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CLINICAL REPORT

Clinical characteristics and identification of a novel TGFB1 variant in three unrelated Chinese families with Camurati-Engelmann disease

Xiao-Hui Tao[1] | Xing-Guang Yang[2] | Zi-Yuan Wang[1] | Yang Xu[1] | Xiao-Yun Lin[1] | Tian Xu[1] | Zhen-Lin Zhang[1] | Hua Yue[1]

[1]Shanghai Clinical Research Center of Bone Disease, Department of Osteoporosis and Bone Diseases, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China

[2]Department of Orthopedic Surgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China

Correspondence
Hua Yue, Shanghai Clinical Research Center of Bone Disease, Department of Osteoporosis and Bone Diseases, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai 200233, China.
Email: yueyinglonghua@163.com

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Abstract

Background: To investigate the clinical characteristics and molecular diagnosis of Camurati-Engelmann disease (CAEND) in Chinese individuals.

Methods: We recruited six patients aged 14 to 45 years in three unrelated families with CAEND, including five females and one male. Clinical manifestations, biochemical tests, and radiographic examinations were analyzed. The *TGFB1* gene variants were further identified by Sanger sequencing. In addition, one female patient was followed up for 5 years.

Results: The onset age of the patients ranged from 1 to 6 years. All of them had family histories and consisted of an autosomal dominant inheritance pattern. Gait disturbance, fatigue, progressive bone pain, muscle atrophy, and weakness were the main complaints. Laboratory examinations revealed that the inflammatory markers were at high levels, in addition to the increased bone metabolism indicators. The thickened diaphysis of long bones and the narrowed medullary cavity was observed by radiography. Furthermore, bone scintigraphy detected abnormal symmetrical radioactive concentrations in the affected regions of bone. Sanger sequencing identified a missense heterozygous variant in exon 4 of the *TGFB1* gene in families 1 and 2, resulting in Arg218Cys, which confirmed CAEND. Moreover, one novel variant c.669C > G in exon 4 of the *TGFB1* gene harboring Cys223Trp was detected in family 3. Subsequent bioinformatics software predicted that the novel variant was pathogenic. Of interest, III:2 in family 3 experienced heart valve defects and tachycardia at birth, which had never been reported in CAEND patients before. Moreover, the response to drug treatment is also full of contradictions and is worthy of further study.

Conclusion: Besides the typical CAEND manifestations, the new phenotypic characteristics of tachycardia and heart valve defects were first reported in one woman carrying the novel variant p.Cys223Trp in *TGFB1* the gene. In addition, we demonstrated that increased bone metabolism indicators and inflammatory markers may possess auxiliary diagnosis for CAEND.

Xiao-Hui Tao and Xing-Guang Yang contributed equally to this work.

KEYWORDS
Camurati-Engelmann disease, genotypes, phenotypes, TGFB1

1 | INTRODUCTION

Camurati-Engelmann disease (CAEND, OMIM: 31300) or progressive diaphyseal dysplasia is a rare sclerosing bone dysplasia characterized by waddling gait, pain in extremities, muscular weakness, and cortical thickening of the diaphysis of the long bones (Cromer et al., 2012; Wallace et al., 2004). If the lesions involve the skull, they will lead to cranial nerve damage, such as hearing impairment and vision loss (Janssens et al., 2006; Van Hul et al., 2019). Some cases have systemic manifestations or mimic some syndromes accompanied by anemia, leukopenia, and hepatosplenomegaly, for instance (Crisp & Brenton, 1982). It is known that dominantly inherited heterozygous mutations in *TGFB1* (OMIM: 190180) gene cause CAEND.

To date, 300 cases of CAEND have been reported worldwide (Baroncelli et al., 2017; Castro et al., 2005; Hughes et al., 2019; Janssens et al., 2000; Janssens et al., 2006; Saraiva, 1997, 2000; Wang et al., 2013). Therefore, the diagnosis of CAEND is challenging among clinicians owing to its rarity and extensive phenotypic variations.

The present study analyzed the clinical characteristics and identified the major causative gene variants in six patients from three unrelated families with CAEND. The relevant reported literature about the disease was reviewed to improve awareness of the rare disease in the clinicians. It is interesting to note that we identified a novel heterozygous variant p.Cys223Trp of the *TGFB1* gene in one 14-year-old woman with a slight phenotype. Our findings help to gain a better understanding and expand the genotypic spectrum of this rare disease.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

The protocol was approved by the Ethics Committee of the Shanghai Sixth People's Hospital affiliated to Shanghai Jiao Tong University, and all subjects signed informed consent forms.

2.2 | Subjects

One male and five female patients aged 14 to 45 (from three unrelated families) were enrolled in the present study (Table 1).

2.3 | Clinical features, biochemical and imaging evaluation

Anthropology information (height, weight, age, etc.), comprehensive clinical evaluation, and family history were obtained. Pedigrees of the three families are shown in Figure 1.

Serum total alkaline phosphatase (ALP), phosphorus, calcium, erythrocyte sedimentation rate (ESR), high-sensitivity C-reactive protein (hsCRP), and other biochemical indices were assessed by a Hitachi 7600–020 automatic biochemistry analyzer (HITACHI, Japan). Serum intact parathyroid hormone (iPTH), 25-hydroxyvitamin D (25OHD), β-CrossLaps of type 1 collagen-containing cross-linked C-telopeptide (β-CTX), and osteocalcin (OC) were measured by an automated Roche electrochemiluminescence system (Roche Diagnostic GmbH, Germany).

Bone scintigraphy with ^{99m}Tc-methylene diphosphonate and radiological examinations were performed. Bone mass density (BMD, g/cm^2) of the lumbar spine 1–4 (L1–4), left femoral neck and total hip were measured by a lunar prodigy dual-energy X-ray absorptiometry densitometer (GE Healthcare, Madison, USA).

2.4 | Genetic analysis and variant prediction

Genomic DNA was extracted from the 3-ml peripheral blood sample of each participant using phenol extraction and isopropanol precipitation. All exons and intron-exon boundaries of the *TGFB1* gene were amplified by PCR using one pair of primers (F: 5′-GGGTTTGCTCCTTCCTTCCT-3′, R: 5′-CCTGAGCCCTCCAAGCTAAA-3′), which was designed by Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) for amplification (Rozen & Skaletsky, 2000). Subsequently, the amplified PCR products were sequenced on the ABI3730XL platform with the BigDye3.1 Kit (ABI company, USA). The sequencing files were analyzed by Polyphred software, and the results were obtained after manual proofreading. All variants were mapped on transcript NM_000660.7 and protein NP_000651.3.

The PolyPhen-2, PROVEAN and UniProt databases were used to predict the pathogenicity of the variants and analyze the amino acid conservation (Adzhubei et al., 2010; Choi & Chan, 2015). Furthermore, the ExAC and 1000 Genomes Project databases were used to identify the novel variant. The protein sequence of TGFβ1 was

TABLE 1 Clinical data of the six cases with CAEND

	Family 1 III:5	Family 1 III:6	Family 1 II:8	Family 2 III:2	Family 3 III:2	Family 3 II:1
Gender/Age (years)	F/22	F/16	F/45	F/34	F/14	M/38
Age of onset (years)	4	6	3	1	5	–
Height (m)	159.1	148.5	158.0	171.3	155.0	172.3
Weight (kg)	33.1	34.4	59.4	50.8	31.0	63.0
Pain in extremities	Marked	Mild	Mild	Marked	–	–
Waddling gait	Marked	Marked	Mild	Marked	Mild	–
Muscle weakness	Marked	Mild	Mild	Marked	Mild	–
Skin temperature increase	Mild	Mild	–	Mild	–	–
Cranial nerve impairment	–	–	–	Marked	–	–
Dizziness	Mild	–	–	Marked	–	–
Vision impairment	–	–	–	Marked	–	–
Exophthalmos	Mild	–	–	Marked	–	–
Hearing loss	–	–	–	–	–	–
Infrequent menses	Mild	–	–	Marked	–	–

Note: "–" means no symptom.

Abbreviations: CAEND, Camurati-Engelmann disease; F, female; M, male.

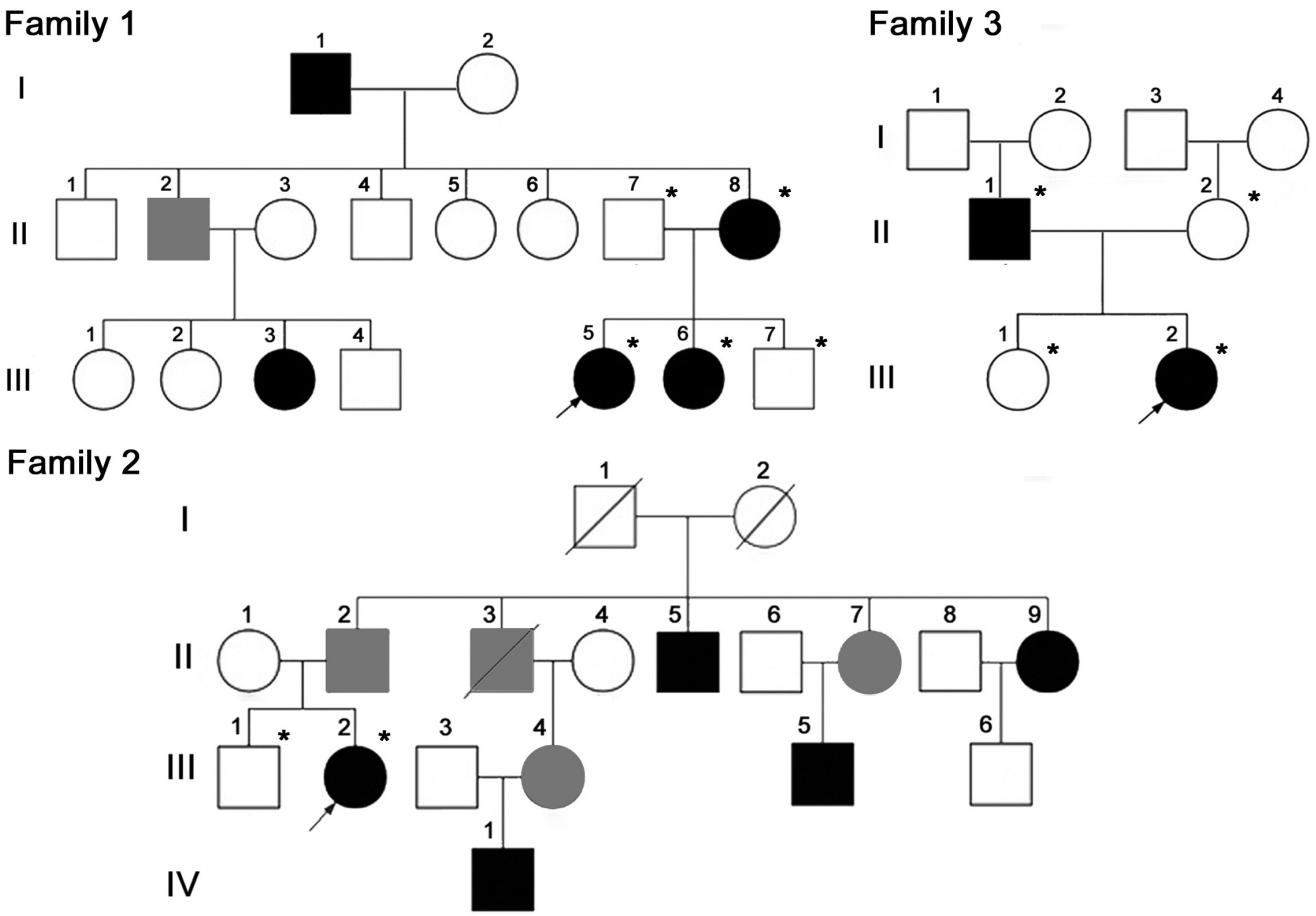

FIGURE 1 Pedigrees of three unrelated Chinese families with CAEND. Members marked with asterisk (*) indicate the subjects have received the genetic confirmation. Black symbols: CAEND cases with typical syndromes (Family 3, II: 1 is asymptomatic); Gray symbols: asymptomatic suspected patients; White symbols: healthy subjects. CAEND, Camurati-Engelmann disease

obtained from the UniProt database in FASTA file form. The three-dimensional structure homology modeling and visualization of the native and mutant proteins were deduced using the online SWISS-MODEL system and PyMol software.

3 | RESULTS

3.1 | Family characteristics

3.1.1 | Family 1

Proband 1 was a 22-year-old woman born at full-term from a non-consanguineous family. She began to walk at 14 months with a normal gait. At the age of 4, a waddling gait occurred. Two years later, she had difficulty in climbing stairs, generalized weakness, increased local skin temperature, and gradually aggravated pain in both lower extremities. The proband underwent glucocorticoid treatment for many years, but her pain did not improve. At the age of 18, she was treated with zoledronate yearly for the diagnosis of "fibrous dysplasia" in another hospital. However, bone pain became more serious. Physical examination revealed muscular atrophy of the lower limbs and valgus knee deformity (Figure 2a). Her younger sister (III:6) could walk by age 2, and waddling gait and mild leg pain occurred at 6 and 12 years old, respectively. Their 45-year-old mother (II:8) complained that she had similar symptoms in adolescence. It was of interest to note that the intensive bone pain gradually relieved and muscle mass and strength of both lower limbs increased with weight gain (bone mass index was increased from approximately 16.1 kg/m^2 to 23.6 kg/m^2) at approximately 30 years of age. Now she only experienced mild arthralgia and fatigue occasionally, along with the average muscle mass of limbs. The proband's cousin (III:3) also developed intensive bone pain and symptoms improved after glucocorticoid treatment (details were not available). The uncle of proband 1 (II:2) was an asymptomatic suspected patient.

3.1.2 | Family 2

Proband 2 was a 34-year-old female patient who was married but never gave birth. She was able to walk at the age of 1, and simultaneous waddling gait and abnormal knee joints were observed. Since then, she fell many times, whereas no fractures occurred. From the age of 29, fusiform enlargement was found in her forearm, followed by the lower limbs. Concurrently, the skin temperature of the affected bone increased. In recent years, she gradually developed bilateral visual field defects accompanied by headache and dizziness. She also complained

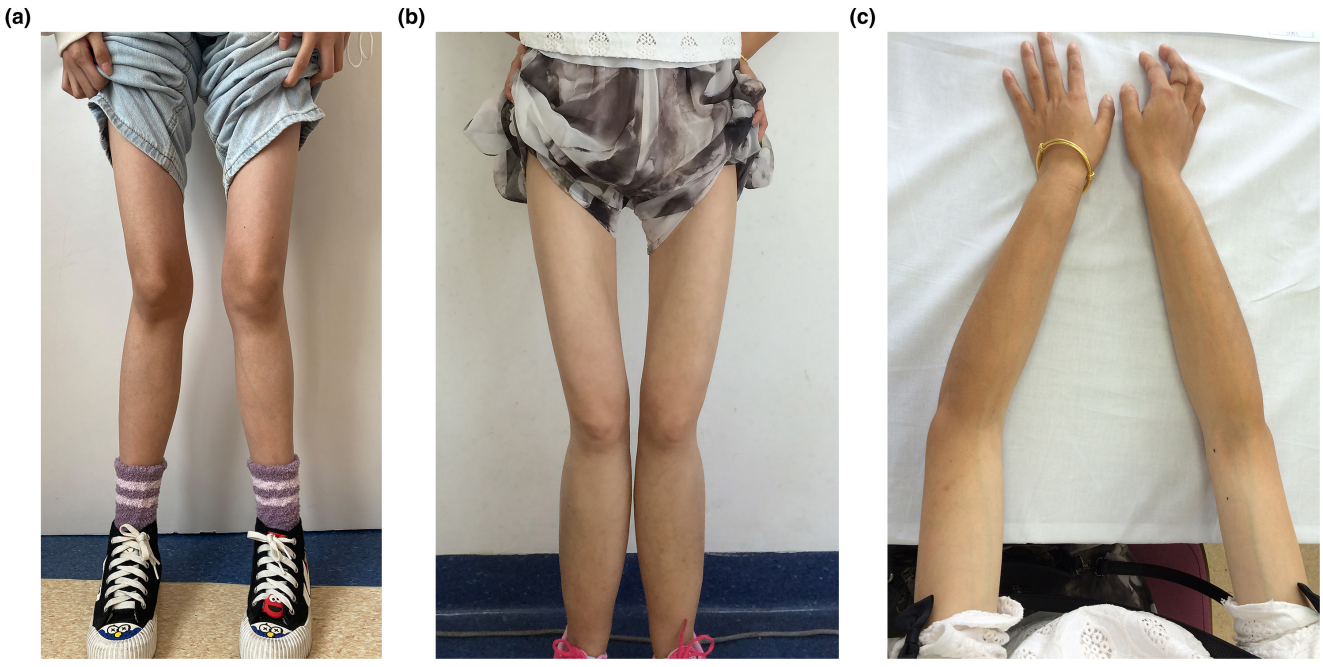

FIGURE 2 Classic clinical features of subjects with CAEND. (a) Proband 1 (22 years, woman) was thin and presented less subcutaneous fat, genu valgus deformity, and muscular atrophy of both lower limbs; (b) Proband 2 (34 years, woman) had similar clinical manifestations with the above-mentioned subject, she reported thickened long bones; (c) Elbow joint of proband 2 (34 years, woman) was unable to straighten

of hypomenorrhea and a delayed cycle (four or five per year) since menarche at 17 years old. Physical examinations showed muscle atrophy in the lower limbs, genu valgus deformity (Figure 2b), and limited multijoint mobility (Figure 2c). Her uncle (II:5), aunt (II:9), cousin (III:5), and nephew (IV:1) all presented with wide-based gait and no other symptoms. The cousin (III:4) of the proband suffered from severe headache and impaired vision for almost 20 years. She was misdiagnosed as "fibrous dysplasia of the skull" and underwent titanium alloy replacement after frontal bone resection.

3.1.3 | Family 3

Proband 3 was a 15-year-old woman who developed tachycardia and heart valve defects at birth. She underwent cardiac valve repair surgery when she was 5 years old (details were not available). She could walk at 15 months, and limping was noticed at the age of 5. By age 13, she suffered a hairline fracture of the bilateral knee by a fall. Progressive aggravation of pain in her knee appeared 3 months ago, so she came to our department for help. Her father (I:1) was diagnosed with CAEND by genetic screening without symptoms.

3.2 | Blood test and imaging examinations

The laboratory examinations and BMD of the patients are shown in Table 2, except I:1 in family 3 who refused to do any medical tests. Liver and kidney function tests of the five patients were in the normal range. BTMs such as β-CTX and OC were highly elevated in patients III:5 (family 1) and III:2 (family 3). Moreover, most of them showed increased levels of inflammatory biomarkers such as ESR (100%, 5/5) and hsCRP (66.7%, 2/3).

In addition, the BMD of the femoral neck and total hip in proband 2 was significantly increased with high symmetrical radioactive concentrations in the affected sites, especially in the long bone of extremities and skull, by bone scintigraphy. The X-ray manifestations of the five patients were very similar, with cortical bone thickening and irregular periosteal sclerosis of the long bone shaft and the sclerosis of the skull (Figure 3).

The BMD of the femoral neck and total hip in proband 2 was significantly increased. Bone scintigraphy revealed high symmetrical radioactive concentrations in the affected sites, especially in extremity long bones and skull. The X-ray manifestations of the five patients were very similar. All of them showed the cortical thickening of

TABLE 2 Laboratory examinations and BMD of five cases with CAEND

	Family 1 III:5	Family 1 III:6	Family 1 II:8	Family 2 III:2	Family 3 III:2
Gender/Age (years)	F/22	F/16	F/45	F/34	F/14
hsCRP (mg/L)	28.97	6.76	–	32	–
ESR (mm/h)	120	42	57	56	30
ALP (U/L)	301 (RF: 15–112)*	157 (RF: 52–171)*	69 (RF: 15–112)*	369 (RF: 15–112)*	139 (RF: 42–390)*
β-CTX (ng/L)	2878 (RF: 354–470)*	1446 (RF: 350–2620)*	458.2 (RF: 112–479)*	5432 (RF: 230–313)*	855.9 (RF: 350–2620)*
OC (ng/ml)	112.1 (RF: 18.72–22.47)*	50.18 (RF: 13.8–58.7)*	17.44 (RF: 4.91–22.31)*	72.4 (RF: 15.33–18.26)*	62.37 (RF: 13.8–58.7)*
PTH (pg/ml)	115.8	49.8	55.75	75.81	52.89
25OHD (ng/ml)	7.41	13.79	26.94	19.16	32.05
Ca (mmol/L)	2.25	2.37	2.24	2.18	2.32
P (mmol/L)	1.3	1.34	1.16	1.53	1.24
L1-L4 Z-Score	−3.2	–	−1.0	0.3	–
FN Z-Score	1.3	–	1.2	8.2	–
TH Z-Score	−0.3	–	0.9	7.2	–

Notes: Values marked with asterisk (*) indicate the levels of markers were higher than the reference range; "–" means not available. RF: age-specific and age-specific reference range of markers (ALP, β-CTX and OC) (Choi et al., 2019; Diemar et al., 2021; Hu et al., 2013; Zhang et al., 2019). Reference range: hsCRP: 0–10 mg/L; ESR: 0–24 mm/h; PTH: 15–65 pg/ml; 25(OHD): >20 ng/ml; Ca: 2.08–2.6 mmol/L; P: 0.8–1.6 mmol/L.

Abbreviations: ALP, alkaline phosphatase; BMD, bone mass density; CAEND, Camurati-Engelmann disease; ESR, erythrocyte sedimentation rate; F, female; FN, femoral neck; hsCRP, high-sensitivity C-reactive protein; M, male; OC, osteocalcin; PTH, parathyroid hormone; TH, total hip; β-CTX, β-isomerized C-terminal cross-linked telopeptide of type I collagen; 25OHD, 25-hydroxyvitamin D.

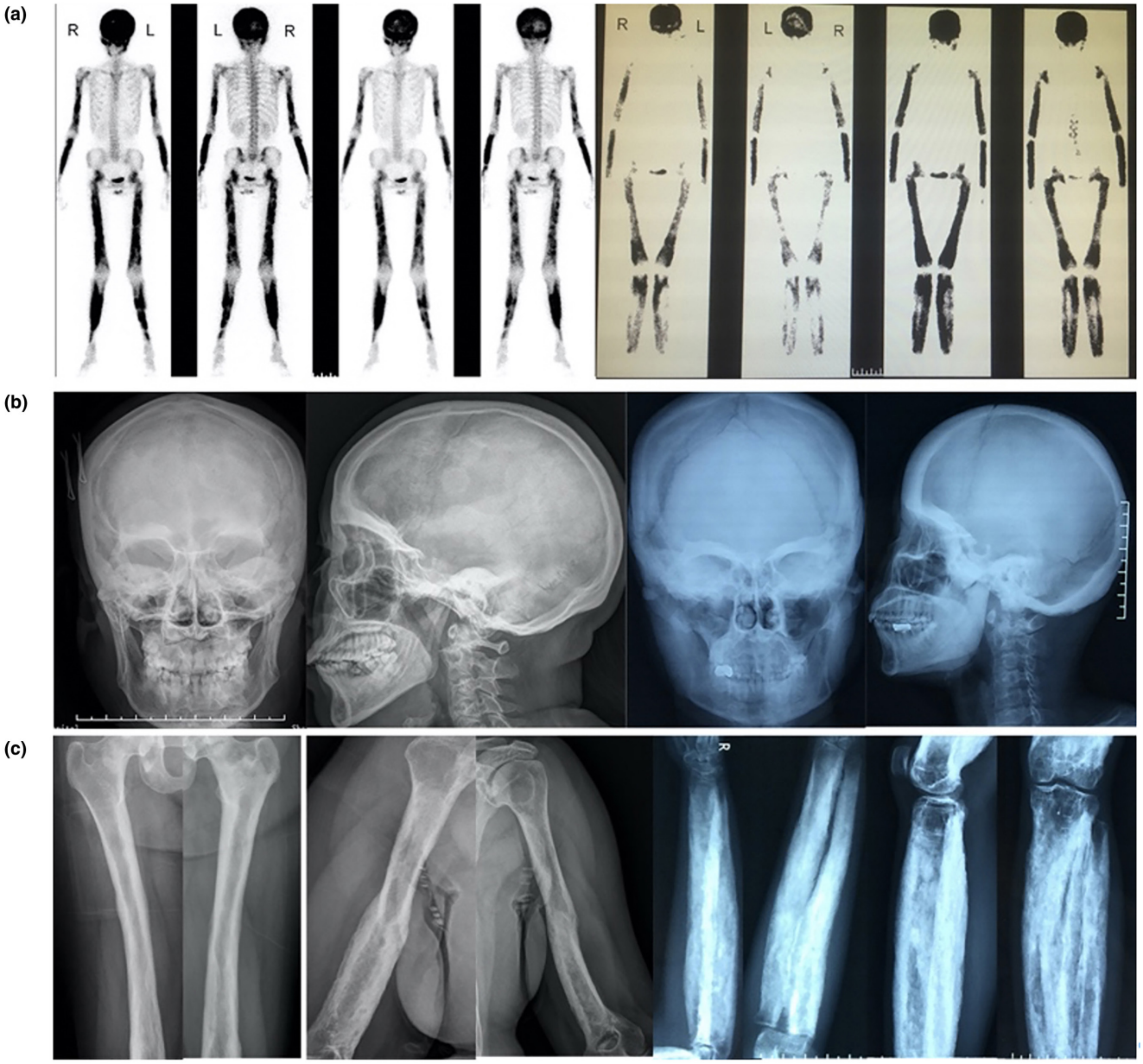

FIGURE 3 Typical radiographic manifestations of CAEND patients. The imaging findings of proband 1 (22 years, woman) and proband 2 (34 years, woman) were shown on the left and right, respectively. (a) Bone scintigraphy with ^{99m}Tc-methylene diphosphonate showed a marked symmetrical increase in radioactivity at the limbs and skull; (b) X rays revealed uneven or significantly increased bone mineral density of cranial bones, along with the thickened diploic bone and sclerosis of the skull; (c) X rays showed periosteal and endosteal thickening of the diaphyseal of the long bones, as well as the enlarged tubular bone diaphysis of extremities and narrowed medullary cavity

the long bone shaft with irregular periosteal sclerosis and sclerosis of the skull (Figure 3).

3.3 | Identification of TGFB1 variants

Molecular analysis demonstrated that proband 1 (III:5, family 1), III:6 (family 1), II8 (Family 1), and proband 2 (III:2, family 2) harbored the variant c.652C > T (p.Arg218Cys) in exon 4 of *TGFB1* gene, whereas proband 3 (III:2, family 3) and II:1 (family 3) possessed the variant c.669C > G (p.Cys223Trp) within exon 4 of *TGFB1* (Figure 4).

3.4 | Variant prediction and structural analysis

Multiple sequence alignment revealed that 223Cys was highly conserved in different species (Figure 5a), with scores of 1.00 and −9.162, respectively predicted by PolyPhen-2 and PROVEAN software (Figure 5b,c). Furthermore, the

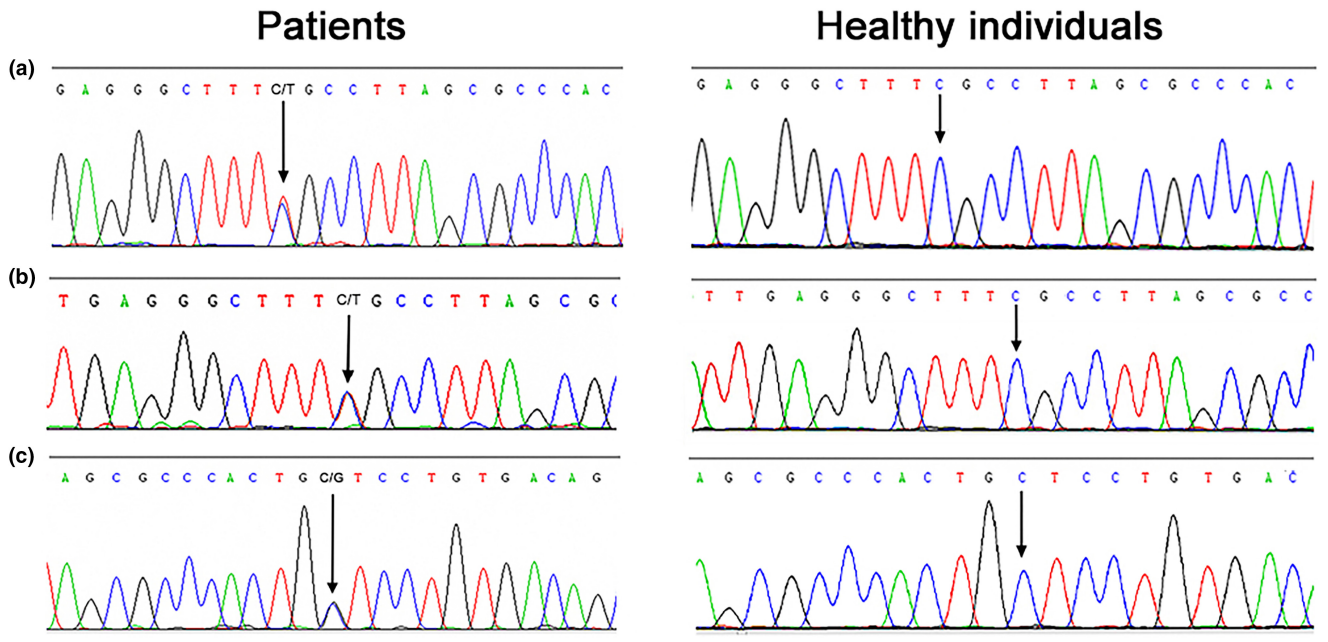

FIGURE 4 Sequencing analysis of the three probands. DNA and Protein GenBank Accession: NM_000660.7, NP_000651.3. (a) In proband 1, a heterozygous missense variant occurred in exon 4 of *TGFB1* gene, resulting in p.Arg218Cys; (b) In roband 2, a heterozygous missense variant occurred in exon 4 of *TGFB1* gene, resulting in p. Arg218Cys; (c) In proband 3, a heterozygous missense variant occurred in exon 4 of *TGFB1* gene, resulting in p.Cys223Trp

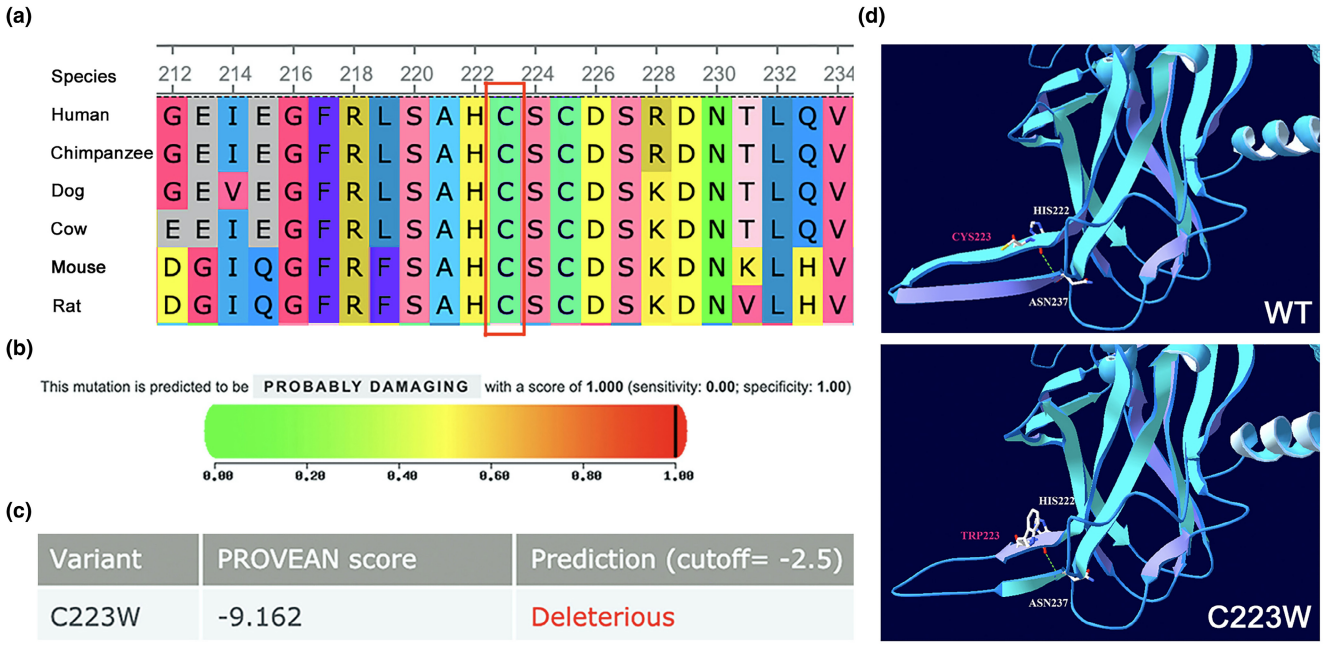

FIGURE 5 Effects of amino acid substitution caused by Cys223Trp on the structure and function of TGFB1. DNA and Protein GenBank Accession: NM_000660.7, NP_000651.3. (a) Multiple sequence alignments revealed that the Cys223 residue in the TGFB1 protein was highly conserved among species; (b) PolyPhen-2 prediction revealed that Cys223Trp variant in TGFB1 was probably damaging; (c) PROVEAN indicated the amino-acid substitution due to the variant was deleterious; (d) Comparison of the Three-Dimensional Modeling of native and mutated local structures

conversion of cysteine to tryptophane disrupted the construction of disulfide bonds between two latency-associated peptide (LAP) molecules, exerting a negative effect on the formation of dimerization and reducing the structural stability of the homodimer by three-dimensional structure prediction (Figure 5d). These results indicated that the novel variant p. Cys223Trp in the *TGFB1* gene played an essential role in the pathogenesis of CAEND.

3.5 | Follow up

Proband 1 complained that her pain worsened after being treated with zoledronic acid intravenously 3 years ago. Additionally, she responded pool to corticosteroids. Therefore, proband 1 (III:5, family 1), III:6 (family 1), and III:2 (family 3) were treated with NSAIDs for pain relief. Proband 2 refused to utilize corticosteroids and NSAIDs; hence, she received the tentative treatment with zoledronate intravenously due to her high level of BTMs. Three months after the administration of zoledronate, laboratory examinations showed that ALP, β-CTX, and OC were 324 U/L (baseline 369 U/L), 4860 ng/L (baseline 5432 ng/L), and 175.9 ng/ml (baseline 72.4 ng/ml), respectively. The mentioned data indicated that the drug failed to suppress the bone turnover, and the patient complained of no improvement in bone pain.

4 | DISCUSSION

TGFB1 is one of the members of the transforming growth factor-ß (TGF-ß) superfamily that encodes the precursor complex, including a signal peptide, LAP, and mature TGFβ1 protein. The LAP and TGFβ1 proteins form a homodimer linked by non-covalent bonds, which are secreted and stored in the extracellular matrix (Janssens et al., 2000). TGFβ1 became active by dimerization via cysteine bridges and cleavage at the dibasic protease site by furin (Hata & Chen, 2016). TGFβ1 is the richest cytokine in the bone matrix, where it becomes the coupling regulator to be involved in bone formation and resorption. In addition, it can promote the differentiation and proliferation of osteoblasts and inhibit the formation of muscle and fat mass (Ignotz & Massagué, 1985; Massagué et al., 1986).

Arg218Cys, Arg218His, and Cys225Arg of the *TGFB1* gene are the most frequently occurring variants in patients with CAEND. Among them, Arg218Cys substitution is the hotspot mutation, accounting for approximately 60% of all variants (Janssens et al., 2006). Previous studies also reported three different variants of Cys at position 223: Cys223Arg, Cys223Ser, and Cys223Gly additionally (Van Hul et al., 2019). Our study detected a novel variant Cys223Trp, which broadened the variant spectrum of pathogenic genes. The hotspot variant Arg218Cys and the novel variant Cys223Trp are both located in exon 4 at the C-terminal region of LAP, it is pivotal for the construction of a dimer cysteine bridge. Therefore, we hypothesized that both Arg218Cys and Cys223Trp variants reduced the binding ability between the LAP and mature TGFβ1 rather than affecting protein secretion, causing the increased activity of TGFβ1.

From our study, it was found that the clinical phenotype of the disease had significant variations, even if it was the same variant site or within the same family. However, patients with the Arg218Cys variant seemed to have more severe symptoms than patients who carried the Cys223Trp variant. The above variant clinical manifestations may be closely related to post-translational modification and other regulatory factors, and mechanical stimulation may also play a partial role (Hering et al., 2002; Hughes et al., 2019). Owing to the rarity and heterogeneity of CAEND, this relationship was deserved to be verified in larger samples. In addition, II:8 (family 1) showed severe typical CAEND symptoms in childhood, whereas she recovered from bone pain at the age of 30 without any treatment. This spontaneous remission was in accordance with previous studies (Collet et al., 2013; Hughes et al., 2019), which may be caused by decreased concentrations of TGFβ1 with aging contributing to the disease (Nicolas et al., 1994; Pfeilschifter et al., 1998).

What interested us, however, was the woman who carried the novel variant C223W with heart valve impairment and tachycardia, which had never been documented in previous studies. Interestingly, cardiac abnormalities are typical clinical manifestations of diseases caused by variants in other genes in TGF-β signaling pathway (Singh et al., 2006; Stheneur et al., 2008). However, since this phenomenon is only a case report and lacks clinical details, whether the atypical cardiac abnormalities are related to CAEND remains to be further investigated.

In our study, high bone turnover markers were detected in patients III:5 (family 1) and III:2 (family 3). Coincidentally, these two patients had more severe phenotypes than the other patients in our study. Moreover, most patients had elevated ESR and hsCRP levels. It is suggested that abnormal inflammatory markers and BTMs should be considered in the diagnosis and prediction of CAEND.

Regarding treatment, there are no effective therapies to date. Bisphosphonate, the powerful anti-resorptive agent, is controversial in the treatment of CAEND (Chérié-Lignière et al., 1999; Inaoka et al., 2001; Janssens et al., 2006). Unfortunately, in the present study, probands 1 and 2 did not ameliorate after the treatment with zoledronic acid and even aggravated bone pain. Intriguingly, it seemed that men tended to experience better therapeutic effects than women (Baroncelli et al., 2017; Castro et al., 2005; Iba et al., 2008; Inaoka et al., 2001; Savoie et al., 2013). Whether sex hormones play a role in the therapeutic response of bisphosphonates to diseases remains unknown and should be studied in the future. In any case, there is no effective treatment for the disease at present. The in-depth investigation of the pathogenesis of CAEND would be helpful to develop targeted treatment drugs.

There are, however, many limitations in our study. On the one hand, the number of patients is not large enough

to extend to phenotypic spectrums of CAEND in China. On the other hand, bioinformatics methods were insufficient to illustrate the function of the novel mutation. Further functional experiments are required to verify this finding.

5 | CONCLUSIONS

In this study, we analyzed the clinical features of CAEND and identified one novel *TGFB1* mutation site. Our findings will contribute to deepening clinicians' understanding of the disease and expanding the mutation spectrum. Additionally, it is recommended that both BTMs and inflammatory biomarkers should be tested to assist in the diagnosis of CAEND and evaluation of the disease activity.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Xiao-Hui Tao and Xing-Guang Yang carried out investigation, formal analysis, data collection, and writing—original draft of the manuscript. **Zi-Yuan Wang** was involved in data collection. **Yang Xu** was involved in data collection. **Yun-Qiu Hu** carried out data curation and resources. **Zhen-Lin Zhang** carried out project administration, validation, supervision, and writing—review and editing; **Hua Yue**: was involved in conceptualization, funding acquisition, supervision, and writing—review and editing of the manuscript.

ETHICS STATEMENT

The study protocol was approved by the Ethics Committee of the Shanghai Sixth People's Hospital of Shanghai Jiao Tong University. The study was conducted consistent with the Declaration of Helsinki.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Zhen-Lin Zhang https://orcid.org/0000-0002-4339-1617
Hua Yue https://orcid.org/0000-0002-9179-5894

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