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Original Article

The Mutant (F310L and V365I) Tissue-Nonspecific Alkaline Phosphatase Gene from Hypophosphatasia

Hiroyuki Takinami[1,2], Masae Goseki-Sone[3], Hisashi Watanabe[1,2], Hideo Orimo[4], Ryoko Hamatani[3], Mariko Fukushi-Irie[3] and Isao Ishikawa[1,2]

1) Department of Hard Tissue Engineering (Periodontology), Graduate School, Tokyo Medical and Dental University, Tokyo, Japan.
2) Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, Tokyo Medical and Dental university.
3) Department of Food and Nutrition, Japan Women's University.
4) Department of Biochemistry and Molecular Biology, Nippon Medical School.

Hypophosphatasia (HOPS) is a heritable disorder characterized by defective skeletal mineralization, deficiency of tissue-nonspecific alkaline phosphatase (TNSALP) activity and premature loss of deciduous teeth. In a previous study, we detected missense mutations in the TNSALP gene of a patient who inherited the F310L and the V365I mutation with severe periodontitis and childhood HOPS. Expression of the mutant V365I TNSALP gene using COS-1 cells demonstrated that the protein translated from the mutant had undetectable ALP activity. In the present study, we characterized another ALP enzyme translated from the mutant F310L and compared it with the ALP in the patient's serum. The COS-1 cells transfected with the F310L and co-transfected with F310L and V365I (F310L/V365I) exhibited levels of 67% and 31%, respectively, with the enzymatic activity of the wild-type taken as 100%. In the thermostability test, TNSALPs in the COS-1 cells transfected with the mutant F310L or F310L/V365I were significantly more heat labile compared with that of the wild-type. Moreover, ALP from the patient's serum was also more heat labile than normal ALP. These results suggest that the protein translated from the mutant F310L, in addition to the mutant V365I, may be responsible for the expression of symptoms of the childhood-type HOPS.

Key words: hypophosphatasia, mutant gene, tissue-nonspecific alkaline phosphatase, site-directed mutagenesis, COS-1 cells

Corresponding Author: Hiroyuki Takinami
Department of Hard Tissue Engineering (Periodontology), Graduate School, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8549, Japan
Tel: +81-3-5803-5488, Fax: +81-3-5803-0196
E-mail: takinami.peri@tmd.ac.jp
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Introduction

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]

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 138 mutations have been reported in previous studies.[6-26] 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, 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]

In this study, we characterized the protein translated from the mutant F310L. The F310L mutant-type TNSALP cDNA expression plasmid was constructed and transfected to COS-1 cells. The properties of the mutant TNSALPs expressed in COS-1 cells and the ALP in the serum of the HOPS patient were studied.

Materials and Methods

Subjects

The proband was a 25-year-old Japanese male (age at onset was 4 years old) with childhood hypophosphatasia. The patient reported premature exfoliation of the deciduous teeth, poor systemic growth (in particular skeletal growth), and severe periodontal destruction in the permanent dentition. Clinical data (x-rays, history of deciduous teeth, other relevant history and findings) and patient management were reported previously.[27] Serum ALP activity was 11 IU/liter (normal: 100-280) and urinary phosphoethanolamine (PEA) excretion was elevated (363 μmol/day, normal: 30-100). ALP activity levels of his parents who showed mild periodontitis were both at the lower limit of normal and those of his healthy brother were normal. 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 inherited a 979T＞C (F310L) in exon 9 from his mother and a 1144G＞A (V365I) in exon 10 from his father.[12,16,25]

Collection of sera

This study approved by the declaration of Helsinki and informed consent has been obtained from all the subjects prior to the experiment. Peripheral blood was collected without anti-coagulants. After centrifugation, sera were used for the experiments.

Site-directed mutagenesis

The normal TNSALP cDNA was obtained from human periodontal ligament cells[28] and was inserted downstream of the human cytomegalo virus gene promoter (PCMV) of the expression plasmid vector pcDNA3 (Invitrogen Corp., San Diego, CA, USA) as described previously.[13] Site-directed mutagenesis was performed with a Unique Site Elimination (USE) Mutagenesis Kit (Amersham-Pharmacia Biotech, Inc., Piscataway, NJ, USA). The normal TNSALP cDNA inserted in pcDNA3 expression vector (wild-type) was a template of mutagenesis. The oligonucleotide of mutagenesis primer 5'-CCCCAAAGGCCTCTTCTTGCT -3' (an underlined nucleotide shows the target mutagenic nucleotide) was used for the mutation of F310L. The primer for the mutation of V365I was as previously reported.[24] *Ssp* I/*Stu* I USE selection primer (37-base primer) in the kit was used as the selection primer. The resultant mutant F310L expression plasmid was selected by restriction digestion with *Stu* I, and confirmed by sequencing using the thermo sequenase Cy 5.5 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.

Preparation of mRNA and reverse transcription-polymerase chain reaction

Total RNA from transfected cells was extracted using the acid granidinium thiocyanate—phenol-chloroform method.[29] 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 9, exon 10 and the housekeeping gene glyceraldehydes 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 9 forward, 5'-ATGAAGGAAAAGCCAAGCAGG-3', reverse, 5'-GCCTGGACCTCGTTGACACCT-3', 297 bp; exon 10 forward 5'-AAGGAGGCAGAATTGACCACG-3', reverse, 5'-CAAAGATAGAGTTGCCACGGG-3', 195 bp; 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).

Enzyme and protein assay

ALP activity was determined with 10 mM *p*-nitrophenylphosphate as a substrate in 100 mM 2-amino-2-methyl-1, 3-propanediole 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 cover slips were stained for alkaline phosphatase activity according to the method of Burstone.[30] 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 of Naphthol AS-MX phosphate as a substrate and 1 mg of 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

Enzyme activity of the samples was assayed in the presence of levamisole (the final concentration; 1 mM).[31,32] For thermostability assay, samples were pretreated at 56°C for 0, 1, 5, 10, 20, or 30 min and then reacted with the substrate.[31,32]

In order to identify possible dominant effects of the mutations, we performed co-transfection of wild-type and mutant F310L.[33]

Statistical analysis

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

Results

RT-PCR analysis

The mutated expression plasmid F310L was confirmed by nucleotide sequencing, and no other mutations were observed in the entire coding region of TNSALP cDNA.

RNA was extracted from the transfected COS-1 cells and RT-PCR analysis using specific primers was performed. We performed digestion of PCR products of exon 9 in TNSALP mRNA using the restriction enzyme *Stu* I in order to confirm successful introduction of the mutation (Fig. 1). The substitution of C for T at codon 310 generates the new *Stu* I site within the amplified 297 bp sequence yielding 240 bp fragment. The 240 bp fragment was only detected in mutant F310L, indicating the presence of the mutation in the PCR products of exon 9 in TNSALP mRNA. The PCR products of exon 10 were directly sequenced and confirmed. PAGE analysis of the PCR products for wild, F310L, wild / F310L, and V365 I /F310L in the COS-1 cells revealed almost equal intensity in exon 9 (Fig. 2). With regard to mRNA expression levels of TNSALP in exon 10, no differences were observed among wild, F310L / V365I, and V365I (Fig. 2).

Expression of mutant ALP cDNA

We measured ALP activity of the expressed protein from the mutant cDNA using the transient expression system shown in Fig. 3A. COS-1 cells transfected with the mutated plasmid F310L exhibited a level 67% that of the wild-type. When co-transfected with the same quantity of mutations (2.5 μg/35-mm diameter dish each), F310L and V365I (F310L / V365I) exhibited a

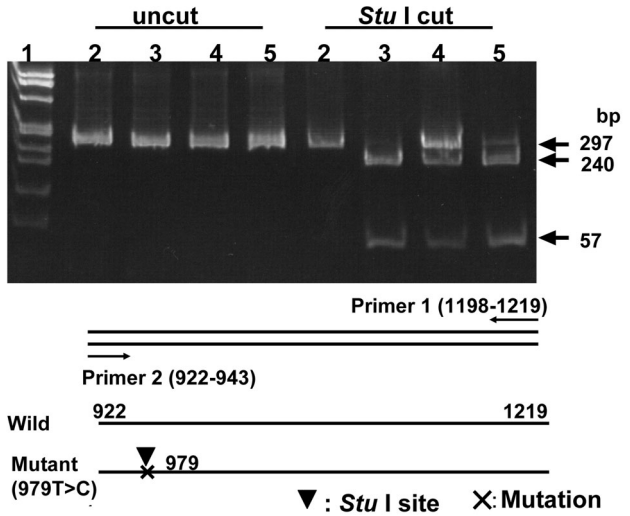

Fig. 1. Verification of introduction of F310L into mRNAs in Exon 9. Digestion of PCR-amplified fragment (297 bp) with *Stu* I generated a smaller 240 bp band in mutants.
Lane 1, molecular size marker (*Hae* III digest of ϕ-X174 RF DNA); lane 2, wild-type expression vector; lane 3, mutated expression vector (F310L); lane 4, mutant wild / F310L; lane 5, mutant F310L / V365I.

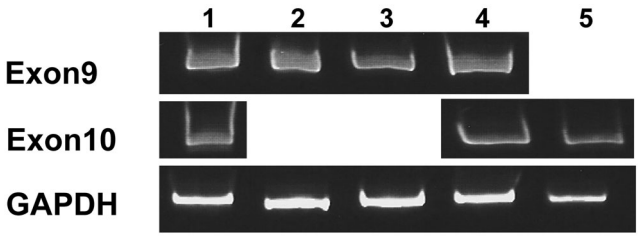

Fig. 2. Detection of mRNAs for Exon 9, Exon 10, and GAPDH. RT-PCR products were analyzed by 10% PAGE. Lane 1, wild-type expression vector; lane 2, mutated expression vector (F310L); lane 3, mutant wild / F310L; lane 4, mutant F310L / V365I, lane 5, V365I.

level of 31% that of the wild-type. COS-1 cells transfected with the mutant V365I exhibited ALP activity corresponding to COS-1 cells transfected with mock expression vector. There were significant differences between the wild-type and the mutated plasmid (F310L or F310L / V365I) ($p< 0.05$) (Fig. 3A). Cytohistochemical staining confirmed the presence of strong alkaline phosphatase activity on the surface of cells transfected with the wild-type or F310L (Fig. 3B). In contrast, cells transfected with the mutant V365I were not positive for staining. We occasionally detected cells expressing alkaline phosphates by co-transfection with the mutant F310L / V365I (Fig. 3B, arrow).

Inhibition studies

Table 1 shows the results of inhibition and thermal

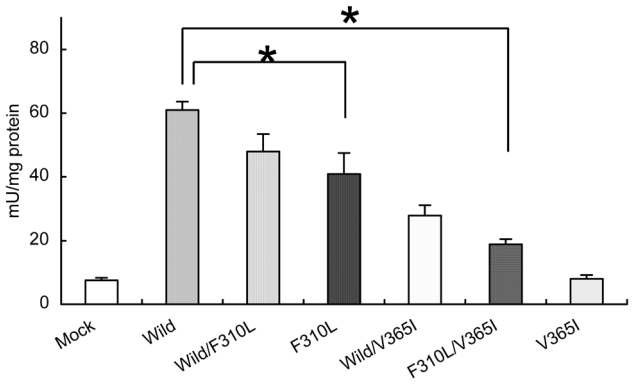

Fig. 3a. COS-1 cells were transfected with the wild-type TNSALP, TNSALP (F310L), TNSALP (V365I), separately or in combination. Mock indicates cells transfected with pcDNA3 plasmid. The transfected cells were homogenized and assayed for ALP activity. The COS-1 cells transfected with the mutated ALP (V365I) expression vector exhibited the level of that of the mock vector.[25] Each experiment was performed in triplicate. *$p<0.05$

inactivation experiments of ALPs in the COS-1 cells transfected with expression vectors such as the wild-type, the wild-type and F310L (wild / F310L), F310L or F310L / V365I. High inhibition of the ALP activity was observed in all mutants by the treatment of levamisole (1 mM). Although the ALP activity of the wild-type after heating at 56°C for 5 min was 40.4%, the activities of the mutants wild-type/F310L and wild-type/V365I, were 29.03 and 32.7%, respectively. Very interestingly, the high inhibition of the ALP activity was still observed in the case of the mutant F310L and F310L/V365I, and their ALP activities were 21.72 and 16.51% compared to those in untreated samples, respectively. The effects of heating on ALPs in COS-1 cells transfected with the wild-type, the mutants F310L and F310L / V365I at 56°C for various durations are shown in Fig. 4. Serum ALP from the HOPS patient who had F310L and V365I in the TNSALP gene was significantly heat labile compared with that of the sera from the healthy subjects (Fig. 5) ($p< 0.05$). Residual activities after heating sera from healthy subjects and the proband at 56°C for 5 min exhibited levels approximately 35.9% and 17.2% compared to unheated sera, respectively.

Identification of recessive effects of mutant F310L

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.[32] This was confirmed in cells co-transfected with

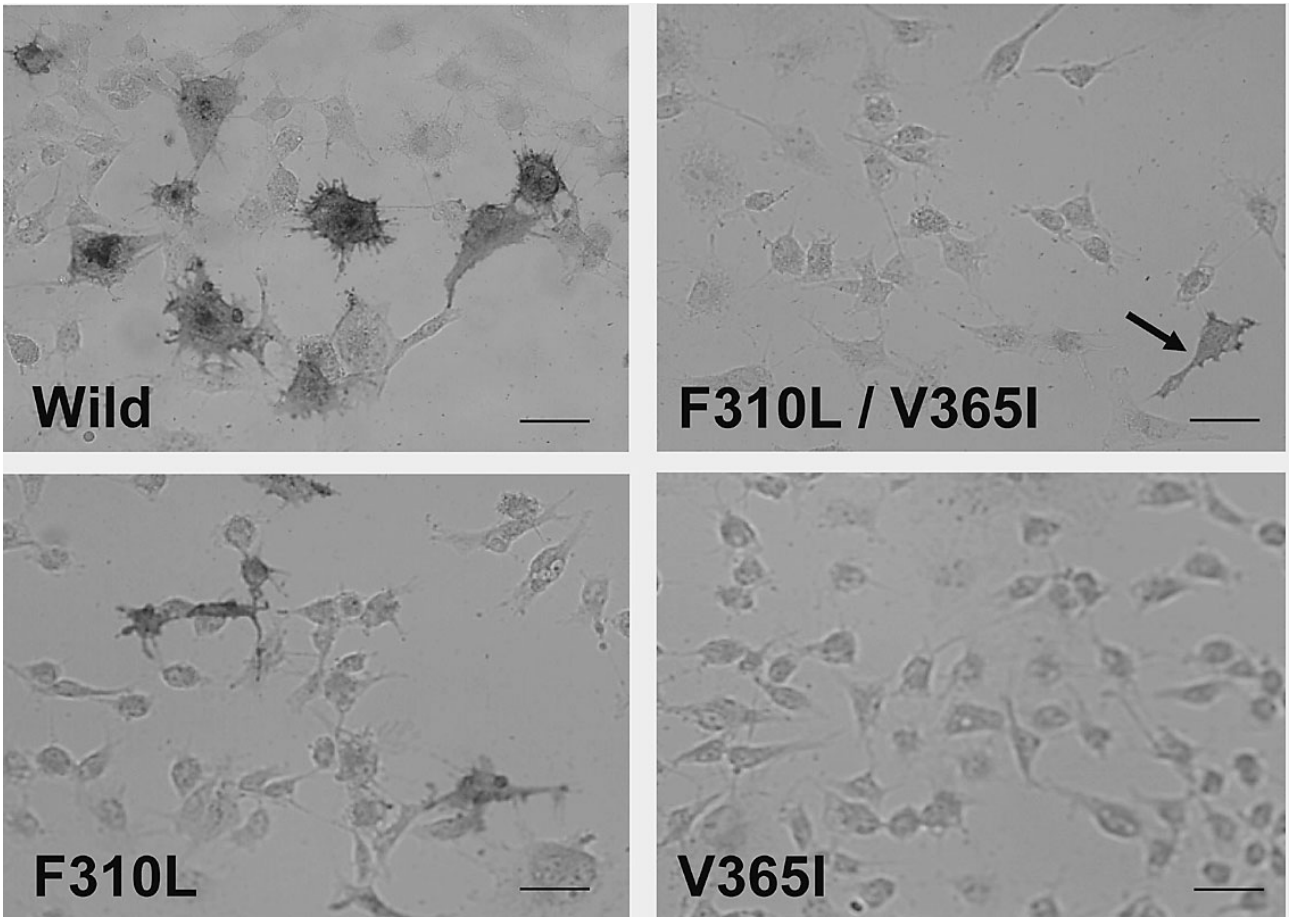

Fig. 3b. (B) Transfected cells were stained for alkaline phosphatase for 10 min at room temperature, as described in the Materials and Methods section. The arrow indicates a single cell showing alkaline phosphatase activity. Scale bars, 50 μm.

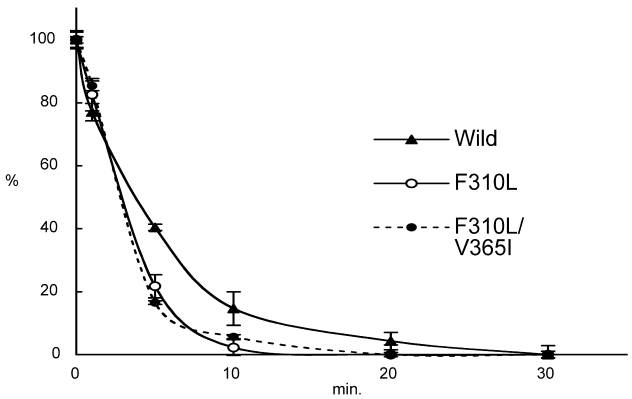

Fig. 4. Effect of heating on ALPs in COS-1 cells transfected with the wild-type TNSALP vector, the mutant F310L and F310L / V365I at 56°C for different periods.
▲; the wild-type TNSALP expression vector, ○; the mutant F310 expression vector, ●; the mutant F310L/V365I.

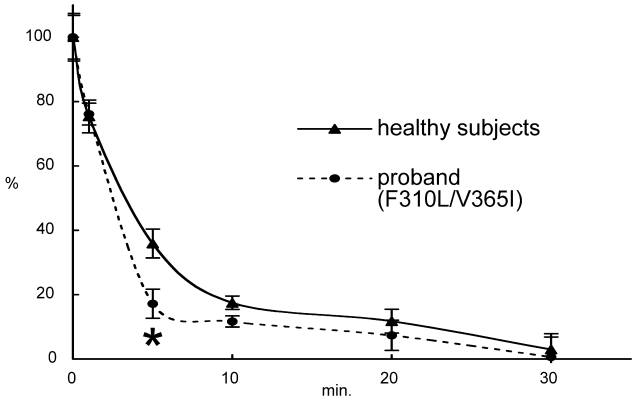

Fig. 5. Effect of heat treatment on serum ALPs at 56°C for different periods. Sera were obtained from the healthy subjects (n=4), and the HOPS patient who had a compound heterozygote that had mutations (F310L and V365I) in the TNSALP gene.
*$p < 0.05$
▲; the healthy subjects, ●; the HOPS patient (F310L and V365I).

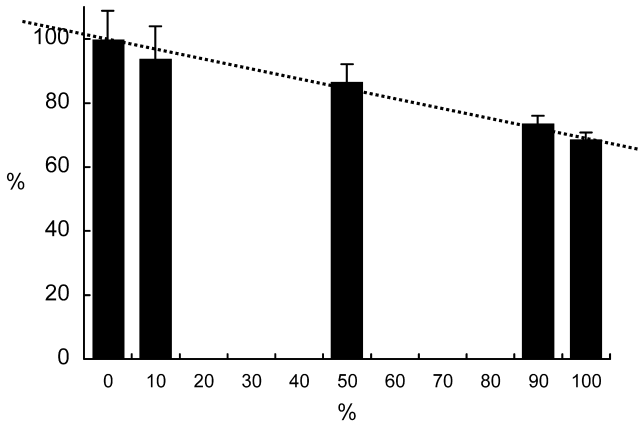

Fig. 6. Correlations between ALP activities of transfected cells expressed as % wild-type (Y axis) and the proportion of mutant (F310L) cDNA expressed in % (X axis).
Co-transfections of wild-type and mutation F310L were performed with three distinct ratios (mutant / normal) 90 / 10, 50 / 50, and 10 / 90. (Each group n=3) Dotted line: Expected variation of enzymatic activity in recessive model.

the wild-type and F310L plasmids, in which enzymatic activities did not statistically differ from the expected 83.5% value (Fig. 6). It is therefore, likely that this mutation is recessively transmitted.

Discussion

HOPS is highly variable in its clinical expression, which ranges from stillbirth, with no radiographically 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 childhood-type HOPS patient in the present study had a compound heterozygote that had a 979T＞C mutation in exon 9 from the mother and a 1144G＞A mutation in exon 10 from the father.[12] The 979T＞C mutation in exon 9 resulted in a substitution of Phe-310 with Leu (F310L) in the mature TNSALP polypeptide, and the G1144A mutation resulted in a substitution of Val-365 with Ile (V365I) in the mature TNSALP polypeptide. Phe-310 is highly conserved in rat TNSALP, human intestinal, and placental ALPs and *Escherichia coli* ALP.[34] Val-365 is also highly conserved in rat TNSALP, human intestinal and placental ALPs.[34] Both the F310L and V365I mutations were near the metal binding ligand region. From the crystal structure, the region of the F310L mutation is buried and important for secondary structure.[35] V365I may be directly involved in homodimer interactions or play a role in maintaining the correct fold to allow these interactions to form.[35] According to our results, the mutation F310L is recessively transmitted. (Fig. 6)

In Japanese HOPS patients, deletion of T at nucleotide 1559 (1559delT) and F310L were distinct from these found in HOPS patients in North America and appear to be mutational hot spots in Japanese HOPS patients.[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 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 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., who reported that the mutant V365I enzyme was completely devoid of catalytic activity.[36] Ozono et al. 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.[10,37] Fukushi-Irie et al. 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.[38,39] In the present study, expression of the mutant TNSALP gene using COS-1 cells showed that the proteins translated from the mutant F310L alone and the combination of F310L and V365I (F310L / V365I) had levels 67% and 31%, respectively, with the enzymatic activity of the wild-type taken as 100% (Fig. 3A). Expression levels of TNSALP mRNA were almost identical among wild-type, F310L, wild / F310L, F310L / V365I, and V365I (Fig. 2).

TNSALPs in human bone, liver, kidney, and dental tissues show similar enzymatic properties such as susceptibility to various inhibitors and thermostability,

Table Properties of the ALP proteins transiently expressed in cos-1 cells

		The remaining ALP activity (%)	
Mutant	n	Levamisole (1 mM)	Heat stability (56°C, 5min)
wild-type	3	3.53 ± 0.46	40.40 ± 1.01
wild-type / F310L	3	0.86 ± 1.49	29.03 ± 0.95
F310L	3	2.93 ± 3.88	21.72 ± 3.70*
wild-type / V365I	3	0.72 ± 1.24	32.70 ± 0.70
F310L / V365I	4	2.96 ± 4.50	16.51 ± 0.58*

Activity was assayed by the rate of hydrolysis of *p*-nitrophenylphosphate. The effect of the inhibitor was determined in the presence of 5 mM $MgCl_2$ in the assay mixture. n indicates number of the experiments. The ALP activities in untreated cells were designated as 100%. *$p < 0.05$ compared with the wild-type.

and are clearly different from human intestinal and placental type ALPs.[30,31] 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.[30,31] Levamisole is an effective inhibitor for TNSALPs, but it is not effective against intestinal and placental ALPs.[30,31] The enzymatic properties of ALPs of the mutant F310L and the present patient's serum were comparatively investigated (Table, Fig. 4 and 5). There is no significant difference among them by treatment of levamisole (1 mM). In this study, high variation was observed among the samples in each group. This variation may be eliminated by increasing the sample size. Amazingly, the F310L alone and the combination of F310L and V365I mutants were inhibited significantly by the heating (56°C) compared with the wild-type (Fig. 4). Serum ALP activity of the HOPS patient was also significantly lost by the heating (56°C) compared with those of the healthy subjects (Fig. 5).

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.[31] 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 (F310L) enzyme partly lost its enzymatic activity, and resulted in the synthesis of thermo-unstable TNSALP molecules that were expressed in the patient's serum. It was suggested that this mutation was the cause of the childhood-type HOPS. Further analysis of HOPS mutations will help to elucidate the molecular and cellular functions of TNSALP.

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