Subject data for those with the p.T273M variant.

reduction in enzyme activity alone or when co-expressed with the wild-type enzyme [36]. However, patients in this cohort had a serum AlkP less than the lower limit of normal (40 IU/L) and a similar proportion had an orthopedic manifestation. The lack of correlation between enzymatic activity and phenotypic manifestations in this cohort suggests that better understanding the protein structure changes induced by these variants outside of their enzymatic function may be needed to more fully explain the pathogenic mechanisms in this disease process.

Of note, while previous observations have suggested that HPP is more prevalent in women [13, 21], our sample contains equal numbers of women and men who had both clinical criteria associated with diagnosis. While not definitive due to a lack of complete clinical information and the small sample size of our cohort, this suggests that reports of higher rates of HPP diagnosis among women may be a product of our current diagnostic assumptions and not a characteristic of the underlying disease.

Taken together, our data have several implications for clinical practice and future study. In contrast to previous work with patients with pediatric onset HPP, our sample of mostly adults who have less severe HPP-associated disease shows minimal, if any, concordance between serum AlkP levels and clinical disease [15]. While one might expect that lower serum alkaline phosphatase activities would predict more severe musculoskeletal disease, this was not the case among our cohort. Indeed, among our cohort, even those with mean AlkP within the normal range were noted to have symptoms consistent with disease, while, on the other hand, several subjects with mean AlkP activities well below the lower limit of normal had no skeletal manifestations discovered during chart review. This lack of concordance between in vitro activity and clinical disease suggests that other pathways may be able to either exacerbate or diminish the effects of even small changes in TNSALP enzymatic activity. Furthermore, while potentially an artifact of our relatively small sample size, the observation that the mean AlkP activity among those with the p.T273M was lower than in those with the p.D294A variant despite in vitro assays requires further study to better understand the molecular basis of this observation

Our data have several strengths. Because our cohort is drawn from all patients presenting to our medical facility, and inclusion is solely based on genotype, we are better able to capture the entire spectrum of disease manifestations and not only those with severe disease seen in subspecialty clinics. As a retrospective review of the entire medical records of these subjects, we were able to retrospectively capture clinical data for decades, and review radiology reports and subspecialty notes, ensuring dense medical history. Similarly, most subjects also had AlkP values from multiple time points, ensuring an accurate reflection of the AlkP trends over time. However, our study has multiple limitations. First, our genotype data are the result of GWAS screening, and while it includes multiple loci within the TNSALP gene, it is by no means comprehensive, and without further sequencing we cannot exclude the possibility that our patients may have additional undetected variants. Additionally, as a retrospective study, our sample is vulnerable, particularly among the

younger subjects in the cohort, to the future discovery of clinical manifestations of disease which might change subject categorization past the conclusion of our analysis. Also, because radiologic studies and physical exams of these subjects were performed by practitioners performing general exams not focused particularly on HPP manifestations, it is likely that certain disease manifestations and classic radiographic findings are underreported within our cohort, as these are less commonly the focus of general medical exams. Finally, the musculoskeletal search terms (*scoliosis, long bone fractures, low bone mineral density, spinal fractures,* and *dental disease*) are nonspecific and could be due to other etiologies. Furthermore, because our data is from a single tertiary referral center and patients had inconsistent follow-up, we are unable to directly calculate prevalence of these outcomes but suspect they are an underestimate of these rates. However, a comparison of rates of fracture and scoliosis in our cohort to epidemiologic data suggests that our cohort has excess risk of these complications compared with their expected background prevalence [40-42]. Despite these limitations, this study demonstrates that the lack of a tight genotype/phenotype correlation in HPP should give clinicians pause when using genotypic information to guide patient care in this disease.

Acknowledgments

Additional Information

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Disclosure Summary: The authors declare nothing to disclose.

Data Availability: The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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