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**Research Article**

# Newborn screening for hypophosphatasia: development of a high-throughput tissue nonspecific alkaline phosphatase activity assay using dried blood spots

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## Abstract

Hypophosphatasia (HPP) is an inherited metabolic disease caused by deficiency of tissue nonspecific alkaline phosphatase (TNAP) caused by pathogenic variants of the *ALPL* gene (MIM 171760). The clinical manifestations of HPP vary, ranging from a lethal perinatal-onset type to a moderate late-onset type presenting with nonspecific symptoms, such as arthropathy and musculoskeletal pain. HPP is characterized by low TNAP activity and defective bone mineralization, leading to bone deformity and skeletal abnormalities. Moreover, this disease can cause systemic complications, such as muscle weakness, seizures, pain, and respiratory failure, leading to premature death in infants. This study aimed to evaluate whether measuring TNAP activity in dried blood spots (DBSs) can identify patients with HPP. We developed an assay to assess TNAP activity using DBSs and screened 45 632 newborns born between February 2019 and March 2022 in Kumamoto Prefecture in Japan for HPP. We detected a single heterozygous variant of the *ALPL* gene in 5 newborns. During the clinical course follow-up, one newborn presented with HPP-related clinical manifestations. This is the first study on newborn screening (NBS) for HPP worldwide. NBS for HPP using DBSs may be practical and beneficial, as it is a high-throughput method. Moreover, the DBSs used for the TNAP assay are the same as those used for public-funded NBS worldwide. In the future, this system may be implemented as standard NBS for HPP.

**Keywords:** hypophosphatasia, *ALPL* gene, newborn screening, dried blood spots, tissue nonspecific alkaline phosphatase

## Lay Summary

Hypophosphatasia (HPP) is an inherited metabolic disease caused by deficiency of tissue nonspecific alkaline phosphatase (TNAP) as a result of pathogenic variants of the *ALPL* gene. HPP is characterized by low TNAP activity and defective bone mineralization, leading to bone deformity and skeletal abnormalities. We developed an assay to assess TNAP activity using dried blood spots and screened 45 632 newborns born between February 2019 and March 2022 in Kumamoto Prefecture, Japan. We detected a single heterozygous variant of the *ALPL* gene in 5 newborns. This is the first study on newborn screening for HPP worldwide.

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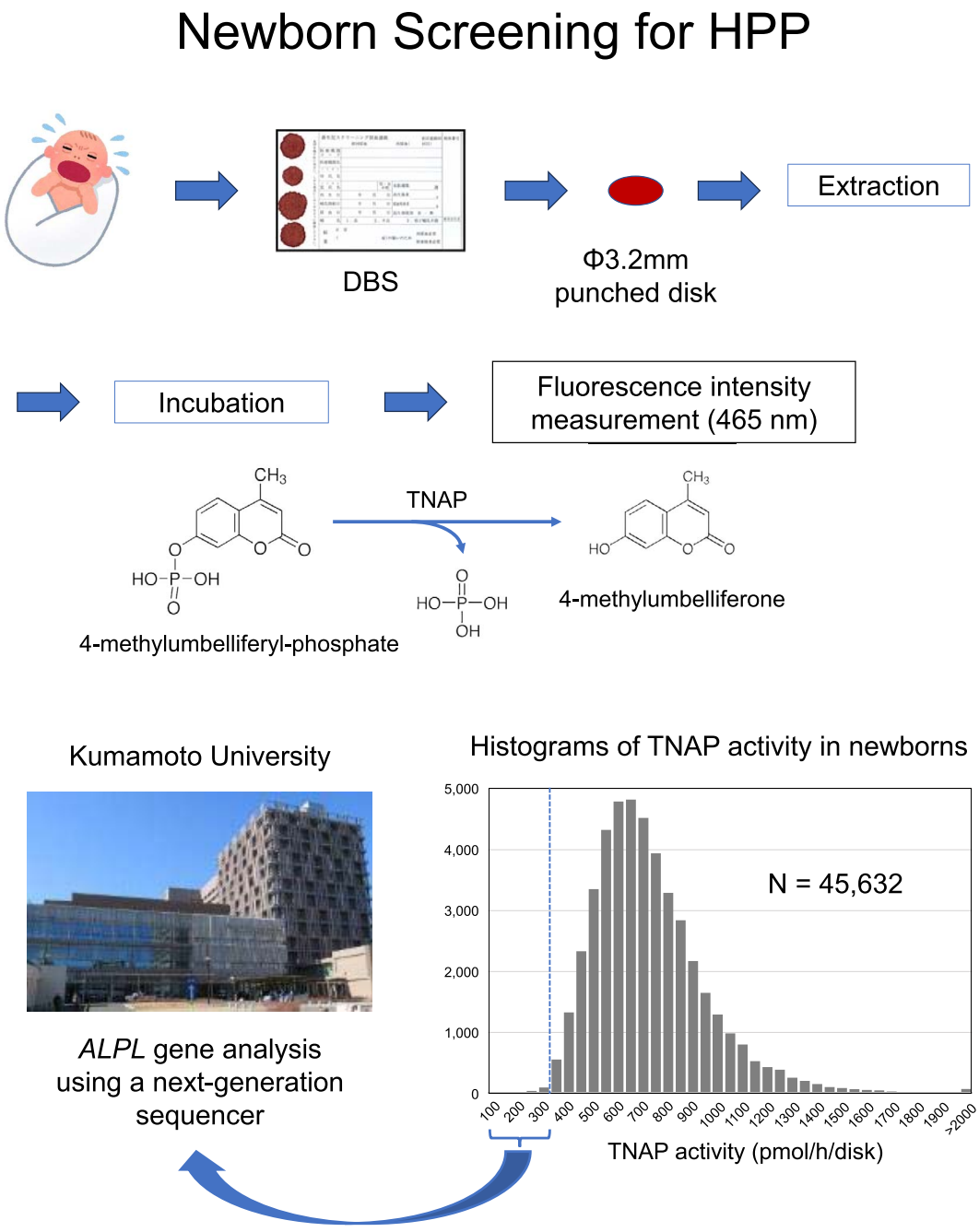
## Graphical Abstract




## Introduction

Hypophosphatasia (HPP) is an inherited metabolic disease caused by the loss of function of tissue-nonspecific alkaline phosphatase (TNAP) encoded by the *ALPL* gene (MIM 171760). The *ALPL* gene is located on chromosome 1p36.12, has a length of 69.4 kb of DNA containing 12 exons, and encodes a 524 amino acid protein.[1]

TNAP is a cell-surface homodimeric phosphohydrolase expressed in the liver, kidneys, skeleton, and developing teeth. Inorganic pyrophosphate (PPi), a potent inhibitor of mineralization, accumulates outside these tissues. The accumulation of extracellular PPi causes complications, such as premature loss of deciduous teeth, rickets, osteomalacia, and calcific arthropathy.[2]

HPP clinical manifestations vary, ranging from a lethal perinatal-onset type to a moderate late-onset type presenting with nonspecific symptoms, such as arthropathy and musculoskeletal pain. HPP is classified into 6 clinical types according to severity and age at onset: perinatal severe, perinatal benign, infantile (OMIM 241500), childhood (OMIM 241510), adult (OMIM 146300), and odontohypophosphatasia.[3] HPP is characterized by low TNAP activity and defective bone mineralization, leading to bone deformity and skeletal abnormalities. Moreover, HPP can cause systemic

manifestations, such as muscle weakness, seizures, pain, and respiratory failure, leading to premature death in infants.[4]

The prevalence of severe HPP in Europe and Japan was estimated based on patient data, with approximately 1 per 300 000 births in Europe[5] and 1 per 150 000 births in Japan.[6,7] Severe-type HPP is inherited in an autosomal recessive manner, whereas moderate types are considered to be inherited in an autosomal recessive or dominant manner, and some variants have been reported to have dominant-negative effects.[8–11]

Mornet et al. estimated the prevalence of moderate-type HPP, associated with genotypes such as severe dominant (Sd)/moderate, severe/moderate, and Sd/WT. These genotypes encompass infantile, childhood, odontohypophosphatasia, and adult type with specific manifestations. The prevalence was calculated as approximately 1 per 2430 births through assessing the proportion of Sd alleles and estimating the penetrance of the Sd alleles in heterozygotes at 50%.[5]

Currently, large human genome databases, such as Tohoku Medical Megabank Organization (ToMMo; https://www.megabank.tohoku.ac.jp/)[12] and Genome Aggregation Database (GnomAD; https://gnomad.broadinstitute.org)[13] are available and contribute to calculating the theoretical prevalence of patients with HPP. Moreover, Michigami et al. reported unique clinical and genetic features of Japanese patients with HPP, including a high prevalence of perinatal severe and perinatal benign forms, which are associated with c.1559delT (p.Leu520Argfs*86) and c.979T>C (p.Phe327Leu), respectively.[14]

The enzyme replacement therapy ([EZRT], asfotase alfa [Strensiq; Alexion Pharmaceuticals, Inc.]) was approved in Japan, Europe, and the USA in 2015. EZRT has been shown to improve long-term clinical outcomes, including survival, bone mineralization, pulmonary function, calcium homeostasis, height, weight, and mobility.[15,16] Moreover, EZRT could improve dental problems in infants with HPP.[17]

In cases of adults with HPP, many patients are misdiagnosed with conditions, such as osteomalacia, rheumatoid arthritis, fibromyalgia, chronic back pain, pseudogout, calcific tendinitis, osteoarthritis, spondyloarthropathy, or undifferentiated connective tissue diseases, leading to diagnostic delays.[18,19] Moreover, some patients diagnosed with "osteoporosis" are treated with bisphosphonates, which may exacerbate skeletal complications due to the risk of worsening bone mineralization.[20] Misdiagnosis and diagnostic delays may impair patients' quality of life (QOL) and long-term outcomes,[18] particularly in cases of severe-type HPP.

Newborn screening (NBS) can help identify patients with HPP before clinical manifestations appear, enabling early diagnosis, timely treatment, and improved clinical outcomes.

There have been trials of NBS using gene analysis in dried blood spots (DBSs)[21] and measuring urinary phosphoethanolamine (PEA).[22] TNAP in DBSs reflects TNAP activity in whole blood, including blood cells and serum. Serum TNAP is derived from various tissues, such as those of the liver, kidneys, and bones.

Here, we aimed to evaluate whether measuring TNAP activity in DBSs from newborns can help identify patients with HPP. Further, we discuss the characteristics of TNAP assay results and the genotypes identified in newborns with positive DBS screening results for HPP. To the best of our knowledge, this is the first study of NBS for HPP using TNAP activity in DBSs.

## Materials and methods

### Study population

In total, 45 632 newborns born between February 2019 and March 2022 in Kumamoto Prefecture in Japan were enrolled. Informed consent was obtained from the parents of all the newborns. The DBS samples of newborns were routinely collected at each maternity clinic or obstetric department as part of a public health program, using a heel-prick procedure 4-6 days after birth. Blood spots were blotted onto filter paper (Toyo Roshi Kaisha Ltd.) and dried at room temperature for at least 4 h. The DBSs were mailed to the Newborn Screening Center at KM Biologics Co., Ltd. (Kumamoto, Japan), where NBS for public health programs was conducted. The DBS samples were then transferred to the Kumamoto University for the TNAP assay.

### NBS for HPP

TNAP activity was assayed in the DBS samples. Newborns whose initial TNAP activities were below the cutoff level were recalled, and DBS samples were prepared again approximately 1 mo after birth for a second TNAP assay. Based on preliminary studies ($n = 2656$, average level: 914 pmol/h/disk), the cutoff level was set at <300 pmol/h/disk, which is equivalent to approximately 0.3% recall rate. For the first and second TNAP assays conducted between February 2019 and January 2020, the cutoff level was set at <300 pmol/h/disk. Between February 2020 and March 2022, the cutoff levels were changed to <320 pmol/h/disk for the first assay and < 400 pmol/h/disk for the second assay. Newborns whose TNAP activity was still below the cutoff level in the second TNAP assay were referred to the hospital for clinical assessment, and physical examination and biochemical assays were performed to detect HPP-related features or manifestations. The *ALPL* gene of the newborn was sequenced to confirm the diagnosis (Figure 1).

### TNAP assay of DBS

The TNAP activity assay was developed in collaboration with KM Biologics Co., Ltd. (see details on intellectual property JP6989871). Briefly, a 3.2-mm diameter disk was punched from the DBS card and placed in each 96-well plate (Greiner 96-well Plate; Sigma-Aldrich Japan K.K., Tokyo, Japan). We added 200 $\mu$L extraction buffer (38 mM potassium chloride, 5 mM magnesium chloride, 0.18 mM dithiothreitol, 0.1% Triton X-100, 0.05% sodium azide in 5 mM acetate buffer, pH = 5.2) into the well, sealed, and rotated the plate for 60 min at 1500 rpm at room temperature. Furthermore, 20 $\mu$L aliquot of the extract was transferred into a 96-well black plate (96 Well FluoroNunc Black, ThermoFisher Scientific K.K.) and 40 $\mu$L of substrate solution (0.1 mM 4-methylumbelliferyl-phosphate, 10 mM sodium succinate, 10 mM L-phenylalanine, 1 mM magnesium chloride, 0.05% Triton X-100 in 0.1 M Tris buffer, pH 10.0) was added. After 3 h of incubation at 38°C, 200 $\mu$L of the reaction stop solution (10 mM EDTA in 300 mM glycine/NaOH buffer, pH = 10.6) was added. Fluorescence intensity was analyzed at excitation and emission wavelengths of 370 and

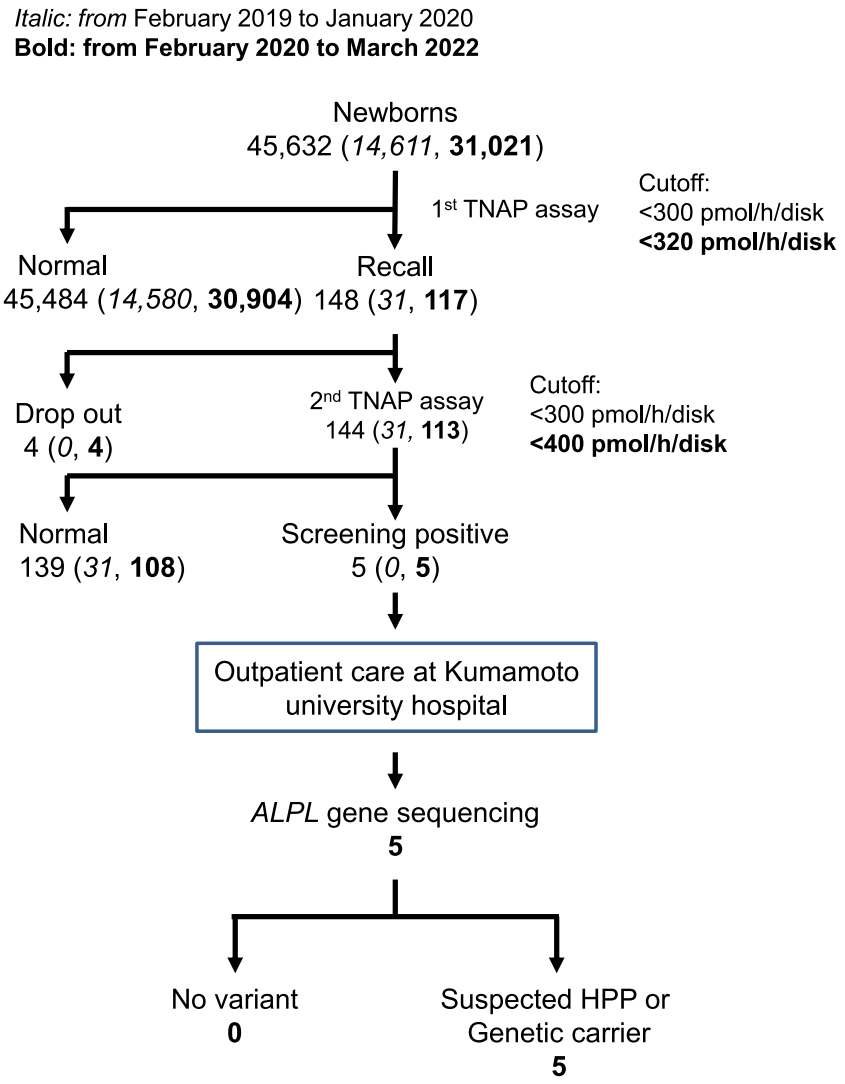



**Figure 1.** Flow chart of newborn screening for hypophosphatasia.

465 nm, respectively. Enzyme activity was calculated as picomoles of 4-methylumbelliferone released per hour per disk (pmol/h per disk).

## Molecular analysis of the ALPL gene

The *ALPL* gene and its flanking regions were amplified by long-range PCR using 3 forward and reverse primer sets (Data S1). The PCR was performed as follows: 94 °C for 2 min, followed by 30 cycles at 98 °C for 10 s, 58 °C for 30 s, and 68 °C for 7 min using KOD FX (Toyobo) and a Veriti Thermal Cycler (Applied Biosystems). The PCR products (amplicons) were purified using an Agencourt AMP XP PCR Purification Kit (Beckman Coulter) and quantified with a Qubit dsDNA HS Assay Kit (Life Technologies) using a Qubit 2.0 Fluorometer (Life Technologies). Three amplicons were mixed in equimolar amounts, followed by simultaneous fragmentation and adaptor ligation, using a Nextera XT Kit (Illumina). The reaction product (library) was validated with a High Sensitivity D1000 ScreenTape (Agilent Technologies), using an Agilent 2200 TapeStation, and quantified using a Qubit dsDNA HS Assay Kit with a Qubit 2.0 Fluorometer to allow for library normalization. Sequencing was performed using a MiSeq Reagent Kit v3 (Illumina) with 150 cycles on a MiSeq sequencer, using the "paired-end" sequencing run method. Sequence data analysis, mapping, and variant calling were streamlined using MiSeq Reporter v2 (Illumina). The reads were aligned to the reference genome sequence of chromosome 1 (NC_000001.11) using algorithm bwa-0.6.1. Single-nucleotide polymorphism and insertion/deletion identifications were performed using the Genome Analysis Toolkit (GATK v1.6; Broad Institute). The reads were visualized using IGV_2.8.0 (Broad Institute).[23] Variants detected by next-generation sequencing (NGS; MiSeq) were confirmed using Sanger sequencing.

## Significance analysis of the variants

The mRNA reference sequence NM_000478.6 was used for this study, wherein the "A" nucleotide of the start codon ATG constituted a + 1 numbering of the cDNA sequence. Methionine, encoded by the start codon ATG, represented +1 for amino acid numbering, as set forth by the preprotein sequences (NP_000469.3). The variant nomenclature followed the guidelines established by the Human Genome Variation Society (http://varnomen.hgvs.org/). ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/)[24] and *ALPL* gene variant database (https://alplmutationdataba

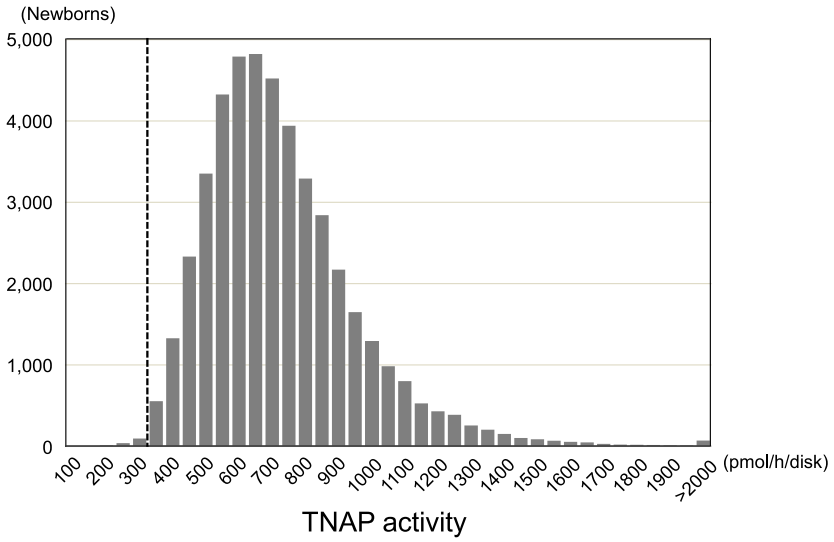



**Figure 2.** Histogram of tissuenonspecific alkaline phosphatase activity in newborns. Dashed line: cutoff level (300 pmol/h/disk).

se.jku.at/)[25] were used to classify each variant. The allele frequency of each variant in the general population was referred to as ToMMo and GnomAD. The bioinformatics tool PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2)[26] was used to predict the potential effect of amino acid alterations in missense variants on the function of each variant.

### Literature review

We surveyed the genetic information of patients with HPP in Japan available on PubMed (https://pubmed.ncbi.nlm.nih.gov), Google Scholar (https://scholar.google.com), and Medical Online (http://mol.medicalonline.jp), using the keywords "hypophosphatasia" and "ALPL pathogenic variant or mutation." We evaluated the variants in patients with HPP as reported in 53 articles (Data S2).

### Ethics

This study was approved by the Ethics Committee of Kumamoto University (approval no. genome 414). Written informed consent was obtained from the parents or legal guardians of newborns.

## Results

### NBS for HPP

The flowchart and results of NBS for HPP are shown in Figure 1. A histogram of the first TNAP assay is shown in Figure 2. Between February 2019 and January 2020, 14 611 newborns were screened, and 31 newborns were recalled for a second TNAP assay. No newborn had TNAP activity below the cutoff level; therefore, the cutoff level was changed to $<320$ pmol/h/disk for the first assay and $<400$ pmol/h/disk for the second assay. Between February 2020 and March 2022, 31 021 newborns were screened, 117 newborns were recalled for a second TNAP assay, and 5 newborns showed positive DBS screening results for HPP. Figure 3 shows the first and second TNAP assays of 144 newborns with TNAP activity below the cutoff level. The mean $\pm$ SD of TNAP activity in the first and second TNAP assays was $256.6 \pm 44.6$ and $691.1 \pm 229.3$ pmol/h/disk, respectively. TNAP activity in the second TNAP assay was 2.7-fold higher than that in the first TNAP assay.

A total of 45 632 newborns were screened, and 5 newborns with low TNAP activity below the cutoff levels, as determined by the second TNAP assay, were examined at the outpatient clinic and underwent DNA analysis. These newborns were evaluated for physical and biochemical manifestations related to HPP at the Kumamoto University Hospital. Moreover, *ALPL* gene sequencing was performed using NGS for these 5 newborns. Each of the 5 newborns had a different variant, and they were diagnosed and followed up as suspected HPP or genetic carriers (Table 1). Three of the 5 variants—c.979T>C (p.Phe327Leu), c.1144G>A (p.Val382Ile), and c.1559delT (p.Leu520Argfs*86)—have been reported in Japanese patients with HPP and are classified as "pathogenic" or "likely pathogenic" in the ClinVar and *ALPL* gene variant databases. The c.734C>T (p.Thr245Met) variant has been detected in French patients with perinatal benign type[5] and is classified as a variant of "uncertain significance" in the ClinVar. The 1304A>G (p.Asp435Gly) variant represents a novel variant not previously reported in the literature.

### Clinical outcomes in newborns with low TNAP activity

Only one newborn presented with significantly lowered serum ALP level, which was measured 5 times (69.3, 57.8, 110.0, 58.0, and 39.0 U/L), and developed mild clinical bone manifestations (Table 1; Figure 4) without a loss of deciduous teeth. The other 4 patients presented with mildly lowered serum ALP levels; however, they did not develop any clinical manifestations. Moreover, PEA increased in cases 1, 3, 4, and 5 (Table 1).

### Literature review

Information on 179 patients with HPP from 167 families was acquired from 53 articles published in Japan. We evaluated the age, sex, clinical outcome (phenotype), ALP activity level, and variant information (Data S2). Of the 179 patients, 63 were males and 63 were females. The information on sex for the remaining 53 patients was not available. Phenotypic classification includes "childhood"

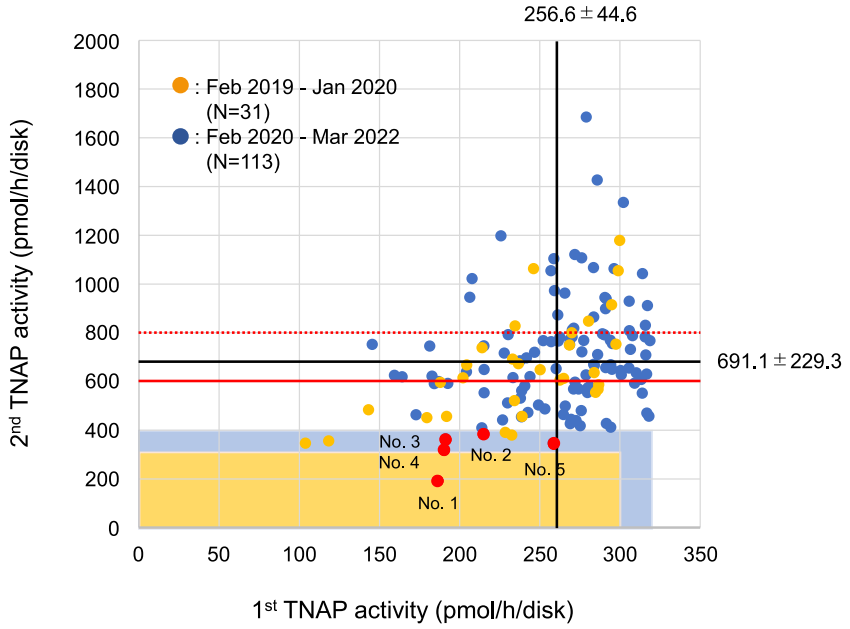



**Figure 3.** TNAP activity below the cutoff level in the first and second TNAP assays of newborns. Overall, 52 (0.11%), 82 (0.18%), and 111 (0.24%) newborns have TNAP activity of <600, <700, and <800 (pmol/h/disk), respectively, in the second assay. Yellow: cutoff level, first assay: 300 pmol/h/disk; second assay: 300 pmol/h/disk. Pale blue: cutoff level, first assay: 320 pmol/h/disk; second assay: 400 pmol/h/disk. Yellow circle: newborn with TNAP activity below 300 pmol/h/disk in the first assay. Blue circle: newborn with TNAP activity below 320 pmol/h/disk in the first assay. Red circle: newborn with TNAP activity below cutoff level in both the first and second assays. TNAP, Tissuenonspecific alkaline phosphatase.

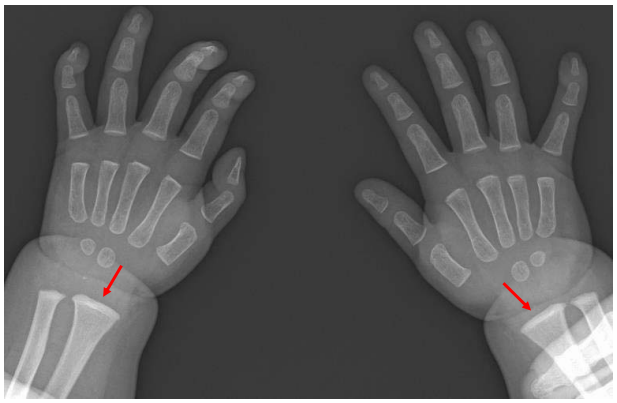


**Figure 4.** Radiography findings of the hand in case 1. The distal end of the radius is thick, and the center of the radial metaphysis is slightly thin.

(51/179, 28.5%), "perinatal" (47/179, 26.3%), "infantile" (24/179, 13.4%), "odontohypophosphatasia" (22/179, 12.3%), "adult" (18/179, 10.1%), and "perinatal benign" (15/179, 8.4%) (Data S3). Thirty patients from 28 families had homozygous variants, 37 patients from 34 families had single heterozygous variants, and 112 patients from 107 families had compound heterozygous variants; phenotypic information was not available for 2 patients (Table 2).

Sixty-four variants were reported in 179 patients with HPP. The first and second most common variants in the patients were c.1559delT (allele frequency: 34.9%, 125/358) and c.979T>C (9.5%, 34/358), respectively (Table 2). Of the 23 patients with the homozygous severe type, 20 had c.1559delT (p.Leu520Argfs*86), and 3 had c.1471G>A (p.Gly491Arg). Of the 7 patients with the homozygous moderate type, 3 had c.787T>C (p.Tyr263His), 2 had c.1559delT (p.Leu520Argfs*86), and 2 each had c.319G>A (p.Val107Ile) and c.1483G>A (p.Gly495Ser). Of the 3 patients with a single heterozygous severe type, 2 had c.1559delT (p.Leu520Argfs*86), and 1 had c.442A>G (p.Thr148Ala). Moreover, of the 21 patients with the compound heterozygous severe type, 20 had c.1559delT (p.Leu520Argfs*86) in the lateral allele (Table 2).

### Theoretical prevalence of HPP in Japan

There are differences in the classification in the ClinVar and *ALPL* gene variant databases. Of the 64 variants reported in Japanese patients with HPP, 32 were classified as "pathogenic or likely pathogenic" in both ClinVar and *ALPL* gene variant databases (Data S4). Of the 32 variants, 15 were registered in ToMMo, which was constructed by collecting the genome data of approximately 54 000 Japanese individuals. The sum of the allele frequencies of 15 variants in the ToMMo was calculated to be 0.005068; therefore, the frequency of a carrier who has any one of the 15 variants was calculated as one per 99 (formula: (1/0.005068) ÷ 2) = 1/99). Therefore, the theoretical minimal prevalence of HPP in patients with homozygote or compound heterozygote in Japan was calculated as 1 per 39, 204 (formula: 1/99 × 1/99 × 1/4) = 1/39 204).

## Discussion

In this study, we performed NBS for HPP on 45 632 newborns between February 2019 and March 2022. We detected 5 newborns with decreased TNAP activity in DBSs and analyzed their *ALPL* gene. Among these 5 newborns, 3 harbored pathogenic variants, while 2 had variants of uncertain significance in the lateral allele. Moreover, only one newborn developed HPP-related clinical manifestations, whereas the remaining 4 may require follow-up to distinguish whether they were genetic carriers or patients.

**Table 1.** Characteristics of suspected patients with HPP or genetic carriers.

| Subject No. | Gender | TNAP (pmol/h/disk) | | ALP (U/L) | PEA (nmol/mgCre) | Variants | | | Follow-up at Jan 2024 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | First | Second | | | Allele 1 | Classification | Allele 2 | Age | Symptoms |
| 1 | M | 186.3 | 192.7 | 69.3<br>39.0* | 836.7 | c.1304A>G/p.Asp435Gly | C3 | ND | 3yr6m | The distal end of the radius was thick, and the center of the radial metaphysis was slightly thin. |
| 2 | F | 215.1 | 387.5 | 201.3 | NA | c.979T>C/p.Phe327Leu | C4/C5 | ND | 3yr5m | No |
| 3 | F | 191.3 | 364.4 | 166.3 | 1086.7 | c.734C>T/p.Thr245Met | C3 | ND | 3yr4m | No |
| 4 | M | 190.3 | 318.5 | 166.6 | 546.4 | c.1559delT/p.Leu520Argfs*86 | C5 | ND | 3yr2m | No |
| 5 | F | 258.5 | 347.5 | 132.0 | 550.0 | c.1144G>A/p.Val382Ile | C4/C5 | ND | 2yr11m | No |

Classification: C3: variant of unknown significance (VUS), C4: likely pathogenic, C5: pathogenic. Abbreviations: ALP, alkaline phosphatase (reference value: 185.5-563.5 U/L); HPP, hypophosphatasia; PEA, phosphoethanolamine (reference value: 7-70 nmol/mg-Cre); NA, not available; ND, not detected. ALP and PEA were measured at age of 1 mo. PEA, phosphoethanolamine; TNAP, tissue nonspecific alkaline phosphatase. *Measured at 3yr6m.

The TNAP assay of DBSs was successful as shown in Figure 2. At least 2 attempts were needed to implement the TNAP assay in DBSs. First, as DBSs contain 2 other alkaline phosphatases different from TNAP phosphatases—intestinal-type and placental-type alkaline phosphatases—inhibition of these 2 alkaline phosphatases was needed. Second, DBSs contain Fe ions derived from RBCs, which degrade the fluorescent substrate, 4-methylumbelliferyl-phosphate. To address this, a substrate solution composition was needed to protect the fluorescent substrate from Fe ions. Moreover, the cutoff level for TNAP activity was changed because no newborns had TNAP activity below the cutoff level in the second TNAP assay between February 2019 and January 2020. Moreover, the second TNAP assay, which was performed at a median of 24 (interquartile range: 14-26) days after the first TNAP assay at 4-6 days after birth, showed increased TNAP activity by 2.7 times compared with that of the first TNAP assay ($p < .001$) (Figure 3). Therefore, we decided to change the cutoff level to <320 pmol/h/disk for the first assay and < 400 pmol/h/disk for the second assay from February 2020. Three newborns from February 2019 to January 2020 had TNAP activity below the cutoff level (< 400 pmol/h/disk) in the second TNAP assay, and we should have performed their follow-up and gene analysis.

Although serum ALP levels may decrease with age, from birth to age 7 yr,[27] TNAP activity in the DBSs may increase with time during the neonatal stage (Data S5). Data S5 suggests that 80% of newborns (112/140) underwent a second TNAP assay more than 10 days after the first, and the activity increased more than twice that obtained in the first assay. The newborns with TNAP activity less than twice that of the first assay in the second assay (20%, 28/140) may be a candidate for gene analysis.

Among 144 newborns, 139 were excluded because they had TNAP activity above the cutoff levels in the second assay. It was suggested that some these 139 newborns might have heterozygous variants, and the cutoff level in the second assay may need to be increased to allow for gene analysis in approximately 0.1% of newborns undergoing NBS. If the ratio of TNAP activity in the second and first TNAP assays is <2.0, 34 newborns (0.072 %, 33/45 632) will be able to undergo gene analysis.

This present NBS system for HPP is considered suitable for detecting severe HPP. The prevalence of severe HPP is estimated to be 1 in 150 000 births.[6,7] This study did not identify newborns with severe-type HPP or demonstrate the real prevalence of severe-type HPP in western Japan because we could only perform this study on 45 632 newborns in Kumamoto Prefecture.

The c.1559delT (p.Leu520Argfs*86) is the most common pathogenic variant in Japan, with an estimated prevalence of 1/480.[6] Patients homozygous for the c.1559delT (p.Leu520Argfs*86) variant develop severe type HPP.[28] Moreover, patients with a single heterozygous c.1559delT (p.Leu520Argfs*86) variant may develop severe HPP (Data S2C). The c.979T>C (p.Phe327Leu) variant is the second most common variant in Japan and has only been reported in patients with compound heterozygosity (Table 2). According to previous reports of compound heterozygous variants, patients with this variant on the lateral side may develop all clinical types of HPP. Among 34 patients, 13 developed the childhood type, while 11 developed the perinatal benign type (Data S2D). Moreover,

**Table 2.** Allele frequency in patients with HPP with homozygous, single heterozygous, and compound heterozygous variants.

| Variant no. | Variant | Allele number (frequency, %) | | | |
|---|---|---|---|---|---|
| | | Homozygote ($n = 30$) | Heterozygote ($n = 37$) | Compound heterozygote ($n = 112$) | Total ($n = 179$) |
| 64 | c.1559delT (p.Leu520Argfs*86) | 44 (73.3) | 10 (13.5) | 71 (31.7) | 125 (34.9) |
| 27 | c.787T>C (p.Tyr263His) | 6 (10.0) | | 13 (5.8) | 19 (5.3) |
| 61 | c.1471G>A (p.Gly491Arg) | 6 (10.0) | | 2 (0.9) | 8 (2.2) |
| 9 | c.319G>A (p.Val107Ile) | 2 (3.3) | | 3 (1.3) | 5 (1.4) |
| 63 | c.1483G>A (p.Gly495Ser) | 2 (3.3) | | | |
| 19 | c.550C>T (p.Arg184Trp) | | 7 (9.5) | 4 (1.8) | 11 (3.1) |
| 3 | c.211C>T (p.Arg71Cys) | | 2 (2.7) | 2 (0.9) | 4 (1.1) |
| 40 | c.1015G>A (p.Gly339Arg) | | 2 (2.7) | 2 (0.9) | 4 (1.1) |
| 54 | c.1366G>A (p.Gly456Arg) | | 2 (2.7) | 3 (1.3) | 5 (1.4) |
| 4 | c.244G>A (p.Gly82Arg) | | 1 (1.4) | | |
| 5 | c.247G>A (p.Glu83Lys) | | 1 (1.4) | | |
| 10 | c.331G>A (p.Ala111Thr) | | 1 (1.4) | | |
| 12 | c.395C>T (p.Ala132Val) | | 1 (1.4) | | |
| 15 | c.442A>G (p.The148Ala) | | 1 (1.4) | | |
| 16 | c.512A>G (p.His171Arg) | | 1 (1.4) | | |
| 17 | c.526G>A (p.Ala176Thr) | | 1 (1.4) | | |
| 37 | c.984_986del (p.Phe328del) | | 1 (1.4) | 6 (2.7) | 7 (2.0) |
| 42 | c.1130C>T (p.Ala377Val) | | 1 (1.4) | | |
| 44 | c.1183A>T (p.Ile395Phe) | | 1 (1.4) | | |
| 53 | c.1354G>A (p.Glu452Lys) | | 1 (1.4) | | |
| 55 | c.1375G>A (p.Val459Met) | | 1 (1.4) | | |
| 60 | c.1466G>A (p.Cys 489Tyr) | | 1 (1.4) | | |
| 62 | c.1482_1532del (p.Gly495_Leu511del) | | 1 (1.4) | | |
| 35 | c.979T>C (p.Phe327Leu) | | | 34 (15.2) | 34 (9.5) |
| 22 | c.572A>G (p.Glu191Gly) | | | 12 (5.4) | 12 (3.4) |
| 14 | c.407G>A (p.Arg136His) | | | 6 (2.7) | 6 (1.7) |
| 2 | c.119C>T (p.Ala40Val) | | | 5 (2.2) | 5 (1.4) |
| 25 | c.670A>G (p.Lys224Glu) | | | 5 (2.2) | 5 (1.4) |
| 18 | c.529G>A (p.Ala177Thr) | | | 3 (1.3) | 3 (0.8) |
| 43 | c.1144G>A (p.Val382Ile) | | | 3 (1.3) | 3 (0.8) |
| 56 | c.1403C>T (p.Ala468Val) | | | 3 (1.3) | 3 (0.8) |
| 11 | c.382G>A (p.Val128Met) | | | 2 (0.9) | 2 (0.6) |
| 20 | c.562T>C (p.Ser188Pro) | | | 2 (0.9) | 2 (0.6) |
| 21 | c.568_570del (p.Asn190del) | | | 2 (0.9) | 2 (0.6) |
| 24 | c.668G>A (p.Arg223Gln) | | | 2 (0.9) | 2 (0.6) |
| 31 | c.885G>A (p.Met295Ile) | | | 2 (0.9) | 2 (0.6) |
| 32 | c.892G>A (p.Glu298Lys) | | | 2 (0.9) | 2 (0.6) |
| 33 | c.896T>C (p.Leu299Pro) | | | 2 (0.9) | 2 (0.6) |
| 45 | c.1183A>G (p.Ile395Val) | | | 2 (0.9) | 2 (0.6) |
| 47 | c.1276G>A (p.Gly426Ser) | | | 2 (0.9) | 2 (0.6) |
| 48 | c.1276G>T (p.Gly426Cys) | | | 2 (0.9) | 2 (0.6) |
| 50 | c.1307A>G (p.Tyr436Cys) | | | 2 (0.9) | 2 (0.6) |
| 58 | c.1436A>G (p.Tyr479Cys) | | | 2 (0.9) | 2 (0.6) |
| 1 | c.88C>T (p.Arg30*) | | | 1 (0.4) | 1 (0.3) |
| 6 | c.258G>A (p.Arg86=) | | | 1 (0.4) | 1 (0.3) |
| 7 | c.283G>A (p.Val95Met) | | | 1 (0.4) | 1 (0.3) |
| 8 | c.299C>T (p.The100Met) | | | 1 (0.4) | 1 (0.3) |
| 10 | c.331G>A (p.Ala111Thr) | | | 1 (0.4) | 1 (0.3) |
| 13 | c.406C>T (p.Arg136Cys) | | | 1 (0.4) | 1 (0.3) |
| 17 | c.526G>A (p.Ala176Thr) | | | 1 (0.4) | 1 (0.3) |
| 23 | c.613G>A (p.Ala205Thr) | | | 1 (0.4) | 1 (0.3) |
| 26 | c.678G>T (p.Met226Ile) | | | 1 (0.4) | 1 (0.3) |
| 28 | c.814C>T (p.Arg272Cys) | | | 1 (0.4) | 1 (0.3) |
| 29 | c.855C>G (p.Tyr285*) | | | 1 (0.4) | 1 (0.3) |
| 30 | c.876A>G (p.Pro292=) | | | 1 (0.4) | 1 (0.3) |
| 34 | c.920C>T (p.Pro307Leu) | | | 1 (0.4) | 1 (0.3) |
| 36 | c.982T>C (p.Phe328Leu) | | | 1 (0.4) | 1 (0.3) |
| 38 | c.1013A>G (p.His338Arg) | | | 1 (0.4) | 1 (0.3) |
| 39 | c.1014C>T (p.His338=) | | | 1 (0.4) | 1 (0.3) |
| 41 | c.1022A>G (p.His341Arg) | | | 1 (0.4) | 1 (0.3) |
| 46 | c.1225C>G (p.Pro409Ala) | | | 1 (0.4) | 1 (0.3) |
| 49 | c.1282C>T (p.Arg428*) | | | 1 (0.4) | 1 (0.3) |
| 51 | c.1333T>C (p.Ser445Pro) | | | 1 (0.4) | 1 (0.3) |
| 52 | c.1348C>T (p.Arg450Cys) | | | 1 (0.4) | 1 (0.3) |
| 57 | c.1418G>A (p.Gly473Asp) | | | 1 (0.4) | 1 (0.3) |
| 59 | c.1446C>G (p.His482Gln) | | | 1 (0.4) | 1 (0.3) |

Abbreviation: HPP, hypophosphatasia.

Michigami et al. reported that 45.2% of 98 unrelated Japanese patients were classified as having the perinatal severe form, 22.4% as having the perinatal benign form, and 12.2% as having the infantile form. Among these 196 alleles, c.1559delT (p.Leu520Argfs*86) was detected in 89 alleles and c.979T>C (p.Phe327Leu) in 23 alleles.[14] Based on the results from ToMMo (Data S4), the allele frequencies of c.979T>C (p.Phe327Leu) and c.1559delT (p.Leu520Argfs*86) in Japanese participants were 0.002772 and 0.001390, respectively. Nagata et al. also reported that the allele frequencies of c.979T>C and c.1559delT in the general Japanese population were 0.0040 and 0.0014, respectively,[29] and the carrier with c.979T>C or c.1559delT had low ALP activity in serum. Truly, the prevalence of these 2 variants is very high in the Japanese population, and individuals with these 2 variants may show impaired TNAP activity in DBSs. Careful judgment is needed when diagnosing HPP in individuals with heterozygous variants.

The c.1144G>A (p.Val382Ile) variant was classified as pathogenic/likely pathogenic in ClinVar. Three patients with HPP (2 childhood and one odontohypophosphatasia) with a compound heterozygous variant of c.1144G>A (p.Val382Ile) have been reported (Data S2A and D).

The c.734C>T (p.Thr245Met) variant was classified as of uncertain significance and unknown in both the ClinVar and *ALPL* gene variant databases. This variant is considered to be of unknown significance according to the ACMG/AMP guidelines (PM2_ classified as uncertain significance in gnomAD, and PP3_ predicted by PolyPhen-2 as probably damaging).[30]

The c.1304A>G (p.Asp435Gly) is a novel variant and is also considered to have uncertain significance according to the ACMG/AMP guidelines (PM2_ was not found in gnomAD and PP3_ predicted by PolyPhen-2 as probably damaging).[30]

Case 1 showed impaired serum ALP levels and mild bone manifestations. Careful follow-up and intervention are needed, as reduced ALP activity can lead to more severe clinical manifestations. Dominant transmission of HPP has been reported based on pedigree studies,[8,31–34] and a dominant-negative effect has been shown through in vitro transfection studies.[35,36] Therefore, HPP diagnosis is more difficult in newborns with a single heterozygous variant. In the present study, 4 of the 5 identified newborns (Case Nos. 2, 3, 4, and 5) may be carriers of autosomal recessive HPP. Even carriers of autosomal recessive HPP may have biochemical abnormalities such as low ALP activity and increased PEA excretion. However, patients are diagnosed with HPP only when they present HPP-related clinical manifestations along with ALPL variants.

In this study, we did not identify any patients with severe HPP. EZRT is very effective for the severe type, and early initiation of EZRT can significantly improve the QOL of patients with this type.[16] Therefore, the starting time and induction of EZRT should be sufficiently discussed because EZRT is expected to improve patients' clinical manifestations, including QOL, even in patients without severe HPP.[37–40]

This study has some limitations. While this study has significance in terms of developing an assay to be used in NBS for HPP, it was performed only in Kumamoto Prefecture, Japan. Therefore, this study needs to be replicated in other populations. This screening system for HPP can detect not only individuals with life-threatening forms of the disease (such as perinatal [severe] and infantile forms) but also individuals with mild types and carriers. Of course, individuals with perinatal severe HPP may be detected with markedly impaired skeletal mineralization before and immediately after birth, even before the results of NBS are available.

In the future, NBS for HPP may be performed in Japan, allowing patients with HPP to receive early interventions that can improve their long-term clinical outcomes. However, a definite diagnosis of HPP is difficult in newborns with a single heterozygous variant, and clinicians should carefully monitor the clinical course in such patients. Moreover, the indication for EZRT should be considered, as most patients with HPP, except for those with perinatal (severe) and infantile forms, may not need EZRT.

The cutoff levels for TNAP activity need to be reconsidered to further reduce false-negative results, particularly in the second TNAP assay. Moreover, the second TNAP activity/first TNAP activity ratio 10 days after the first TNAP assay is very useful for selecting patients for gene analysis.

In conclusion, we established an NBS system for HPP using a TNAP assay in DBSs. Of the 45 632 newborns in Kumamoto Prefecture, we detected 5 newborns with a single heterozygous variant. Three of these 5 variants were classified as likely pathogenic or pathogenic according to genetic assessment.

This TNAP assay using DBSs for NBS of HPP may be practical and beneficial for newborns undergoing expanded NBS, which target lysosomal storage disease, severe combined immune deficiency, and spinal muscular atrophy.

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## Author contributions

All authors have read and approved the final manuscript for submission. All authors have agreed to be personally accountable for their contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even those in which the author was not personally involved, were appropriately investigated and resolved, and the resolution was documented in the literature.

Yusuke Noda (Data curation, Writing—original draft), Jun Kido (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing—original draft, Writing—review & editing), Takaaki Sawada (Data curation, Investigation), Kenichi Tanaka (Investigation), Kousuke Kumeda (Methodology), Shinichiro Yoshida (Data curation, Methodology), Keishin Sugawara (Data curation, Formal analysis, Investigation, Writing—review & editing), and Kimitoshi Nakamura (Conceptualization, Supervision).

## Supplementary material

Supplementary material is available at *JBMR Plus* online.

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## Conflicts of interest

Yusuke Noda, Jun Kido, Takaaki Sawada, Kenichi Tanaka, Kousuke Kumeda, Keishin Sugawara, and Kimitoshi Nakamura declared no conflict of interest.

## Data availability

Data supporting the findings of this study are available upon request from the corresponding author. The data are not publicly available because of privacy and ethical restrictions.

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