よろしければ貴施設についてお答えください。

質問 12：病院全体の病床数は？　　（　　）床

質問 13：小児科の病床数は？　　（　　）床

質問 14：小児科医は何人ですか？

常勤　（　　）人

非常勤（　　）人

質問 15：小児科の入院患者は一年間で何人くらいですか？

（　　）人

質問 16：分娩数は？

（　　）人

その他ご意見があればご自由にお書きください（二次調査に期待すること等）

ご回答ありがとうございました。

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特許

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Ⅶ. 業績別刷

CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

Czech Dysplasia Occurring in a Japanese Family

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Czech dysplasia (OMIM 609162) is a recently established COL2A1 disorder characterized by normal height, early-onset osteoarthritis, platyspondyly, short metatarsals, and the absence of ophthalmological complications or cleft palate. A specific missense mutation (c.823C > T, R275C) in the exon 13 of the *COL2A1* gene, coding for the triple helical domain of the alpha 1 chain of the type II collagen, has been linked to Czech dysplasia, which is quite a unique situation among the COL2A1 disorders. Since all of the 11 families and patients reported to date were of European ancestry, an ancient single origin of the R275C mutation was speculated about. Here we report on a Japanese family consisting of three patients with Czech dysplasia, each member showing valgus knees in addition to remarkably uniform manifestation of the clinical and radiological abnormalities. Mutation analysis documented the *COL2A1* c.823C > T mutation in all affected individuals. In conclusion, this report provides novel evidence for the independent occurrence of Czech dysplasia among the populations.

Key words: COL2A1; Czech dysplasia; Japanese population; valgus knee

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Am J Med Genet Part A 149A:2285–2289.

INTRODUCTION

Czech dysplasia (OMIM 609162) is an autosomal dominant skeletal dysplasia characterized by normal height, early-onset, progressive pseudorheumatoid arthritis, platyspondyly, and short third and fourth toes [Kozlowski et al., 2004; Marik et al., 2004]. Recently, a specific missense mutation (c.823C > T, R275C) in the exon 13 of the *COL2A1* gene, coding for the triple helical domain of the alpha 1 chain of the type II collagen, was identified in six unrelated patients with Czech dysplasia [Hoornaert et al., 2007; Tzschach et al., 2008]. The identical mutation had been reported before in patients from five other families whose condition was, retrospectively, compatible with Czech dysplasia [Williams et al., 1993; Reginato et al., 1994; Bleasel et al., 1995, 1996; Lopponen et al., 2004; Hoornaert et al., 2006]. R275C corresponds to R75C in the older publications where the numbering of the amino acid residues started at the first glycine of the triple helical domain of the alpha 1 chain of the type II collagen. The new codon numbering of COL2A1 starts at the first methionine (start codon for translation) [Hoornaert et al., 2007].

Since all families and patients reported to date were of European ancestry and were not shown to be de novo, an ancient single origin of the R275C mutation was speculated about. Here, we report on a Japanese family in which Czech dysplasia was identified in three members, and the detection of a R275C mutation.

CLINICAL REPORTS

The pedigree of this Japanese family is shown in Figure 1, and the clinical details of the affected individuals are listed in Table I. The proband (III-1) was referred to our hospital at the age of 3 years due to valgus alignment of the bilateral lower extremities. She had hearing problems from infancy, and physical examination revealed "broad" as well as valgus knees, and mildly short fingers and toes. The height was normal. Facial appearance included depressed nasal bridge. X-ray examination at the age of 7 years showed mild platyspondyly with irregular endplates, and mildly short metacarpals and metatarsals (Fig. 2). The younger brother (III-2) showed

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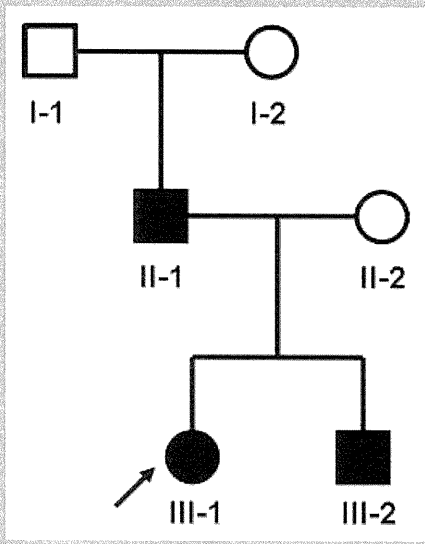


FIG. 1. Pedigree of the Japanese family. Closed symbols indicate patients with Czech dysplasia. Proband (III-1) is identified with an arrow.

remarkably similar clinical and X-ray manifestations at the age of 5 years, including "broad" as well as valgus knees, mildly short fingers and toes, normal height, depressed nasal bridge, mild platyspondyly with irregular endplates, and mildly short metacarpals and metatarsals (Fig. 3). The father (II-1) had been suffering from hip pain since adolescence, and underwent the right hip replacement at the age of 35 years. On examination at the age of 37 years, the height was relatively tall for standard Japanese, the alignment of the bilateral lower extremities was valgus, and bilateral shortness of the fingers and toes was noticed. He had stiffness on his neck and shoulders, suffered from pain in the low back, the left hip, and the knees after mild exercise, and used analgesics regularly. X-ray examination showed mild platyspondyly with irregular endplates, osteoarthritic changes and synovial osteochondromatosis in the knees, and short metacarpals and metatarsals (Fig. 4). Hearing problems had been noticed from adolescence. Audiological investigations revealed sensorineural hearing loss particularly of higher and lower frequencies in the father, and that particularly of lower frequencies in the proband and in the younger brother.

MATERIALS AND METHODS

Although literature lack Czech dysplasia of non-European ancestry, the above described phenotype of the Japanese family seemed compatible with Czech dysplasia, which prompted us to analyze the R275C mutation of COL2A1. DNA of the family members (II-1, II-2, III-1, and III-2 in Fig. 1) was extracted from peripheral blood after informed consent. Targeted sequencing of exon 13 of the *COL2A1* gene was performed by PCR amplification of the area of interest using a set of primers set within the franking introns [Hoornaert et al., 2007]. After purification, the PCR products were directly sequenced using the PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and one of each primer set used for the PCR amplification. The sequencing data were compared with the wild-type *COL2A1* sequence (GenBank accession number NM_001844). For cDNA numbering, +1 corresponds to the A of the ATG translation initiation codon.

RESULTS

Targeted sequencing of exon 13 of the *COL2A1* gene revealed a heterozygous C to T transition at nucleotide 823 (c.823C > T) which predicts an arginine to cysteine missense mutation at codon 275 (R275C) in the proband, the younger brother, and the father

TABLE I. Clinical and Radiographic Features of the Patients With Czech Dysplasia

Manifestations	II-1	III-1	III-2	Literature (n = 11)[a]
Age	37	7	5	
Origin	Japan	Japan	Japan	Europe
Normal height	+	+	+	11/11
Exact height	180 cm	121 cm	115 cm	
Sensorineural hearing loss	+	+	+	3/11
Joint pain in childhood	+	+	+	11/11
Limited joint mobility	+	−	−	11/11
Hip replacement	+	−	−	8/11
Short toes	+	+	+	11/11
Radiographic features				
Platyspondyly	+	+	+	11/11
Irregular vertebral endplates	+	+	+	11/11
Osteoarthritis	+	−	−	11/11
Osteochondromatosis	+	−	−	8/11
Short metacarpals	+	+	+	3/11
Short metatarsals	+	+	+	11/11

[a]Williams et al. [1993], Bleasel et al. [1995, 1996], Lopponen et al. [2004], Hoornaert et al. [2006, 2007], and Tzschach et al. [2008].

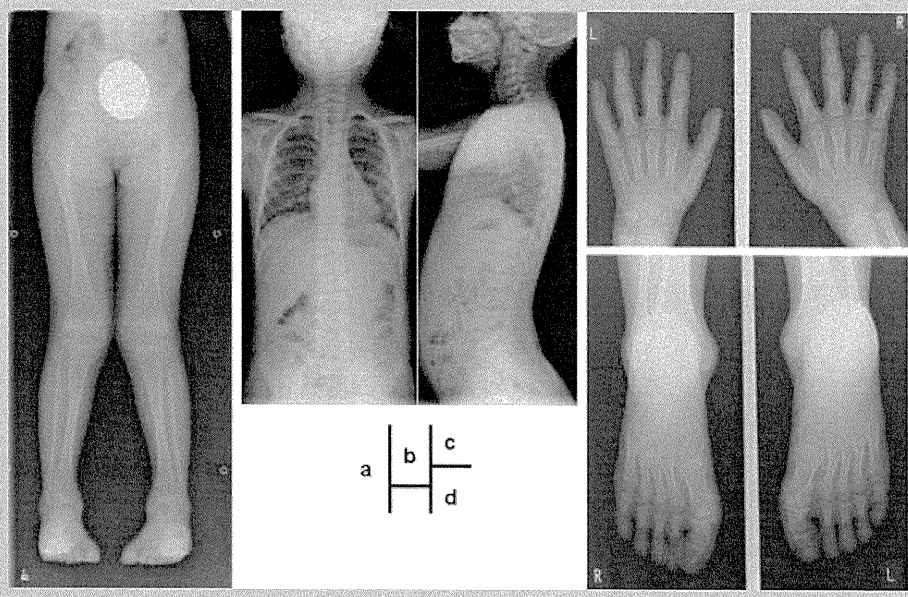


FIG. 2. Radiographs of the proband (III-1 in Fig. 1) at the age of 7 years. a: Lower extremities. Note the marked valgus alignment. b: Spine. Note mild platyspondyly with irregular endplates. c: Hands. Metacarpals are mildly short. d: Feet. Metatarsals are mildly short.

(III-1, III-2, and II-1 in Fig. 1, respectively) (Fig. 5). The healthy mother (II-2 in Fig. 1) did not carry this mutation.

DISCUSSION

The Japanese family reported here, including three patients, is the first one reported out of European ancestry. In addition, the family history clearly indicated a de novo occurrence of the disease (Fig. 1). Together, this report provides novel evidence for the independent presence of Czech dysplasia among the populations.

Previously reported patients with Czech dysplasia, all from European ancestry, showed remarkably similar phenotypic expression [Williams et al., 1993; Bleasel et al., 1995, 1996; Lopponen et al., 2004; Hoornaert et al., 2006, 2007; Tzschach et al., 2008]. The main clinical features were normal height, joint pain in childhood, limited joint mobility, hip replacement in young adult, and short

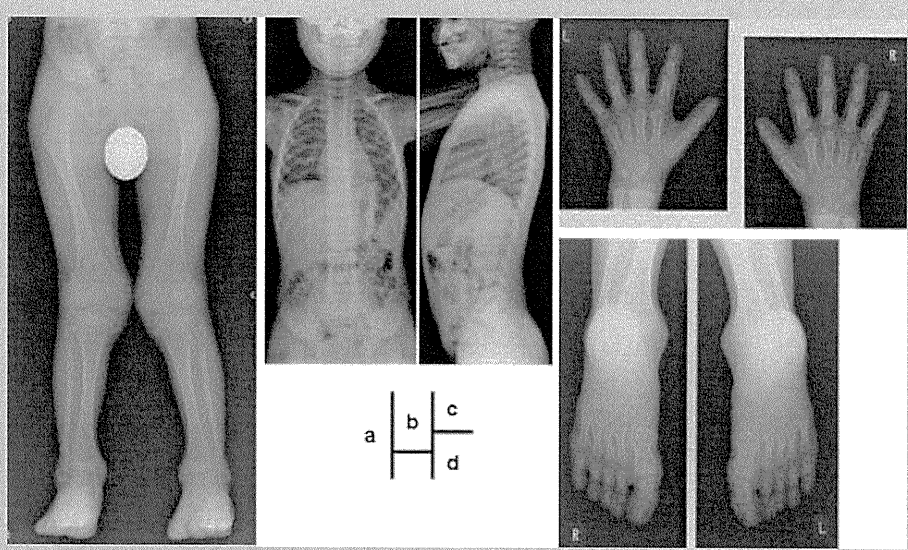


FIG. 3. Radiographs of the younger brother (III-2 in Fig. 1) at the age of 5 years. a: Lower extremities. Note the marked valgus alignment. b: Spine. Note mild platyspondyly with irregular endplates. c: Hands. Mildly short metacarpals are recognized. d: Feet. Mildly short metatarsals are present.

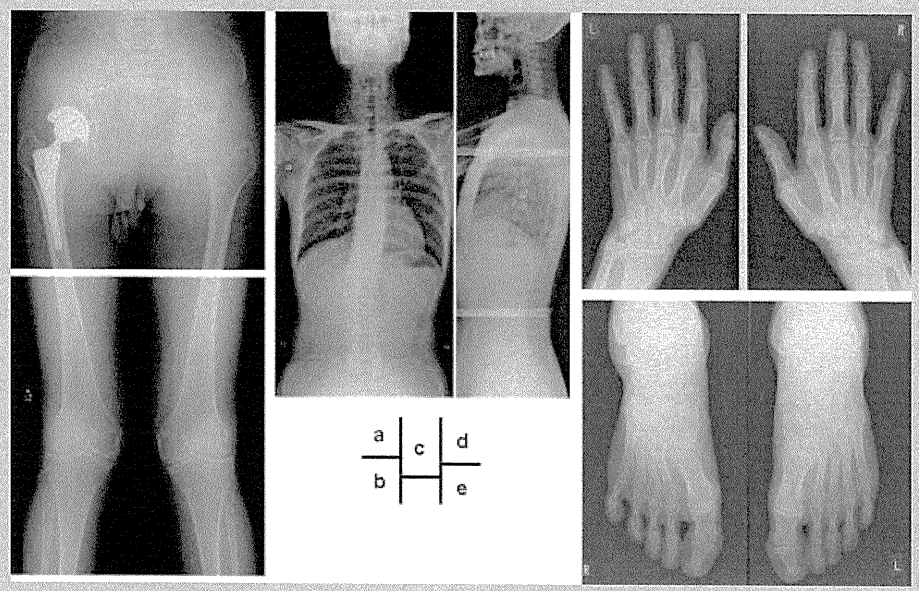
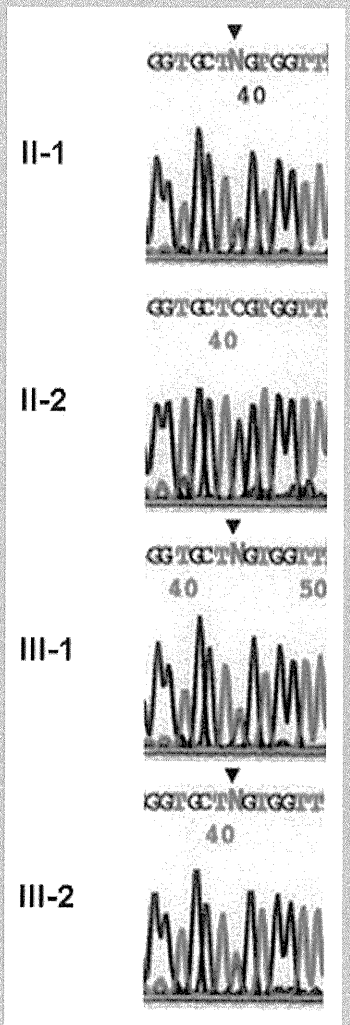


FIG. 4. Radiographs of the father (II-1 in Fig. 1) at the age of 37 years. a: Hips. The right hip has been replaced and the left hip shows advanced osteoarthritic changes. b: Knees. Apparently valgus alignment, osteoarthritic changes as well as osteochondromatosis are present. c: Spine. Note mild platyspondyly with irregular endplates. d: Hands. Metacarpals are mildly short. e: Feet. Metatarsals are mildly short.



toes. Three Japanese patients reported here also showed these manifestations (Table I). Hearing problems, emphasized by Tzschach et al. [2008], were also recognized in the three Japanese patients. Radiographically, previously reported features included platyspondyly, irregular vertebral endplates, osteoarthritis, osteochondromatosis, as well as short metacarpals and metatarsals, which were common to the three Japanese patients. The fact, that Czech dysplasia is the only *COL2A1* disorder that seems to be associated with a specific missense mutation (c.823C > T, R275C), may explain the unusual phenotypic similarity among the populations.

Valgus knee, which was a common feature in the three Japanese patients with Czech dysplasia, was not announced in the previous reports. At this moment, we have no grounds to determine whether this manifestation is a part of Czech dysplasia or not. Malalignment of the lower limbs is known risk factor accelerating the development of osteoarthritis [Sharma, 2001]. If marked valgus alignment of the lower limbs is a part of Czech dysplasia, the precocious joint replacement due to osteoarthritis could be postponed by early diagnosis of the disease and surgical correction in adolescence.

FIG. 5. Targeted sequencing of the PCR product from the family members (II-1, II-2, III-1, and III-2 in Fig. 1). Part of the sequence from exon 13 of the *COL2A1* gene is shown. Arrowheads indicate the heterozygous C to T transition at nucleotide 823 (c.823C > T) which predicts an arginine to cysteine missense mutation at codon 275 (R275C), in the proband (III-1), as well as in the younger brother (III-2) and the father (II-1). Unaffected mother (II-2) did not carry this mutation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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Original Article

Clinical Characteristics of Perinatal Lethal Hypophosphatasia: A Report of 6 Cases

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Abstract. Hypophosphatasia is a rare inherited disorder caused by deficient tissue-nonspecific alkaline phosphatase activity. It is classified into 6 subtypes, and the perinatal lethal form of hypophosphatasia is the most severe. Patients with this form suffer from various symptoms, including respiratory failure, premature craniosynostosis, rachitic changes in the metaphyses, convulsions and hypercalcemia. This report presents 6 cases of the perinatal lethal form of hypophosphatasia. All of the patients showed shortening of the long bones in *utero* in ultrasonographic examinations. Two of the six patients died at birth because they could not establish spontaneous breathing. Three of the remaining four patients also died before 1 yr of age. The major cause of death was respiratory failure due to hypoplastic lung. All of the patients, except for the two who died at birth, experienced convulsions in their clinical courses. Vitamin B6 therapy effectively reduced the frequency and severity of convulsions. However, it could not always make the patients convulsion free. Three patients underwent a genetic analysis. The 1559delT mutation, which abolishes Alkaline Phosphatase (ALP) activity, was a hot spot. A homozygous 1559delT mutation was observed in two patients. However, they differed in severity of symptoms. Although a good genotype-phenotype correlation has been reported in hypophosphatasia, the genotype alone does not always predict the life span of the patients. These cases therefore suggested the importance of genetic counseling.

Key words: Hypophosphatasia, perinatal lethal form, alkaline phosphatase, ALP, *ALPL*

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Introduction

Hypophosphatasia is caused by deficient tissue-nonspecific alkaline phosphatase (TNSALP) activity, thus resulting in hypomineralization of

bone. The prevalence of severe forms is estimated at 1/100,000 (1). It has been classified into five subtypes based on the age of onset and the severity as follows: perinatal, infantile, childhood, adult and odontohypophosphatasia. Recently, the perinatal type was divided into lethal and benign types (2). Patients affected with the perinatal lethal form of hypophosphatasia tend to die around the time of birth due to impaired development of the lung and severe hypomineralization of their bones. On the other hand, patients with the perinatal benign form have a spontaneous improvement of skeletal defects despite the prenatal symptoms (3, 4).

Patients with the perinatal form of hypophosphatasia suffer from various symptoms, including respiratory failure associated with a narrow chest, premature craniosynostosis, rachitic changes in the metaphyses, uncontrollable convulsions and hypercalcemia. X-ray examination reveals shortening of the long bones, osteochondral spurs protruding from the forearms or legs and rachitic deformities of the chest (5). Laboratory examination shows markedly reduced serum alkaline phosphatase (ALP) activity as a characteristic finding. Furthermore, an increased urine phosphoethanolamine (PEA) levels are also a supportive finding.

Hypophosphatasia is caused by mutations in the *ALPL* gene, which encodes the TNSALP. More than 100 mutations have been reported in *ALPL* (1). Although some patients with the mild phenotype present autosomal dominant inheritance, the severe type of hypophosphatasia, including the perinatal lethal form, presents autosomal recessive inheritance (2).

This report presents 6 cases with the perinatal lethal form of hypophosphatasia.

Patient Reports

The characteristics of the 6 patients with the perinatal lethal form of hypophosphatasia are summarized in Table 1. None of the patients had an obvious family history suggesting hypophosphatasia, except for mildly low levels of serum ALP activity. After obtaining written informed consent during genetic counseling, a genomic analysis was performed in 3 patients. Genetic analysis was not performed in patient 1, 2 and 5 because we could not obtain approval for genetic analysis from their parents.

Shortening of the long bones and hydramnion was detected in Patient 1 in *utero* at 28 wk of gestation in an ultrasonographic examination. Aspiration of the amniotic fluid was performed at 36 wk of gestation. He was delivered by natural childbirth method at 37 wk of gestation. He presented bowing of the limbs, a narrow chest and hydrocele testis at birth. He could not establish spontaneous breathing and died within several hours. His serum ALP was 2 IU/l (normal range: 419–1,110). Severe hypomineralization of his bones was detected by X-ray and CT examination at birth.

Patient 2 manifested limb shortening, an enlarged biparietal diameter and hydramnion in *utero* at 29 wk of gestation in an ultrasonographic examination. She was born at 38 wk of gestation by Cesarean section. She presented bowing of the limbs and a narrow chest at birth. Her serum ALP was 7 IU/l. Her mother's ALP was 175 IU/l. She could not establish spontaneous breathing and died within several hours.

Patient 3 had shortening of the long bones, which was detected in *utero* at 19 wk of gestation in an ultrasonographic examination. She was born at 38 wk of gestation by natural childbirth. She presented with bowed limbs, a narrow chest, an enlarged anterior fontanelle and a short right fourth finger at birth. Skeletal radiographs showed a narrow chest, undermineralized bones and fraying metaphyses. Her serum ALP was 10 IU/l on day 0. Her urine PEA was 10,174 nmol/mg creatinine (normal range: 80–220 nmol/mg creatinine). Artificial respiration was needed from birth to treat respiratory failure. She presented with sunset phenomenon and generalized seizures on day 1 and was treated with phenobarbital (PB). Vitamin B6 therapy

Table 1 Summary of the patients

	Patients					
	1	2	3	4	5	6
Sex	Male	Female	Female	Male	Male	Female
Gestational age	37 wk 0 d	38 wk 3 d	38 wk 6 d	38 wk 5 d	40 wk 5 d	38 wk 4 d
BW at birth	2,560 g	3,060 g	2,808 g	2,384 g	2,384 g	2,434 g
	−1 SD	0 SD	−0.5 SD	−1.8 SD	−1.8 SD	−1.5 SD
BH at birth	48 cm	ND	43 cm	43 cm	45.5 cm	44.5 cm
	−0.47 SD		−2.6 SD	−2.9 SD	−1.7 SD	−1.9 SD
Apgar Score	3/1	1/1	5/5	9/10	6/8	9/9
ALP (IU/L)	2	7	10	27	3	34
Age at diagnosis	Day 0	Day 0	Day 0	Day 100	Day 0	Day 0
Urine PEA	ND	ND	10,175	7,760	10,510	8,975
(Normal range: 80–220 nmol/mg creatinine)						
Symptoms at birth						
Short limbs	+	+	+	+	+	+
Narrow chest	+	+	+	−	+	−
Other findings	Hydrocele testis		Hypoplastic fourth finger			
Hypercalcemia	ND	ND	+	+	+	+
Serum calcium level			5.7	5.2	12.7	6.2
(Normal range)			(4.3–5.2)	(4.3–5.1)	(8.6–10.4)	(4.3–5.2)
Convulsion type			Tonic seizure Setting sun sign	Setting sun sign	−	Absence attack
Onset of convulsion			Day 1	Day 25	Day 11	8 mo
Anticonvulsant drug			PB, DZP	PB,VPA	PB, DZP	−
Beginning days of vitamin B6			Day 13		Day 19	Day 21
Effectiveness of vitamin B6			Partially effective		Partially effective	Not evaluable
Prognosis	Day 0	Day 0	Day 117	Day 131	Day 383	Day 826
ALPL			G456D 1559delT	1559delT 1559delT	ND	1559delT 1559delT

PB: Phenobarbital. VPA: valproic acid. DZP: diazepam.

(20 mg/kg/d) was started at 13 d of age. The vitamin B6 therapy was effective in reducing the frequency and severity of convulsion. She underwent a tracheotomy at 62 d of age. Despite the intensive treatment, she developed a pulmonary hemorrhage on day 102 and died from respiratory failure at 117 d of age. Sequence analyses revealed that the patient had compound heterozygous mutations, a single base pair substitution, G to A, at nucleotide 1418, 1418G>A (G456D) and a single base pair deletion at nucleotide 1559, 1559delT, in *ALPL*. And, her parents were heterozygous carrier.

Patient 4 was found to have short limbs in *utero* in an ultrasonographic examination. He was born at 38 wk of gestation. He presented with shortening of the limbs and was suspected to have osteogenesis imperfecta (OI). His first

convulsion was observed on day 25. Artificial respiration was started at that time. Although multiple antiepileptic drugs, including valproic acid (VPA), PB, lidocaine and clonazepam were administered, they could not sufficiently suppress the convulsions. A low level of serum ALP (27 IU/l) was noted on day 100. Unfortunately, this was the first sampling of the serum ALP level. This data led to diagnosis of hypophosphatasia. His urine PEA level (7,760 nmol/mg creatinine) was elevated, and he and his parents were subjected to genetic testing. A sequence analysis revealed that the patient had homozygous 1559delT mutations in *ALPL*, and his parents were heterozygous carrier. In spite of the treatment, he died from respiratory failure at 131 d.

Patient 5 had short-limbs, which were detected in *utero* at 28 wk of gestation in an ultrasonographic examination. He was born at 40 wk of gestation by Cesarean section. He could not establish spontaneous breathing and was resuscitated by intubation. He presented a narrow chest and bowing of the limbs at birth. His serum ALP was 3 IU/l day 0, and his urine PEA level (10,510 nmol/mg creatinine) was elevated. The serum ALP level of the mother was 53 IU/l. His first convulsion was observed on day 11. Although multiple antiepileptic drugs, including VPA, PB and diazepam, were administered, none could sufficiently suppress his convulsions. Thereafter, vitamin B6 therapy was started on day 19. The vitamin B6 therapy was able to effectively reduce the frequency and severity of convulsion. He underwent a tracheotomy at 62 d of age. He died from respiratory failure at 383 d of age.

Patient 6 had short limbs and intra-uterine growth retardation, which were detected in *utero* at 32 wk gestation in an ultrasonographic examination. She was born at 38 wk of gestation by Cesarean section. Bowing of the long bones and rachitic changes in the metaphysis were detected by X-ray examination on day 1. She presented with short-limb syndrome and an enlarged anterior frontalle at birth. She had low levels of serum ALP (34 IU/l) and an elevated level of urine PEA (8,975 nmol/mg creatinine). Vitamin B6 therapy was started on day 21 to prevent convulsions. On day 87, she fractured a right rib. Subsequently, her respiratory distress progressed, and artificial ventilation was started on day 121. She received a tracheotomy on day 131. Her first convulsion was observed on day 144. The dose of vitamin B6 was increased from 10 mg/kg/d to 20 mg/kg/d. Thereafter, her convulsions were controlled. At 4 mo of age, she developed an enlarged liver and exhibited a deterioration of hepatic function. A prolonged prothrombin time and low level in the hepaplastin test were observed at 6 mo of age. Very low levels of clotting factor activity in V, VII, IX, XI and XII were observed. Vitamin K did not improve the low clotting activity. The laboratory findings for the levels of cholinesterase, fibrinogen, serum albumin and total bilirubin did not reflect severe cirrhosis of the liver, except for a relatively low platelet count (around 100,000 /μl). Therefore, the cause of the low clotting activity was unclear. In spite of these laboratory data, her general conditions gradually became stable at around 1 yr of age. She was able to eat food and hold toys with her hands at 2 yr of age. Digitate impressions were observed by cranial X-ray examinations at 2 yr old. A head CT scan revealed that she had a narrow cerebral ventricle and a brain fissure. Bulging of the anterior fontanelle was also observed. These findings suggested that the patient had developed craniostenosis. On day 822, she began to vomit and had a poor appetite without fever. She subsequently experienced intractable convulsions. A CT image taken after this convulsion episode did not show any remarkable changes from the previous study. In addition, an electroencephalogram study showed hypsarrhythmia. Therefore, it was not clear that the convulsions originated from either an elevation of the intracranial pressure or complications due to the primary disease. She died on day 826 due to respiratory failure

associated with convulsions and a lung hemorrhage. A necropsy was not performed because consent could not be obtained from her parents. Sequence analyses revealed that the patient had homozygous deletions of 1559delT in *ALPL*. And, her parents were heterozygous carrier.

Discussion

This manuscript summarized 6 cases presenting with the perinatal lethal form of hypophosphatasia. All of the patients demonstrated findings such as shortening of the long bones, and 2 patients had hydramnion in *utero* according to ultrasonographic examinations. Zanki *et al.* noted that osseous spurs, a patchy ossification pattern, undermineralization of the thoracic spine and cupped metaphyses are specific ultrasonographic features of this condition (6). Patient 5 was suspected to have OI at birth. Unfortunately, the serum ALP level was not measured in this patient until day 100. Therefore, the diagnosis of hypophosphatasia was late. OI, rickets, achondrogenesis and hypochondrogenesis are thought to be among the major differential diagnoses of hypophosphatasia (2, 6). The lower levels of serum ALP are a helpful indicator to identify hypophosphatasia. Therefore, measurement of serum ALP activity is very important when ultrasonography reveals shortening of the long bones in *utero*.

The characteristic symptoms of the perinatal lethal form are respiratory failure, hypercalcemia and uncontrollable convulsion. Two of the six patients died at birth because they could not establish spontaneous breathing. The other 4 patients also needed artificial ventilation during their clinical courses. In spite of intensive care, including artificial ventilation, 3 of the remaining 4 patients died before one year of age. The major cause of death was respiratory failure. Hypercalcemia was observed in all of the patients except for the patients that died at birth. The use of low-calcium milk was effective in improving hypercalcemia. No other treatments of hypercalcemia, e.g., calcitonin and diuretic drugs, were needed to control hypercalcemia in the current study.

Three of the four patients, patients 3, 4 and 5, demonstrated refractory convulsions in their clinical courses. Such symptoms are thought to be a severe and frequent complication in patients with the perinatal lethal form of hypophosphatasia. Vitamin B6 therapy was selected for patients 3, 5 and 6. In order to control the convulsions, patients 3 and 5 were administered vitamin B6 in addition to other anticonvulsants. The vitamin B6 therapy effectively reduced the frequency and severity of their convulsions, but it could not always achieve complete resolution of the convulsions. Vitamin B6 was also administered to patient 6 to prevent convulsions. This patient experienced her first convulsions at day 144. The convulsions were suppressed by an increased dose of vitamin B6. Her first convulsions occurred later than those of the other 3 patients. However, considering the comparatively mild clinical course of this patient, it was not possible to verify whether the vitamin B6 therapy effectively prevented convulsions. Litmanovitz *et al.* reported a case of the perinatal lethal form that was effectively treated with vitamin B6 (7). The convulsions of the patient were immediately suppressed, and the vitamin B6 therapy resulted in her electroencephalogram to normal. The mechanisms of vitamin B6 action have not yet been fully elucidated; however, TNSALP is known to play a role in vitamin B6 metabolism (7, 8). Vitamin B6 therapy should therefore be considered one of the alternative treatments against uncontrollable convulsions associated with hypophosphatasia.

Craniostenosis is thought to be the one of major complications, and it was observed in one patient. Kozlowski *et al.* conducted a radiographic analysis of 24 cases of hypophosphatasia and suggested that decreased growth of the skull, a tense fontanelle and a palpable sutural ridge

should be regarded as signs of craniostenosis, whatever the radiographic findings (9). They suggested a craniectomy before craniostenosis. Although the sudden exacerbation of the general condition of patient 6 was not suspected to be related to the uncontrollable elevated intracranial pressure based on a CT study, she may have benefitted from an early craniectomy.

We performed a genomic analysis for three patients, and all three of them had biallelic mutations in *ALPL*. Two of the patients had homozygous mutations of 1559delT, and one patient had compound heterozygous mutations of G456D/1559delT. These results suggest that the 1559delT mutation is a hot spot among Japanese patients with the perinatal lethal form of hypophosphatasia. Michigami *et al.* reported that the F310L and 1559delT mutations are hot spots in hypophosphatasia in Japan (1). In that study, three patients were identified with homozygous 1559delT mutations, and all three died shortly after birth. Their ALP enzymatic activity *in vitro* proved that the 1559delT mutation completely eliminated the activity of this enzyme (1). Previous reports have indicated a good correlation between the severity and *in vitro* enzymatic activity of the mutant protein (2). However, in the current study, homozygous 1559delT mutations were also identified in patient 6, who presented with a relatively mild phenotype of the perinatal lethal form of hypophosphatasia. The clinical course of patient 6 indicated that the prognosis could not always be predicted based on the results of a genetic analysis alone. In addition, similar phenomena have also been reported in previous studies. Ozono *et al.* reported sibling cases carrying the same *ALPL* compound heterozygous mutations, but who demonstrated different clinical courses from each other (10). Brun-Heath *et al.* reported the 1133A>T mutation in *ALPL* to be associated with an increase in the level of *ALPL* mRNA (11). Based on these data, an mRNA analysis, e.g., quantification of mRNA, should therefore be considered when the nature of a mutation cannot be fully explained the phenotype. Furthermore, Fauvert *et al.* reported that a particular haplotype, which derived from a sequence variation of the *ALPL* gene, could play a role as an aggravating factor (12). These results suggest that an additional genetic modifier and/or an environmental factor may therefore influence the severity in patients. Therefore, genetic counseling for hypophosphatasia should be carefully carried out.

This manuscript detailed 6 cases of the perinatal lethal form of hypophosphatasia. All of the cases exhibited shortening of the long bones and hydramnion in *utero* in ultrasonographic examinations. Measurement of serum ALP was a helpful indicator for diagnosis of hypophosphatasia. Although there is no curative treatment for hypophosphatasia, some treatments are useful for treating its symptoms. Recently, other challenging effective treatments, e.g., enzyme replacement therapy, transplantation therapy using bone fragments and cultured osteoblasts, have been reported (2, 13). Further study is needed to improve the prognosis and quality of life of patients with hypophosphatasia.

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Signaling of extracellular inorganic phosphate up-regulates cyclin D1 expression in proliferating chondrocytes via the Na^+/Pi cotransporter Pit-1 and Raf/MEK/ERK pathway

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ABSTRACT

As chondrocytes mature, the concentration of inorganic phosphate (Pi) increases in the extracellular milieu. It was demonstrated that the progressive accumulation of Pi started from the proliferative zone and peaked in the hypertrophic zone of growth plate. Although extracellular Pi is reported to be involved in the apoptosis and mineralization of mature chondrocytes, its role in proliferating chondrocytes remains unclear. Here we investigated this role utilizing ATDC5, an established cell model of chondrocytic differentiation. In proliferating ATDC5 cells, we found that the expression of cyclin D1 was up-regulated, and that of alkaline phosphatase (ALP) was down-regulated in response to an increase in extracellular Pi within 24 h. Moreover, an increase in extracellular Pi-induced activation of the Raf/MEK/ERK pathway, and treatment with a MEK inhibitor PD98059 abolished the effects on the expression of cyclin D1 and ALP, indicating that extracellular Pi regulates the expression of these genes through the Raf/MEK/ERK pathway. Consistent with its up-regulation of cyclin D1 expression, the extracellular Pi facilitated the proliferation of ATDC5 cells. Treatment with phosphonoformic acid (PFA), an inhibitor of sodium/phosphate (Na^+/Pi) cotransporters, abrogated the activation of the Raf/MEK/ERK pathway and gene expression induced by the increase in extracellular Pi. Knocking down of the type III Na^+/Pi cotransporter Pit-1 diminished the responsiveness of ATDC5 cells to the increase in extracellular Pi. Interestingly, the increased extracellular Pi induced the phosphorylation of fibroblast growth factor receptor substrate 2α (FRS2α), which was also cancelled by knocking down of the expression of Pit-1. In primary chondrocytes isolated from mouse rib cages as well, increased extracellular Pi induced the phosphorylation of ERK1/2 and alterations in the expression of cyclin D1 and ALP, both of which were abolished by treatment with PFA. These results suggest that signaling by extracellular Pi is mediated by Pit-1 and FRS2α, and leads to activation of the Raf/MEK/ERK pathway and increased expression of cyclin D1, which facilitates the proliferation of immature chondrocytes.

Introduction

In vertebrates, long bones form through endochondral bone formation, a multistep process involving the mesenchymal condensation of undifferentiated cells, the proliferation of chondrocytes, and differentiation into hypertrophic chondrocytes, followed by mineralization [1]. As chondrocytes mature, they change morphologically and exhibit alterations in the production of extracellular matrix proteins. Proliferating chondrocytes produce collagen types II and IX, while hypertrophic chondrocytes are characterized by a high level of alkaline phosphatase (ALP), diminished levels of collagen types II and IX, and the production of type X collagen, a hypertrophic chondrocyte-specific product. Evidence has suggested that terminally differentiated chondrocytes undergo programmed cell death in mammals. Various signaling molecules, including Indian hedgehog, parathyroid hormone-related protein (PTHrP) and fibroblast growth factors (FGFs), have been revealed to regulate the maturation of chondrocytes [2–8].

It is well established that inorganic phosphate (Pi) plays critical roles in the skeletal mineralization. The predominant mineral crystal phase present in bone extracellular matrix is hydroxyapatite, which contains calcium (Ca) and Pi ions and is deposited both within and

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