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Characterization of the mutant (A115V) tissue-nonspecific alkaline phosphatase gene from adult-type hypophosphatasia

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Abstract

Hypophosphatasia (HOPS) is a clinically heterogeneous heritable disorder characterized by defective skeletal mineralization, deficiency of tissue-nonspecific alkaline phosphatase (TNSALP) activity, and premature loss of deciduous teeth. To date, various mutations in the TNSALP gene have been identified. Especially, A115V located in exon 5 has been detected in a Japanese patient with severe periodontitis and adult-type HOPS. In this study, we have characterized the protein translated from the mutant A115V gene. Wild-type and A115V mutant-type TNSALP cDNA expression vector pcDNA3 have been constructed and transfected to COS-1 cells by lipofectin technique. After 48-h transfection, the cells were subjected to assay ALP activity. In order to identify possible dominant effect of the mutation, we performed co-transfections of wild-type and mutated cDNA, and evaluated the residual activities of each mutation. Detection of TNSALP synthesized by COS-1 cells transfected with the wild- or the mutated-type was also performed by using an immunofluorescent method. ALP activity of cell transfected with the mutant cDNA (A115V) plasmid after 48-h transfection exhibited 0.399 ± 0.021 U/mg. As the enzymatic activity of the wild type was taken as 100%, the value of the mutant was estimated as 16.9%. When co-transfected this mutant showed no inhibition of the wild-type enzyme. TNSALP in COS-1 cells with transfected with the mutant exhibited strong fluorescence at the surface of cells as wild-type. This study indicated that the mutant (A115V) TNSALP gene produced the defective ALP enzyme and it could be recessively transmitted and be a disease-causing mutation of the adult-type hypophosphatasia.

Keywords: Hypophosphatasia; Mutant gene; Tissue-nonspecific alkaline phosphatase; Site-directed mutagenesis; COS-1 cells

Hypophosphatasia (HOPS) is an inheritable disorder characterized by defective skeletal bone mineralization and premature loss of deciduous teeth associated with a deficiency of alkaline phosphatase activity (ALP; orthophosphoric monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1.). This disease is highly variable in its clinical expression, due to various mutations in the tissue-nonspecific ALP (TNSALP) gene, but in early life the severity tends to reflect the age of onset. HOPS is classified based on its age at diagnosis: perinatal, infantile, childhood, and adult types [1]. Patients with only premature loss of deciduous teeth but not with skeletal bone loss are regarded as having odonto-type HOPS [1].

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In humans, there are four isozyme forms of alkaline phosphatase: TNSALP, intestinal, placental, and placental-like types, and each isozyme is encoded by a separate gene [2–5]. The gene for TNSALP is located on chromosome 1p34–36.1 and consists of 12 exons and 11 introns distributed over 50 kb with the coding sequence beginning in the second exon [5]. Up to 130 mutations have been reported in previous studies [6–27]. We previously reported the TNSALP gene from five unrelated Japanese patients using the polymerase chain reaction-single strand conformation polymorphism method (PCR-SSCP) and found five new missense mutations (331G > A, 529G > A, 572A > G, 979T > C, and 1144G > A) [12]. In the case of childhood-type HOPS, the proband had a 979T > C mutation in exon 9 from the mother and a 1144G > A mutation in exon 10 from the father. 979T > C mutation causes amino acid substitution at 310-Phe with Leu (F310L) and 1144G > A mutation in exon 10 causes amino acid substitution at 365-Val with Ile (V365I) [12]. Our previous study confirmed that the mutation V365I produced the inactive ALP enzyme and would be a disease-causing mutation [25].

The point mutation A115V in exon 5 was first detected in a Japanese patient with severe periodontitis and adult-type HOPS [22]. The proband had a 395C > T mutation in exon 5. 395C > T mutation causes amino acid substitution at 115-Ala with Val (A115V).

The purpose of this study was to characterize the protein translated from the mutant A115V from adult type HOPS and analyze its function.

Materials and methods

Subjects. The proband was a 52-year-old Japanese female diagnosed with adult onset HOPS. Clinical data and patient management were reported previously [22]. Serum ALP activity was 44 IU/L (normal: 100–280) and urinary phosphoethanolamine (PEA) excretion was elevated (215 μmol/day, normal 30–100). She had bone fractures twice in her forties, her upper and lower extremities were short for her height, and curving of the lower extremities was also seen. The patient manifested advanced periodontitis and severe vertical bone resorption in the permanent dentition in spite of maintaining relatively good oral hygiene and having received meticulous routine tooth scaling. Her father's serum ALP activity was normal, but he had early periodontitis. In addition, her healthy brothers and sisters also had normal serum ALP levels. Her mother's sister, however, had a serum ALP of 67 IU/L but increased urinary PEA excretion was not found. Previously, we screened the genomic DNA from the patient, his parents, and his brother by PCR-SSCP analysis using 12 sets of primers to cover the entire coding region of the TNSALP gene, as described previously [12]. The proband had a mutation 395C > T (A115V) in exon 5 [22].

Site-directed mutagenesis. The normal TNSALP cDNA was obtained from human periodontal ligament cells [12] and was inserted downstream of the human cytomegalovirus gene promoter (PCMV) of the expression plasmid vector pcDNA3 (Invitrogen, San Diego, CA, USA) as described previously [12].

Site-directed mutagenesis was performed with Unique Site Elimination (U.S.E.) Mutagenesis Kit (Amersham–Pharmacia Biotech). TNSALP cDNA inserted in pcDNA3 expression vector (Invitrogen, San Diego, CA, USA) was a template of mutagenesis. The oligonucleotide of mutagenesis primer 5′-GGGGGTAAGCGTAGCCACT GA-3′ (an underlined nucleotide shows the target mutagenic nucleotide) was used for the mutation of A115V. *Ssp*I/*Stu*I USE selection primer (37-base primer) in the kit was used as the selection primer. The resultant mutant A115V expression plasmid was selected by restriction digestion with *Alw*NI, and confirmed by sequencing using the Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit (Amersham–Pharmacia Biotech) with a Gene Rapid sequencer (Amersham–Pharmacia Biotech).

Transfection of the mutant plasmid. The expression vectors of normal or mutated ALP cDNA (5 μg per 35-mm diameter dish) were transfected into COS-1 cells using a lipofectin technique (Gibco-BRL, GRAND Island, NY, USA). After 48-h transfection, cells were collected and homogenized using a polytron homogenizer (Kinematica, Switzerland) with 10 mM Tris-buffered saline (pH 7.4) containing 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 10,000*g* for 5 min, the supernatant was assayed.

Enzyme activity and protein assay. ALP activity was determined with 10 mM *p*-nitrophenylphosphate as a substrate in 100 mM 2-amino-2-methyl-1,3-propanediol HCl buffer containing 5 mM $MgCl_2$, pH 10.0, at 37 °C. Enzyme activity was determined from the rate of hydrolysis of *p*-nitrophenyl-phosphate and was expressed in units (U = μmol *p*-nitrophenol formed/min). Protein concentrations were determined using BCA protein assay reagent (Pierce, Rockford, IL, USA).

Enzyme histochemistry. COS-1 cells expressing TNSALP mutants on coverslips were stained for alkaline phosphatase activity according to the method of Burstone [28]. Cells were fixed with 4% (w/v) paraformaldehyde in PBS for 10 min on ice and washed three times with 10 mM TBS. Cells were then incubated with a mixture of 0.2 mg naphthol AS-MX phosphate as a substrate and 1 mg Fast Red Violet LB salt dissolved in 2 ml of 0.1 M Tris–HCl (pH 8.8) at room temperature for 10 min.

Inhibition studies. In order to identify possible dominant effects of the mutations, we performed co-transfection of wild-type and mutant A115V, and the enzyme activity of the samples was assayed [31].

Preparation of mRNA and reverse transcription-polymerase chain reaction. Total RNA from transfected cells was extracted using the acid guanidinium thiocyanate–phenol–chloroform method [32]. As a template for PCR, single strand cDNA was prepared from 2 μg using the Ready-To-Go You Prime First–Strand Beads (Amersham–Pharmacia Biotech, NJ, USA). PCR primers for exon 5, exon 10, and the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) were used. The PCR conditions were as follows: 5 cycles of 94 °C (1 min), 50 °C (1 min), and 72 °C (1 min), and 25 cycles of 94 °C (30 s), 55 °C (30 s), and 72 °C (30 s), followed by 10 min at 72 °C. The primer sequences were exon 5 forward, 5′-AGGCTGGAGATGGA CAAGTTC-3′, reverse, 5′-GACAACGAGATGCCCCCTGAG-3′, 330 bp, exon 10 forward 5′-AAGGAGGCAGAATTGACCACG-3′, reverse, 5′-CAAAGATAGAGTTGCCACGGG-3′, 195 bp; and GAPDH forward, 5′-ACCACAGTCCATGCCATCATCAC-3′ reverse, 5′-TCCACCACCCTGTTGCTGTA-3′, 452 bp. The amplified sample (10 μl) was analyzed using 10% polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gel was stained with ethidium bromide solution (0.5 μg/ml). Stained gels were observed under UV light. PCR products were directly sequenced using the Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit (Amersham–Pharmacia Biotech, NJ, USA) with a Gene Rapid sequencer (Amersham–Pharmacia Biotech, NJ, USA).

Immunofluorescence. COS-1 cells, grown on coverslips in a 35-mm dish, were transfected with 5.0 μg plasmid, as described above. After 24 h, the cells were fixed with 4% (w/v) paraformaldehyde (Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS) (pH 7.2) for 30 min at 4 °C. The fixative was aspirated and the cells were rinsed with PBS, and cells were incubated with PBS containing 5% (w/v)

skimmed milk at 4 °C overnight. A polyclonal antibody (PCA-SWKD) recognizing purified swine kidney ALP was raised in rabbits, as described previously [33]. Fixed cells were incubated first with purified antibody against TNSALP (PCA-SWKD) (0.05 mg/ml) for 1 h at room temperature, and then with rhodamine-conjugated goat anti-rabbit immunoglobulin G for 1 h at room temperature. Coverslips were washed with PBS, mounted on a slide glass with 90% (w/v) glycerol in PBS, and sealed. Slides were viewed using a confocal laser scanning microscope (Leica, Heidelberg, Germany), and images were analyzed and prepared for publication using Photoshop 6.0 (Adobe Systems Incorporated, USA).

Statistical analysis. Values are shown as means ± SE, and significance was determined by Mann–Whitney *U* test (StatView-J5.0; Abacus Concepts, Berkeley, CA, USA). Statistical significance was set at the $p < 0.05$ level.

Results

Expression of mutant ALP cDNA

The mutated expression plasmid A115V was confirmed by nucleotide sequencing, and no other mutations were observed in the entire coding region of TNSALP cDNA. We measured ALP activity of the expressed protein from the mutant cDNA using the transient expression system shown in Table 1. COS-1 cells transfected with the mutated plasmid A115V exhibited a level 16.9% that of the wild type. Cytohistochemical staining confirmed the presence of strong alkaline phosphatase activity on the surface of cells transfected with the wild type (Fig. 1). On the contrary, we scarcely detected cells expressing alkaline phosphates in the cells transfected with the mutant A115V (Fig. 1, arrow).

Identification of dominant effects of mutant A115V

In the recessive model, it is assumed that the enzymatic activity of cells co-transfected with equal quantities of wild-type and mutant plasmids is 50% or more, depending on the residual activity of the mutant, and is linearly correlated with the proportion of wild-type cDNA [31]. This was confirmed in cells co-transfected with the wild-type and A115V plasmids, in which enzymatic activities did not statistically differ from the expected 83.5% value (Fig. 2). It is, therefore, likely that this mutation is recessively transmitted.

Table 1
ALP activity of the mutant proteins transiently expressed in COS-1 cells

Mutant	*n*	ALP activity	%WT
Mock	4	0.059 ± 0.043	2.5
Wild-type	5	2.366 ± 0.361	100
A115V	4	0.399 ± 0.021	16.9
V365I	4	0.063 ± 0.012	2.7

Mock indicates cells transfected with pcDNA3 plasmid. The *n* indicates the number of experiments. Each experiments was performed in triplicate. The values of ALP activity are in units per milligram of protein and they are expressed as means ± SE. The percentage of WT shows the percentage of activity in cells transfected with wild-type cDNA. We previously reported that a mutant V365I in the TNSALP gene caused the childhood type HOPS. Thus, we compared the ALP activity of V365I as a negative control.

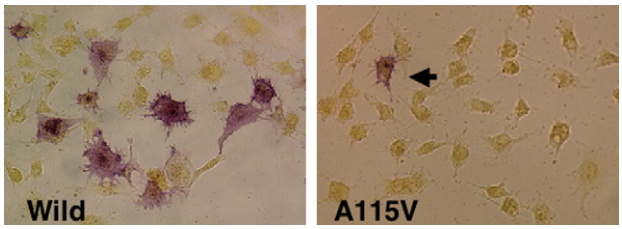

Fig. 1. Transfected cells were stained for alkaline phosphatase for 10 min at room temperature, as described in Materials and methods. The arrow indicates a single cell showing alkaline phosphatase activity.

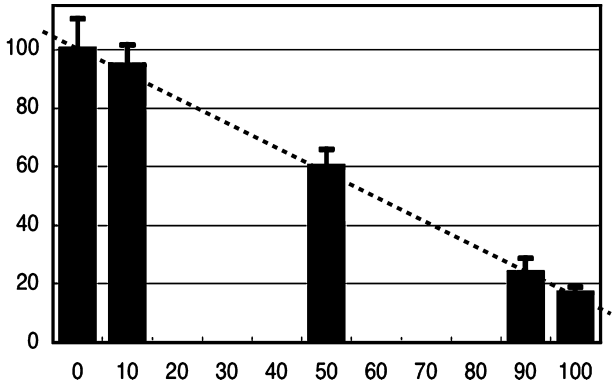

Fig. 2. Correlations between ALP activities of transfected cells expressed as percentage of wild-type (*Y* axis) and the proportion of mutant (A115V) cDNA expressed in percentage (*X* axis). Co-transfections of wild-type and mutation A115V were performed with three distinct ratios (mutant/normal) 90/10, 50/50, and 10/90. Dotted line: expected variation of enzymatic activity in recessive model.

RT-PCR analysis

RNA was extracted from the transfected COS-1 cells and RT-PCR analysis using specific primers was performed. PAGE analysis of the PCR products for wild, wild/A115V, A115V, and V365I in the COS-1 cells revealed almost equal intensity in exon 5 and exon 10 (Fig. 3). We performed digestion of PCR products of exon 5 in TNSALP mRNA using the restriction enzyme *Alw*NI in order to confirm successful introduction of the mutation (Fig. 4). The substitution of C for T at codon 395 generates deletion of the *Alw*NI site indicating the presence of the mutation in the PCR products of exon 5 in TNSALP mRNA. The PCR products of exon 5 were directly sequenced and confirmed.

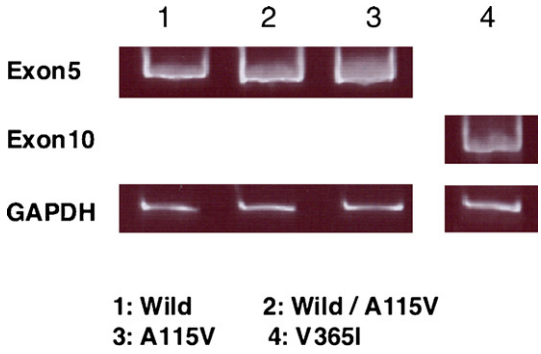

Fig. 3. Detection of mRNAs for exon 5, exon 10, and GAPDH. RT-PCR products were analyzed by 10% PAGE. Lane 1, wild-type expression vector; lane 2, mutant wild/A115V; lane 3, mutant A115V; and lane 4, V365I.

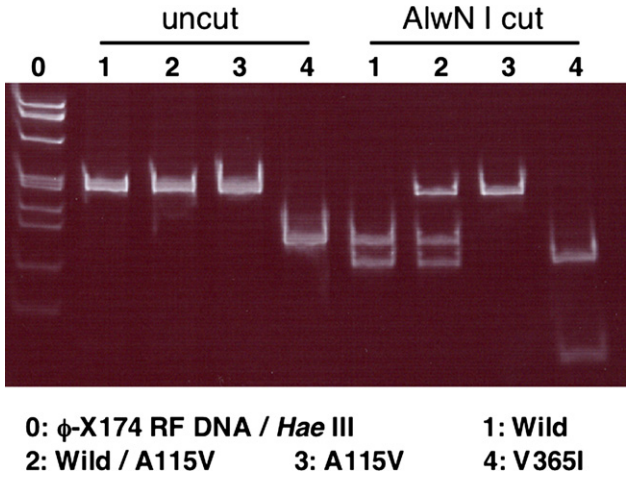

Fig. 4. Verification of introduction of A115V into mRNAs in exon 5. The *Alw*NI site (CAGNNNCTG) was deleted by the substitution of C for T at codon 395. We performed the digestion of PCR products of exon 5 in TNSALP gene by restriction enzyme *Alw*NI and confirmed the introduction. Lane 0, molecular size marker (*Hae*III digest of ϕ-X174 RF DNA); lane 1, wild-type expression vector; lane 2, mutant wild/A115V; lane 3, mutated expression vector (A115V); and lane 4, mutated expression vector (V365I).

Immunohistochemical staining of COS-cells transfected with mutant gene

We performed immunofluorescent staining of intact COS-1 cells transfected with the mutant gene for TNSALP (Fig. 5) using a specific antibody against TNSALP (PCA-SWKD). TNSALP is an ectoenzyme anchored to the plasma membrane. COS-1 cells transfected with the mutant A115V exhibited strong fluorescence at the surface, which was comparable to that seen for wild-type cells.

Discussion

HOPS is highly variable in its clinical expression, which ranges from stillbirth, with no radiographically

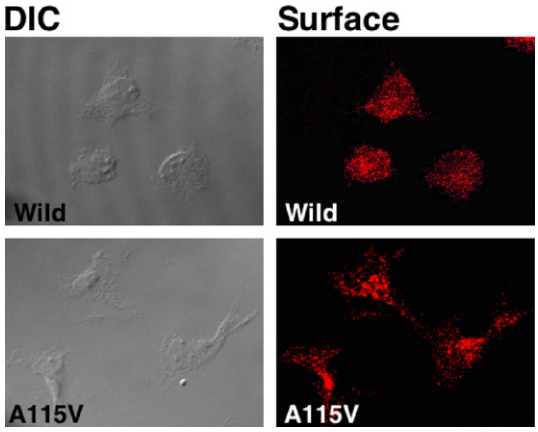

Fig. 5. Immunolocalization of TNSALPs using confocal laser-scanning microscope. Immunofluorescent detection of TNSALP synthesized by COS-1 cells transfected with cDNA encoding the wild-type or A115V. DIC: differential interference contrast.

apparent mineralized bone, to pathologic fractures that occur only during late adulthood. In general, infantile-type HOPS is considered to be a disease with autosomal recessive inheritance. However, the inheritance pattern for milder forms of HOPS is less clear, and both autosomal dominant and autosomal recessive patterns have been proposed [1]. We previously suggested the presence of dominant mutations in milder cases (odonto-type and adult-type HOPS patients) [12,22].

The adult-type HOPS patient in the present study had a heterozygote that had a 395C > T mutation in exon 5 from the mother [22]. The 395C > T mutation in exon 5 resulted in a substitution of Ala-115 with Val (A115V) in the mature TNSALP polypeptide. Ala-115 is highly conserved in rat TNSALP, human intestinal, and placental ALPs and *Escherichia coli* ALP [34]. Most of the effect of this mutation may be because of reduced cofactor binding as nucleotide 395 is near the metal-binding region in the enzyme [22]. According to our results, the mutation A115V is recessively transmitted (Fig. 2).

In Japanese HOPS patients, deletion of T at nucleotide 1559 (1559delT) and F310L were distinct from those found in HOPS patients in North America [35] and appear to be mutational hot spots [11–13,16,24]. In 1559delT cases, a frame shift is observed downstream from codon L503, and we previously revealed that the 1559delT mutant protein in COS-1 cells exhibited no enzymatic activity, and its molecular size was larger than that of the wild-type protein [13]. Interestingly, a compound heterozygote with the trinucleotide deletion of CTT at nucleotide 978–980 (F310del) and the deletion of T at nucleotide 1559 was found in an infantile-type HOPS patient [11]. These findings suggest that the Phe-310 is an important amino acid position in TNSALP gene.

Recently, we revealed that the mutation V365I in TNSALP gene produced the inactive ALP enzyme using reconstructive experiments [25]. This is in agreement with the results of Di Mauro et al. [36] who reported that the mutant V365I enzyme was completely devoid of catalytic activity. Ozono and co-workers [10,37] identified F310L, 1559T del, and G439R (Gly 439 to Arg) in a neonatal HOPS patient and they demonstrated that 1559T del and G439R caused a loss of ALP activity and F310L caused a slight reduction in the ALP activity, with a relatively low level of messenger RNA. Fukushi-Irie et al. [38,39] reported that the mutant TNSALP G317D (Gly 317 to Asp) and R54C (Arg 54 to Cys) were largely confined to the endoplasmic reticulum (ER) in the steady-state and were degraded rapidly by the ER quality-control system. In the present study, expression of the mutant TNSALP gene using COS-1 cells showed that the proteins translated from the mutant A115V had levels 16.9%, with the enzymatic activity of the wild type taken as 100%. Expression levels of TNSALP mRNA were almost identical among wild type, A115V, wild/A115V and V365I (Fig. 3). As shown in Fig. 5, the surface ALP immunofluorescent staining of COS-1 cells transfected with the A115V was not inhibited.

TNSALPs in human bone, liver, kidney, and dental tissues show similar enzymatic properties such as susceptibility to various inhibitors and thermostability, and are clearly different from human intestinal and placental-type ALPs [29,30]. Previously, we reported that the human intestinal and placental-type ALPs showed heat stability, but TNSALPs lost greater than 50% of their activity within 5 min at 56 °C [29,30]. Levamisole is an effective inhibitor for TNSALPs, but it is not effective against intestinal and placental ALPs [29,30].

TNSALP is expressed at high levels in cells within mineralizing tissues, such as osteoblasts and odontoblasts. Human bone and dental pulp ALPs show similar properties being slightly more heat labile than other TNSALPs, such as liver ALP [30]. It is suggested that the onset of HOPS symptoms may be related to enzymatic thermostability. Although the physiological role of TNSALP is still largely unknown, TNSALP may play important roles in active bone metabolism by hydrolyzing phospho-compounds to supply free inorganic phosphate.

In summary, the present study revealed that the mutant (A115V) enzyme fairly lost its enzymatic activity, and its expression level of mRNA and localization on the surface of transfected COS-1 cells were similar to those of wild type. In addition, it was likely that this mutant was not transmitted by a dominant negative manner but was transmitted recessively. It was suggested that this mutation was the cause of the adult-type HOPS. Further analysis of HOPS mutations will help us to elucidate the molecular and cellular functions of TNSALP.

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References

[1] M.P. Whyte, Hypophosphatasia, in: W.A. Peck (Ed.), Bone and Mineral Research, vol. 6., Elsevier Science Publishers, New York, USA, 1989, pp. 175–218.
[2] M.J. Weiss, P.S. Henthorn, M.A. Lafferty, C. Slaughter, M. Raducha, H. Harris, Isolation and characterization of a cDNA encoding a human liver/bone/kidney-type alkaline phosphatase, Proc. Natl. Acad. Sci. USA 83 (1986) 7182–7186.
[3] P.S. Henthorn, M. Raducha, T. Kadesch, M.J. Weiss, H. Harris, Sequence and characterization of the human intestinal alkaline phosphatase gene, J. Biol. Chem. 263 (1988) 12011–12019.
[4] B.J. Knoll, K.N. Rothblum, M. Longley, Nucleotide sequence of the human placental alkaline phosphatase gene, J. Biol. Chem. 263 (1988) 12020–12027.
[5] M.J. Weiss, K. Ray, P.S. Henthorn, B. Lamb, T. Kadesch, H. Harris, Structure of the human liver/bone/kidney alkaline phosphatase gene, J. Biol. Chem. 263 (1988) 12002–12010.
[6] M.J. Weiss, D.E. Cole, K. Ray, M.P. Whyte, M.A. Lafferty, R.A. Mulivor, H. Harris, A missense mutation in the human liver/bone/kidney alkaline phosphatase gene causing a lethal form of hypophosphatasia, Proc. Natl. Acad. Sci. USA 85 (1988) 7666–7669.
[7] P.S. Henthorn, M. Raducha, K.N. Fedde, M.A. Lafferty, M.P. Whyte, Different missense mutations at the tissue-nonspecific alkaline phosphatase gene locus in autosomal recessively inherited forms of mild and severe hypophosphatasia, Proc. Natl. Acad. Sci. USA 89 (1992) 9924–9928.
[8] C.R. Greenberg, C.L. Taylor, J.C. Haworth, L.E. Seargeant, S. Philipps, B. Triggs-Raine, B.N. Chodirker, A homoallelic Gly317 → Asp mutation in ALPL causes the perinatal (lethal) form of hypophosphatasia in Canadian mennonites, Genomics 17 (1993) 215–217.
[9] H. Orimo, Z. Hayashi, A. Watanabe, T. Hirayama, T. Hirayama, T. Shimada, Novel missense and frameshift mutations in the tissue-nonspecific alkaline phosphatase gene in a Japanese patient with hypophosphatasia, Hum. Mol. Genet. 3 (1994) 1683–1684.
[10] K. Ozono, M. Yamagata, T. Michigami, S. Nakajima, N. Sakai, G. Cai, K. Satomura, N. Yasui, S. Okada, M. Nakayama, Identification of novel missense mutations (Phe310Leu and Gly439Arg) in a neonatal case of hypophosphatasia, J. Clin. Endocrinol. Metab. 81 (1996) 4458–4461.
[11] H. Orimo, M. Goseki-Sone, S. Sato, T. Shimada, Detection of deletion 1154–1156 hypophosphatasia mutation using TNSALP exon amplification, Genomics 42 (1997) 364–366.
[12] M. Goseki-Sone, H. Orimo, T. Iimura, Y. Takagi, H. Watanabe, K. Taketa, S. Sato, H. Mayanagi, T. Shimada, S. Oida, Hypophosphatasia: identification of five novel missense mutations (G507A, G705A, A748G, T1155C, G1320A) in the tissue-nonspecific alkaline phosphatase gene among Japanese patients, Hum. Mutat. Suppl. 1 (1998) S263–S267.
[13] M. Goseki-Sone, H. Orimo, T. Iimura, H. Miyazaki, K. Oda, H. Shibata, M. Yanagishita, Y. Takagi, H. Watanabe, T.

Shimada, S. Oida, Expression of the mutant (1735T-del) tissue-nonspecific alkaline phosphatase gene from hypophosphatasia patients, J. Bone Miner. Res. 13 (1998) 1827–1834.
[14] N. Sugimoto, S. Iwamoto, Y. Hoshino, E. Kajii, A novel missense mutation of the tissue-nonspecific alkaline phosphatase gene detected in a patient with hypophosphatasia, J. Hum. Genet. 43 (1998) 160–164.
[15] E. Mornet, A. Taillandier, S. Peyramaure, F. Kaper, F. Muller, R. Brenner, P. Bussiere, P. Freisinger, J. Godard, M. Le Merrer, J.F. Oury, H. Plauchu, R. Puddu, J.M. Rival, A. Superti-Furga, R.L. Touraine, J.L. Serre, B. Simon-Bouy, Identification of fifteen novel mutations in the tissue-nonspecific alkaline phosphatase (TNSALP) gene, Eur. J. Hum. Genet. 6 (1998) 308–314.
[16] H. Watanabe, M. Goseki-Sone, T. Iimura, S. Oida, H. Orimo, I. Ishikawa, Molecular diagnosis of hypophosphatasia with severe periodontitis, J. Periodontol. 70 (1999) 688–691.
[17] A. Taillandier, L. Zurutuza, F. Muller, B. Simon-Bouy, J.L. Serre, L. Bird, R. Brenner, O. Boute, J. Cousin, D. Gaillard, P.H. Heidemann, B. Steinmann, M. Wallot, E. Mornet, Characterization of eleven novel mutations (M45L, R119H, 544delG, G145V, H154Y, C184Y, D289V, 862+5A, 1172delC, R411X, E459K) in the tissue-nonspecific alkaline phosphatase (TNSALP) gene in patients with severe hypophosphatasia, Hum. Mutat. 13 (1999) 171–172.
[18] L. Zurutuza, F. Muller, J.F. Gibrat, A. Taillandier, B. Simon-Bouy, J.L. Serre, E. Mornet, Correlations of genotype and phenotype in hypophosphatasia, Hum. Mol. Genet. 8 (1999) 1039–1046.
[19] H. Mochizuki, M. Saito, T. Michigami, H. Ohashi, N. Koda, S. Yamaguchi, K. Ozono, Severe hypercalcaemia and respiratory insufficiency associated with infantile hypophosphatasia caused by two novel mutations of the tissue-nonspecific alkaline phosphatase gene, Eur. J. Pediatr. 159 (2000) 375–379.
[20] E. Mornet, Hypophosphatasia: the mutations in the tissue-nonspecific alkaline phosphatase gene, Hum. Mutat. 15 (2000) 309–315.
[21] H. Orimo, H.J. Girschick, M. Goseki-Sone, M. Ito, K. Oda, T. Shimada, Mutational analysis and functional correlation with phenotype in German patients with childhood-type hypophosphatasia, J. Bone Miner. Res. 16 (2001) 2313–2319.
[22] H. Watanabe, M. Hashimoto-Uoshima, M. Goseki-Sone, H. Orimo, I. Ishikawa, A novel point mutation (C571T) in the tissue-non-specific alkaline phosphatase gene in a case of adult-type hypophosphatasia, Oral Dis. 7 (2001) 331–335.
[23] A. Taillandier, A.S. Lia-Baldini, M. Mouchard, B. Robin, F. Muller, B. Simon-Bouy, J.L. Serre, A. Bera-Louville, M. Bonduelle, J. Eckhardt, D. Gaillard, A.G. Myhre, S. Kortge-Jung, L. Larget-Piet, E. Malou, D. Sillence, I.K. Temple, G. Viot, E. Mornet, Twelve novel mutations in the tissue-nonspecific alkaline phosphatase gene (ALPL) in patients with various forms of hypophosphatasia, Hum. Mutat. 18 (2001) 83–84.
[24] H. Orimo, M. Goseki-Sone, M. Inoue, Y. Tsubakio, T. Sakiyama, T. Shimada, Importance of deletion of T at nucleotide 1559 in the tissue-nonspecific alkaline phosphatase gene in Japanese patients with hypophosphatasia, J. Bone Miner. Metab. 20 (2002) 28–33.
[25] H. Watanabe, M. Goseki-Sone, H. Orimo, R. Hamatani, H. Takinami, I. Ishikawa, Function of mutant (G1144A) tissue-nonspecific ALP gene from hypophosphatasia, J. Bone Miner. Res. 17 (2002) 1945–1948.
[26] H. Watanabe, M. Umeda, T. Seki, I. Ishikawa, Clinical and laboratory studies of severe periodontal disease in an adolescent associated with hypophosphatasia, J. Periodontol. 64 (1993) 174–180.
[27] M. Goseki-Sone, S. Oida, K. Takeda, Y. Ogata, T. Iimura, Y. Maruoka, S. Sasaki, Identification of bone-type alkaline phosphatase mRNA from human periodontal ligament cells, J. Dent. Res. 74 (1995) 319–322.
[28] M.S. Burstone, in: Enzyme Histochemistry and its Application on the Study of Neoplasms. Academic Press, New York, 1962, pp. 275–276.
[29] M. Goseki-Sone, S. Oida, S. Sasaki, Detection of minor immunological differences among human "universal-type" alkaline phosphatases, J. Cell. Biochem. 38 (1988) 155–163.
[30] M. Goseki-Sone, S. Oida, S. Sasaki, Properties of alkaline phosphatase of the human dental pulp, J. Dent. Res. 69 (1990) 909–912.
[31] A.S. Lia-Baldini, F. Muller, A. Taillandier, J.F. Gibrat, M. Mouchard, B. Robin, B. Simon-Bouy, J.L. Serre, A.S. Aylsworth, E. Bieth, S. Delanote, P. Freisinger, J.C.-.C. Hu, H.-.P. Krohn, M.E. Nunes, E. Mornet, A molecular approach to dominance in hypophosphatasia, Hum. Genet. 109 (2001) 99–108.
[32] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction, Anal. Chem. 162 (1987) 156–159.
[33] S. Oida, M. Sone (Goseki), S. Sasaki, Purification of swine kidney alkaline phosphatase by immunoaffinity chromatography, Anal. Biochem. 140 (1984) 117–120.
[34] E.E. Kim, H.W. Wyckoff, Structure of alkaline phosphatases, Clin. Chim. Acta 186 (1990) 175–187.
[35] E. Mornet, E. Stura, A.S. Lia-Baldini, T. Stigbrand, A. Menez, M.H. Le Du, Structural evidence for a functional role of human tissue nonspecific alkaline phosphatase in bone mineralization, J. Biol. Chem. 276 (2001) 31171–31178.
[36] S. Di Mauro, T. Manes, L. Hessle, A. Kozlenkov, J.M. Pizauro, M.F. Hoylaerts, J.L. Millán, Kinetic characterization of hypophosphatasia mutations with physiological substrates, J. Bone Miner. Res. 17 (2002) 1383–1391.
[37] G. Cai, T. Michigami, T. Yamamoto, N. Yasui, K. Satomura, M. Yamagata, M. Shima, S. Nakajima, S. Mushiake, S. Okada, K. Ozono, Analysis of localization of mutated tissue-nonspecific alkaline phosphatase proteins associated with neonatal hypophosphatasia using green fluorescent protein chimeras, J. Clin. Endocrinol. Metab. 83 (1998) 3936–3942.
[38] M. Fukushi-Irie, N. Amizuka, K. Hoshi, H. Ozawa, H. Kumagai, S. Omura, Y. Misumi, Y. Ikehara, K. Oda, Intracellular retention and degradation of tissue non-specific alkaline phosphatase with a $Gly^{317} \rightarrow Asp$ substitution associated with lethal hypophosphatasia, Biochem. Biophys. Res. Commun. 246 (1998) 613–618.
[39] M. Fukushi-Irie, M. Ito, Y. Amaya, N. Amizuka, H. Ozawa, S. Omura, Y. Ikehara, K. Oda, Possible interference between tissue-non-specific alkaline phosphatase with an $Arg^{54} \rightarrow Cys$ substitution and a counterpart with an $Asp^{277} \rightarrow Ala$ substitution found in a compound heterozygote associated with severe hypophosphatasia, Biochem. J. 348 (2000) 633–642.