PBS, and transferred to a solution of 50 mM $MgCl_2$ in 0.05 M Tris–maleic acid buffer (pH 7.4) for 30 minutes for the reactivation of ALP.[37] The sections then were incubated in a freshly prepared mixture of Naphthol AS-MX phosphate disodium salt (Sigma-Aldrich) and Fast Blue BB Salt (Sigma-Aldrich) as described previously.[38] Methyl green served as the counterstain.

Statistical analysis

Data are expressed as mean ± SD. Differences between two groups were tested for statistical significance using Student's *t* test. p values $< .05$ were considered statistically significant. Kaplan-Meier curves were produced and analyzed using SPSS for Windows, Version 14.0J (SPSS Japan, Tokyo, Japan).

Results

Growth and survival of *Akp2*$^{-/-}$ mice

The growth of the *Akp2*$^{+/-}$ HET mice appeared indistinguishable from that of the WT mice. The *Akp2*$^{-/-}$ HPP mice were born with a normal appearance and weight. However, HPP mice showed apparent growth failure and became progressively exhausted (Fig. 1*B*). Most of the HPP mice also developed spontaneous seizures with various clinical presentations, including tonic-clonic convulsions and abnormal running and vocalization. The mice usually died 1 to 2 days after the epileptic seizures began. The average life span of the HPP mice was 12.0 ± 4.4 days ($n = 13$). Pyridoxine supplementation of the food for the nursing mother delayed the onset of the epileptic attacks in the neonates and extended their survival to postnatal day 18.1 ± 7.6 ($n = 15$).

Lentiviral vector containing bone-targeted human *TNALP* cDNA (HIV-TNALP-D10) was injected into the jugular vein of the neonatal HPP mice on days 1 through 3 ($n = 6$). The weight and growth rates of the treated HPP mice were improved compared with the untreated HPP mice and were indistinguishable from those of their WT and HET littermates ($n = 13$; Fig. 1*B*). The long-term follow-up was done for 7 treated mice. Compared with untreated mice ($n = 12$), the life spans of treated mice ($n = 7$) were significantly extended up to at least 160 days of age, except that one treated animal died on day 6 from unknown causes (Fig. 1*D*). In the long survivors ($n = 6$), 3 were euthanized on day 160 for X-ray analysis, whereas the remaining 3 animals survived for more than 400 days with normal appearance and physical activity. Seizures were not observed in the treated mice throughout the experimental period. The average body weights of treated HPP ($n = 6$) and WT/HET ($n = 13$ to 18) were compared on days 1, 10, 30, 60, and 160 (Fig. 1*C*). The body weights differed between male and female mice after 60 days. In either gender, the slight but significant growth retardation was detected in treated HPP mice on days 60 and 160.

Lentivirus-mediated expression of ALP

At 10 to 12 days after birth, ALP activity in the plasma of WT and HET mice was 0.25 ± 0.07 U/mL ($n = 9$) and 0.16 ± 0.05 U/mL ($n = 21$), respectively, whereas that of the HPP mice was less than 0.1 U/mL ($n = 5$; Fig. 1*E*). A single injection of HIV-TNALP-D10 into the neonatal HPP mice ($n = 6$) on days 1 through 3 resulted in extremely high levels of plasma ALP (2.67 ± 0.56 U/mL). The plasma ALP activity in the WT and HET mice decreased slowly with aging, whereas the lentivirus-mediated expression of ALP remained stable, and the high levels of ALP activity persisted for at least 6 months. At 60 days of age, the average ALP activity in the treated HPP mice was 73-fold higher than that of the WT mice (5.14 ± 2.66 versus 0.07 ± 0.02).

Biodistribution of lentiviral vector was determined using quantitative PCR (qPCR) on genomic DNA isolated from the injected WT littermate mice 14 days after injection (Fig. 1*F*). The highest copy number of integrated vector was detected in liver samples (0.40 copy/diploid). Low levels of lentiviral integration also were observed in the spleen and the heart. Transduction of the bone tissue, including bone marrow cells, was very low (<0.001 copy/diploid).

Radiographic analysis

Since radiographic changes in the HPP mice were not apparent during the first 8 days of life, we examined X-ray images of the feet and legs of mice at approximately 20 days after birth. The severity of the mineralization defects in the untreated HPP mice was found to be highly variable. In the most severely affected cases, the metacarpal and digital bones were significantly shorter than those of the WT mice, and their epiphyses were not detected. In addition, some of the carpal bones were absent. We also observed the absence of secondary ossification centers in the feet (Fig. 2). The most severe phenotype was observed in approximately 10% of the *Akp2*$^{-/-}$ homozygous neonates, and these mice usually died by 10 days of age. In the milder cases, the epiphyses and all the digital bones were significantly mineralized, even though the HPP mice were smaller than the HET and WT mice. Heterogeneous radiographic changes between these two extreme phenotypes were observed in the untreated HPP mice.

X-ray images of treated HPP mice showed that mineralization was accelerated following lentivirus-mediated expression of TNALP-D10. Secondary ossification centers were detected in the feet of all treated animals at 15 days of age, although the intensity of the mineralization was variable. Ossification of the carpal bones also was improved. All the untreated HPP mice died, with an average survival of 18.1 days. No differences in skeletal structure and mineralization were observed between the long survivors after treatment and the WT mice at 100 days of age. These results indicate that mineralization defects in HPP mice can be corrected efficiently by gene therapy.

Histochemical examination of the bone

The proximal tibias were analyzed histochemically for ALP activity using the azo-dye technique with methyl green counterstaining (Fig. 3). Strong ALP activity was detected in both the bone and hypertrophic cartilage zones of the WT mice (Fig. 3*A*), whereas no ALP signal was observed in the epiphysis of the HPP mice (Fig. 3*B*). After treatment with lentiviral vector, faint ALP staining was observed on the surface of the endosteal bone (Fig. 3*C*, *D*).

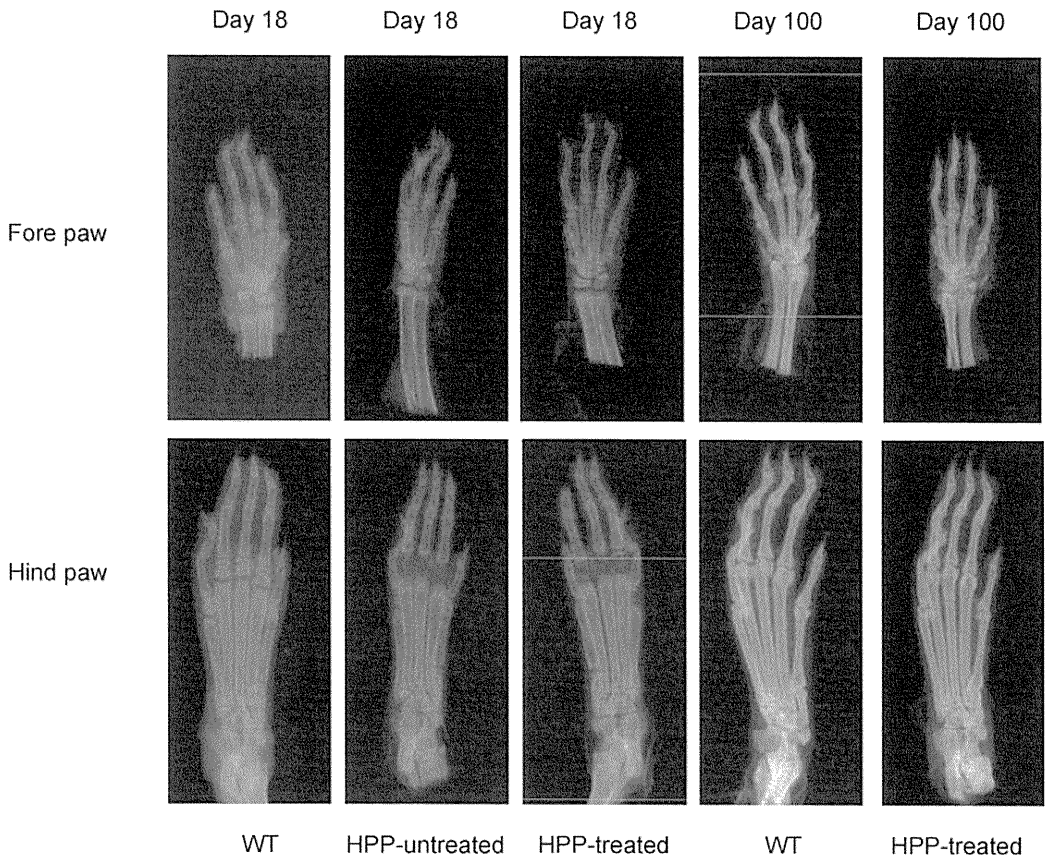

Fig. 2. X-ray images of the feet. Secondary ossification centers in the hind paws were absent in untreated HPP mice but were detectable in the treated mice at 18 days after birth. No differences in skeletal mineralization were observed between treated long survivors and WT mice at 100 days of age.

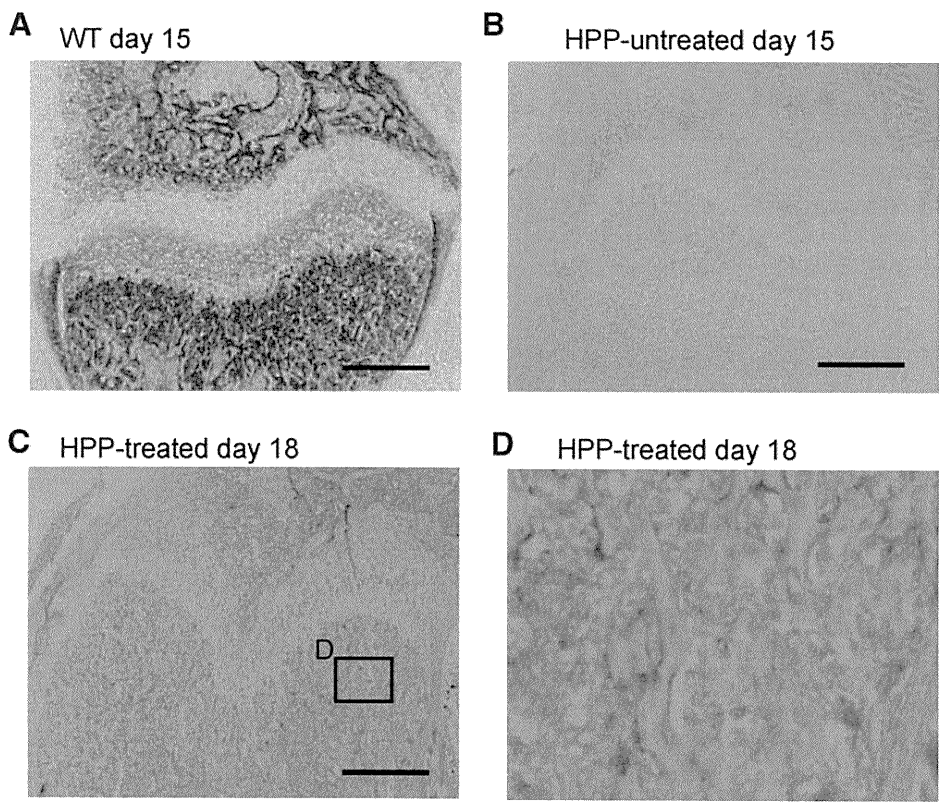

Fig. 3. Histochemical staining of ALP activity in the tibias. ALP activity was detected in WT (*A*) but not HPP mice (*B*) at 15 days after birth. Following treatment with lentiviral vector, (*C*) ALP activity was detected on the surface of the endosteal bone at 18 days after birth. (*D*) Magnified image of the square in panel *C*. Bars = 1 mm.

Discussion

TNALP is an ectoenzyme that is known to be particularly abundant on the cell surfaces of osteoblasts and hypertrophic chondrocytes, including their shed matrix vesicles.[6,39] Since ALP functions on the exterior of the cell, enzyme replacement following repeated administration of soluble ALP has been hypothesized as a potential approach to treat ALP deficiencies. However, the outcomes of previous clinical trials of enzyme-replacement therapy have proven disappointing. Intravenous infusions of ALP-rich serum from patients with Paget disease[13,14] and purified soluble ALP from human liver[16] and placenta[17] have shown no significant clinical benefits in patients with HPP. Recently, Millán and colleagues[25] demonstrated that daily injections of high-dose bone-targeted TNALP significantly extended the lifespan and corrected the abnormal phenotypes of HPP mice, suggesting that HPP could be treated by enzyme replacement if sufficient amounts of TNALP were able to reach the sites of skeletal mineralization. Based on these data, new clinical trials involving enzyme-replacement therapy for HPP patients have been initiated.[28]

A general problem of enzyme-replacement therapy is the short half-life of the administered protein in patients. A pharmacokinetic study showed that the half-life of bone-targeted TNALP is 34 hours in the plasma of adult mice, but in bone tissue the half-life is extended to more than 300 hours.[25] Nevertheless, repeated administration of large amounts of the enzyme is required for long-term correction. In the initial clinical trials, HPP patients received subcutaneous injections of bone-targeted TNALP three times weekly.[28] The preparation of adequate amounts of clinical-grade purified enzyme is a limitation, and repeated injection is highly invasive and not optimal for small children. In this study we demonstrated that a single injection of lentiviral vector resulted in sustained expression of ALP and phenotypic correction in HPP neonatal mice. As such, viral vector–mediated enzyme replacement may prove to be more practical than classic enzyme replacement by repeated injection.

One of the concerns of gene therapy is the safety of the viral vector. We used an HIV-1-based lentiviral vector in this study.[30] Lentivirus-mediated gene transfer has proven to be effective for long-term expression of transgenes in nondividing cells. Although the pathogenicity of HIV-1 was almost negligible in the current modified version of lentiviral vector, insertional mutagenesis is still a major concern for all integrating vectors.[40] To minimize the possibility of protooncogene activation, our novel self-inactivating lentiviral vector contains the insulator element from the chicken β-globin locus.[30] So far, lymphoproliferative complications owing to insertional mutagenesis have been detected in ex vivo hematopoietic stem cell gene therapy only. For the treatment of HPP, lentiviral vector was injected directly into the circulation of neonatal mice. After this in vivo systemic gene therapy, the lentiviral sequence was detected in the liver, lung, and heart. The oncogenicity of the integrated lentiviral vector in these differentiated tissues requires further examination in a long-term follow-up study.

We also found that the epileptic seizures were completely inhibited and the lifespan was significantly extended in the treated HPP mice. Without treatment, HPP mice died by 20 days of age.[22,35] The major cause of death in the untreated HPP mice was apnea, most likely resulting from their severe epileptic convulsions.[21] Pyridoxine-responsive seizures in HPP patients and HPP model mice are thought to be caused by reduced levels of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in the brain.[21,35] PLP is an essential cofactor of glutamate decarboxylase, which is responsible for the synthesis of GABA.[41] Diminished hydrolysis of extracellular PLP in HPP causes decreased intracellular pyridoxal levels in cells. This results in a reduction in the rephosphorylation of pyridoxal to PLP, and thus biosynthesis of GABA within the brain cells is reduced.[10] The seizure phenotype can be rescued in part by administration of pyridoxal.[23] We demonstrated that epileptic seizures were efficiently inhibited by either systemic infusion of TNALP[25] or viral vector–mediated expression of TNALP, suggesting that the defective metabolism of PLP in the brain could be corrected by replacement of soluble TNALP.

Although epileptic seizures are observed in some severely affected patients, the major clinical complications in human HPP patients are directly related to defective skeletal mineralization.[3] Patients with severe infantile HPP usually die from respiratory failure caused by skeletal diseases in the chest, such as flail chest, rachitic deformity, and rib fractures.[3] Compared with severe infantile HPP in human patients, bone defects in our mouse model are relatively mild. The *Akp2*$^{-/-}$ HPP mice were born with a normal appearance and bone mineral deposition. Hypomineralization becomes apparent after around 10 days of age, although the severity of mineralization defects varies widely.[22] Lentivirus-mediated expression of bone-targeted ALP efficiently prevents the progressive skeletal demineralization, as well as the lethal epilepsy.

Since enzyme replacement also was effective,[25] the major mechanism of successful gene therapy for HPP mice seems to be due to continuous supply of bone-targeted TNALP from the vector-infected liver to the circulation. Another possibility is that osteoblasts and chondrocytes may be directly transduced with lentiviral vector. However, since the copy number of integrated vector in the whole bone tissue was very low, the ALP staining in treated mice is mainly due to the circulating TNALP-D10 in the bone matrix. The contribution of in situ expression of TNALP in bone cells to bone mineralization is not likely to be significant.

A major physiologic role for TNALP has been shown to be the restriction of the extracellular pool of PP_i, which is a strong inhibitor for mineralization.[24,39] Localization of TNALP to the skeleton should be important for the treatment of HPP. TNALP with a repetitive C-terminal extension of 10 Asp was shown to display high affinity for bone tissue both in vitro[26] and in vivo.[25] The use of the bone-targeted TNALP construct in a clinical setting is currently under investigation in clinical trials.[28]

The efficacy of gene therapy to correct hypomineralization was evaluated by radiographic examination. The major problem is that the severity of the mineralization defects in untreated infantile *Akp2*$^{-/-}$ mice is highly variable. In addition, X-rays of infantile mice could be taken only after euthanization. The time course of mineralization in a single animal could not be

examined under the condition used. Further studies using more reliable histomorphometric and micro-computed tomographic (μCT) techniques may be required to optimize the gene therapy protocol to rescue the skeletal phenotype.

In conclusion, we found that severe infantile HPP in *TNALP* knockout mice can be treated with a single injection of lentiviral vector during the neonatal period. Lentiviral-mediated gene therapy may prove to be an important option in the treatment of human hypophosphatasia.

Disclosures

JLM is a consultant for Enobia Pharma, Inc. All the other authors state that they have no conflicts of interest.

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SHORT COMMUNICATION

Prevalence of c.1559delT in *ALPL*, a common mutation resulting in the perinatal (lethal) form of hypophosphatasia in Japanese and effects of the mutation on heterozygous carriers

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Hypophosphatasia (HPP) is an inherited disorder caused by mutations in *ALPL* that encodes an isozyme of alkaline phosphatase (ALP), TNSALP. One of the most frequent *ALPL* mutations is c.1559delT, which causes the most severe HPP, the perinatal (lethal) form (pl-HPP). c.1559delT has been found only in Japanese and its prevalence is suspected to be high; however, the allele frequency of c.1559delT in Japanese remains unknown. We designed a screening system for the mutation based on high-resolution melting curve analysis, and examined the frequency of c.1559delT. We found that the c.1559delT carrier frequency is 1/480 (95% confidence interval, 1/1562–1/284). This indicates that ~1 in 900 000 individuals to have pl-HPP caused by a homozygous c.1559delT mutation. In our analysis, the majority of c.1559delT carriers had normal values of HPP biochemical markers, such as serum ALP and urine phosphoethanolamine. Our results indicate that the only way to reliably detect whether individuals are pl-HPP carriers is to perform the *ALPL* mutation analysis.
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Keywords: *ALPL*; c.1559delT; perinatal form of hypophosphatasia; serum alkaline phosphatase; skeletal dysplasia; urine phosphoethanolamine

INTRODUCTION

Hypophosphatasia (HPP) is an inherited disorder characterized by defective mineralization of the bone and low activity of alkaline phosphatase (ALP; EC 3.1.3.1).[1,2] HPP is a clinically heterogeneous disease and classified into five forms according to severity and age of onset: perinatal (lethal), infantile (OMIM 241500), childhood (OMIM 241510), adult (OMIM 146300) and odontohypophosphatasia.[1] All forms of HPP display reduced activity of unfractionated serum ALP and the presence of either one or two pathologic mutations in *ALPL*, the gene encoding an ALP isozyme (TNSALP).

The perinatal (lethal) form of HPP (pl-HPP) is the most severe HPP with an autosomal recessive mode of inheritance. pl-HPP is more common in Japan than in other countries.[3] Parents of pl-HPP are heterozygous carriers of *ALPL* mutations. They show no clinical symptoms, but have reduced serum ALP activity and increased urinary phosphoethanolamine (PEA).[4–8]

ALPL is the only gene known to be associated with HPP.[1] More than 200 *ALPL* mutations have been described, accounting for most phenotype variabilities.[9] HPP is frequently caused by p.E191K and p.D378V in Caucasians,[1] whereas p.F327L[10] and c.1559delT[10,11] are more common in Japanese.[1] To date, c.1559delT has only been found in Japanese.[11] Some patients with pl-HPP are homozygous for c.1559delT, with parents who are heterozygous carriers for the mutation but with no evidence of consanguinity.[12,13]

To identify c.1559delT genotype and to examine its frequency in Japanese, we designed a screening system based on a high-resolution melting curve analysis.[14] In addition, we examined serum ALP activity and urine PEA in heterozygous c.1559delT carriers to determine whether these markers can identify the HPP carriers.

MATERIALS AND METHODS

This study was approved by the Institutional Genetic Research Ethics Committee at Nippon Medical School and RIKEN, Center for Genomic Medicine. Blood samples were collected under written informed consents from 3844 healthy Japanese without HPP and its related findings confirmed by orthopedic surgeons. Genomic DNA was extracted from peripheral blood leukocytes using standard protocols. The c.1559delT genotype screening was performed by the small amplicon genotyping method based on high-resolution melting curve

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analysis.[14] PCR primers for c.1559delT were designed to flank the mutation leaving only single base, including the mutation between the primers: 5′-TT TAAATTCTCGCGCTGGCCCTCTACCCC-3′ (forward) and 5′-TTTAAATTCC CTCAGAACAGGACGCTC-3′ (reverse). PCR conditions were as follows: initial denaturation at 95°C for 2 min, followed by 45 cycles at 94°C for 30 s and annealing at 67°C for 30 s. After PCR, high-resolution melting was performed in a 96-well plate LightScanner (Idaho Technology, Salt Lake City, UT, USA), which collected data from 55°C to 97°C at a ramp rate of 0.10°C sec^{-1}. The observed number of c.1559delT carriers was divided by the total number of individuals tested to determine the carrier frequency. Serum ALP activity and urine PEA were measured in c.1559delT-heterozygous parents of pl-HPP patients.

RESULTS

Three *ALPL* c.1559delT genotypes (wt/wt, wt/c.1559delT and c.1559delT/c.1559delT) were distinguished by the modified small amplicon genotyping method (Figure 1). A heterogeneous c.1559delT mutation (wt/c.1559delT) was detected in 8 of 3844 healthy Japanese subjects, indicating a carrier frequency of 1/480 in the Japanese population (95% confidence interval, 1/1562–1/284).

The numerical value of ALP activity and urinary PEA varied in heterozygous c.1559delT carriers in parents of perinatal HPP patients. The majority of heterozygous c.1559delT carriers had normal levels of both ALP activity (five out of six males and three out of four females) and urinary PEA (three out of six males and four out of five females) (Figure 2).

DISCUSSION

Based on our results, we estimated the frequency of c.1559delT-homozygous individuals (for example, those with pl-HPP) to be 1/900 000. Previous studies showed that all Japanese pl-HPP patients carried the c.1559delT mutation in at least one allele; half (10/20) were homozygous for c.1559delT and half (10/20) were compound heterozygous for c.1559delT,[9–13,15] which gives a pl-HPP prevalence of 1/450 000 for patients that are homozygous or compound heterozygous for c.1559delT mutation. The other common mutation on *ALPL* in Japan, p.F327L, is a mild allele whose product retained ~70% of its enzymatic activity. Patients compound heterozygous for c.1559delT and p.F327L are not associated with pl-HPP.[10]

Biochemical markers, serum ALP activity and urinary PEA levels fell within their normal ranges in the majority of the c.1559delT carriers examined in this paper, whereas heterozygous carriers of the severe forms in other *ALPL* mutations were reported to have reduced serum ALP activity and increased urinary PEA.[4–8] Some possible reasons why c.1559delT carriers display normal marker levels are as follows: the first is the protein properties caused by the different mutation positions. The c.1559delT mutation causes a frameshift downstream of codon L503, resulting in the elimination of the termination codon at 508 and the addition of 80 amino acids at the C-terminus. The mutant protein forms an aggregate that is polyubiquitinated and then degraded in the proteasome. However, the aggregates possess enzyme activity, and may, therefore, influence physiological processes before their destruction.[16] Second, serum ALP activity is affected by some other factors. The genetic modifier of ALP is reported to have a potential influence on serum ALP activity.[17] Total ALP value is also elevated by some environmental factors, in vitamin D deficiency[2] or in the third trimester of gestation by the increasing placental ALP, which is not affected by TNSALP.[18] Recently, it was shown that patients who are homozygous for the c.1559delT mutation differed in the severity of HPP, including both their symptoms and serum ALP activity.[15]

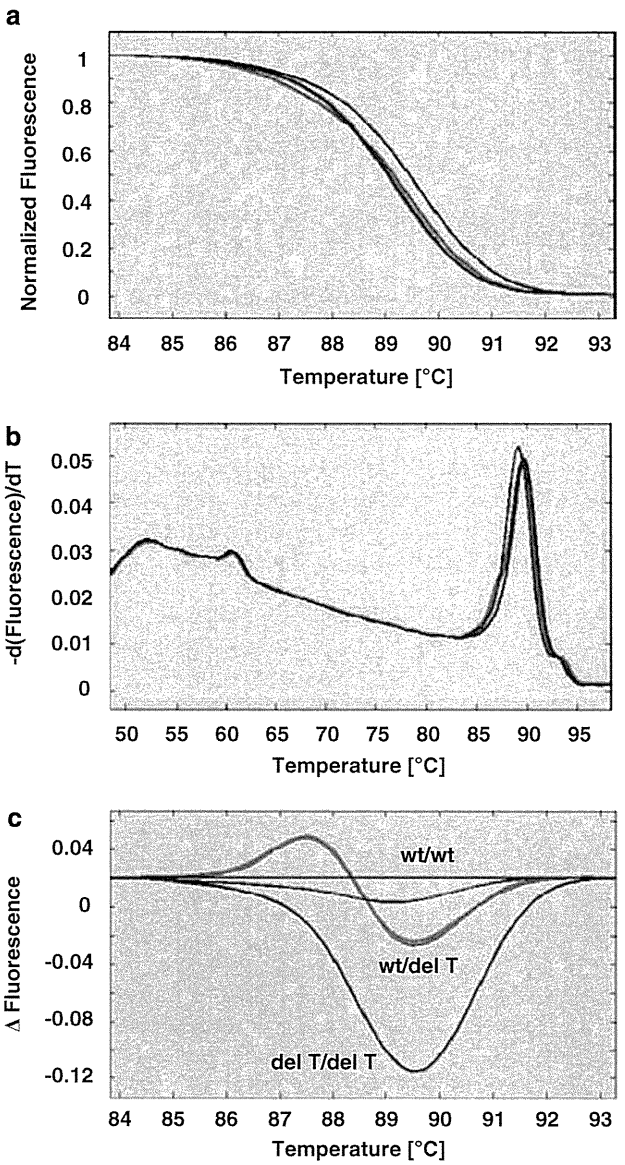

Figure 1 Identification of c.1559delT mutation in *ALPL* by small amplicon genotyping (SAG) method. (**a**) Normalized fluorescence plots. (**b**) d(fluorescence)/dT plot. (**c**) The corresponding fluorescence difference plots. Wild-type (wt/wt) samples are in gray; samples heterozygous for c.1559delT (wt/c.1559delT) are in red; and samples homozygous for c.1559delT (c.1559delT/c.1559delT) are in blue. The three genotypes were clearly distinguishable in the SAG method.

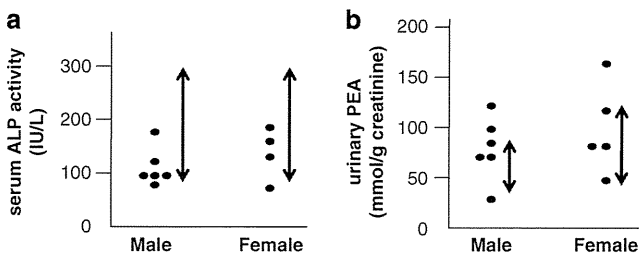

Figure 2 Biochemical marker levels in heterozygous carriers of the *ALPL* c.1559delT mutation. The serum ALP activity (**a**) and urinary PEA (**b**) levels in the majority of heterozygous carriers (wt/c.1559delT) fell within normal ranges (indicated by arrows).

Thus, the only way to reliably detect the pl-HPP carriers is to perform the *ALPL* mutation analysis. The small amplicon genotyping method in this study using the high-resolution melting curve analysis is a one-step, single-tube method for detection of specific mutations and faster, simpler and less expensive than the approaches requiring separations or labeled probes.[19]

The screening for c.1559delT in *ALPL* may be useful for diagnosis of pl-HPP in Japanese to provide optimum genetic counseling for fetal skeletal dysplasia. pl-HPP occasionally could not be diagnosed with sonographic examination in the first trimester because incomplete ossification is an usual finding at this stage of development.[20] To diagnose pl-HPP, collaborations between obstetricians and clinical geneticists are important and could provide support for parents of prenatal patients suspected of having skeletal dysplasia.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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