Journal of Bone and Mineral Metabolism (2021) 39:804–809
https://doi.org/10.1007/s00774-021-01219-0

ORIGINAL ARTICLE

# Novel mutation in the *ALPL* gene with a dominant negative effect in a Japanese family

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Received: 23 October 2020 / Accepted: 25 February 2021 / Published online: 5 April 2021


## Abstract

**Introduction** Hypophosphatasia (HPP) is caused by mutations in the *ALPL* gene encoding tissue nonspecific alkaline phosphatase (TNSALP) and inherited in either an autosomal recessive or autosomal dominant manner. It is characterized clinically by defective mineralization of bone, dental problems, and low serum ALP levels. In the current report, we demonstrate a novel mutation in the *ALPL* gene (c.244G > A p.Gly82Arg) in a Japanese family with low serum ALP levels.

**Materials and methods** The *ALPL* gene analysis using hybridization capture-based next-generation sequencing was performed. The expression plasmids of the wild type and mutated TNSALP were introduced into COS-7 cells. The enzymatic activity of ALP in the cell lysates was measured using p-nitrophenylphosphate as a substrate.

**Results** TNSALP with the novel *ALPL* mutation (c.244G > A p.Gly82Arg) completely lost its enzymatic activity and suppressed that of wild-type TNSALP, corroborating its dominant negative effect. The diagnosis of autosomal dominant HPP was confirmed in three members of the family.

**Conclusion** Our approach would help to avoid the inappropriate use of bone resorption inhibitors for currently mis- or underdiagnosed HPP, given that the presence of further, yet undetected mutations of the *ALPL* gene are plausible.

**Keywords** Hypophosphatasia · Adult hypophosphatasia · *ALPL* gene · Novel mutation

## Introduction

Hypophosphatasia (HPP) is caused by mutations in the *ALPL* gene, which encodes tissue nonspecific alkaline phosphatase (TNSALP), and characterized by defective mineralization of bone, dental problems, and low serum ALP levels. TNSALP forms a homodimer which is required to exert an enzymatic activity. HPP is generally classified into the following six subtypes based on the age of onset, clinical features and disease severity; perinatal severe, perinatal benign, infantile, childhood, adult and odonto HPP. To date, more than 400 *ALPL* gene mutations have been identified and listed in the *ALPL* gene mutation database (http://alplmutationdatabase.hypophosphatasie.com/). HPP results from either autosomal recessive or autosomal dominant inheritance, depending on the residual enzymatic activity and the dominant negative effect of each mutated TNSALP. Patients with perinatal severe HPP generally have homozygous or compound heterozygous mutations with decreased enzymatic activities of ALP, whereas odonto HPP, the least severe form of HPP lacking musculoskeletal abnormalities, is frequently caused by a single

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heterozygous mutation with a dominant negative effect [1, 2]. We herein report a novel mutation in the *ALPL* gene in a Japanese family which was incidentally found in routine clinical practice and confirmed to have a dominant negative effect by in vitro transfection experiments.

## Materials and methods

The *ALPL* gene analysis using hybridization capture-based next-generation sequencing was performed at Kazusa DNA Research Institute (Kisarazu, Japan) and was covered by insurance as usual clinical practice.

The expression plasmid for green fluorescent protein (GFP)-tagged TNSALP (pcDNA-GFP-ALP) was constructed using ALP cDNA provided by Dr. Henthorn, as previously reported [3]. The identified *ALPL* mutation (c.244G > A; p.Gly82Arg) was introduced using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Palo Alto, CA, USA).

The expression plasmids of the wild type (WT) and mutated TNSALP and pGreenLantern encoding GFP alone (Life Technologies) were introduced into COS-7 cells using the FuGENE HD Reagent (Roche, Indianapolis, IN, USA). Three days later, cell lysates were harvested in 10 mM Tris–Cl and 0.05% Tris–Triton X-100 after repeated freeze and thaw. The enzymatic activity of ALP in the cell lysates were measured using p-nitrophenylphosphate as a substrate and normalized based on the protein amount. To confirm the expression of GFP-tagged TNSALP proteins, Western blotting was performed using aliquots of the lysates and anti-GFP mouse monoclonal antibody (Roche).

Subcellular distribution of GFP-tagged TNSALP proteins in living cells was determined by detecting GFP fluorescence under Olympus IX51 fluorescence microscope. To examine the presence of GFP-tagged TNSALP proteins on the plasma membrane, cells fixed in 3.7% formaldehyde were subjected to immunofluorescent staining using anti-GFP rabbit polyclonal antibody (Proteintech) and Alexa Fluor® 555 antirabbit IgG (Molecular Probes) without permeabilization.

Statistical analysis was performed using one-way analysis of variance (ANOVA) and the method of Tukey–Kramer for post hoc tests.

The study was approved by institutional review board in Osaka Women's and Children's Hospital.

## Results

### Case

A 29-year-old woman presented Raynaud's phenomenon and purpura on her legs and was confirmed to have cutaneous polyarteritis nodosa by skin biopsy. Besides CRP elevation (0.63 mg/dL), blood tests incidentally revealed a low serum ALP level (52 IU/L [normal range: 105–330 IU/L]). Re-examination with an interval of three months confirmed the low serum ALP levels (59 IU/L). Although she had no history of fracture nor tooth loss, increased urinary phosphoethanolamine (PEA) (281.6 µmol/L [normal range: 5.9–76.6 µmol/L]) suggested the presence of HPP. Genetic analysis using hybridization capture-based next-generation sequencing identified a novel missense mutation in her *ALPL* gene (c.244G > A p.Gly82Arg) in a heterozygous fashion (Fig. 1a). In silico analyses, including SIFT, Polyphen-2, and CADD, all predicted the novel mutation as damaging. To characterize the novel *ALPL* mutation (c.244G > A p.Gly82Arg) and to confirm the diagnosis of HPP in the case, we performed in vitro transfection experiments. As shown in Fig. 1b, TNSALP with the novel *ALPL* mutation (c.244G > A p.Gly82Arg) completely lost its enzymatic activity and suppressed that of WT TNSALP, corroborating its dominant negative effect as well as the diagnosis of autosomal dominant HPP in our case (Fig. 1b). Although the dominant negative effect of p.Gly82Arg was not dose-dependent (comparable ALP activity with 0.25 and 0.5 g of GFP-ALP[p.G82R] as shown in Fig. 1b), the same pattern was observed with p.Ala377Val [2]. GFP fluorescence was observed in the cytoplasm and on the plasma membrane of living cells transfected with GFP-ALP[WT] and those transfected with GFP-ALP[p.G82R] (Fig. 2a). We confirmed the presence of GFP-ALP[WT] and ALP[p.G82R] on the plasma membrane by immunofluorescent staining of non-permeabilized cells using anti-GFP antibody (Fig. 2b). She had normal bone mineral density at the lumbar spine and the femoral neck (107% and 105% of young adult mean, respectively), but taking into account the current use of glucocorticoid (prednisolone of 10 mg/day), treatment was started with eldecalcitol (0.75 µg/day).

### Family study

Among the family members of the case, her father, mother, and sister agreed to undergo genetic analysis. After obtaining informed consent, we performed blood test, urine amino acid analysis, and hybridization capture in the *ALPL* gene. Her mother and her sister also had the novel *ALPL* mutation (c.244G > A p.Gly82Arg) in a heterozygous fashion (Table 1). Increased urinary PEA was observed in both, whereas her sister, but not her mother, exhibited a low serum ALP level (36 and 127 IU/L, respectively). In her mother, the extremely high level of bone resorption marker TRACP-5b (738 mU/dL [standard range: 120–420 IU/L]) and an ALP of around lower limit of the normal range (127 IU/L) suggest an impaired compensation for the increased bone resorption, presumably due to postmenopause, by bone formation, supporting the presence of HPP. Her father had another *ALPL*

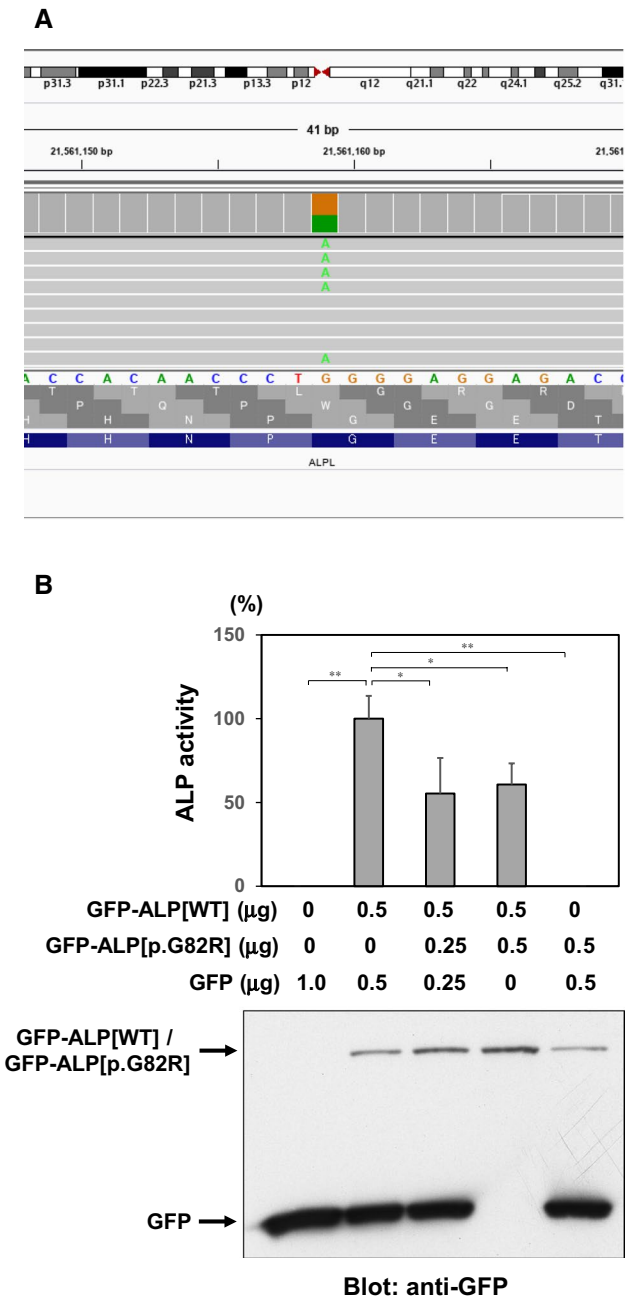


**Fig. 1** The novel *ALPL* gene mutation in the case (**a**). Hybridization capture-based next-generation sequencing identified a heterozygous missense variant at chromosome 1: 21,561,159 bp (c.244G > A p.Gly82Arg). Dominant negative effect of the p.Gly82Arg mutant of TNSALP (**b**). The indicated amounts of the plasmids for wild type (GFP-ALP[WT]) and/or the p.Gly82Arg mutant (GFP-ALP[p.G82R]) TNSALP were introduced into COS-7 cells. Total amounts of the plasmids were adjusted by adding pGreenLantern encoding GFP alone. Three days after the transfection, cell lysates were harvested to determine the enzymatic activity using p-nitrophenylphosphate as a substrate. The activity in the cells transfected with GFP-ALP[WT] without GFP-ALP[p.G82R] was designated as 100%. The data are shown as the mean ± S.D. ($N = 3$). *$p < 0.05$, **$p < 0.01$. The expression of the GFP-tagged TNSALP proteins was confirmed by Western blotting using the aliquots of the lysates and anti-GFP antibody

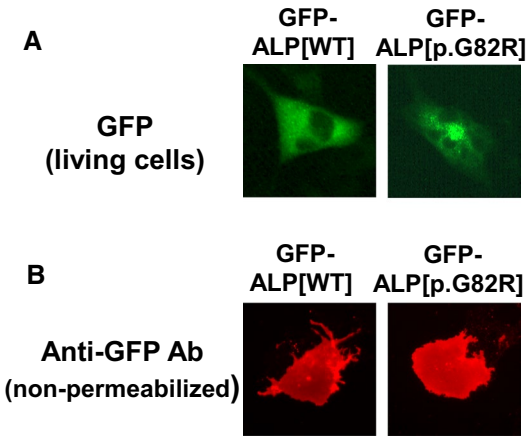


**Fig. 2** Subcellular distribution of GFP-ALP[WT] and GFP-ALP[p.G82R] in transient transfections to COS-7 cells. In living cells, GFP fluorescence derived from the TNSALP fusion proteins was detected in the cytoplasm and on the plasma membrane (**a**). The presence of GFP-ALP[WT] and GFP-ALP[p.G82R] on the plasma membrane was confirmed by immunostaining using anti-GFP antibody of non-permeabilized cells (**b**)

mutation (c.613G > A p.Ala205Thr, minor allele frequency 0.04%), and a slightly decreased ALP (103 IU/L), but did not have an increase in urinary PEA (61.9 μmol/L [normal range: 5.9–76.6 μmol/L], 45 μmol/gCr [normal range: 7–70 μmol/gCr]). Besides these mutations, four single nucleotide polymorphisms (SNPs) in the *ALPL* gene with minor allele frequencies of 7–27% were detected, but the carriage of these SNP alleles was not different among the family members. These SNPs were predicted to be benign by in silico analyses including SIFT, Polyphen-2, and CADD (Table 1 and Supplementary Table 1). Her mother started treatment with eldecalcitol (0.75 μg/day) and raloxifene (60 mg/day). Her sister was planned to undergo bone mineral density testing annually and to initiate treatment with eldecalcitol if progressive bone loss would be observed.

## Discussion

The phenotype of HPP varies greatly in patients, even among those with identical *ALPL* genotype [4, 5]. It is plausible that not a few patients with HPP and mild symptoms are currently mis- or under-diagnosed [6]. The identification of HPP is of great clinical significance particularly in osteoporotic individuals since the use of bone resorption inhibitors, such as bisphosphonates and denosumab, may increase the risk of atypical femoral fractures in patients with HPP regardless of the disease severity [7, 8]. Bisphosphonates inhibit ALP activity by competing for binding to divalent metal ions, such as zinc or magnesium, which is vital for ALP to exert an effect on bone mineralization [9].

**Table 1** Characteristics, laboratory data, X-ray findings, and mutations and single nucleotide polymorphisms (SNPs) in the *ALPL* gene of the present family

| | | | Father | Mother | Case | Sister |
|---|---|---|---|---|---|---|
| Age (year) | | | 63 | 54 | 29 | 22 |
| Height (cm) | | | 174 | 156 | 163 | 165 |
| Weight (kg) | | | 79 | 70 | 71 | 50 |
| Disease | | | HT, DL, HU | HT, DL | cPAN | - |
| Anti-Osteoporotic Treatment | | | – | – | – | – |
| Fracture | | | – | – | – | – |
| Tooth Loss | | | – | – | – | – |
| Musculoskeletal Pain | | | – | – | – | – |
| Laboratory Data ([Standard Value] Unit) | | | | | | |
| ALP ([106–322] IU/L) | | | 103 | 127 | 52 | 36 |
| Alb ([4.1–5.1] g/dL) | | | 4.0 | 4.3 | 4.1 | 4.4 |
| Ca ([8.8–10.1] mg/dL) | | | 9.2 | 9.5 | 9.8 | 9.2 |
| P ([2.7–4.6] mg/dL) | | | 3.1 | 5.2 | 3.5 | 3.6 |
| Mg ([1.8–2.3] mg/dL) | | | 1.9 | 2.0 | 1.7 | 1.7 |
| BAP ([2.9–14.5] µg/L*) | | | N.A | 5.6 | 2.0 | N.A |
| PINP ([17.1–64.7] µg/L*) | | | N.A | 71.9 | 15.5 | 51.6 |
| TRACP-5b ([120–420] mU/dL*) | | | N.A | 738 | 118 | 250 |
| 25(OH)D ([> 20.0] ng/mL) | | | N.A | 17.7 | 19.9 | 13.6 |
| PTH-I ([9.3–74.9] pg/mL) | | | N.A | 25 | 17 | 15 |
| FGF23 ([< 30.0] pg/mL) | | | N.A | 40.7 | 77.7 | 37.9 |
| U-PEA ([5.9–76.6] µmol/L) | | | 61.9 | 159.2 | 281.6 | 86.9 |
| X-ray | | | | | | |
| Bone Bowing | | | N.A | – | – | – |
| BMD (YAM) | Lumber Spine | | N.A | 81% | 107% | 82% |
| BMD (YAM) | Femoral Neck | | N.A | 72% | 105% | 95% |
| Mutations and SNPs in the ALPL gene | | | | | | |
| | | In silico analysis** | | | | |
| c.244G > A (p.Gly82Arg) | Novel mutation | Damaging | – | +, Hetero | +, Hetero | +, Hetero |
| c.330 T > C (p.Ser110Ser) | rs1780316 (MAF 7%) | Benign | +, Homo | +, Homo | +, Homo | +, Homo |
| c.613G > A (p.Ala205Thr) | rs751455369 (MAF 0.04%) | Damaging | +, Hetero | – | – | – |
| c.787 T > C (p.Tyr263His) | rs3200254 (MAF 27%) | Benign | +, Hetero | +, Hetero | +, Hetero | +, Hetero |
| c.863-7 T > C (Intron Variant) | rs74063111 (MAF 26%) | Benign | +, Hetero | +, Hetero | +, Hetero | +, Hetero |
| c.876A > G (p.Pro292Pro) | rs3200255 (MAF 27%) | Benign | +, Hetero | +, Hetero | +, Hetero | +, Hetero |

*Mean value ± 1.96 S.D. of healthy premenopausal women. **Details are presented in Supplementary Table 1. HT, hypertension; DL, dyslipidemia; HU, hyperuricemia; cPAN, cutaneous polyarteritis nodosa; BAP, bone-specific alkaline phosphatase; PINP, procollagen type I N-terminal propeptide; TRACP-5b, tartrate-resistant acid phosphatase 5b; 25(OH)D, 5-hydroxyvitamin D; PTH-I, intact parathyroid hormone; FGF23, fibroblast growth factor 23; U-PEA, urinary phosphoethanolamine; BMD, bone mineral density; YAM, young adult mean; MAF, minor allele frequency based on the SNP database or Human Genetic Variation Database; N.A., not available

Conversely, increased bone mineral density of the lumbar spine was observed in adult HPP patients, particularly those with lower ALP activity and higher levels of ALP substrates including urinary PEA [10]. Therefore, more information on the characteristics of HPP, particularly those with adult and odonto HPP, would help to prevent the development of atypical femoral fractures by avoiding inappropriate use of bone resorption inhibitors. The current report provides additional knowledge of adult HPP and suggests the presence of further, yet undetected mutations of the *ALPL* gene.

Referring to the analysis of 58 mammalian TNSALP by Silvent et al. [11], the position Gly82 has been conserved through 220 million years of mammalian evolution. Although Gly82 is not located in known functionally important regions, such as homodimeric interface, active site, N-terminal α-helix domain, crown domain, calcium site, conservation during long geological periods suggests its indispensable role in the biological function of TNSALP. Glu83, Glu84, and Thr85 are in homodimeric interface [11], suggesting some roles of their adjacent

amino acid Gly82 in dimer formation. Consistent with these findings, the substitution of Arg82 for Gly82 resulted in the complete loss of ALP enzymatic activity in our in vitro analysis. Although the subcellular distribution of p.Gly82Arg was not different from that of WT TNSALP (Fig. 2), the dominant effect of mutant TNSALP may also be due to the sequestration of the WT protein by the mutated one into the Golgi apparatus, preventing it from being transported to the membrane [12, 13].

The current data also indicate an imbalance between the genotype and phenotype of HPP, in particular that of adult HPP [4]. Consistent with the intrafamilial phenotypic variability in HPP as previously reported [5, 8, 14], bone turnover markers, and bone mineral densities differed largely between our case and her sister (Table 1). A recent family study reported asymptomatic mother despite extremely low levels of serum ALP, whereas her daughter had several HPP-related symptoms, such as tooth loss, fractures, short stature, with slightly decreased ALP levels [8]. SNPs in the *ALPL* gene, variants in other genes, and epigenetic modifications may alter the penetration of the disease. Previous reports included some information regarding one of the *ALPL* SNPs (rs3200254 c.787 T > C p.Tyr263His), which was also identified in the current case and her family (Table 1). In a Chinese family, two members with premature deciduous tooth loss and low serum ALP levels had a heterozygous substitution on rs3200254 without any other *ALPL* mutations causing amino acid substitution [15]. Japanese case series reported low serum ALP levels in individuals with a homozygous substitution on rs3200254 and more severe phenotypes in patients with a heterozygous substitution on rs3200254 and another *ALPL* mutation as compared to those with the *ALPL* mutation alone [16]. Conversely, a substitution on rs3200254 was associated with a high bone mineral density among postmenopausal Japanese women [17]. A recent study using next-generation sequencing indicated a SNP in the *COL1A2* gene (rs42524 c.1645C > G p.Pro549Ala) as a modifier of HPP [18]. *COL1A2* encodes the pro-alpha2 chain of type I collagen and its mutations are responsible for osteogenesis imperfecta. Moreover, TNSALP is known to interact with type I collagen and this interaction is believed to contribute to bone mineralization [19, 20]. Female predominance in HPP, particularly that in adult HPP observed in the recent cohorts [6, 10, 18], also suggests HPP as a multifactorial disease rather than a single gene disorder.

In conclusion, we demonstrate a novel mutation in the *ALPL* gene with a dominant negative effect and its heterozygous inheritance as a cause of HPP. Although further evidence is required to identify the modifiers of HPP phenotype, the current report provides additional knowledge of adult HPP. In addition, our approach, including genetic testing, in silico analysis, and in vitro transfection experiments, would help to avoid the inappropriate use of bone resorption inhibitors for currently mis- or under-diagnosed HPP.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00774-021-01219-0.

**Acknowledgements** We thank Dr. Olga Amengual for proofreading.

**Funding** No funding was received.

## Compliance with ethical standards

**Conflict of interests** Masaru Kato has received research grants from AbbVie, Actelion, and GlaxoSmithKline and speaking fees from Eli Lilly. Tatsuya Atsumi has received research grants from Astellas, Takeda, Mitsubishi Tanabe, Chugai, Daiichi-Sankyo, Otsuka, Pfize, Alexion, Bayer, Otsuka, Chugai, Takeda, Eisai, Bristol-Myers Squibb, Daiichi Sankyo, Mitsubishi Tanabe and AsahiKasei, consultant fees from Ono, Sanofi, Daiichi Sankyo and Pfizer and speaking fees from Mitsubishi Tanabe, Chugai, Astellas, Takeda, Pfizer, Daiichi Sankyo, Bristol-Myers Squibb and Eli Lilly. Other authors have nothing to declare.

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