III. 研究成果の刊行に関する一覧表

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雑誌

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Takagi M, Kaneko-Schmitt S, Suzumori N, Nishimura G, Hasegawa T.	Atypical achondroplasia due to somatic mosaicism for the common thanatophoric dysplasia mutation R248C.	Am J Med Genet A.	158A	247-250	2011
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IV. 研究成果の刊行物・別冊

CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

Heterozygous C-Propeptide Mutations in *COL1A1*: Osteogenesis Imperfecta Type IIC and Dense Bone Variant

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Osteogenesis imperfecta type IIC (OI IIC) is a rare variant of lethal OI that has been considered to be an autosomal recessive trait. Twisted, slender long bones with dense metaphyseal margins and normal vertebral bodies in OI IIC contrast with crumpled, thick long bones and multiple vertebral compression fractures in OI IIA. Here, we report on two sporadic patients with classical OI IIC and a pair of siblings, with features of OI IIC but less distortion of the tubular bones (OI dense bone variant). One case with OI IIC and the sibs had novel heterozygous mutations in the C-propeptide region of *COL1A1*, while the second patient with clear-cut OI IIC had no mutation in this region. Histological examination in the two sporadic cases showed a network of broad, interconnected cartilaginous trabeculae with thin osseous seams in the metaphyses. These changes differed from the narrow and short metaphyseal trabeculae found in other lethal or severe cases of OI. Our experience sheds light on the genetics and etiology of OI IIC and on its phenotypic spectrum.

Key words: osteogenesis imperfecta; type IIC; dense bone; *COL1A1*; C-propeptide

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Am J Med Genet Part A 155:2269–2273.

INTRODUCTION

Osteogenesis imperfecta (OI; OMIM 166200, 166210, 259420, and 166220) comprises a heterogeneous group of connective tissue disorders characterized by fragile bones with susceptibility to fractures. Most cases of OI are caused by heterozygous mutations in *COL1A1* or *COL1A2*, the genes encoding the two type I procollagen alpha chains, proα1 (I) and proα2 (I). Each chain consists of three domains including a core triple helical domain composed of uninterrupted repeats of the Gly-Xaa-Yaa tripeptide, and N and C propeptide domains flanking the triple helical domain at both the amino- and carboxyl-terminal ends. Germ cell mosaicism and

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intrafamilial recurrence have been reported in *COL1A1* and *COL1A2* associated OI. The most common mutation pattern is a single nucleotide transition that substitutes a glycine residue in the triple helical domain. Although uncommon, mutations in the C-propeptide domain of *COL1A1* and *COL1A2* have also been observed [Chessler et al., 1993; Lamandé et al., 1995; Pace et al., 2001, 2002].

The Sillence clinical/radiological classification divides OI into a non-lethal type I with blue sclera, lethal types IIA, IIB, and IIC, progressively deforming type III, and non-lethal type IV with white sclera [Sillence et al., 1979]. Among the lethal forms, OI IIC is especially rare. It manifests with severe calvarial demineralization, discontinuous beading of the ribs, normal vertebral bodies, misshapen scapulae and ischia, and slender, twisted long bones with fractures [Sillence et al., 1984; Spranger, 1984; Thompson et al., 1987; van der Harten et al., 1988]. OI IIC is hallmarked by absence of vertebral compression fractures and slender long bones despite multiple fractures, which contrast with the compressed vertebral bodies and thick, crumpled long bones seen in other lethal or severe types of OI (OI IIA, IIB, and III). Chondroosseous histology is also distinctive in OI IIC exhibiting a network of relatively broad and irregularly arranged cartilaginous trabeculae with many interconnections and thin osseous seams in the metaphyseal spongiosa, which differs from the narrow and short metaphyseal trabeculae seen in other types [van der Harten et al., 1988]. It has been thought that OI IIC, unlike other forms, is inherited as an autosomal recessive trait [Sillence et al., 1984]. The etiology of OI IIC has not been elucidated.

Here, we report on two sporadic patients with OI IIC and a pair of siblings, who had features of OI IIC but less distortion of the tubular bones. One case with OI IIC and the sibs had novel heterozygous mutations in the C-propeptide region of *COL1A1*, while the second patient with clear-cut OI IIC had no mutation in this region. Our experience sheds light on the genetics and etiology of OI IIC and on its phenotypic spectrum.

PATIENT REPORTS

The four patients were unrelated and of Japanese origin. No consanguinity was reported in their parents. Family history was unremarkable in all families.

Patient 1 was the newborn female and was the first child of healthy parents. Prenatal ultrasonography at 26 weeks of gestation revealed short limbs, multiple fractures of the long bones, and a hypoplastic thorax. She was vaginally delivered at 36 weeks' gestation after breech presentation. She succumbed shortly after birth. Birth weight was 1,638 g (below 3rd centile), length 40.0 cm (below 3rd centile), and OFC 30.5 cm (10th centile). Physical findings included disproportionately short, bent limbs with relatively long fingers and toes, a hypoplastic thoracic cage, relative macrocephaly with caput membraneceum, and triangular face with hypertelorism, protruding eyes, and low set, malformed ears. Postmortem radiographs demonstrated severe calvarial demineralization and small, dense facial bones (Fig. 1A). The skull base was also dense. The clavicles and ribs showed discontinuous beading with multiple fractures. The diaphyses of the long bones appeared slender and twisted despite many fractures. Broad gap and sclerosis of the fracture surfaces resembled those of pseudoarthrosis. The metaphyses of the long bones were flared with dense transverse bands. The scapular, ischial, and iliac margins were irregular and sclerotic. Despite conspicuous deformities of the tubular bones and flat bones, the spine appeared unremarkable.

Patient 2-1 (older sister of Patient 2-2) was born to healthy parents who had one healthy child. Fetal ultrasound at 34 weeks' gestation showed bowing of lower limbs and a hypoplastic thorax. She was delivered by caesarian at 37 week's gestation. Birth weight

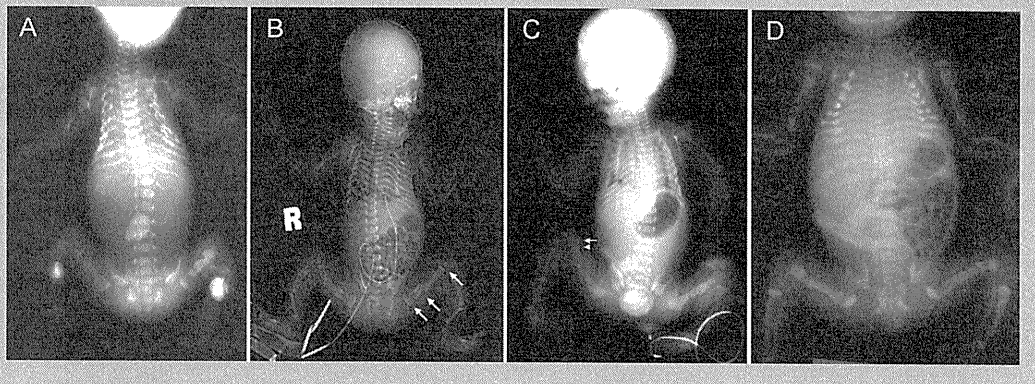

FIG. 1. Radiographs of Patients 1 (A), 2-1 (B), 2-2 (C), and 3 (D). A: There were severe calvarial demineralization with small, dense facial bones, discontinuous beading of the ribs, and twisted long bones with multiple fractures. The metaphyses of the long bones were flared with dense transverse bands. The scapular, ischial, and iliac margins appeared irregular and sclerotic. The height of vertebrae was normal. B: Dense sclerotic bands were found in the metaphyses and fracture sites of the long bones (arrows). No vertebral compression fracture was found. The manifestation of the long bones was milder than that seen in Patient 1. C: Note mild metaphyseal sclerosis (arrow) and adjacent radiolucency (arrowhead). D: Alternating bands of radiodensity and radiolucency were found in the long bones. Osteosclerosis was particularly conspicuous in Patient 3. Discontinuous beading of the ribs and twisted long bones were noted. [Color figure can be seen in the online version of this article, available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-4833]

was 2,523 g (3rd to 10th centile), length 38.5 cm (below 3rd centile), and OFC 33.4 cm (50th to 75th centile). She had dysmorphic facial features including micrognathia and a triangular face. Radiographs showed wormian bones of the skull, and slender long bones and ribs with multiple fractures (Fig. 1B). Dense transverse bands were found in the metaphyses and fracture surfaces of the long bones. No vertebral compression fracture was found. She had respiratory distress requiring a ventilation support, and died at age 4 months.

Patient 2-2 (younger brother of Patient 2-1) is the third child of the couple and currently 10 years old. Fetal ultrasound at 24 weeks' gestation showed deformity of lower limbs and ribs. He was delivered by caesarian at 37 weeks' gestation. Birth weight was 2,776 g (10th to 25th centile), length 47.0 cm (3rd to 10th centile), and OFC 36.0 cm (97th centile). He had micrognathia and a triangular face. Radiographs showed wormian bones of the skull and slender bones with multiple fractures in the long bones and ribs. Unlike his sister, he showed very mild metaphyseal sclerosis (Fig. 1C). The metadiaphyseal junction was radiolucent, and the diaphyses were relatively dense. He required mechanical ventilation followed by nasal continuous positive airway pressure for several months. After discharge from the hospital at age 18 months, he still necessitated respiratory support (oxygen and biphasic positive airway pressure). He had chronic lung disease and was susceptible to pulmonary infections. Pamidronate treatment was started at age 11 months. No new fracture was recorded for latest 3 years. Gross motor development was delayed and he never sat without support.

Patient 3 was the newborn female and delivered at 29 weeks' gestation. She died soon after birth. Polyhydroamnios was noted. Birth weight was 1,170 g (10th to 50th centile), length 36.0 cm (10th to 50th centile), and OFC 27.0 cm (50th to 90th centile). Physical findings included caput membranaceum, a small face with mild hypertelorism, white sclerae, and bent limbs. On radiographs, discontinuous beading of the ribs and irregular clavicles, scapulae, pubic and ischial bones were similar to those of Patient 1. The long bones were twisted and slender with incomplete fractures. The metaphyses were significantly dense (Fig. 1D). The diaphyses presented with alternating radiodense and radiolucent bands.

Histological Findings

The samples from Patients 1 and 3 were processed according to the standard procedure, and the paraffin-embedded sections were stained with hematoxylin and eosin. In Patients 1 (Fig. 2A) and 3 (Fig. 2B), chondroosseous junction of the proximal femur showed a network of relatively broad, interconnected cartilaginous trabeculae with thin osseous seams in the metaphyseal spongiosa. Thick, cartilaginous trabeculae (cartilaginous cores) were also found in the diaphyseal spongiosa (data not shown). Chondrocyte columnization appeared somewhat irregular.

MATERIALS AND METHODS

PCR-Based Mutation Screening

Approval for this study was obtained from the Institutional Review Board of Keio University School of Medicine. We obtained written informed consent for molecular studies from the parents.

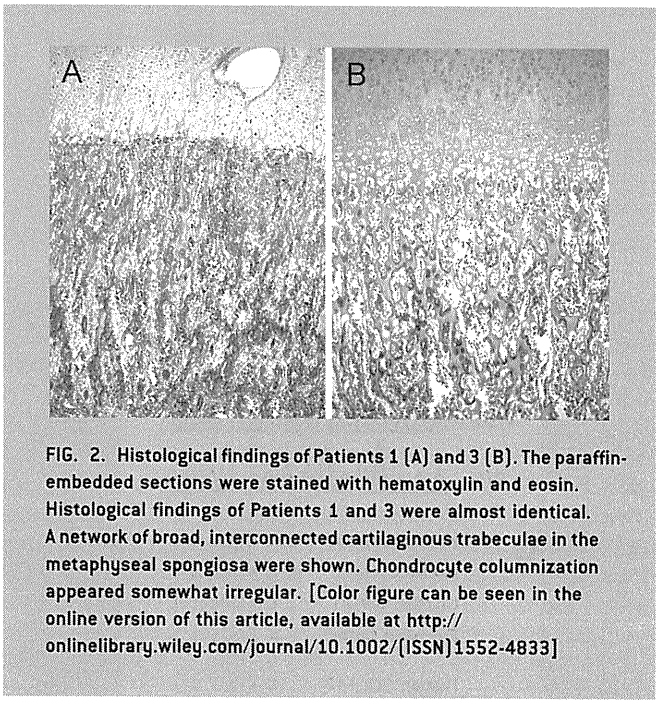

FIG. 2. Histological findings of Patients 1 (A) and 3 (B). The paraffin-embedded sections were stained with hematoxylin and eosin. Histological findings of Patients 1 and 3 were almost identical. A network of broad, interconnected cartilaginous trabeculae in the metaphyseal spongiosa were shown. Chondrocyte columnization appeared somewhat irregular. [Color figure can be seen in the online version of this article, available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-4833]

Genomic DNA was extracted from blood of the umbilical cord (Patient 1), Epstein–Barr virus-transformed lymphoblasts (Patient 2-1), and peripheral blood of Patient 2-2 together with the unaffected parents and older sister of the Patients 2-1 and 2-2 by a standard technique. In Patient 3, only formalin-fixed liver was available for the analysis, and thus we extracted genomic DNA by using TaKaRa DEXPAT (Takara Shuzo, Otsu, Japan) according to the manufacture's instructions. In Patients 1, 2-1, and 2-2, we analyzed all coding exons and flanking introns of *COL1A1*, *COL1A2*, *LEPRE1*, *CRTAP*, *PPIB* by PCR and direct sequencing. Deletion/duplication involving *COL1A1* and *COL1A2* were checked by multiplex ligation-dependent probe amplification (MLPA) analyses (SALSA MLPA KIT P271, P272; MRC-Holland, Amsterdam, The Netherlands). In Patient 3, PCR amplification was difficult; thus, we could examine only the C-propeptide region of *COL1A1*.

Subcloning

To demonstrate paternal somatic mosaicism for the unaffected father of the Patients 2-1 and 2-2, we subcloned PCR products of DNA from his peripheral blood and nail, took 100 colonies of each, screened the mutation, and calculated the ratio of mutant to wild-type alleles.

RESULTS

Molecular Analysis

The sequence analysis revealed novel heterozygous *COL1A1* mutations, c.4247delC (p.T1416RfsX10) in Patient 1 and c.4160C>T (p.A1387V) in Patients 2-1 and 2-2 (Fig. 3A,B). These mutations are located in the C-propeptide region of proα1(I). The A1387 is evolutionarily highly conserved in all vertebrate proα1(I) and in

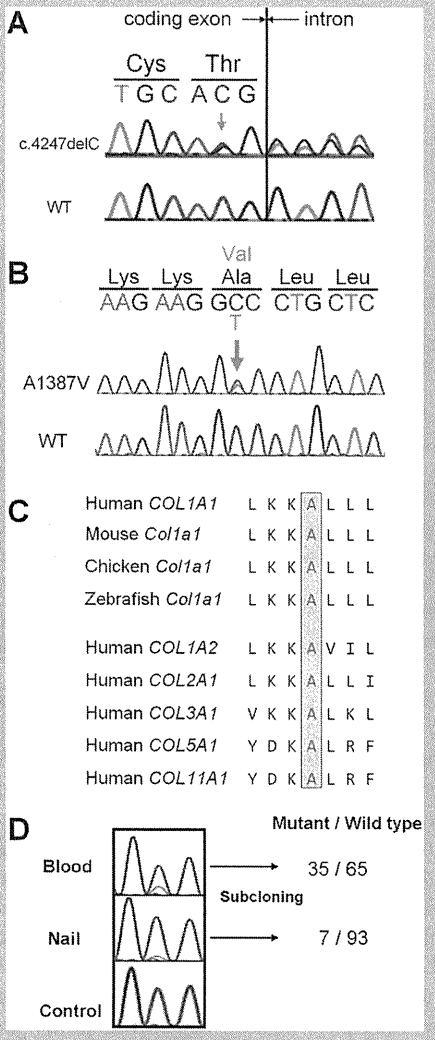

FIG. 3. Identification of mutations in the C-propeptide region of *COL1A1*. A,B: Partial sequences of PCR products of Patients 1, 2-1, and 2-2 are shown. Heterozygous single base pair deletion (c.4247delC) in Patient 1 and heterozygous missense mutation (c.4160C>T; p. A1387V) in Patients 2-1 and 2-2 are indicated by arrows. C: Alanine at codon 1387 is conserved among all human fibrillar procollagens and several other species. D: Subcloning of the PCR products from the father of Patients 2-1 and 2-2. The ratio of mutant to wild-type alleles was 35/65 in the peripheral blood and 7/93 in the nail, suggesting somatic mosaicism. [Color figure can be seen in the online version of this article, available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-4833]

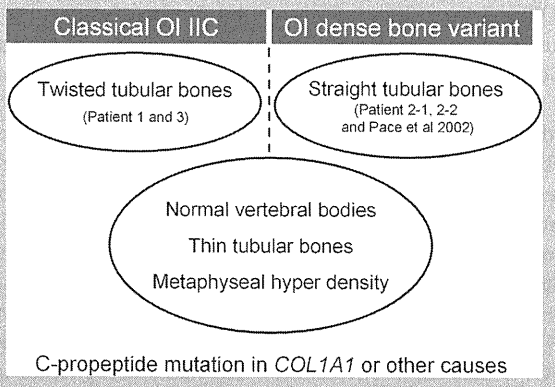

FIG. 4. Radiological variation and consistency of OI IIC spectrum. OI dense bone variant differs from classic OI IIC by the better preservation of the long bone shape (twisted or straight). Normal vertebral bodies, thin tubular bones and metaphyseal hyper density are consistent in OI IIC spectrum. [Color figure can be seen in the online version of this article, available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-4833]

all human fibrillar procollagens (Fig. 3C). T1416RfsX10 and A1387V were not found in 100 control individuals. In Patients 1, 2-1, and 2-2, no sequence variation was found in *COL1A2*, *LEPRE1*, *CRTAP*, and *PPIB*, and neither exon-level deletion nor duplication was detected by the MLPA analyses. Familial gene analysis of Patients 2-1 and 2-2 revealed that clinically unaffected father also had the A1387V. Mother and healthy older sister did not have the mutation. We did not found any sequence variation in the C-propeptide region of *COL1A1* in Patient 3.

Subcloning

The subcloning analysis revealed somatic mosaicism of A1387V in the father of the Patients 2-1 and 2-2. The ratio of mutant to wild-type alleles was 35/65 in the peripheral blood and 7/93 in the nail (Fig. 3D).

DISCUSSION

We found novel heterozygous mutations in the C-propeptide region of *COL1A1* in three of four cases. The heterozygosity of the mutations argues against an autosomal recessive trait that has been assumed in OI IIC. The sib case was caused by germinal mosaicism of the unaffected father. We could not find a *COL1A1* C-propeptide mutation in Patient 3 suggesting genetic heterogeneity of OI IIC. Unfortunately, poor quality of available DNA precluded analyses for other known OI genes.

Radiological variation and consistency of OI IIC spectrum are illustrated in Figure 4. Patients 1 and 3 fulfilled the previously reported radiological and histological criteria of OI IIC, as outlined above. Patient 2-1 exhibited radiological changes similar to those in the "OI dense bone variant" previously reported in a patient with a C-propeptide mutation in *COL1A1* [Pace et al., 2002]. This phenotype differs from classic OI IIC by the better preservation of the long bone shape. Skeletal changes were even less severe in the second sib with only discrete metaphyseal hyperdensity.

The dense bone segments and absence of vertebral compression fracture in OI IIC deserve comment. Mild metaphyseal hyperdensity is seen in many newborns with severe OI. However, they are more prominent in classic OI IIC and in the so-called "OI dense

bone variant" [Pace et al., 2002, our Patients 2-1 and 2-2]. Histologic sections showed a network of broad and irregularly arranged trabeculae with retained cartilage cores in the metaphyseal spongiosa. It contrasts with the narrow and short metaphyseal trabeculae in other lethal or severe cases of OI [van der Harten et al., 1988]. Hypothetically, the cartilaginous trabeculae may be resistant to compression forces but susceptible to bending forces explaining the long bone distortion and absence of vertebral compression fractures in OI IIC.

The pathogenesis of metaphyseal hyperdensity is more difficult to explain. Previous reports on C-propeptide mutations of *COL1A1* showed that trimer assembly was delayed, secretion was diminished, and a total amount of procollagen production was reduced [Chessler et al., 1993; Lamandé et al., 1995]. Pace et al. [2002] claimed that the dense bones in their reported case reflected both diminished amounts of secreted type I procollagen and the presence of overmodified, yet stable, molecules. C-propeptide modulation of TGF-beta and collagen synthesis of osteoblastic cells at the early stage of differentiation [Mizuno et al., 2000a,b] may also contribute to the extra bone formed in the metaphyses of patients with OI IIC (with or without twisted long bones). However, in what way C-propeptide mutations influence that mechanism and stimulate metaphysesal bone formation, remains to be established.

In conclusion, heterozygous C-propeptide mutations in *COL1A1* may result in OI IIC with or without twisting of the long bones. Absence of vertebral compression fractures and presence of conspicuous metaphyseal sclerosis in these patients seem to be related to a distinctive histology with broad, cartilaginous, rather than thin osseous trabeculae. OI IIC appears to be inherited as an autosomal dominant trait. Failure to detect *COL1A1* propeptide mutations in one patient points to genetic heterogeneity of OI IIC.

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RESEARCH LETTER

AMERICAN JOURNAL OF medical genetics PART A

Atypical Achondroplasia Due To Somatic Mosaicism for the Common Thanatophoric Dysplasia Mutation R248C

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TO THE EDITOR:

Heterozygous gain-of-function mutations in fibroblast growth factor receptor-3 (*FGFR3*) give rise to a group of skeletal dysplasias comprising lethal thanatophoric dysplasia (TD; OMIM 187600), prototypic achondroplasia (ACH; OMIM 100800), hypochondroplasia (HCH; OMIM 1463000), and other rare disorders. Common mutations have been identified in these conditions [Rousseau et al., 1994; Shiang et al., 1994]. A missense mutation (R248C) is one of two common mutations that cause TD [Tavormina et al., 1995; Rousseau et al., 1996]. The R248C mutation has been identified in two patients with non-TD phenotypes [Camera et al., 2001; Hyland et al., 2003]. It is logical to assume that these phenotypes are related to somatic mosaicism. Here, we report on another encounter with skeletal dysplasia caused by somatic mosaicism of the R248C. The phenotype of the affected boy was compatible with that of an FGFR3 disorder, but not typical for ACH or one of the other well-defined FGFR3 conditions. We were given the opportunity to examine the degree and distribution of cells carrying the mutation.

The patient, a 2-year-old boy, was the first child of non-consanguineous healthy Japanese parents. Prenatal ultrasonography had revealed polyhydramnios, short limbs, and a hypoplastic thorax. He was born by elective cesarean at 36 weeks of gestation. Apgar scores were 7 and 8 at 1 and 5 min, respectively. His birth weight was 3,090 g (90th centile), length 47.5 cm (50–90th centile), and OFC 36.0 cm (97th centile). He had frontal bossing, a mildly flattened nasal bridge, mildly short limbs, and a striking shortening of the hands and feet with a trident configuration of the hands (Fig. 1). Frequent apneic spells ensued shortly after birth, and he required mechanical ventilation for 2 days. He exhibited hypotonia and his gross motor development was delayed. He could sit without support at 2 years of age, but was unable to walk unaided. In early infancy, he presented generalized seizures, which have been well controlled with sodium valproate.

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Am J Med Genet Part A.

Radiological examination in the neonatal period (Fig. 2) and at 2 years of age (Fig. 3) showed essentially the same findings. The skeletal manifestations overlapped with those of ACH, but there were a few differences. Platyspondyly in the neonatal period was more severe than that usually seen in ACH. Unlike ACH though, the long bones were not significantly short or broad, but rather slender and without metaphyseal flaring.

These clinical and radiological observations led us to a molecular analysis of *FGFR3*.

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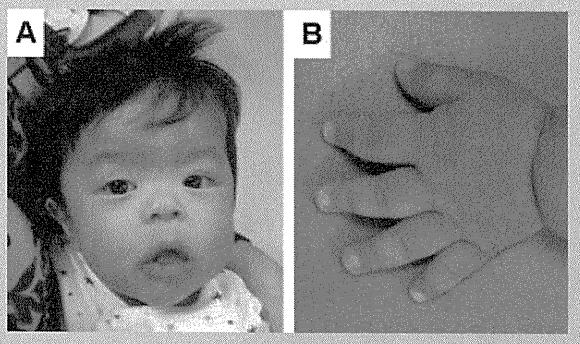


FIG. 1. Photographs of the propositus at 8 months of age. A: Facial features demonstrated frontal bossing, mildly flattened nasal bridge. B: Note the trident configuration of the hands.

After genetic counseling, we obtained written informed consent from the parents for molecular studies. Approval for this study was obtained from the institutional review board of the Keio University School of Medicine. Genomic DNA of the patient and unaffected parents were extracted from peripheral blood by a standard technique. We checked all 17 coding exons and flanking introns of *FGFR3*, and found a heterozygous c.742C>T transition (R248C) in the proband (Fig. 4A). The unaffected parents did not have the mutation. To determine the presence of somatic mosaicism, we subcloned PCR products of DNA from his peripheral blood and hair root, took 100 colonies of each, screened for the mutation, and calculated the ratio of mutant to wild-type alleles. The ratio of mutant to wild-type alleles was 43/57 in the peripheral blood and 11/89 in the hair root, demonstrating somatic mosaicism of the R248C (Fig. 4B).

The manifestations of the present patient differed from those of the non-TD cases with R248C mutations reported previously. Other than the presence of mild femoral and tibial bowing, the phenotype of the affected boy reported by Camera et al. [2001] was almost identical with that of ACH. The R248C substitution was identified in both peripheral blood and mucous membranes of the mouth, but other tissues were not examined. The skeletal manifestation in the affected woman reported by Hyland et al. [2003] resembled that of HCH. However, she had limb asymmetry, developmental delay, and redundant, thickened, pigmented skin similar to that of acanthosis nigricans. Somatic mosaicism of the R248C in peripheral lymphocytes was confirmed using denaturing high-performance liquid chromatography (DHPLC). However, other tissues were not examined.

Thus, this is the first report of the relation between an atypical manifestation of FGFR3-pathy and somatic mosaicism of the R248C mutation by examining the degree and distribution of cells carrying the mutation. Based on the report by Hyland et al. and our own observation, somatic mosaicism of the R248C is responsible for atypical ACH- or HCH-like phenotypes.

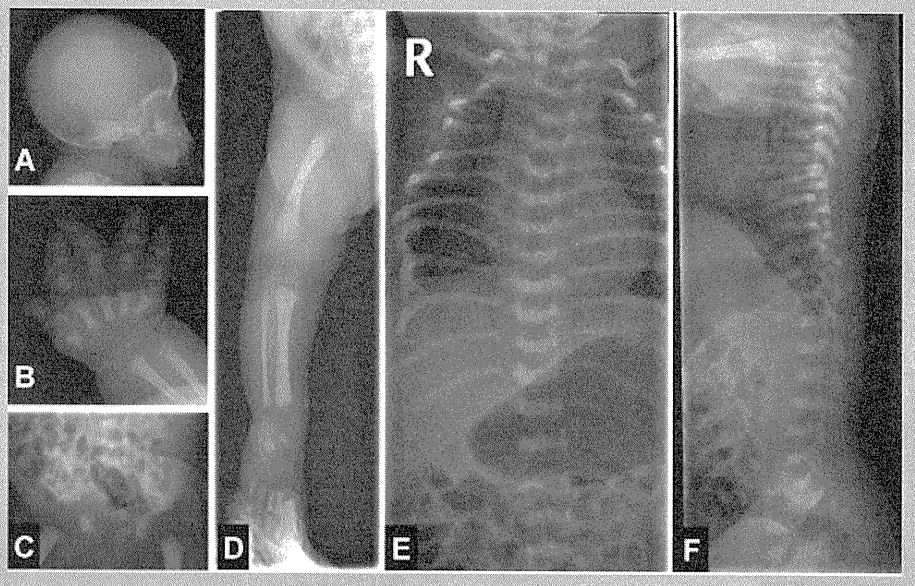


FIG. 2. Radiographs as a neonate. A: The facial bones and skull base are mildly hypoplasitc. B: The short tubular bones are very short and bullet-shaped with "trident hand." C: The ilia are short and somewhat wide with short greater sciatic notches. D: The long bones are relatively slender without metaphyseal flaring. E,F: The thorax is narrow and anterior costal ends are cupped. There is platyspondyly with central notches. The lumbar interpediculate distance is somewhat narrow.

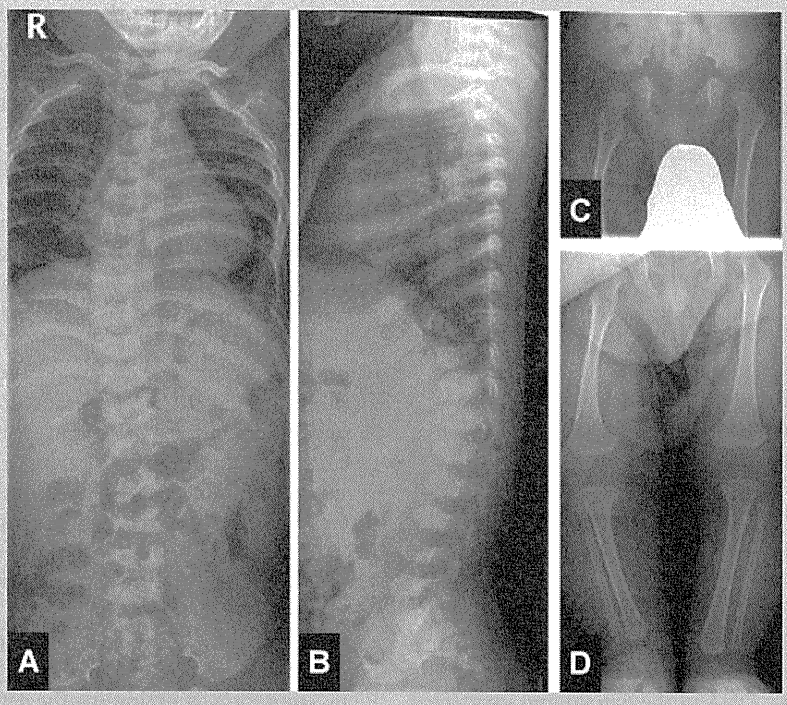
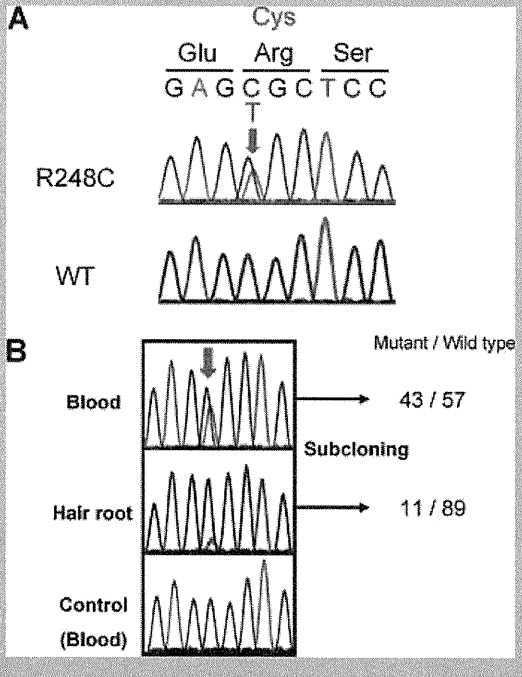


FIG. 3. Radiographs at 2 years of age. The overall findings are essentially the same as those seen as a neonate, but there are some differences. A,B: Thoracolumbar gibbus developed. Note dorsal wedging of the vertebral bodies show anterior pointing and dorsal wedging and. The pedicles are relatively long. C,D: Mild constriction of the proximal femora and a convex appearance of the distal femoral, proximal tibial, and distal tibial metaphyses became apparent.



In conclusion, the entire coding region of FGFR3 should be screened in patients with strong features of ACH or HCH but negative *FGFR3* results for common mutations. Analyses for more than one tissue may be required.

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We thank the patients and their families for participation in this study. We also thank Prof. Takao Takahashi for fruitful discussion. This work was supported by Grant-in-Aid for Young Scientists (B) (22790999) from Japan Society for the Promotion of Science, and was partly supported by the Health and Labour Sciences Research Grants for the Research on Intractable Diseases (H22-Nanji-Ippan-195 and H22-Nanji-Ippan-194) from Ministry of Health, Labour and Welfare of Japan.

FIG. 4. Identification of the R248C. Thanatophoric dysplasia common mutation in *FGFR3*. A: A partial sequence of PCR product of patient is shown. Heterozygous missense mutation (c.742C>T, p.R248C) is indicated by arrows. B: Subcloning of the PCR products from the patient. The ratio of mutant to wild-type alleles was 43/57 in the peripheral blood and 11/89 in the hair root, suggesting somatic mosaicism of the R248C.

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A novel mutation in *LEPRE1* that eliminates only the KDEL ER- retrieval sequence causes non-lethal osteogenesis imperfecta

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ABSTRACT

Prolyl 3-hydroxylase 1 (P3H1), encoded by the *LEPRE1* gene, forms a molecular complex with cartilage-associated protein (CRTAP) and cyclophilin B (encoded by *PPIB*) in the endoplasmic reticulum (ER). This complex is responsible for one step in collagen post-translational modification, the prolyl 3-hydroxylation of specific proline residues, specifically α1(I) Pro986. P3H1 provides the enzymatic activity of the complex and has a Lys-Asp-Glu-Leu (KDEL) ER-retrieval sequence at the carboxyl terminus. Loss of function mutations in *LEPRE1* lead to the Pro986 residue remaining unmodified and lead to slow folding and excessive helical post-translational modification of type I collagen, which is seen in both dominant and recessive osteogenesis imperfecta (OI). Here, we present the case of siblings with non-lethal OI due to novel compound heterozygous mutations in *LEPRE1* (c.484delG and c.2155dupC). The results of RNA analysis and real-time PCR suggest that mRNA with c.2155dupC escapes from nonsense-mediated RNA decay. Without the KDEL ER-retrieval sequence, the product of the c.2155dupC variant cannot be retained in the ER. This is the first report of a mutation in *LEPRE1* that eliminates only the KDEL ER-retrieval sequence, whereas other functional domains remain intact. Our study shows, for the first time, that the KDEL ER-retrieval sequence is essential for P3H1 functionality and that a defect in KDEL is sufficient for disease onset.

Key words: Osteogenesis imperfecta; *LEPRE1*; KDEL ER- retrieval sequence

INTRODUCTION

Osteogenesis imperfecta (OI; MIM #166200, #166210, #259420, #166220, #610967, #610968, #610682, #610915, #259440, #613848 and #613982) comprises a heterogeneous group of connective tissue disorders characterized by fragile bones with susceptibility to fractures. Most cases of OI are caused by heterozygous mutations in *COL1A1* or *COL1A2*, the genes encoding the two type I procollagen alpha chains, proα1 (I) and proα2 (I) [1]. Mutations in these genes result in quantitative and/or qualitative defects in type I collagen production by osteoblasts [2-4].

Recurrence of severe OI in families with unaffected parents results from either dominant (parental mosaicism) or recessive inheritance [5-7]. Recent investigations have discovered several genes responsible for OI inherited as an autosomal recessive trait [8-18]. Among these genes, *LEPRE1* encodes prolyl 3-hydroxylase 1 (P3H1), which forms a molecular complex with cartilage-associated protein (CRTAP) and cyclophilin B (CypB, encoded by *PPIB*) in the endoplasmic reticulum (ER) that is responsible for one step in collagen post-translational modification, the prolyl 3-hydroxylation of specific proline residues, specifically α1(I) Pro986 [19]. P3H1 provides the enzymatic activity of the complex and is the only component of the complex with a Lys-Asp-Glu-Leu (KDEL) ER-retrieval sequence at the carboxyl terminus [20]. Loss of function mutations in either *LEPRE1* or *CRTAP* lead to loss of both proteins in the cell, leave the Pro986 residue unmodified, and lead to slow folding and excessive helical post-translational modification of type I collagen [21].

To date, more than 20 *LEPRE1* mutations have been described [10, 21-25]. With the exception of only one missense mutation, Leu489Pro [25], all *LEPRE1* mutations result in a premature termination codon (PTC) with mRNA that is destroyed by the process of nonsense-mediated RNA decay. Here we present the case of siblings with OI due to novel compound heterozygous mutations in *LEPRE1* (c.484delG and c.2155dupC). Without the KDEL ER- retrieval sequence, the product of the c.2155dupC variant cannot be retained in the ER. Our

study shows, for the first time, that the KDEL ER- retrieval sequence is essential for P3H1 functionality and that a defect in KDEL is sufficient for disease onset.

RESULTS

PATIENT REPORTS

Patient II-2 was a 5-year-old female born to healthy parents who already had one healthy child (Fig 1A). Prenatal ultrasonography at 28 weeks of gestation showed deformity of the lower limbs. She was delivered with multiple fractures by caesarian section at 35 weeks' gestation. Birth weight was 1966 g (below 3rd percentile), length 42.2 cm (below 3rd percentile), and OFC 31.2 cm (3rd-10th percentile). She did not have blue sclera or dysmorphic facial features, such as micrognathia or a triangular face. She had no neonatal respiratory distress. Radiographs showed multiple rib fractures, healed fractures of both femora and the right humerus, and a subacute fracture of the left humerus (Fig 1B). Metaphyseal osteopenia was significant. A diagnosis of OI type III was made. At least 10 fractures occurred in the first 6 months of life. Pamidronate treatment was initiated at 2 months of age. The pamidronate was initially administered by infusion every 2 months and was changed to every 3 months at the age of 2 years. The bone mineral density (BMD) of the lumber spine (L2–L4) was 0.336 gm/cm^2 (Z score of –2.2), 0.429 g/cm^2 (Z score of –2.7), 0.479 g/cm^2 (Z score of –4.9), and 0.514 g/cm^2 (Z score of –5.9) at the ages of 1 year, 2 years, 4 years, and 5 years respectively (We used BMD reference data [26] in Spanish children). She did not have severe deformity of the long bones at age 5 years, and her skin was normal in extensibility. She had white sclerae and normal dentition. She was able to walk with difficulty while holding on to a table. Her intellectual development was normal.

Patient II-3 was the product of couple's next pregnancy; this pregnancy was electively terminated. Postmortem radiographs showed bilateral femoral bowing, a healed fracture of the right femoral shaft, thin ribs, and metaphyseal demineralization (Fig 1C).

Patient II-3 Bone Histology

Bone samples, obtained at autopsy, from Patient II-3 were processed according to standard procedure, and the formalin fixed paraffin-embedded sections were stained with hematoxylin and eosin. Irregular trabeculae of woven bone rimed by osteoblasts were observed in the humerus (Fig 1D) and spine (Fig 1E). The stroma surrounding the woven bone was mildly to moderately cellular and consisted of fibroblasts and collagen. These histological features resembled those of osteofibrous dysplasia.

Detection of *LEPRE1* Mutations

Sequence analysis revealed novel compound heterozygous *LEPRE1* mutations (c.484delG, p.A162LfsX22 and c.2155dupC, p.E719RfsX11) in both patients (Fig 2A). Their father carried c.484delG and their mother carried c.2155dupC. These mutations were not found in 200 control alleles. No sequence variation was found in *COL1A1, COL1A2*, *CRTAP*, or *PPIB*, and neither exon-level deletion nor duplication involving *COL1A1* and *COL1A2* was detected by MLPA analysis. The p.E719RfsX11 mutation creates a PTC in the last exon and results in the lack of only the KDEL ER-retrieval sequence, whereas other functional domains, such as the tetratricopeptide domain and Prolyl/Lysyl hydroxylase domain, remain intact (Fig 2B).

***LEPRE1* transcripts and P3H1 protein in probands**

Only the allele with c.2155 dupC was successfully amplified and sequenced at the cDNA level. Real-Time PCR revealed that the level of *LEPRE1* transcripts of Patient II-3 was about one-half the control level (Fig 3A).

Western blot analysis of fibroblast lysates confirmed the absence of intracellular P3H1 in Patient II-3 (Fig 3C). Fluorescent microscopy showed the expected colocalization of P3H1 and

CRTAP with GRP94 in control cells. Both P3H1 and CRTAP proteins were absent in fibroblasts from Patient II-3 (Fig 3D), reflecting mutual protection in the complex.

Collagen post-translational modification

In both the cell layer and media, steady-state fibroblast collagen of Patient II-3 displayed helical overmodification, detected as back-streaking of collagen alpha chain bands on gel electrophoresis (Fig 3B).

Tandem mass spectrometry analysis of tryptic peptides of Patient II-3 secreted α1 (I)-collagen chains revealed only a slight reduction (85% in proband, 95-98% in control collagen) of Pro986 3-hydroxylation (data not shown) despite the absence of detectable mutant P3H1 protein in the cell.

DISCUSSION

ER-resident proteins must be distinguished from newly synthesized secretory proteins, which pass through this compartment as they transit the secretory pathway toward the extracellular space. One of the mechanisms by which this is achieved is the selective retrograde transport of soluble ER-resident proteins from the cis-Golgi to the ER [27]. Receptors in post-ER compartments recognize a C-terminal motif that marks proteins that are to be retained in the ER. The KDEL motif binds to this salvaging receptor (KDEL receptor) in the Golgi, resulting in this ligand-receptor complex being returned to the ER [27]. Soluble ER-resident proteins such as molecular chaperones and components of the control quality machinery, e.g. immunoglobulin heavy-chain binding protein, calreticulin, and protein disulfide isomerase, contain the KDEL motif at the carboxyl terminus. P3H1, encoded by *LEPRE1*, forms a molecular complex with CRTAP and CypB in the ER, and provides the enzymatic activity of the complex. P3H1 is the only component of the complex with a KDEL ER-retrieval sequence at the carboxyl terminus [20]. One splice mutation, c.2055+18G>A,