Transforming growth factor-β1 gene mutations and phenotypes in pediatric patients with Camurati-Engelmann disease

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Abstract. The aim of the present study was to investigate the clinical characteristics and major causative gene in pediatric patients with Camurati-Engelmann disease (CED). Biochemical and radiographic examinations, bone scintigraphy and genetic analyses were performed in two affected males and their parents. The two patients experienced waddling gait, muscular weakness and growth developmental delay. X-ray radiography revealed typical fusiform thickening of the diaphyseal portions of the long bones. The abnormal uptake of tracer Tc-99m was visualized in the skull and both sides of the upper humeri, ulnas, radii, femurs and tibias using bone scintigraphy. Serum levels of the bone formation marker procollagen type I N-terminal propeptide (PINP) and the bone resorption marker β-isomerized C-terminal cross-linked telopeptide of type I collagen (β-CTX) in the 6-year-old patient were significantly increased compared with the normal value range, while only the β-CTX levels were elevated in the 16-year-old patient. A heterozygous missense mutation p.Arg218Cys in exon 4 of the transforming growth factor β1 (*TGFβ1*) gene was detected in the two patients, while their parents had normal wild-type genotypes. In conclusion, the p.Arg218Cys mutation was shown to contribute to the clinical phenotypes in two pediatric patients with CED. The results of this study suggest that abnormal bone turnover marker levels, typical radiological findings and mutations in the *TGFβ1* gene are three important factors in the diagnosis of sporadic CED cases.

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Introduction

Camurati-Engelmann disease (CED, OMIM 131300) is a rare autosomal dominant disease with variable clinical manifestations. The main clinical and laboratory manifestations include painful limbs, waddling gait, muscular weakness, joint contracture, cranial nerve impingement, delayed pubertal development and increased serum alkaline phosphatase (ALP) levels. Fusiform thickening of the diaphyseal and metaphyseal cortex of the long bones is the typical radiographic signature. Occasionally, a number of patients experience blindness or deafness due to thickened skull bones. The incidence of this disease is ~1:1 million births.

The primary causative gene in CED is the transforming growth factor β1 (*TGFβ1*) gene (1), which contains seven exons. TGFβ1, encoded by the *TGFβ1* gene, is a member of the TGFβ1 signaling pathway and regulates cell proliferation, migration, differentiation and apoptosis. TGFβ1 is particularly abundant in the bone matrix, where it is involved in the regulation of bone formation and resorption. The inactive form of the TGFβ1 protein (pre-pro-TGFβ1) is composed of three subunits; the signal peptide, the latency-associated peptide (LAP) and the mature peptide. The activation procedure includes the cleavage of the signal peptide followed by cleavage of the LAP from the mature peptide. Mutations in different domains of the *TGFβ1* gene lead to inherited sclerosing bone disorder or osteoporosis (2).

To date, >40 CED families from Europe, Australia, Israel, Japan, Korea, South America, the USA and China with ~10 mutations have been reported. The majority of these mutations are located in the LAP region (3). The first Chinese family with CED was reported in 2006 (4); the heterozygous missense mutation p.Arg218His (R281H) in exon 4 of the *TGFβ1* gene was detected in the affected patient.

The present study aimed to investigate the cases of two Chinese males diagnosed with CED, using clinical and X-ray examinations, bone scintigraphy and *TGFβ1* gene mutation screening.

Patients and methods

Patients. Patient one (P1) was a 6-year-old male (height, 114 cm; weight, 19 kg) who had been born at full term and was the third

child born to the mother. P1 was unable to walk independently until 18 months of age and experienced waddling gait, muscular weakness and left pelvic limb pain during the last 4 years.

Patient two (P2) was a 16-year-old male (height, 147 cm; weight, 21 kg). The parents of P2 noted that the patient was short and underweight at the age of 6, compared with others of the same age. P2 had a waddling gait, mild muscular weakness and no signs of sexual development.

No fractures, auditory or visual impairments or family history have been reported in either patient. The two patients and their healthy non-consanguineous parents were enrolled in this study by the Department of Osteoporosis and Bone Diseases Outpatient Clinic (Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China). The present study was approved by the Ethics Committee of the Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Informed consent was obtained from the parents of the two patients. Two hundred age- and gender-matched healthy donors were used as controls for the mutation analysis after being recruited for a previous study (5).

Methods. Biochemical parameters, including the complete blood count and levels of serum calcium (Ca), phosphonium (P), ALP, blood urea nitrogen (BUN), serum creatinine (Scr), β-isomerized C-terminal cross-linked telopeptide of type I collagen (β-CTX), procollagen type I N-terminal propeptide (PINP), 25-hydroxy D [25(OH)D] and parathyroid hormone (PTH) were determined in the two patients and their parents. X-ray radiography of the thoracic and lumbar vertebrae, limbs, hips and skull was performed individually. A lunar prodigy dual-energy X-ray absorptiometry (DXA) densitometer (Lunar Corporation, Madison, WI, USA) was used to measure the bone mineral density (BMD) values of the left proximal femur, including the femoral neck and total hip, and the anteroposterior lumbar spine 1-4 (L1-4). The machine was calibrated daily. Prodigy enCORE version 6.70 software was used to analyze the data (standard-array mode; GE Healthcare, Madison, WI, USA). DXA measurements were obtained from triplicate measurements at L1-4 and the total hip, femoral neck and trochanter in 15 individuals; the coefficient of variability (CV) values for the DXA measurements were 1.39, 0.70, 2.22 and 1.41%, respectively (6). Weekly repeated phantom measurements were carried out; these determined that the long-term reproducibility of the DXA data during the study was 99.55% (7). Standardized equipment were used to determine the body weight and height of subjects. The body mass index (BMI) was calculated as the weight/height^2 (kg/m^2). Bone scintigraphy with Tc-99m hydroxymethylene diphosphonate (HMDP) was performed to detect abnormal bone metabolism.

For the mutation analysis, genomic DNA was isolated from peripheral blood leukocytes using the conventional phenol-chloroform extraction method. The entire coding region and adjacent splice sites of the *TGFβ1* gene were amplified and sequenced directly from the two patients, their parents and 200 healthy donors.

Results

Biochemical and bone turnover markers. Serum levels of ALP, Ca, P, PTH, 25(OH)D, PINP and β-CTX in P1 and P2

Table I. Biochemical parameters, bone turnover markers and bone mineral density (BMD) values of lumbar spine 1-4 (L1-4) and hip sites in patients one (P1) and two (P2).

Patient	Height (cm)	Weight (kg)	BMI (kg/m^2)	ALP (U/l)	Ca (mmol/l)	P (mmol/l)	PTH (ng/l)	25(OH)D (ng/ml)	β-CTX (ng/l)	PINP (ng/ml)	L1-4 (g/cm^2)	Femoral neck (g/cm^2)	Trochanter (g/cm^2)	Total hip (g/cm^2)
P1	114	19	14.62	435	2.33	1.57	56.19	10.66	3,770	>1200	0.591	0.596	0.413	NA
P2	147.5	21	9.65	157	2.16	1.34	21.42	12.13	1,590	70.99	0.512	0.422	0.407	0.406

BMI, body mass index; ALP, alkaline phosphatase; Ca, calcium; P, phosphonium; PTH, parathyroid hormone; 25(OH)D, 25-hydroxy D; β-CTX, β-isomerized C-terminal cross-linked telopeptide of type I collagen; PINP, procollagen type I N-terminal propeptide.

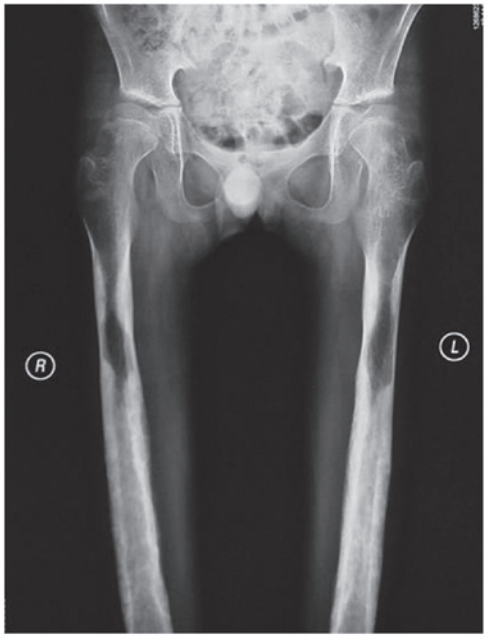

Figure 1. X-ray examination of the 16-year-old patient (P2) revealed the symmetrical fusiform thickening on both femurs. R, right; L, left.

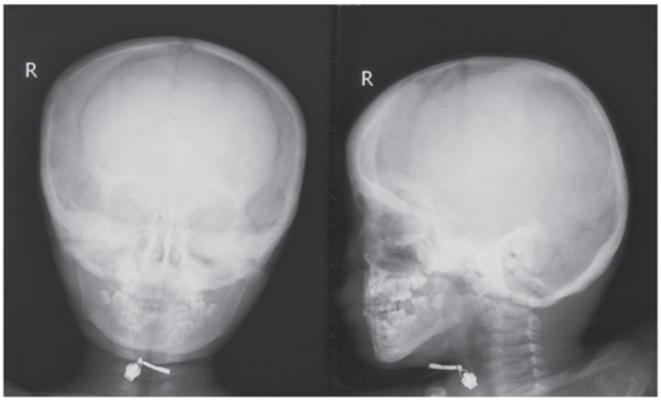

Figure 2. X-ray examination of the 6-year-old patient (P1) revealed the cortical thickening at the base of skull. R, right.

are shown in Table I. Notably, levels of the bone resorption marker β-CTX were markedly elevated 7-fold compared with the upper limit of normal (ULN) in P1, while only a slight increase was observed in P2. The results of two bone formation markers, ALP and PINP, were inconsistent in the two patients. In P1, the serum PINP level exceeded the measurement range, while the serum ALP level was only mildly increased (normal range in childhood, 85-400 U/l). However, in P2, serum ALP and PINP levels were within the normal range. Furthermore, the complete blood count and renal function markers in the two patients were normal (data not shown).

Radiographic examinations. The X-ray examinations revealed typical fusiform thickening of the diaphysis of the long bones among the tibias, humeri, femurs, ulnas and radii (Fig. 1). The skulls of the two patients exhibited cortical thickening located at the base (Fig. 2).

Abnormal tracer uptake was observed in the skull and both sides of upper humeri, ulnas, radii, femurs and tibias using bone scintigraphy in the two patients (Fig. 3).

BMD. The BMD values of the two patients at L1-4 and hip sites are shown in Table I. The Z scores of P1 were imponderable due to the lack of reference BMD values for Chinese children aged <10 years. The BMD values of L1-4 and femoral neck in P1 were similar to those in a study by Wu *et al* (8). For P2, the BMD values at each site were lower compared with the age- and gender-matched mean reference values (8,9).

TGFβ1 gene mutation. A heterozygous missense mutation p.Arg218Cys (R218C) in exon 4 was detected in the two patients, while their parents and the 200 healthy donors had normal wild-type genotypes (5).

Discussion

CED, also termed progressive diaphyseal dysplasia (PDD), is a type of inherited sclerosing bone disorder characterized by hyperostosis on the periosteal and endosteal surface of the long bones. The age of onset varies greatly; however,

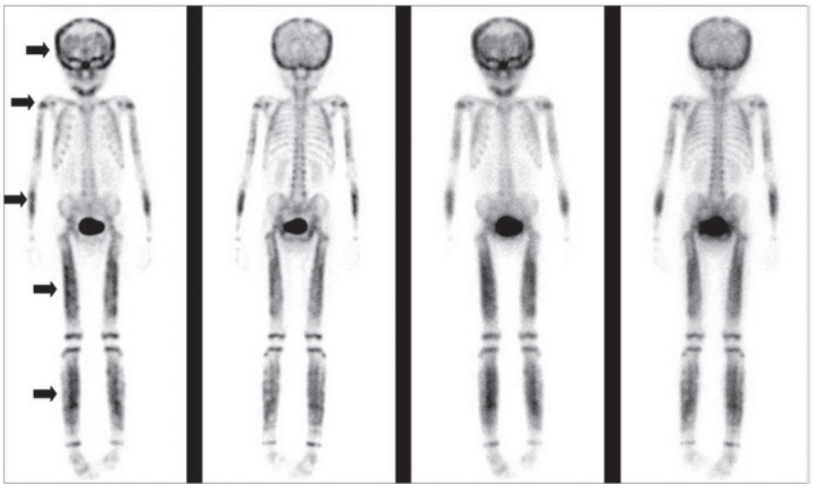

Figure 3. Whole-body bone scintigraphy of the 6-year-old patient (P1) revealed the symmetrical distribution of the disease. Increased tracer uptake was observed in the skull, the diaphyseal portion of the upper humeri, ulnas, radii, femurs and tibias (black arrows).

the majority of patients initially exhibit symptoms, including pain and weakness, by adolescence. The typical radiological characteristic is fusiform thickening of the diaphyseal portions of the long bones. Vanhoenacker *et al* (10) reported that the radiographic manifestations were typically detected before the age of 30 and were usually more extensive with increasing age. Concomitant broadening of the diaphyses of long bones and the narrowing of the medullary canal suggest that an excessive periosteal apposition of bone and a defective resorption of bone at the endosteal side of the long bones exist.

The *TGFβ1* gene has been identified as the causative gene of CED; numerous mutations in *TGFβ1* have been detected in CED patients worldwide. The majority of the mutations detected in CED are missense mutations, including the arginine residue at position 218 (R218C), R218H, H222D, C223S and C225R, located in exon 4 at the C-terminal region of LAP, close to or within the two cysteine residues (3). Additional mutations, including E169K and R156C in exon 2, Y81H in exon 1 and L10-L12dup and LLL12-13ins in the signal region, have been identified in CED family studies (3,11-14). Among them, R218C is the most common mutation hotspot in CED patients, accounting for >60% of the mutations (3,11). However, to date, no CED cases caused by the *TGFβ1* gene R218C mutation have been reported in Chinese patients. Furthermore, the correlation between polymorphisms and clinical variability has not yet been elucidated (11).

Mutations located in the LAP region have not been demonstrated to lead to the overproduction of TGFβ1 in functional studies; however, they are able to increase its activity. Two possible mechanisms may explain this; firstly, the destabilized disulphide bridging of the LAPs leads to premature activation of the mature peptide mediated by exon 4 mutations. Secondly, mutations in exon 1 lead to intracellular retention of the mutant protein, which affects secretion (15,16). Overactive TGFβ1 proteins lead to increased bone density and decreased body fat and muscle tissue (15,16); this contributes to the signs and symptoms of CED.

An animal study by Tang *et al* (17) showed that TGFβ1 was involved in bone resorption and formation through an SMAD signaling pathway that mediates bone mesenchymal stem cell (BMSCs) migration. A high level of active TGFβ1 was detected in the bone marrow of CED mice carrying *TGFβ1* gene mutations, and typical progressive diaphyseal dysplasia manifestations were observed.

Occasionally, individuals may possess the gene mutation that causes CED yet never develop the characteristic features of this condition, supporting the incomplete penetrance of CED (11). A number of individuals with clinical manifestations of CED with no identified mutations in the *TGFβ1* gene are diagnosed with CED type II (OMIM 606631) (18).

The two patients in the present study harbored the most frequently detected R218C mutation in exon 4. Their clinical manifestations, X-ray signatures and bone scintigrapy results were consistent with previously reported phenotypes. With regard to the 6-year-old patient (P1), the ALP level was slightly increased compared with the normal range in children aged 0-6 years. Generally, serum ALP level is higher in childhood and adolescence than in adults, due to the increased bone turnover associated with growth. However, the markedly elevated levels of the bone formation marker PINP indicated an upregulated bone formation process in CED. Notably, the bone absorption marker β-CTX was also significantly increased in the same patient. The 16-year-old patient (P2) also had increased serum β-CTX levels, while the bone formation markers ALP and PINP were within the normal ranges. To date, no studies have reported increased β-CTX levels in CED patients. This novel result requires further investigation in future studies.

In conclusion, the present study reported the cases of two Chinese pediatric patients with CED caused by the heterozygous missense mutation R218C in the *TGFβ1* gene. The results of this study suggest that abnormal bone turnover marker levels, typical radiological findings, bone scintigraphy results and mutations in the *TGFβ1* gene are important factors for diagnosis and appropriate genetic counseling in apparently sporadic CED cases.

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