Clinical Characteristics And Identification of Novel TGF-β1 Mutation In Three Unrelated Chinese Families With Progressive Diaphyseal Dysplasia

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Research Article

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Abstract

Background

To investigate the clinical characteristics and molecular diagnosis of progressive diaphyseal dysplasia (PDD) in three unrelated Chinese families.

Methods

The present study recruited six patients aged 14 to 45 from three unrelated families with PDD, including five females and one male. Clinical manifestations, biochemical tests, radiographic examinations were analyzed and the *TGF-β1* gene mutation was further identified by Sanger sequencing. In addition, data of treatment and follow-up were also collected.

Results

The onset age of the patients ranged from 1 to 6 years. All the affected patients had family histories and were consisted with autosomal dominant inheritance pattern. All of them exhibited gait disturbance, fatigue, progressive bone pain, as well as muscle atrophy and weakness in limbs. Notably, there was one 15-year-old girl who experienced heart valve defects and tachycardia at birth. Laboratory examinations revealed the inflammatory markers were in high level, besides the extremely increased bone metabolism indicators. The thickened diaphysis of long bones and the narrowed medullary cavity were observed by radiography. Furthermore, radionuclide bone scan detected abnormal symmetrical radioactive concentration in the affected regions of bone. Sanger sequencing identified a missense heterozygous mutation in exon 4 of *TGF-β1* gene, resulting in R218C, which confirmed PDD eventually. More important, a novel mutation c.669C>G in exon 4 of *TGF-β1* gene harboring C223W were detected in family 3. Subsequent bioinformatics software predicted that the novel mutation was pathogenic. Our study also showed that zoledronic acid was not effective in the control of bone turnover markers and the relief of bone pain in patients with PDD.

Conclusion

In addition to the typical PDD manifestations, the new phenotypic characteristics such as tachycardia and heart valve defect were firstly reported in one female patient carried the novel mutation p.Cys223Trp in *TGF-β1* gene. In addition, our study indicated that the increased bone metabolism indicators and inflammatory markers may possess auxiliary diagnosis for PDD. More importantly, zoledronic acid was used to treat PDD patients in this study. After one-year follow-up, it was proved that the drug effect was not satisfactory, and new drugs need to be developed to treat the disease.

Background

Progressive diaphyseal dysplasia (PDD), also known as Camurati-Engelmann disease (CED, OMIM 131300) is an autosomal dominant disorder, usually caused by *transforming growth factor-beta1* (*TGF-*

β1) gene mutations. Waddling gait, pain in extremities, muscular weakness, and cortical thickening of the diaphysis of the long bones[1, 2] are the hallmark clinical manifestations and imaging features. If the lesions involve skull, will lead to cranial nerve damage, such as hearing impairment and vision loss[3, 4]. Some cases have systemic manifestations or like some kind of syndromes, accompanied with anemia, leukopenia and hepatosplenomegaly for instance[5]. Since PDD is a kind of rare disease, to date, there are altogether 300 cases with PDD have been reported over the world[4, 6–12]. Therefore, the diagnosis of PDD is a great challenge for clinicians owing to its rarity and extensive phenotypic variations. The present study fully analyzed the clinical characteristics and identified the major causative gene mutations in 6 patients from 3 unrelated families with PDD. Meanwhile, the relevant reported literatures about the disease were reviewed simultaneously, in order to improve the awareness of the rare disease in the clinicians. It is of interest to note that we identified one novel mutation p.Cys223Trp of *TGF-β1* gene in one of the female patients with a heart valve defect and tachycardia. The research result will provide important help for us to further understand the rare disease, and also expand the pathogenic gene mutation spectrum and phenotype of the disease.

Methods

2.1 Subjects

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

Table 1. Clinical data of the six cases with PDD

	Family 1	Family 1	Family 1	Family 2	Family 3	Family 3
	III5	III6	II8	III2	III2	II1
Gender/Age (year)	F/22	F/16	F/45	F/34	F/14	M/38
Age of onset (year)	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	Mild	Mild	Marked	Mild	-
Muscle weakness	Marked	Mild	Mild	Marked	Mild	-
Skin temperature increase	Mild	-	-	Mild	-	-
Cranial nerve impairment	-	-	-	Marked	-	-
Dizziness	Mild	-	-	Marked	-	-
Vision impairment	-	-	-	Marked	-	-
Exophthalmos	Mild	-	-	Marked	-	-
Hearing loss	-	-	-	-	-	-
Infrequent menses	Mild	-	-	Marked	-	-

PDD: Progressive diaphyseal dysplasia.

"–" means no symptom.

2.2 Medical history collection

Anthropology information (height, weight, age, *etc.*), comprehensive clinical evaluation and family history were obtained. Pedigrees of the three families were showed in Figure1.

2.3 Laboratory and imaging evaluation

The clinical routine biochemistry tests, bone turnover markers (BTMs), bone scintigraphy and radiological examinations were detected in the subjects with PDD. Bone mineral density (BMD) of the lumbar spine (L1–4), left femoral neck and total hip were gauged by a lunar prodigy dual-energy X-ray absorptiometry (DXA) densitometer (GE Healthcare, Madison, USA).

2.4 Genetic analysis and mutation prediction

3mL peripheral blood sample was collected from each subject and genomic DNA was extracted using the conventional method. Primers of *TGF-β1* gene were designed using Primer3 software for the

amplification. After 95ºC pre-denature for 2 min, PCR amplifications were performed for 35 cycles * (96ºC 10 s, 68 ºC 1min, 72°C 1min). Subsequently, all exons together with the exon and intron boundaries of *TGF-β1* gene 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.

PolyPhen-2, PROVEAN and UniProt database were used to predict the variant and analyze the amino acid conservation. Furthermore, the ExAC and 1000 Genomes Project databases were used to identify the novel mutations. The protein sequence of TGF-β1 was obtained from the UniProt database in FASTA file form. The three-dimensional structure homology modeling and visualization of the native and mutant protein were deduced using the online SWISS-MODEL system and PyMol software.

Results

3.1 Family characteristics

3.1.1 Family 1

Proband 1 was a 22-year-old girl, born from a non-consanguineous family at full term. She began to walk at 14-month with normal gait. At the age of 4, waddling gait occured. Two years later she was difficulty in climbing stairs, along with generalized weakness, increased local skin temperature and gradually aggravated pain in four limbs. 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 other hospital. However, bone pain became even serious. Physical examination revealed the muscular atrophy of lower limbs and valgus knee deformity (Figure 2A). Her little sister (III6) could walk by age 2, waddling gait and mild leg pain occurred at 6 and 12 years old, respectively. Their 45-year-old mother (II8) complained that she had similar symptoms in adolescence. It was of interest to note that intensive bone pain gradually relieved and muscle mass and strength of both lower limbs increased with weight gain (BMI 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 normal muscle mass of limbs. The proband's cousin (III3) also developed intensive bone pain and symptoms improved after glucocorticoid treatment. The uncle of the proband 1 (II2) was an asymptomatic suspected patient.

3.1.2 Family 2

Proband 2 was a 34-year-old female who was married but never giving birth. She was able to walk at the age of 1, simultaneously waddling gait and abnormal knee-joint were observed. Since then, she fell down many times, whereas no fractures happened. 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 developed bilateral visual field defects gradually, accompanied with headache and dizziness. She also complained of hypomenorrhea and delayed cycle (four or five per year)

since menarche at 17-year-old. Physical examinations showed muscle atrophy in lower limbs, genu valgus deformity (Figure 2B), as well as the limitation of multi-joint mobility (Figure 2C). Her uncle (II5), aunt (II9), cousin (III5), and nephew (IV1) all presented with wide-based gait and no other symptoms. The cousin (III4) of the proband had 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 girl who developed tachycardia and heart valve defect at birth. She underwent cardiac valve repair surgery at 5 (details were not available). She could walk at 15-month, 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 three months ago, so she came to our department for help. While her father (I1) was diagnosed with PDD by genetic screening with a normal phenotype.

3.2 Blood test and imaging examinations

The laboratory examinations and BMD of the patients were shown in Table 2, except I1 in Family 3 who refused to do any medical tests. BTMs such as β-C-telopeptides of type I collagen (β-CTX) and osteocalcin (OC) were highly elevated in the above patients. Moreover, most of them showed increased levels of inflammatory biomarkers such as erythrocyte sedimentation rate (ESR, 100%, 5/5) and high-sensitivity C-reactive protein (hsCRP, 66.7%, 2/3).

Table 2. Laboratory examinations and BMD of five cases with PDD

	Family 1	Family 1	Family 1	Family 2	Family 3	Normal range
	III5	III6	II8	III2	III2	
CRP (mg/L)	28.97	6.76	-	32	-	0-10
ESR (mm/h)	120	42	57	56	30	0-26
ALP (U/L)	301	157	69	369	139	15-112
β-CTX (ng/L)	2878	1446	458.2	5432	855.9	Male (30-50 yrs: <84, 50-70 yrs: 704, > 70 yrs: <854); Female (premenopausal: <573, postmenopausal: <1008)
OC (ng/ml)	112.1	50.18	17.44	72.4	62.37	Male (18-30 yrs: 24-70, 30-50 yrs: 14-42,> 50 yrs: 14-46); Female (premenopausal: 11-34, postmenopausal: 15-46)
PTH (pg/ml)	115.8	49.8	55.75	75.81	52.89	15-65
25OHD (ng/ml)	7.41	13.79	26.94	19.16	32.05	>20
Ca (mmol/L)	2.25	2.37	2.24	2.18	2.32	2.08-2.6
P (mmol/L)	1.3	1.34	1.16	1.53	1.24	0.8-1.6
L1-L4 Z-Score	-3.2	-	-1.0	0.3	-	-
FN Z-Score	-3.2	-	1.2	8.2	-	-
TH Z-Score	-0.3	-	0.9	7.2	-	-

“–” means not available.

Abbreviations: PDD: Camurati-Engelmann disease; CRP: high-sensitivity C-reactive protein; ESR: erythrocyte sedimentation rate; ALP: alkaline phosphatase; β-CTX: β-isomerized C-terminal cross-linked telopeptide of type I collagen; OC: osteocalcin; PTH: parathyroid hormone; 25OHD: 25-hydroxyvitamin D; FN: femoral neck; TH: total hip.

In addition, the BMD of 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 the cortical bone thickening and irregular periosteal sclerosis of the long bone shaft as well as the sclerosis of skull (Figure 3)

3.3 Identification of *TGFβ1* mutations

As shown in Figure 4, the proband 1 (III5), III6, and II8 in Family 1, along with the proband 2 (III2) in Family 2 harbored the c.652C> T mutation in exon 4 of *TGF-β1* leading to p.Arg218Cys (R218C, NM_000660.7) by Sanger sequencing. Whereas proband3 (III2) and II1 in Family 3 possessed the c.669C>G mutation in exon 4 of *TGF-β1* leading to p.Cys223Trp (C223W, NM_000660.7), which was a novel mutation, never found in ExAC nor 1000 Genomes Project databases.

3.4 Gene Mutation Damaging Prediction and Structural Analysis

Multiple sequence alignment revealed that 223Cys were highly conserved in different species (Figure 5A), with scores 1.00 and -9.162, respectively predicted by PolyPhen-2 and PROVEAN software (Figure 5B and 5C). Furthermore, the conversion of Cysteine to Tryptophane disrupted the construction of disulfide bond between two latency associated peptide (LAP) molecules, exerting a negative effect on the formation of dimerization and reducing the structural stability of homodimer by three-dimensional (3D) structure prediction (Figure 5D). The above mentioned data indicated that the novel mutation played an important role in the pathogenesis of PDD.

3.5 Follow up

Proband 1 in family 1 claimed that her bone pain couldn’t be relieved after been treated with zoledronate three years ago, so she declined to accept the treatment once more. Whereas III5, III6 in Family 1 and III2 in Family 3 were treated with pain killer (such as NSAIDs). However, proband 2 was treated with zoledronate due to her high levels of BTMs. Three months later, laboratory examinations showed ALP, β-CTX and OC was 324u / L (baseline 369u / L), 4860ng / L (baseline 5432ng / L) and 175.9ng/ml (baseline 72.4ng / ml), respectively. The above data indicated that the curative effect of zoledronate was inadequate.

Discussion

PDD was reported by Cockayne[13] in 1920 for the first time, Camurati[14] determined the disease was hereditary two years later. In 1929, Engelmann[15]described a typical case with muscle atrophy and bone involvement. Since then, the disease had been widely known as “Camurati-Engelmann disease”. In 1948, Neuhauser found that the clinical characteristics of CED were sclerosis and thickening of the long bones, hence he coined the term" progressive diaphyseal dysplasia". With the rapid development of high-throughput sequencing and linkage inheritance technologies, the etiology of PDD was considered to be the heterozygous mutations of *TGF-β1* gene in 2000[12-14].

TGF-β1 is one of the members of TGF-β superfamily encodes the precursor complex, including signal peptide, LAP and mature TGF-β1 protein. The LAP and TGF-β1 protein form the homodimer linked by non-covalent bonds, which is secreted and stored in the extracellular matrix[10]. TGF-β1 became active by dimerization via cysteine bridges and cleavage at the dibasic protease site by furin[16]. TGFβ1 is the richest cytokine in bone matrix, where it becomes the coupling regulator to be involved in the process of bone formation and resorption. It can promote osteogenesis and inhibit the formation of muscle and fat mass[17, 18]. In addition, TGF-β1 can also regulate cell proliferation and differentiation, along with embryogenesis, angiogenesis and immunosuppression[19].

R218C, R218H and C225R of *TGF-β1* gene are the most frequently occurred mutations in patients with PDD, among them R218C substitution is the hotspot, accounting for approximately 60% of all the mutations[4]. Previous studies also reported three different mutations of Cys at position 223: C223R, C223S and C223G additionally[3]. In our study, a novel mutation C223W was detected, which broadened the mutation spectrum of pathogenic genes in Chinese. Since the hotspot mutation R218C and the novel mutation C223W are both located in exon 4 at the C-terminal region of LAP, which is pivotal for the construction of dimer cysteine bridge. Therefore, the mutations reduced the binding ability between the LAP and mature TGF-β1 rather than affecting the protein secretion, causing the increased activity of TGF-β1.

Our study demonstrated the clinical features of PDD, and furthermore analyzed the relationship between the genotype and phenotypes. From our study, it was found that the clinical phenotypes of the disease had great variations, even it was the same mutation site or within the same family. However, it seemed that patients with R218C mutation had more severe symptoms than patients carried C223W mutation. 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[9, 20]. Previous study in large pedigrees had described a tendency of PDD to occur earlier and more severe in the younger generations [7, 11, 12], which was genetically named “anticipation”. In the present study, family 1 and family3 were consistent with the findings of the above. As for family 2, we harbored the idea that IV1 in the younger generation might become serious later, which supported the presence of anticipation partially. Owing to the rarity and heterogeneity of PDD, this relationship was deserved to be verified in larger samples. In addition, II8 in Family 1showed typical PDD symptoms in childhood, whereas she recovered from bone pain at the age of 30 without any treatment. This spontaneous remission was in accordance with the previous studies[9, 21]. Our study indicated that the decreased concentration of TGF-β1 with aging may contribute to this condition[22, 23].

What interested us, however, was the girl who carried the novel mutation C223W with the heart valve impairment and tachycardia, which had never been documented in the previous studies. Interestingly, cardiac abnormalities were typical clinical manifestations of the diseases caused by mutations in other genes in TGF-β signaling pathway[24, 25]. This atypical heart abnormality broadened the clinical phenotype spectrum of the disease and provided practical basis for further study of the pathogenesis of PDD.

In our study, high bone turnover markers were detected in all five patients. However, it was observed that patients with more severe clinical phenotypes had higher bone turnover markers, excepted II8 in Family 1 with spontaneous remission presented mild increased BTMs. Meanwhile, it is suggested that the abnormal inflammatory markers and BTMs should be paid attention to in the diagnosis and prediction of PDD. As to the treatment, there is no effective therapies to date. Bisphosphonate, the powerful anti-resorptive agent, was controversial in the treatment of PDD[4, 26, 27]. Proband 1 and 2 did not ameliorate after the treatment with zoledronate, even aggravated bone pain. However, our department had ever demonstrated the beneficial utility of both alendronate and zoledronate in two male patients with PDD in 2019[6]. Intriguingly, it seemed that men tended to gain better therapeutic effects better than women[7, 8, 26, 28, 29]. Whether sex hormones play a role in the therapeutic response of bisphosphonates to diseases is unknown, which is also one of the research contents we need to study in the future. In any case, there is no effective treatment for the disease at present. The in-depth investigation of the pathogenesis of PDD would be helpful to develop targeted treatment drugs.

Conclusions

In this study, the clinical features were fully analyzed and *TGF-β1* gene mutations were identified, and one novel pathogenic site was detected in a 15-year-old girl with cardiac abnormalities and mild joint pain, which would contribute to expanding the phenotype and mutational spectrum of PDD in Chinese. Meanwhile, it is recommended that both BTMs and inflammatory biomarkers should be tested to assist the diagnosis CED and evaluation of the disease activity.

Abbreviations

PDD: Progressive Diaphyseal dysplasia

TGF-β1: *transforming growth factor-beta1*

PDD: progressive diaphyseal dysplasia

DXA: dual-energy X-ray absorptiometry

BMI: body mass index

BMD: bone mineral density

BTMs: bone turnover markers

β-CTX: β-isomerized C-terminal cross-linked telopeptide of type I collagen

OC: osteocalcin

LAP: latency associated peptide

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the Shanghai Sixth People's Hospital of Shanghai Jiao Tong University. All methods were carried out used in this study adhere to the tenets of the Declaration of Helsinki.

Written informed consent was obtained from the patients or their legally authorized representatives who participated in this study. A copy of the written consent is available for review by the Editors-in-Chief of this journal.

Consent for publication

Written informed consent was obtained from the patients or their legally authorized representatives for publication of this paper and accompanying images.

Availability of data and materials

The data cannot be publicly shared because permission was not obtained from the participants but are available from the corresponding author on reasonable request.

Conflict of Interest

The authors declare no conflicts of interest.

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Author Contributions

Xiao-Hui Tao and **Xing-Guang Yang**: Investigation, Formal analysis, Data collection, Writing - original draft; **Zi-Yuan Wang**: Data collection; **Yang Xu**: Data collection; **Zhen-Lin Zhang**: Project administration, Validation, Supervision, Writing - review & editing; **Hua Yue**: Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

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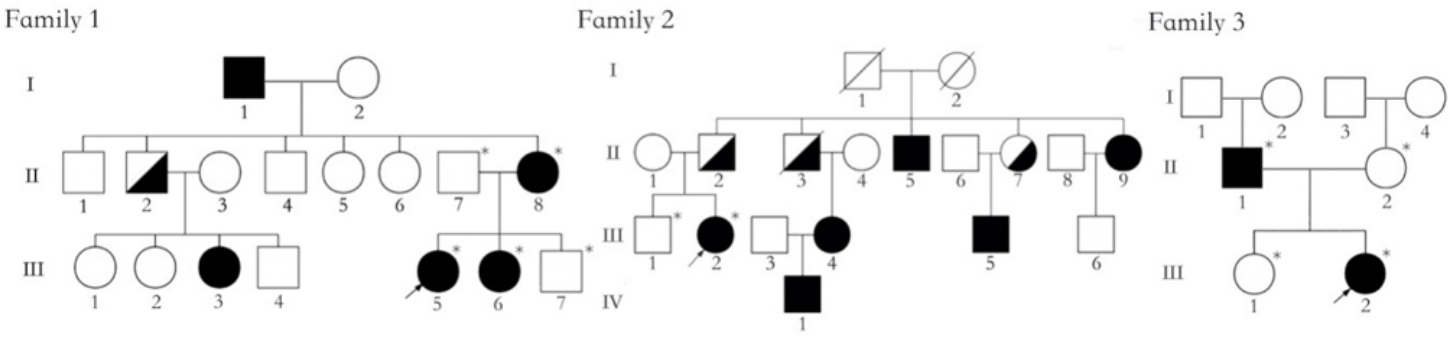
Figures



The arrow indicated the proband. ● ■ Indicated the affected female or male, ◪ ◪ indicated the asymptomatic suspected patients; members marked with asterisk (*) represented subjects included in Sanger sequencing.

PDD: Progressive diaphyseal dysplasia.

Figure 1

Pedigrees of three unrelated Chinese families with PDD

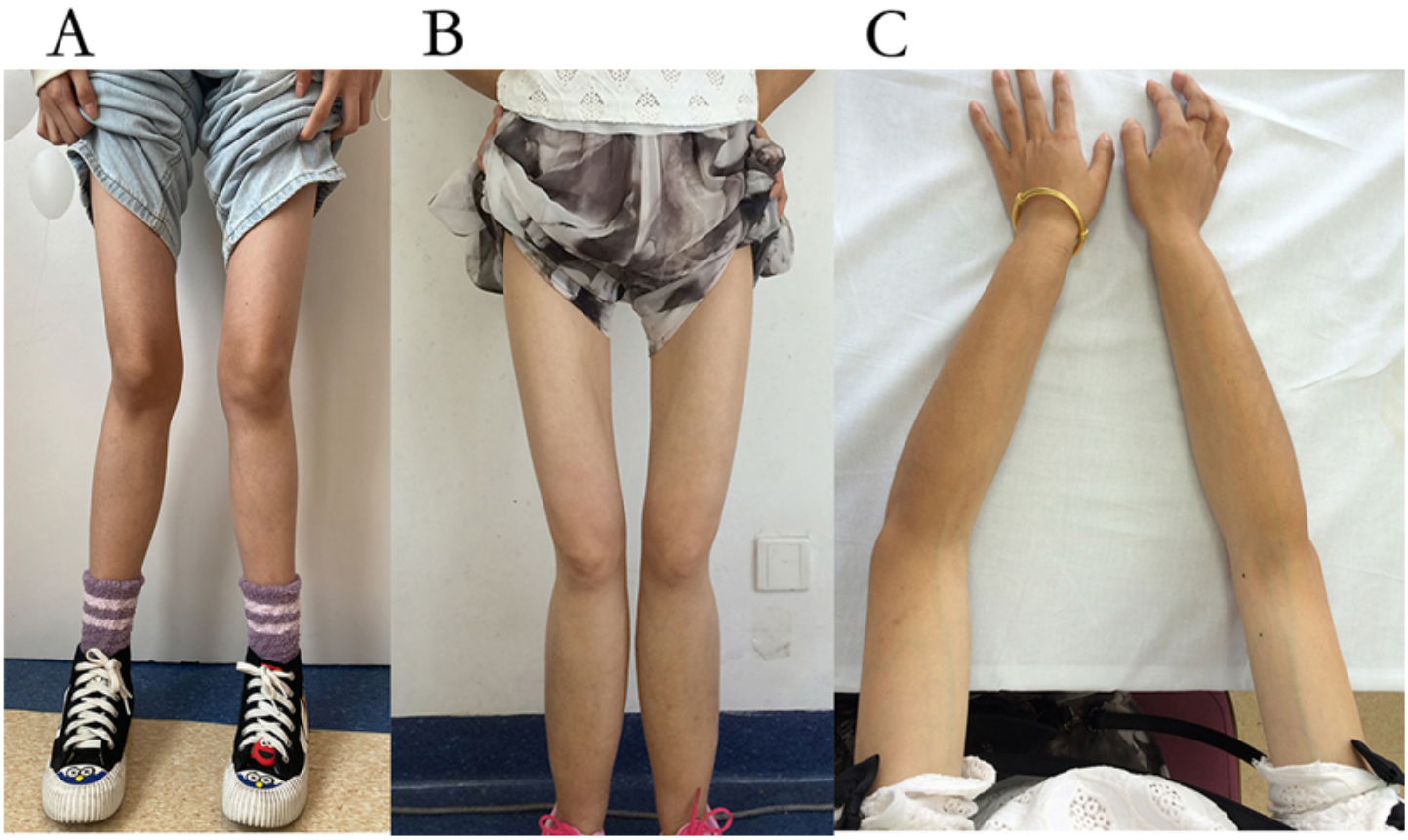


Figure 2

Classic clinical features of subjects with PDD A. Proband 1 was extremely thin and presented the decreased subcutaneous fat, genu valgus deformity, and muscular atrophy of both lower limbs. B. Proband 2 had similar clinical manifestations with the subjects as mentioned earlier, and she reported thickened long bones. C. Elbow joint of proband 2 was unable to straighten.

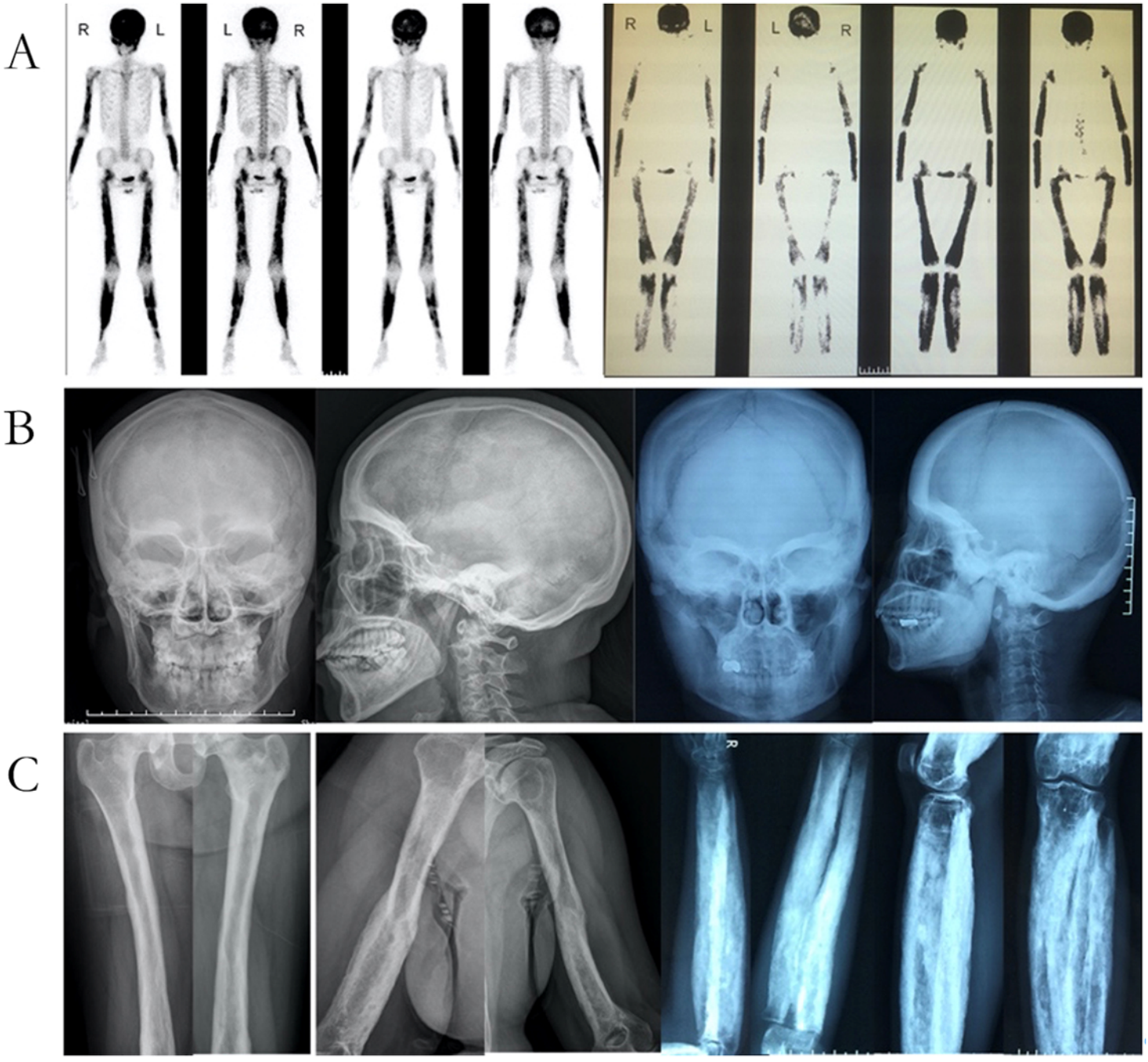


Figure 3

Typical radiographic manifestations of PDD patients A. Bone scintigraphy with 99mTc-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.

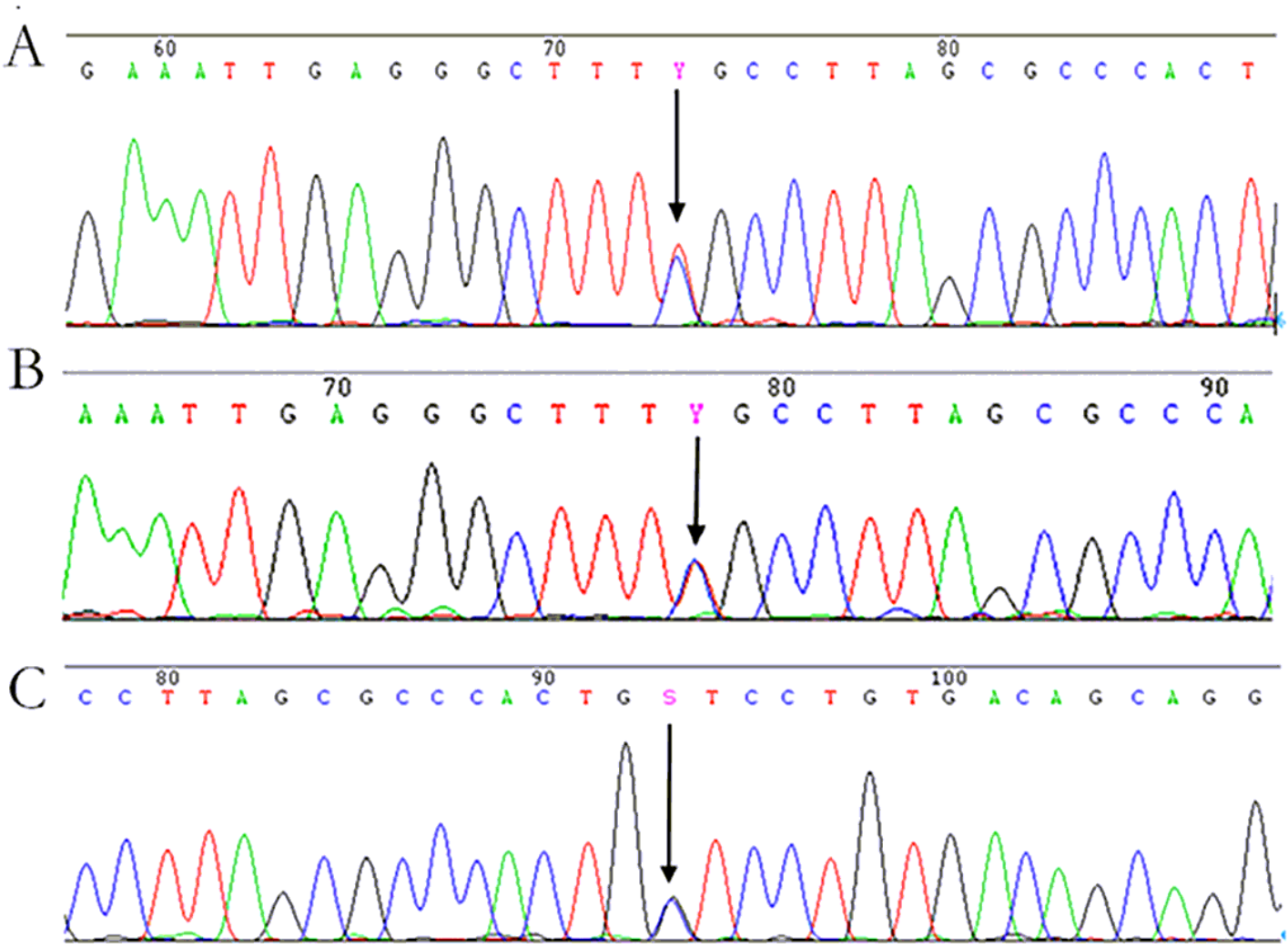


Figure 4

Sequencing analysis of the three probands A. In proband 1, a heterozygous missense mutation occurred in exon 4 of TGF-β1 gene, resulting in p.Arg218Cys; B. In roband 2, a heterozygous missense mutation occurred in exon 4 of TGF-β1 gene, resulting in p. Arg218Cys; C. In proband 3, a heterozygous missense mutation occurred in exon 4 of TGF-β1 gene, resulting in p.Cys223Trp.

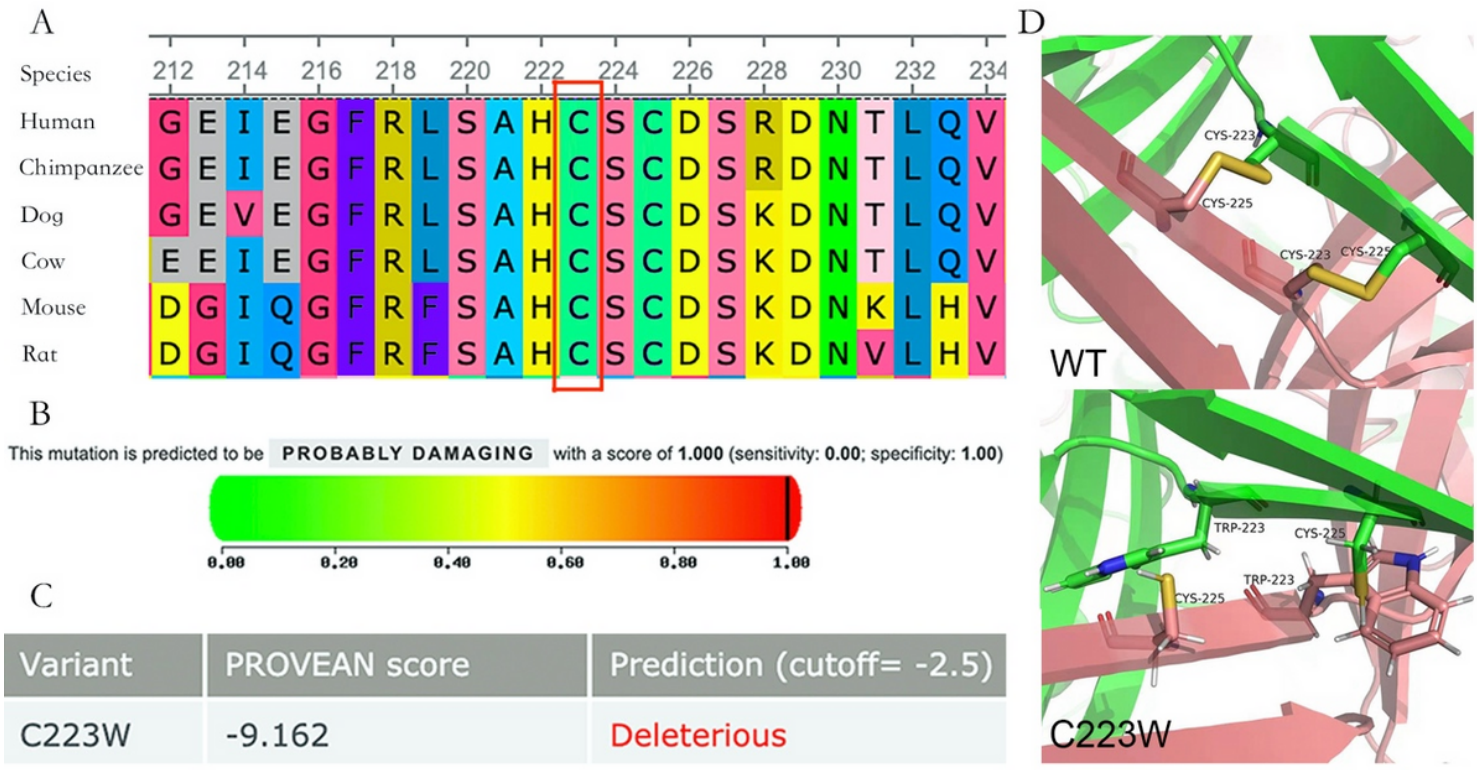


Figure 5

Effects of amino acid substitution caused by C223W on the structure and function of TGF-β1 A. Multiple sequence alignments revealed that the Cys223 residue in the TGF-β1 protein was highly conserved among species. B. PolyPhen-2 prediction revealed that C223W mutation in TGF-β1 was probably damaging. C. PROVEAN indicated the amino-acid substitution due to the mutation was deleterious. D. Comparison of the Three-Dimensional Modeling of native and mutated local structures.