Exome sequencing identified two novel COL10A1 heterozygous mutations in two irregular dominance Chinese pedigrees with Schmid-type metaphyseal chondrodysplasia

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Abstract
Background Schmid-type metaphyseal chondrodysplasia (MCDS) is an autosomal dominant disorder caused by COL10A1 mutations, which is characterized by short stature, waddling gait, coxa vara and bowing of the long bones. However, the research about unusual phenotype features of MCDS is rare.
Methods Two probands and available family members affected with MCDS were subjected to clinical and radiological examination. Genomic DNA of all affected individuals underwent whole-exome sequencing, and candidate mutations were verified by Sanger sequencing in all available family members and 250 donors. Then the spatial model of type X collagen (α1) C-terminal noncollagenous (NC1) domain was further constructed.
Results We found that the affected family members exhibited evident irregular dominance. Mutation analysis indicated that there were two novel heterozygous missense mutations c.1765T>A (p.Phe589Ile) and c.1846A>G (p.Lys616Glu) in the COL10A1 gene in family 1 and 2, respectively. The two novel substitution sites were highly conserved and the mutations were predicted to be remarkably deleterious in silico analysis. Furthermore, protein modeling revealed that the two substitutions located in the NC1 domain of collagen X (α1), which potentially impacted the trimerization of collagen X (α1) and combination with molecules in pericellular matrix.
Conclusion Two novel mutations were identified in the present study, which facilitated to diagnose MCDS and further expanded the spectrum of the COL10A1 mutations associated with MCDS patients. In addition, our research preliminarily elaborated the phenotype features and heredity characteristics of MCDS based on the two Chinese pedigrees.

Background
Schmid-type metaphyseal chondrodysplasia (MCDS; MIM 156500), the most common type of metaphyseal chondrodysplasia, is an autosomal dominant congenital disorder, which is characterized by short stature, waddling gait, coxa vara and bowing of the long bones (primarily involved in femur) [1,2]. Besides, the distinctive clinical features of MCDS also include leg pain, enlarged capital femoral epiphyses or partly metaphyseal abnormalities of upper limbs [3]. The typical radiographic findings of MCDS are the widening and irregularity of the growth plates, especially in the distal femur and
proximal tibia [4,5,6], but only a small percentage of patients have upper limbs involved [7]. In addition, it should be noted that patients affected with MCDS all exhibit normal extraskeletal manifestations [8]. Despite the significant clinical and radiological features found in MCDS patients, there is still no systematic description in terms of the phenotype and heredity characteristics of MCDS nowadays. COL10A1 (MIM 120110), located on chromosome 6q21-q22.3 [9], is closely associated with MCDS as the only confirmed pathogenic gene [10,11]. The product of COL10A1 gene is α1 of type X collagen, which composes the type X collagen by forming homotrimer [5]. Type X collagen is a member of the collagen superfamily of structural macromolecules, which has a unique expression pattern localized to the hypertrophic chondrocytes of growth plate cartilage [12]. In function, as a short-chain minor collagen of cartilage, type X collagen plays an important role in fetal chondrogenesis and endochondral ossification [5,13]. Therefore, specific mutations in COL10A1 are likely to result in occurrence of MCDS. Previously, a total of 44 mutations including 29 missense or nonsense mutations, 14 small deletions and 1 complex rearrangement in COL10A1 have been reported in Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/) successively (Summarized in supplementary data) [14,15]. Based on different mutation types, the mutations in COL10A1 can be divided into two categories: single amino acid residue lack or substitution resulting from missense mutations or small deletions (Class I) and truncated peptide attributing to nonsense mutations or frameshift mutations (Class II) [16,17]. Of note, the underlying pathogenic mechanisms of both are completely different. The present study describes unique phenotype features of two unrelated Chinese pedigrees with MCDS and elaborates the rules of the disease. Moreover, mutation screening used to support diagnosis was performed in the two unrelated Chinese MCDS families.

Methods

Patient families

Two independent non-consanguineous five-generation families (Fig. 1) containing nineteen MCDS patients, identified by two independent orthopedic surgeons, were recruited from the outpatient department of Shanghai Jiao Tong University Affiliated Sixth People’s Hospital (Shanghai, China) when
they sought treatment for the probands. All the available patients underwent comprehensive clinical and radiological measurements for diagnosis. Written informed consent was obtained from all of the participants. The present study was approved by the Ethics Committees of Shanghai Jiao Tong University Affiliated Sixth People’s Hospital (Shanghai, China).

Whole-exome sequencing and mutation confirmation
Peripheral blood samples from all of the available family members and 250 unrelated healthy donors aged 18 to 45 years old (125 males and 125 females) were collected and stored at -80°C. Genomic DNA was extracted from samples using the QuickGene DNA whole blood kit (Kurabo Industries Ltd., Osaka, Japan). BioAnalyzer 2100 was subsequently employed to assess nucleic acid quantity and quality. Then we sequenced the whole-exome of all available affected individuals and II1 in family 1 in order to determine the mutated gene resulting in this disorder. The SureSelect Human All Exon 57Mb Kit (Agilent Technologies, Inc., Santa Clare, CA, USA) and the HiSeq 2000 platform (Illumina, Inc., San Diego, CA, USA) were used to capture the whole-exome sequence following the manufacturer’s protocol. Illumina base-calling software v1.7 was then applied to convert the raw image files into 90-base-paired-end reads and all of the variants were validated.

In the first step, all detected variants were filtered against six single-nucleotide polymorphism databases, including dbSNP144 (hgdownload.cse.ucsc.edu/goldenPath/hg19/database/snp144), the HapMap Project (ftp.ncbi.nlm.nih.gov/hapmap), the 1000 Genomes Project (www.internationalgenome.org), the YanHuang database (yh.genomics.org.cn), the Exome Variant Server (evs.gs.washington.edu/EVS/) and the Exome Aggregation Consortium database (exac.broadinstitute.org), which rejected common single nucleotide polymorphism (SNP) sites. In the second step, common single nucleotide variants (SNV) among all family members in each family were of interest but synonymous or intronic variants not located within splice site were excluded. In the third step, we further checked the conservation of remained mutations using the UCSC database (https://genome.ucsc.edu/) and the causality of the altered amino acid residues by utilizing Polymorphism Phenotyping version 2 (Polyphen-2; genetics.bwh.harvard.edu/pph2/) and Protein Variation Effect Analyzer (PROVEAN; provean.jcvi.org/index.php/). Finally, the most likely pathogenic
candidate mutation was presented in accordance with gene functions and previous studies (Details in supplementary data).

The identified mutation regions and flanking sequence of the COL10A1 gene were amplified using a standard polymerase chain reaction protocol to facilitate Sanger sequencing among all available family members and donors. The primers were designed using Primer-3 software (bioinfo.ut.ee/primer3-0.4.0/). Direct sequencing was performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the sequence was analyzed with an ABI Prism 3130 automated sequencer.

Mutation was checked using Polyphred program (droog.mbt.washington.edu/poly_get.html).

**Protein spatial model construction**

The wild type ribbon structure of type X collagen (α1) C-terminal noncollagenous (NC1) domain was initially constructed using DeepView and SWISS-MODEL (swissmodel.expasy.org). In addition, to illustrate the positions of the pathogenic mutation and flanking regions, the amino acid residue substitutions were further incorporated into the model.

**Results**

**Clinical features**

Clinical findings in two MCDS pedigrees are primarily summarized in Table 1. The typical manifestations, including short stature, coxa vara, bowing of the femur and widening and irregularity of the growth plates of distal femur and proximal tibia in X-ray image, were partly found in all patients sooner or later after birth. However, the severity of symptoms varies between individuals.

Family 1

Apart from those typical clinical features, the proband 1 (V2), a 2-year-old boy, and his grandfather (III1) also presented remarkable waddling gait and their affected growth plates seemed like “cup” shape in X-ray image (Fig. 2A-C). Interestingly, the mother (IV4) of proband 1 only exhibited relatively short stature without other aforementioned clinical and radiological manifestations. Furthermore, the development and appearance of II1 were absolutely normal without any mental and physical deformity; so were all other unaffected family members.
Family 2

Proband 2, a 18 month-old boy, had exhibited typical clinical manifestations and remarkably slow growth since 6 months after birth (Fig. 2D E). But the clinical features of his mother (IV_4) remained mild, who was similar to IV_4 in family 1 (Fig. 2D F). Notably, most of the affected members in family 2 were male and there was a trend that male patients were significantly more severe than females.

Different from family 1, there was no phenomenon of atavism in family 2.

Genetic analysis

Whole-exome sequencing was followed by systematic screening and bioinformatic analysis. After rejecting common SNP sites (MAF>1%), SNV were captured. Considering the function of the mutant genes and the results of previous studies, the present study focused on the novel heterozygous missense mutation c.1765T>A (p.Phe589Ile) (NM_000493.3) in exon 3 of the COL10A1 gene in family 1 and another novel mutation c.1846A>G (p.Lys616Glu) (NM_000493.3) in identical region of the COL10A1 gene in family 2. Interestingly, the two novel mutations were not found in ExAC and gnomAD database, so the results further supported the point that the two mutations were novel and rare.

Of note, the changed amino acid residues were highly conserved among several species (Fig. 3E). The Polyphen-2 and PROVEAN scores of the variant c.1765T>A (p.Phe589Ile) were 1.000 and -2.69, respectively, which indicated a remarkably deleterious function; and the corresponding scores of the other variant c.1846A>G (p.Lys616Glu) were 1.000 and -1.633.

To confirm the identification of COL10A1 mutations revealed by whole-exome sequencing, we performed Sanger sequencing for all of the available family members and the 250 healthy donors. In accordance with the results of whole-exome sequencing, the mutation c.1765T>A (p.Phe589Ile) and c.1846A>G (p.Lys616Glu) were identified in the eight affected individuals and II_1 in family 1 and nine affected ones in family 2, respectively, but not in other available unaffected family members and the 250 healthy donors (Fig. 3A-D).

Protein structural model
According to the spatial ribbon structure of the protein, both of the mutant p.Phe589Ile and p.Lys616Glu were located in the NC1 domain of type X collagen (α1) (Fig. 4A B), where three identical regions interacted to form collagen X homotrimer. Moreover, the replaced wild type residues are remarkably conserved among various collagen. It is of note that the two substitutions locate on hydrophobic area and the surface of assembled homotrimer, respectively. The mutant p.Phe589Ile evidently weakens hydrophobicity of wild residue, and the other substitution p.Lys616Glu introduces the residue site from strong alkaline to acidic, both of which potentially destroyed the interaction between type X collagen (α1) peptide chains and other molecules or itself.

Discussion

There is no doubt that MCDS is an autosomal dominant inherited disorder, resulting from heterozygous mutation of COL10A1 gene [4,10,16]. In present study, probands with MCDS and affected family members were identified in two large independent Chinese pedigrees by means of typical clinical findings and genetic analysis. Interestingly, although these individuals suffered from identical disease, there were still remarkably variant severity of clinical manifestations among all patients in each family. Moreover, the presentations of II_1 in family 1 was normal absolutely including relatively moderate stature without any deformity, but the radiological examination was not performed for him due to individual reluctance, so we failed to acquire further evidence of clinical diagnosis. However, the mutation c.1765T>A in COL10A1 gene was confirmed in genome of II_1 in family 1, which was similar with a mutation-attacking person who exhibited normal phenotype in a previous report [18]. Therefore, we attributed the phenomenon of atavism and differential performance to irregular dominance based on identical pathogenic mutation in each family, possibly caused by existence of modifier gene and diverse surroundings. Recently, Forouhan et al. proposed that ATF6α and ATF6β played an important role in modulating disease severity in MCDS mice by positively or negatively regulating endoplasmic reticulum stress response [19], which we considered as the associated mechanism of the irregular dominance of these MCDS patients. However, further molecular experiments were needed.

Depending on the differential expressivity of all affected members in two Chinese families (Table 1),
we have managed to summarize the following possible rules on the pedigrees affected with MCDS [6,15]. First, based on the onset age, these patients were characterized by delayed dominance, which occurred until months or even a year after birth, 6 to 18 months old in present study, rather than at birth. Furthermore, the severity of disease was tightly associated with onset age presenting evidently negative correlation, that is, the earlier the onset age, the more severe the condition. For example, decreased quality of life including unstable standing or waddling gait was observed in those patients that onset age was only around 6 months old, but conversely there was only short stature in those abnormal development occurred in 10 months old or later. In addition, we found that there were possibly potential differences in gender susceptibility in MCDS, and 15 patients of 28 males (54%) and 5 patients of 16 females (31%) were affected in the two Chinese pedigrees. Moreover, despite the trend that male patients were more severe than females was presented in family 2, we still could not draw a firm conclusion due to the rather small sample size.

As for the molecular structure of type X collagen, it is a homotrimer of three α1 (X) chains, each comprised of a 463 amino acid Gly-X-Y collagenous domain (COL1) flanked by a 38-residue N-terminal noncollagenous domain (NC2) and a 161-residue C-terminal noncollagenous domain (NC1) (Fig. 4C) [9]. In addition, there is a 18-residue signal peptide ahead of NC2 domain. Up to now, a total of 44 mutations including 29 missense or nonsense mutations, 14 small deletions and a complex rearrangement of COL10A1 gene resulting to MCDS have been reported successively (Supplementary data). Obviously, all of the identified mutation sites of MCDS associated with COL10A1 including mutations in present study were located in NC1 domain [4,8,20,21,22,23], except for 2 missense mutation in signal peptide. The function of NC1 domain is mainly to assist the folding of the peptide chain and combine to form a homotrimer [5]. Therefore once impairment of NC1 domain occurred, the collagen X (α1) chains would be hindered to aggregate and form non-functional polymers instead, which tended to promote harmful accumulation of invalid products and even initiate the endoplasmic reticulum stress response [5,24]. On the other hand, the content of correctly folded homotrimer of collagen X reduced accordingly, and functional haploinsufficiency became the most probable cause of the clinical phenotype in MCDS [13,25,26].
According to the previous experiments, COL10A1 nonsense mutations in cartilage tissue led to removal of the mutant mRNA by nonsense-mediated mRNA decay (NMD), which was the pathogenic molecular mechanism of nonsense mutations in MCDS definitely [17,27]. Nevertheless, it was completely different that the process of COL10A1 missense mutations results in MCDS [16]. In the case of COL10A1 missense mutations, a common consequence appeared to be a disruption of collagen X trimerization, homeostasis, secretion and combination, with consequent intracellular or pericellular degradation [6,16]. In present study, one of the novel substitutions (p.Phe589Ile) locate on hydrophobic area and the other (p.Lys616Glu) on the surface of assembled trimer (Fig. 4A B). The substitution p.Phe589Ile evidently weakens hydrophobicity of wild residue, which seriously impacts the assembly and stability of hydrophobic channel of collagen X trimerization possibly. And the other substitution (p.Lys616Glu) transfers the alkaline residue site to acidity, potentially destroying the combination of trimeric collagen X to supramolecular structures within the cartilage pericellular matrix. In total, these biochemical and pathophysiological processes may explain the underlying mechanisms of MCDS in the two present pedigrees.

To our knowledge, the present study is the first report about COL10A1 missense mutations in Chinese pedigrees with MCDS, which lays the foundation for genetic research of MCDS in Chinese and further elaborates phenotype features and heredity characteristics of MCDS. In addition, dominant negative effect may be playing an important role in development of MCDS [16,28], but it has not been fully confirmed and still needs further experimental verification.

Conclusions
In summary, we identified two novel COL10A1 heterozygous missense mutations [c.1765T>A (p.Phe589Ile)] and [c.1846A>G (p.Lys616Glu)] in two relatively large unrelated Chinese pedigrees with MCDS, respectively. The genetic analysis facilitated to diagnose the disease and further expanded the spectrum of the COL10A1 mutations associated with MCDS patients. Besides, our research preliminarily elaborated several rules on occurrence and development of the disease based on the two MCDS pedigrees including the phenotype of delayed dominance, irregular dominance and the possible mechanism of MCDS. We also summarized the underlying pathogenic mechanisms of
**COL10A1 mutations.**

**Abbreviations**

MCDS: schmid-type metaphyseal chondrodysplasia; COL10A1: type X collagen (α1); COL1: collagenous domain; NC1: C-terminal noncollagenous domain; NC2: N-terminal noncollagenous domain; MAF: minor allele frequencies; NMD: nonsense-mediated mRNA decay

**Declarations**

**Acknowledgement**

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**Authors’ contributions**

L.C.K primarily analyzed the outcomes of whole-exome sequencing and Sanger sequencing, and was a major contributor in writing the manuscript. L.S collected the clinical information and peripheral blood samples of all of the participants, and contributed to the analysis of sequencing outcomes. W.B.W, R.T.Z and M.W.W contributed to the conducting of genetic analysis for the participants. Q.L.K conceived and designed the study and experimental methods. All authors read and approved the final manuscript.

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**Availability of data and materials**

Most of data generated or analyzed during this study are included in the article and supplementary files. The extra data used and analyzed in the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

Written informed consent was obtained from all of the adult participants and the guardians of participants under 16 years old. The present study was approved by the Ethics Committees of Shanghai Jiao Tong University Affiliated Sixth People’s Hospital (Shanghai, China).

**Consent for publication**

Written informed consent was obtained from all of the participants approving this non-commercial
research and the publication of any associated data/images.

Competing interests
The authors declare that they have no competing interests.

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Table
Due to technical limitations, Table 1 is only available as a download in the supplemental file section.

Figures

Figure 1
The pedigrees of the MCDS patients. The phenomenon of atavism was presented in family 1 (A), but not in family 2 (B). Genetic analysis was performed among all patients and available unaffected families members.
The clinical and radiological features of patients in two affected families. The legs of proband in family 1 exhibited evident “O” shape (A) and bowing of the femurs and widening and irregularity of the growth plates of distal femur (white arrows) were shown in X-ray image (B). Moreover, the radiological features of III1 in family 1 also presented bowing of the femurs, deformity of growth plates and even hip (white arrows) (C). In family 2, the boy, proband, showed a deformity of the lower extremities (D) and similar radiological presentations (white arrows) (E) with proband in family 1. However, proband’s mother in family 2 exhibited almost normal appearance in X-ray image (F).
Genetic analysis of patients and unaffected family members. (A) The novel heterozygous mutation site c.1765T>A (black arrows) of patients and II1 in family 1 and (B) corresponding sequence in the other unaffected individuals. (C) The other mutation site c.1846A>G (black arrows) was identified in family 2 patients and (D) wild type site in unaffected family members. (E) Both of the substitution loci p.Phe589 and p.Lys616 were conserved in different species according to the UCSC database.
Protein modeling of type X collagen (α1) NC1 domain and stylized structure of collagen X.

(A) (B) The two substitutions p.Phe589Ile and p.Lys616Glu were illustrated in the ribbon protein modeling with two mutually perpendicular perspectives, which showed that two substitutions were located in different subunits. (C) The stylized structure of type X collagen (α1) is composed of a 18 amino acid signal peptide (S) and a 463 amino acid collagenous domain (HELIX) flanked by a 38-residue NC2 domain and a 161-residue NC1 domain. Furthermore, changes in present study and mostly previous mutations (supplementary data) were located in NC1 domain.

Supplementary Files
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