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ABSTRACT

Hypophosphatasia is caused by mutations of the tissue-non-specific alkaline phosphatase (*TNSALP*) gene with deficiency of dentin structure. The aim of this study was to examine whether *TNSALP* mutation in dental pulp cells contributes to dentin dysplasia in hypophosphatasia. Mutation analysis showed that compound heterozygous mutations of *TNSALP* were identified in three hypophosphatasia patients, including 3 novel mutation sites. Exfoliated teeth from the patients showed abnormal dentin mineralization and loss of cementum, as assessed by ground sections and scanning electron microscope analysis. Dental pulp cells isolated from one of the patients showed a significantly reduced TNSALP activity and mineralization capacity when compared with those in dental pulp cells from the unaffected individuals. Our results suggested that dentin dysplasia in hypophosphatasia may be associated with the decreased mineralization ability of dental pulp cells.

KEY WORDS: hypophosphatasia, tissue-non-specific alkaline phosphatase, gene mutation, dental pulp cells.

INTRODUCTION

Hypophosphatasia is an inherited disorder of bones and teeth, manifested by defective mineralization and a deficiency in alkaline phosphatase activity. Clinical manifestation varies from stillbirth without mineralized bone to early tooth loss without bone symptoms. Systemic phenotypes include osteochondral spurs protruding from the limbs, rachitic deformities of the chest, osteoporosis, and hypercalcemia (Kozlowski *et al*., 1976; Sinico *et al*., 2007). The typical oral manifestation of hypophosphatasia is premature loss of primary teeth. This defect prompted many affected individuals to seek dental care, when hypophosphatasia was subsequently diagnosed (Lundgren *et al*., 1991).

Dysplasia or aplasia of cementum was histologically observed in hypophosphatasia and has been proposed to be the primary reason for the early exfoliation of teeth (Bruckner *et al*., 1962: e1-Labban *et al*., 1991; Olsson *et al*., 1996; Hu *et al*., 2000). Irregular dentin calcification and enlarged pulp chambers were also observed in individuals with hypophosphatasia (Beumer *et al*., 1973; Jedrychowski and Duperon, 1979; Olsson *et al*., 1996).

The tissue-non-specific alkaline phosphatase (*TNSALP*) gene was identified to be the causative gene of hypophosphatasia (Taillandier *et al*., 2001; Watanabe *et al*., 2005; Michigami *et al*., 2005; Brun-Heath *et al*., 2005, 2007). TNSALP cleaves extracellularly to inorganic pyrophosphates (PPi) to release inorganic phosphate (Pi). Since Pi is required for hydroxyapatite crystallization, the ability of TNSALP to hydrolyze PPi to Pi is essential for mineralized tissue formation (Whyte, 1994; Mornet *et al*., 2001; Harmey *et al*., 2004).

Dental pulp cells are capable of differentiating into odontoblast-like cells with excreting extracellular matrix and forming mineralized nodules *in vitro* (Couble *et al*., 2000; Yokose *et al*., 2000). Isolation and culture of dental pulp cells from individuals with hypophosphatasia will help us to evaluate whether the dentin mineralization process was affected in hypophosphatasia.

In this study, to explore whether *TNSALP* alteration in dental pulp cells of hypophosphatasia contributed to dentin dysplasia, we examined the exfoliated teeth from persons with hypophosphatasia and analyzed genetic variations of the *TNSALP* gene in three Chinese children with hypophosphatasia. In addition, we assessed *TNSALP* activity in dental pulp cells of persons with hypophosphatasia.

MATERIALS & METHODS

The protocol for this study was approved by Peking University Health Science Center's Ethical Committee (IRB00001052–06060). Three unrelated Chinese children (aged 5-6 yrs), clinically diagnosed as having hypophosphatasia (Mornet, 2008), were included in this study with informed parental consent.

Gene Mutation Analysis

The patients, their parents, and 112 unaffected individuals with no evidence of hypophosphatasia were examined for *TNSALP* gene mutation.

We collected 2 mL of peripheral blood from each individual. Genomic DNA was isolated from leukocytes by means of the QIAmp Blood Kit (Qiagen, Hilden, Germany). Twelve exons of the *TNSALP* gene were amplified by PCR with specific primers (Appendices 1, 2). The PCR products were sequenced by an autocycle sequencing method (Bioasia, Shanghai, China).

Ground Sections

The exfoliated upper primary central incisors from Patient I were used for ground sections. Avulsed primary central incisors from trauma were collected and used as controls. The teeth were cut along the long axis, and ground into sections of 80 to 100 μm in thickness. After dehydration in a graded ethanol concentration series, and clearing in xylene, the sections were sealed with neutral balsam, and examined by microscopy.

Scanning Electron Microscopy (SEM)

Tooth samples were fixed in 4% paraformaldehyde and ultrasonically cleaned for 30 min in 10% sodium hypochlorite solution, which was changed every 5 min and finally replaced by 0.9% sodium chloride. The samples were dried at 37°C for 24 hrs, then sputter-coated with gold. The root surfaces were observed under SEM, with 15.0 KV (S-4800, Hitachi, Tokyo, Japan).

Dental Pulp Cell Culture

The deciduous upper lateral incisors of Patient I were quite mobile and interfered with the making of the space maintainer and therefore were extracted. The dental pulp was isolated, and the apical 2 mm was discarded. The dental pulp was treated with 3 mg/mL type I collagenase (Sigma, St. Louis, MO, USA) and 4 mg/mL dispase (Sigma), washed, and cengtrifuged. The cells were cultured in alpha-MEM (GIBCO/BRL, Grand Island, NY, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA), 2 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2.

The retained deciduous incisors from three unaffected children (aged 5-6 yrs) were extracted for failure of exfoliation, and their dental pulp cells were isolated as controls.

Mineral Induction

To induce mineralization, we added 10 mM β-glycerophosphate (Fluka, St. Louis, MO, USA), 10^{-7}M dexamethasone (Sigma), and 50 μg/mL L-ascorbic acid-2-phosphate (Sigma) to the culture medium. The cells were subjected to von Kossa staining 3 wks later for evaluation of mineralized nodule formation (Okabe *et al.*, 2006).

Alkaline Phosphatase Activity Test

The dental pulp cells from the patient and unaffected children were cultured and collected at 100% confluence and 3 wks after confluence. The cells were rinsed 3x with PBS and subjected to lysis in 2 mL 0.1% Triton X-100 in 0.01 M PBS at 4°C overnight. The activities of alkaline phosphatase (ALP) in the lysates were measured with the ALP Kit (Biosino Bio-technology and Science Inc., Beijing, China) according to the manufacturer's instructions. Absorbance was measured at 405-nm wavelength with an auto analyzer system (Hitachi 7180, Tokyo, Japan). One unit of the activity was defined as the enzyme-liberated 1 μmol product/min at 37°C. Statistical significance of the differences was evaluated by the paired-samples *t* test.

RESULTS

Phenotype of Hypophosphatasia

All three patients showed premature loss of deciduous teeth. Patient I was of particular interest due to the patient's bone phenotype and 2 newly discovered mutations. The patient's height and weight at birth were within unaffected ranges (50 cm and 3.8 kg, respectively). It was found that the patient had 10 deciduous teeth lost at age 5 (Fig. 1a). Panoramic radiographs showed reduced alveolar bone, enlarged pulp chambers, and abnormal morphology of the unerupted first permanent molars. The enamel of the first permanent molars was very thin compared with that of unaffected controls. The occlusal surface of the crown was rough and granular, with uneven translucency (Fig. 1b). The patient had the deformity known as 'pigeon breast', but no respiratory complications. Radiographs of the patient's legs showed exostosis in the bilateral knee joints (Fig. 1c). The serum alkaline phosphatase levels of the patient, her mother, and father were 8 U/L, 20 U/L, and 48 U/L, respectively. The patient was the only affected one in her pedigree (Appendix 3).

Patient II and Patient III were also the only affected ones in their own pedigrees. These two patients suffered from premature loss of primary teeth as well, but without bone signs (see detail in the Table and Appendices 4,5).

TNSALP Gene Mutated in the Three Hypophosphatasia Patients

Six compound heterozygous mutations in the *TNSALP* gene were identified in the three patients. Each patient carried 2 mutations, 1 of paternal origin and 1 of maternal origin (Table). These nucleotide changes were not detected among 112 unrelated healthy individuals.

Of the 6 mutations identified, 3 are novel for *TNSALP* (Table). Of the 3 novel mutations, 1 was located within exon 10 and led to a predicted tyrosine to histidine amino acid change (Y371H); the other 2 were located at exon-intron junction regions (intron 9, 997+1 G>T and intron 5, 472+1 G>A). Patient I carried 2 of the 3 novel mutations, located in exon 10 and intron 9, respectively.

Characteristics of Teeth from Hypophosphatasia Patients

Ground sections revealed that there was no cementum structure on the root surface of the hypophosphatasia patient. The Tomes' granular layer, which is located near the end of dentinal tubules,
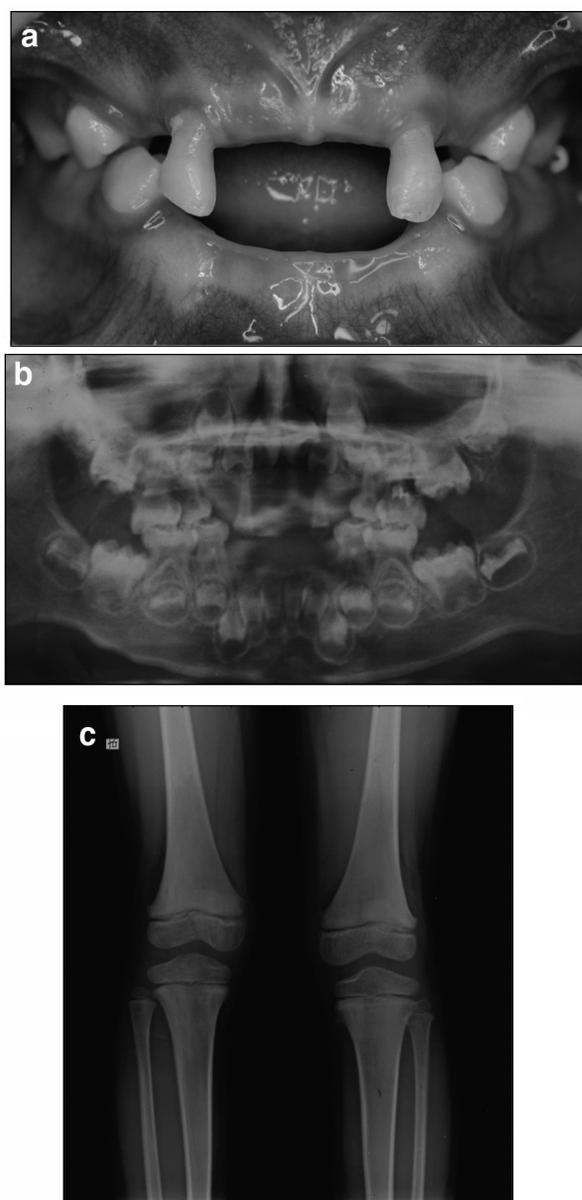
Figure 1. Clinical features of Patient I. **(a)** Intra-oral photograph of Patient I at 5 yrs old, showing premature exfoliation of primary teeth. **(b)** Panoramic radiograph of Patient I at 5 yrs old, showing reduced alveolar bone, enlarged pulp chambers, and abnormal morphology of unerupted first permanent molars. **(c)** Radiograph showing exostosis in the bilateral knee joints in Patient I at 5 yrs old.

was much thicker when compared with that of the healthy children (Figs. 2a, 2b). Interestingly, at the middle regions of the patients' tooth crowns, the terminus of dentinal tubules close to the dentin-enamel junction (DEJ) appeared as a dark granular region (Figs. 2c, 2d). SEM showed that the root surface structure of the patient was partially missing, while the root surfaces of unaffected children were smooth and homogenous (Figs. 2e, 2f).

Decrease of TNSALP Activity and Mineralization of Dental Pulp Cells from the Hypophosphatasia Patient

Dental pulp cells isolated from the patient and an unaffected child showed fibroblast-like morphology. No obvious morphological differences were observed between the cells from the hypophosphatasia patient and the unaffected child.

The TNSALP activity of the dental pulp cells from the unaffected children was dramatically increased at 3 wks post-confluence, while the TNSALP activity of the dental pulp cells from the patient failed to show any increase. The level of TNSALP activity in the patient's dental pulp cells was 10 times lower than that of the unaffected children ($p < 0.01$) (Fig. 3a). Von Kossa staining revealed the presence of mineralized nodules in dental pulp cells when cultured under osteo/odontogenic-inductive conditions. However, there were fewer, and smaller, mineralized nodules in the cells from the patient compared with those from the unaffected children (Figs. 3b, 3c).

DISCUSSION

In the present study, we found 6 compound heterozygous mutations in 3 unrelated hypophosphatasia patients and observed disturbances of dentin structures in these patients. Dental pulp cells isolated from the patient with 2 novel mutations showed lower TNSALP activity and lower capacity for forming mineralized nodules, which may contribute to dentin dysplasia.

Patient I possessed a novel missense mutation of exon 10 and a novel mutation of an exon-intron junction (intron 9, 997+1G>T). The novel mutation within exon 10 led to a predicted tyrosine to histidine (Y 371H), located in the 12-Å sphere around the phosphate group at an active site of TNSALP (Le Du and Millán, 2002). The active site is the center of catalytic activity of TNSALP. Although severe forms of hypophosphatasia usually develop if missense mutations locate within the 15-Å sphere around the phosphate group of TNSALP, there are exceptions whereby several missense mutations within this region showed only moderate hypophosphatasia (Mornet *et al*., 2001). The current novel missense mutation of exon 10 may be another case of the exceptions, due to the fact that patient I did not show severe clinical symptoms.

Patient I showed the most relatively severe clinical manifestations among the three affected individuals in this study. Theoretically, mutations in the exon-intron junction may affect RNA splicing and lead to accumulation of abnormal transcripts and reduction of protein expression (Barese *et al*., 2005; Vetrini *et al*., 2006). The mutation located at this patient's exon-intron junction region (intron 9, 997+1G>T) may combine with the mutation in exon 10 to produce aberrant functional TNSALP. Thus, this patient may have the most severe clinical manifestations among the three affected individuals in this study.

In the present study, the activity of TNSALP of the dental pulp cells from Patient I was significantly lower than that of the dental pulp cells from the unaffected children. Previous study showed that dental pulp cells stop proliferation to differentiate once the cells reach confluence, and then TNSALP activity begins to increase continually and reaches the peak at 2 to 3 wks

Table. Clinical and Genetic Features of the Three Patients in This Study

Patients	Tooth Loss	Pigeon Chest	Exostosis	Short Stature	Mutation			References
					Nucleotide	Origin	Type	
I	++	+	+	+	997+1G>T (intron9)	Father	Splice junction alteration	Novel
					1162T>C (exon10)	Mother	Mis-sense mutation	Novel
II	++	-	-	+	472+1G>A (intron5)	Mother	Splice junction alteration	Novel
					1120G>A (exon10)	Father	Mis-sense mutation	Versailles lab. 2004*
III	+++	-	-	+	668G>A (exon7)	Mother	Mis-sense mutation	Mumm *et al.* (2002)**
					1120G>A (exon10)	Father	Mis-sense mutation	Versailles lab. 2004*

*From http://www.sesep.uvsq.fr/Database.html.
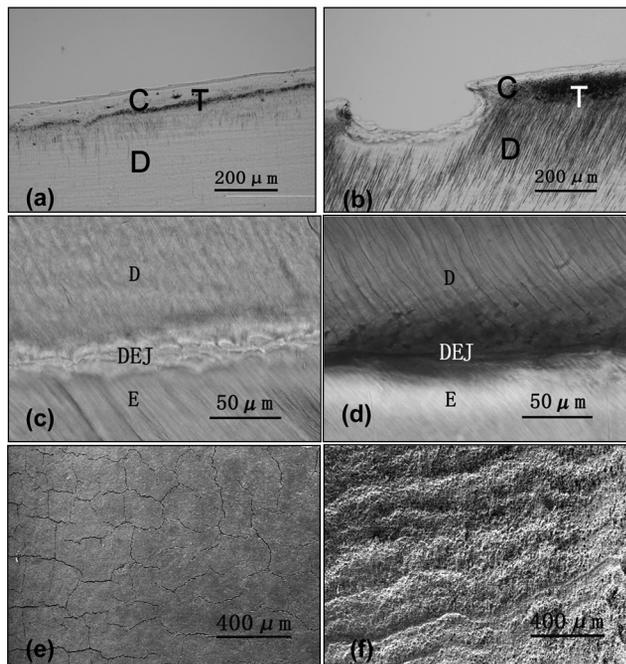**From Mumm *et al.* (2002).

Figure 2. Microstructural changes in the teeth from hypophosphatasia patient compared with those from unaffected children. Ground sections of the teeth at the middle third region of roots (100X) from **(a)** unaffected children and **(b)** hypophosphatasia patients revealed that there are resorbed lacunae at the root surfaces of the patients, and Tomes' layer was abnormally thick with no apparent cementum structure. Ground sections of the teeth at the labial middle region of crowns (400X) from **(c)** unaffected children and from **(d)** hypophosphatasia patients showed that the terminus of dentinal tubules close to the DEJ in the patients displays a dark granular unmineralized region. SEM images of the root surfaces (30X) of teeth from **(e)** unaffected children and from **(f)** hypophosphatasia patients revealed that the root surface of the unaffected control is smooth and homogenous, while the surface structure of the patient was partially missing. Abbreviations: T, Tomes' layer; D, dentin; C, cementum; E, enamel; DEJ, dentino-enamel junction.
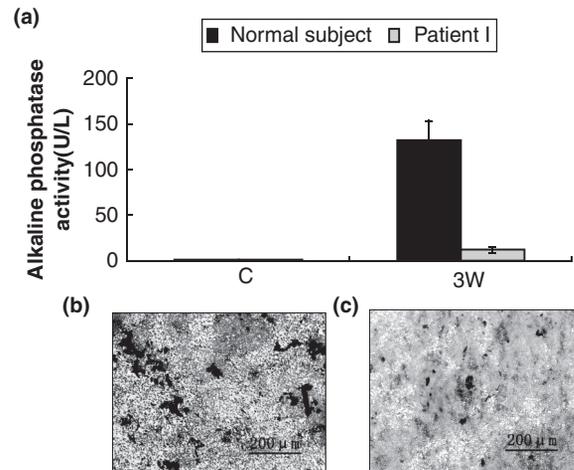

Figure 3. TNSALP activity and mineralization ability of dental pulp cells from a hypophosphatasia patient. **(a)** TNSALP activity of dental pulp cells from patient I was comparable with that of unaffected children when cells reached confluence, but was significantly lower than that of unaffected children at 3 wks after confluence. N = 3. Von Kossa staining of dental pulp cells from **(b)** unaffected children and from **(c)** hypophosphatasia patient I showed that nodules formed in the patient were fewer and smaller than those of the unaffected children. Abbreviations: C, complete confluence; 3W, 3 wks after confluence.

after confluence (Liu *et al.*, 2005). TNSALP activity peaks coinciding with the onset of mineralization (Suri *et al.*, 2008) and cleaves inorganic pyrophosphates (PPi) extracellularly, with release of inorganic phosphate (Pi), which is essential for hydroxyapatite crystallization (Whyte, 1994; Mornet *et al.*, 2001). Defects in TNSALP activity lead to increased levels of pyrophosphate and influence the mineralization process. Consistently in our study, the decrease of TNSALP activity in the dental pulp cells from the patient actually led to reduced mineralization as detected by von Kossa staining. Our results provided evidence to support the hypothesis that mutations of *TNSALP*

may alter the mineralization ability of dental pulp cells and therefore contribute to dentin dysplasia in hypophosphatasia patients.

In conclusion, this study is the first to show that the dental pulp cells from hypophosphatasia patients had lower TNSALP activity along with reduced mineralization capacity, which may be associated with the dentin dysplasia of hypophosphatasia.

ACKNOWLEDGMENT

REFERENCES

Barese CN, Copelli SB, De-Matteo E, Zandomeni R, Salgueiro F, Di-Giovanni D*, et al*. (2005). Molecular characterization of a novel splice site mutation within the CYBB gene leading to X-linked chronic granulomatous disease. *Pediatr Blood Cancer* 44:420-422.

Beumer J 3rd, Trowbridge HO, Silverman S Jr, Eisenberg E (1973). Childhood hypophosphatasia and the premature loss of teeth. A clinical and laboratory study of seven cases. *Oral Surg Oral Med Oral Pathol* 35:631-640.

Bruckner RJ, Rickles NH, Porter DR (1962). Hypophosphatasia with premature shedding of teeth and aplasia of cementum. *Oral Surg Oral Med Oral Pathol* 15:1351-1369.

Brun-Heath I, Taillandier A, Serre JL, Mornet E (2005). Characterization of 11 novel mutations in the tissue non-specific alkaline phosphatase gene responsible for hypophosphatasia and genotype-phenotype correlations. *Mol Genet Metab* 84:273-277.

Brun-Heath I, Lia-Baldini AS, Maillard S, Taillandier A, Utsch B, Nunes ME*, et al*. (2007). Delayed transport of tissue-nonspecific alkaline phosphatase with missense mutations causing hypophosphatasia. *Eur J Med Genet* 50:367-378.

Couble ML, Farges JC, Bleicher F, Perrat-Mabillon B, Boudeulle M, Magloire H (2000). Odontoblast differentiation of human dental pulp cells in explant cultures. *Calcif Tissue Int* 66:129-138.

el-Labban NG, Lee KW, Rule D (1991). Permanent teeth in hypophosphatasia: light and electron microscopic study. *J Oral Pathol Med* 20:352-360.

Harmey D, Hessle L, Narisawa S, Johnson KA, Terkeltaub R, Millan JL (2004). Concerted regulation of inorganic pyrophosphate and osteopontin by akp2, enpp1, and ank: an integrated model of the pathogenesis of mineralization disorders. *Am J Pathol* 164:1199-1209.

Hu JC, Plaetke R, Mornet E, Zhang C, Sun X, Thomas HF*, et al*. (2000). Characterization of a family with dominant hypophosphatasia. *Eur J Oral Sci* 108:189-194.

Jedrychowski JR, Duperon D (1979). Childhood hypophosphatasia with oral manifestations. *J Oral Med* 34:18-22.

Kozlowski K, Sutcliffe J, Barylak A, Harrington G, Kemperdick H, Nolte K*, et al*. (1976). Hypophosphatasia. Review of 24 cases. *Pediatr Radiol* 5:103-117.

Le Du MH, Millán JL (2002). Structural evidence of functional divergence in human alkaline phosphatases. *J Biol Chem* 277:49808-49814.

Liu H, Li W, Shi S, Habelitz S, Gao C, DenBesten P (2005). MEPE is down-regulated as dental pulp stem cells differentiate. *Arch Oral Biol* 50:923-928.

Lundgren T, Westphal O, Bolme P, Modéer T, Noren JG (1991). Retrospective study of children with hypophosphatasia with reference to dental changes. *Scand J Dent Res* 99:357-364.

Michigami T, Uchihashi T, Suzuki A, Tachikawa K, Nakajima S, Ozono K (2005). Common mutations F310L and T1559del in the tissue-nonspecific alkaline phosphatase gene are related to distinct phenotypes in Japanese patients with hypophosphatasia. *Eur J Pediatr* 164:277-282.

Mornet E (2008). Hypophosphatasia. *Best Pract Res Clin Rheumatol* 22:113-127.

Mornet E, Stura E, Lia-Baldini A, Stigbrand T, Ménez A, Le Du MH (2001). Structural evidence for a functional role of human tissue nonspecific alkaline phosphatase in bone mineralization. *J Biol Chem* 276:31171-31178.

Mumm S, Jones J, Finnegan P, Henthorn P, Podgornik M, Whyte M (2002). Denaturing gradient gel electrophoresis analysis of the tissue nonspecific alkaline phosphatase isoenzyme gene in hypophosphatasia. *Mol Genet Metab* 75:143-153.

Okabe T, Sakamoto M, Takeuchi H, Matsushima K (2006). Effects of pH on mineralization ability of human dental pulp cells. *J Endod* 32:198-201.

Olsson A, Matsson L, Blomquist HK, Larsson A, Sjodin B (1996). Hypophosphatasia affecting the permanent dentition. *J Oral Pathol Med* 25:343-347.

Sinico M, Levaillant JM, Vergnaud A, Blondeau JR, Encha-Razavi F, Mornet E*, et al*. (2007). Specific osseous spurs in a lethal form of hypophosphatasia correlated with 3D prenatal ultrasonographic images. *Prenat Diagn* 27:222-227.

Suri L, Damoulis PD, Le T, Gagari E (2008). Expression of MMP-13 (collagenase-3) in long-term cultures of human dental pulp cells. *Arch Oral Biol* 53:791-799.

Taillandier A, Lia-Baldini AS, Mouchard M, Robin B, Muller F, Simon-Bouy B*, et al*. (2001). Twelve novel mutations in the tissue-nonspecific alkaline phosphatase gene (ALPL) in patients with various forms of hypophosphatasia. *Hum Mutat* 18:83-84.

Vetrini F, Tammaro R, Bondanza S, Surace EM, Auricchio A, De-Luca M*, et al*. (2006). Aberrant splicing in the ocular albinism type 1 gene (OA1/GPR143) is corrected in vitro by morpholino antisense oligonucleotides. *Hum Mutat* 27:420-426.

Watanabe H, Takinami H, Goseki-Sone M, Orimo H, Hamatani R, Ishikawa I (2005). Characterization of the mutant (A115V) tissue-nonspecific alkaline phosphatase gene from adult-type hypophosphatasia. *Biochem Biophys Res Commun* 327:124-129.

Whyte MP (1994). Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization. *Endocr Rev* 15:439-461.

Yokose S, Kadokura H, Tajima Y, Fujieda K, Katayama I, Matsuoka T*, et al*. (2000). Establishment and characterization of a culture system for enzymatically released rat dental pulp cells. *Calcif Tissue Int* 66:139-144.