

# Novel collagen VI mutations identified in Chinese patients with Ullrich congenital muscular dystrophy

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**Background:** We determined the clinical and molecular genetic characteristics of 8 Chinese patients with Ullrich congenital muscular dystrophy (UCMD).

**Methods:** Clinical data of probands were collected and muscle biopsies of patients were analyzed. Exons of *COL6A1*, *COL6A2* and *COL6A3* were analyzed by direct sequencing. Mutations in *COL6A1*, *COL6A2* and *COL6A3* were identified in 8 patients.

**Results:** Among these mutations, 5 were novel [three in the triple helical domain (THD) and 2 in the second C-terminal (C2) domain]. We also identified five known missense or in-frame deletion mutations in THD and C domains. Immunohistochemical studies on muscle biopsies from patients showed reduced level of collagen VI at the muscle basement membrane and mis-localization of the protein in interstitial and perivascular regions.

**Conclusions:** The novel mutations we identified underscore the importance of THD and C2 domains in the assembly and function of collagen VI, thereby providing useful information for the genetic counseling of UCMD patients.

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## Introduction

Ullrich congenital muscular dystrophy (UCMD, MIM254090) was first described by Ullrich in 1930 as "congenital scleroatonic muscular dystrophy". He characterized it as generalized muscle weakness and striking hypermobility of the distal joints in conjunction with contractures of more proximal joints, but normal intellectual development.<sup>[1]</sup> Children with UCMD typically cannot walk independently (or walk independently only for short periods).<sup>[2]</sup> Other distinctive features of the disease include: congenital hip dislocations, transient kyphotic deformity at birth, follicular hyperkeratosis over the extensor surfaces of the upper and lower limbs, soft velvety skin on the palms and soles, abnormal wound healing resulting in the formation of keloids or "cigarette paper" scar formation, posterior protruding calcanei; involvement of the facial muscles and high-arched palates.<sup>[3]</sup> As the disease progresses, subjects develop spinal rigidity and scoliosis as well as variable proximal contractures; the distal hyperlaxity can give way to marked long-finger flexion contractures and tight Achilles tendons.<sup>[4]</sup> Respiratory failure in the first or second decade is a common cause of death unless treated with nocturnal respiratory support, but cardiac involvement has not been documented.<sup>[3]</sup> Serum concentrations of creatine kinase (CK) are usually normal or mildly elevated.<sup>[5]</sup>

Collagen VI (COLVI) is a ubiquitous protein in the extracellular matrix that forms a beaded microfibrillar network in close association with the muscle basement membrane. A COLVI molecule comprised three  $\alpha$  chains,  $\alpha 1$ (VI),  $\alpha 2$ (VI) and  $\alpha 3$ (VI). Mutations in *COLVI* are associated with UCMD. Mutations in collagen VI-encoding genes are associated: with substitutions of single amino acids, splice-site mutations resulting in in-frame deletions, frameshift, and the introduction of a premature termination codon (PTC).<sup>[3,6]</sup>

To date, studies on UCMD within a Chinese population have not been reported. We conducted clinical, genetic and immunohistochemical analyses on eight patients with UCMD. The relationships between genotype and phenotype were then analyzed. Our study

will provide a better understanding of UCMD in China.

## Methods

### Ethical approval of the study protocol

The study protocol was approved by the Ethic Committee of the First Hospital of Peking University (Beijing, China). Written informed consent was obtained from all patients.

### Patients

Patients with a clinical diagnosis of UCMD were studied according to the guidelines formulated by the 166th European Neuromuscular Center (ENMC) International Workshop on Collagen Type VI-Related Myopathies.<sup>[7]</sup> Blood samples from the patients and their parents were also analyzed. As controls, DNA samples from 50 subjects without known muscle disease were studied.

### Phenotypic studies

The clinical features of patients were analyzed based on their medical records provided by the attending physicians. The characteristics of patients (including hypotonia, joint contractures or hyperlaxity, congenital hip dislocation, motor retardation and high-arched palates) were collected by the attending physicians. The ages when patients acquired independent ambulation, the ages when patients became wheelchair-bound, and data from pertinent laboratory examinations (e.g., serum levels of CK, electromyography) were recorded.

### Muscle biopsy and immunohistochemical staining

Muscle biopsies from the gastrocnemius muscle were embedded in optimum cutting temperature compound (Tissue-Tek<sup>®</sup>, Sakura, Alphen aan den Rijn, the Netherlands) and frozen in isopentane cooled in liquid nitrogen (VWR, West Chester, PA, USA). Using commercially available monoclonal antibodies against merosin (1:4000 dilution; mAb1922; Chemicon International, Temecula, CA, USA) and COLVI (1:800, mAb3303; Chemicon), we analyzed expression patterns on 6- $\mu$ m-thick cryosections. Collagen status was assessed by two independent observers blinded to the study protocol.

### Sequence analyses of collagen VI genes

Direct sequencing was undertaken on *COL6A1*, *COL6A2* and *COL6A3* of subjects with the clinical manifestations of UCMD. Genomic DNA was extracted from peripheral blood lymphocytes using standard protocols. Polymerase chain reaction (PCR) primers were designed to amplify all the exons of *COL6A1*,

*COL6A2* and *COL6A3* and their flanking intronic regions (primer sequences are available upon request). Amplified fragments were sequenced directly on an ABI3100 automated genetic analyzer. Sequence data were evaluated with the SeqScape (Applied Biosystems, Foster City, CA, USA) program and compared with the genomic sequences of COLVI genes in the GenBank database (gene identification: *COL6A1*, NM\_001848; *COL6A2*, NM\_001849; *COL6A3*, NM\_004369). One-hundred control chromosomes were examined by restriction enzyme analyses and direct sequencing to identify novel mutations in the genomic DNA of the patients.

## Results

### Clinical features

The main clinical characteristics of the eight patients in the present study are summarized in Table 1. These patients displayed clinical features consistent with a diagnosis of UCMD: early onset, proximal muscle weakness, distal hyperlaxity (Fig. 1A), skin changes, normal intellectual development, and normal (or mildly increased) serum levels of CK.

All patients had delayed motor milestones, and presented with hypotonia in the first months of life. Hip dislocation occurred in 6 patients (P2, P4, P5, P6, P7 and P8), torticollis in 5 (P2, P3, P4, P7 and P8), extended talipes in 4 (P2, P5, P7 and P8) (Fig. 1B), decreased fetal movement in 1 (P7), follicular hyperkeratosis presenting as a rough skin rash in 7 (Fig.



**Fig. 1.** Patients display typical clinical feature of Ullrich congenital muscular dystrophy. Patients showed hyperlaxity of the distal joints [including the distal interphalangeal joint (A, P4)], a round face, hip dislocation, torticollis, prominent calcanei, soft plantar skin (B, P4), follicular hyperkeratosis (C, P2) and keloids (D, P2).

1C, except P7), and skin involvement in the form of keloids in 4 (Fig. 1D).

The motor disability of the patients varied from relatively mild to severe: 5 of the 8 patients acquired ambulation but showed a mild delay (16-24 months; P1, P3, P5, P6 and P8), whereas the other 3 patients had severe motor delays, never acquired independent ambulation (P2, P4 and P7). Among the 5 patients who acquired ambulation, 3 achieved independent ambulation (P1, P5 and P8) and 2 could run for short distances (P3 and P6). However, all of these patients except P3 lost ambulation as the disease progressed: P1 became wheelchair-bound before the age of 5 years, whereas for the others this happened after the age of 6 years. P3 is 12 years old now and can still walk independently though gait abnormal. The serum levels of CK in the 8 patients varied between normal and a three-fold increase. Electromyograms were normal or showed myogenic changes. None of the patients had a family history of muscular dystrophy. Based on these clinical characteristics, the patients were divided into three groups: "early-severe" (P2, P4 and P7), "moderate-progressive" (P1, P5, P6 and P8), and "mild" (P3).

#### Analysis of collagen VI expression in muscle biopsies

The muscular biopsies from the UCMD patients were analyzed. All of the biopsied muscles showed abnormal

variations in the diameter of muscle fibers, an increased number of fibers with internal nuclei, and regenerating fibers. Proliferation of connective tissue and fat tissue was confirmed by staining of either hematoxylin & eosin (H&E) (Fig. 2D, G and J) or modified Gomori trichrome (MGT) (Fig. 2H and K).

Three of the muscle biopsies (P1, P6 and P7) were analyzed by immunohistochemical staining with an anti-COLVI antibody (Fig. 2, Table 2). As reported previously,<sup>[2-4]</sup> the normal control muscle samples showed strong staining for COLVI at the basement membrane but no staining elsewhere (Fig. 2C). In contrast, the muscle samples of patients showed a reduction (P1, P6 and P7) of COLVI at the basement membrane. In the muscle samples of P1 and P6, COLVI was instead detected in the interstitial and perivascular regions between muscle fibers (Fig. 2F, I and L).

#### Genomic DNA analyses

Genomic DNA samples from the 8 patients were analyzed by PCR and DNA direct sequencing. All of these patients had mutations in *COL6A1*, *COL6A2* or *COL6A3*. These mutations and the protein domains affected are summarized in Fig. 3 and Table 2.

Splice-site mutations in the triple helical domain (THD) of *COL6A2* were identified in two patients (P1 and P8), one of which was a novel mutation (Cys246\_

**Table 1.** Clinical information of Chinese Ullrich congenital muscular dystrophy patients

No.	Sex	Age of head control (mon)	Age of sitting up (mon)	Age of walking (mon)	Max motor ability	Age at last exam (y)	Age of loss of ambulation (y)	Musculoskeletal					Skin		Max CK (U/L)	EMG
								Proximal contractures	Distal hyperextensibility	Scoliosis	Hip dislocation	Torticollis	Hyperkeratosis	Atrophic scars		
P1	M	3	6	22	Walk	12	4.5	+	+	-	±	-	+	-	499	Normal
P2	M	9	6	N	Sit	8	N/R	+	+	+	+	+	+	+	204	Myopathic
P3	F	3	20	22	Run	12	Not yet	+	+	+	-	+	+	-	234	Myopathic
P4	M	4	9	N	Sit	4	N/R	-	+	+	+	+	-	-	165	Normal
P5	M	3	8	18	Walk	10	6	+	+	+	+	-	+	+	309	Normal
P6	F	3	NA	24	Run	16	8.5	+	+	+	+	-	+	+	328	Normal
P7	M	NA	NA	N	Sit	6	N/R	-	+	-	+	+	-	-	204	Myopathic
P8	F	3	7	16	Walk	13	8	+	+	-	+	+	+	+	222	Myopathic

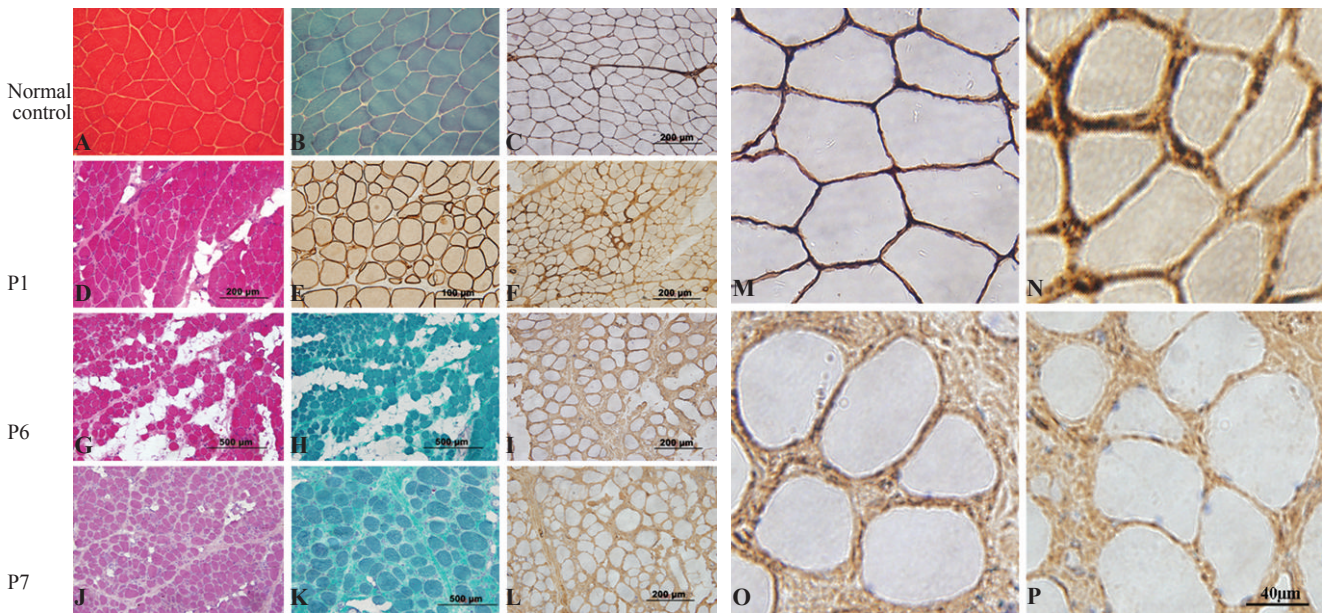
\*: the upper limit for the normal CK value in our research team is 195 U/L. M: male; F: female; NA: not available; N/R: not relevant; N: never; CK: creatine kinase; EMG: electromyography; P: patient.

**Table 2.** Immunohistochemical staining of biopsied muscle and collagen-VI gene mutations of Ullrich congenital muscular dystrophy patients

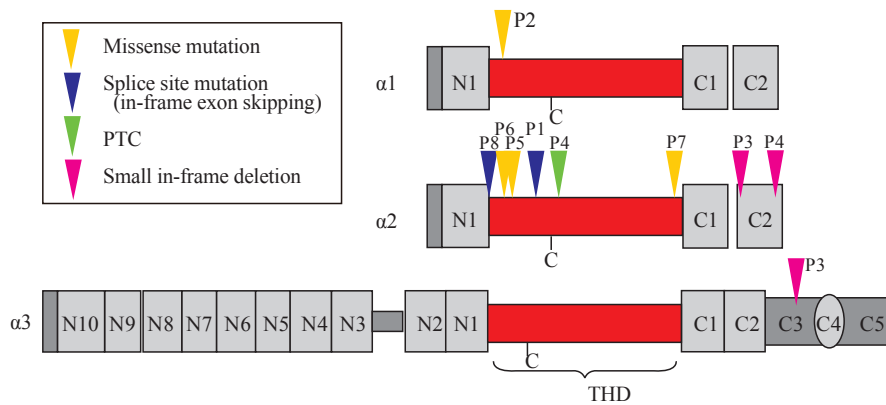
No.	Phenotypes	Collagen VI	Gene mutation	Domain affected	Nucleotide change	Protein change	Source	Reference
P1	Moderate-progressive	Reduced	COL6A2	THD	c.955-2_955-1, delAG (het)	p.Gly319_Lys333del	<i>De novo</i>	3
P2	Early-severe	NA	COL6A1	THD	c.850G>A (het)	p.Gly284Arg	<i>De novo</i>	3, 8, 9
P3	Mild	NA	COL6A2	C2	c.2548-2550del (CAC)	p.850del_His	Paternal	Novel
			COL6A3	C3	c.8877_8879del (TGC)	p.2960del_Ala	Maternal	6
P4	Early-severe	NA	COL6A2	THD	c.1096C>T	p.Arg366	Paternal	Novel
				C2	c.2930_2941del	p.Gly977_Val980del	Maternal	Novel
P5	Moderate-progressive	NA	COL6A2	THD	c.875G>A (het)	p.Gly292Asp	<i>De novo</i>	8
P6	Moderate-progressive	Reduced	COL6A2	THD	c.812G>A (het)	p.Gly271Asp	<i>De novo</i>	8
P7	Early-severe	Reduced	COL6A2	THD	c.1616G>A (het)	p.Arg539Gln	Parental DNA not available	Novel, not found in 100 control chromosomes
P8	Moderate-progressive	NA	COL6A2	THD	c.736-1,G>A (het)	p.Cys246_Lys267del	<i>De novo</i>	Novel

NA: not available; THD: triple helical domain; C2: the second C-terminal globular domain; C3: the third C-terminal globular domain; P: patient.

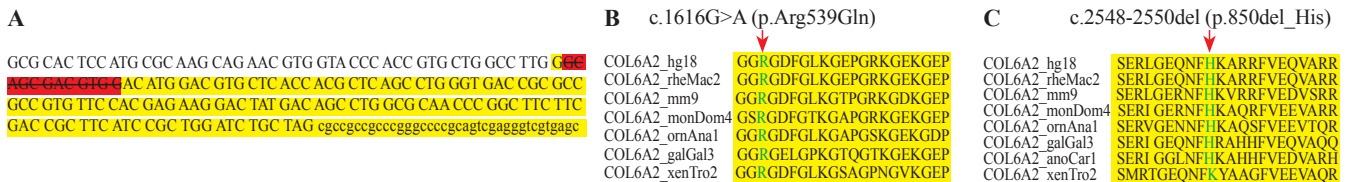




**Fig. 2.** Pathological and immunohistochemical staining in skeletal muscle biopsies. Skeletal muscle samples from normal subjects (A, B, C and M) and Ullrich congenital muscular dystrophy patients (D-L, N-P) were stained with hematoxylin & eosin (A, D, G, J), modified Gomori trichrome (B, H, K) or an antibody against collagen-VI (C, F, I and L) or merosin (E, positive staining). M, N, O, P showed higher magnification of C, F, I and L, respectively.



**Fig. 3.** Domain structure of *COL6A1-3* (schematic). The triple helical domain (THD) contains a single cysteine residue (depicted as "C") that is important for dimer assembly. Mutations in the patients who had a moderate-progressive phenotype are clustered in the N-terminal side of the cysteine residue in the THD. PTC: premature termination codon.



**Fig. 4.** Alignment showing the *COL6A2* regions in which novel mutations were detected, including (A) a 12 base pairs deletion in P4 leading to a in frame deletion mutation in C2; (B) an amino acid substitution in P7 in a region of repeating Gly-Xaa-Yaa sequences that are highly conserved among different species, and (C) a small in-frame deletion in P3.

Lys267del in P8). The mutations resulted in the skipping of exon 10 in P1 and exon 5 in P8 (both in-frame deletions). The mutations were not found in their parents, suggesting that they had occurred *de novo*.

Glycine substitution mutations were identified in the THD in three patients (P2, P5 and P6). The

mutations were not found in their parents, suggesting that these were *de novo* mutations. Arginine substitution mutations were identified in the *COL6A2* of 2 patients (P4 and P7). In P4, an arginine was substituted by a stop codon (Arg366X), which was also found in his father. P4 also had an in-frame deletion (p.Gly977\_Val980del,

**Table 3.** The single-nucleotide polymorphisms (SNPs) found in *COL6A2*

No.	<i>COL6A2</i> SNP				
P1	c.1196G>A (hom)				c.2980G>A (hom)
P2		c.2039G>A (het)	c.2094G>A (het)	c.2097G>A (het)	c.2184G>A (het) c.2724A>G
P3	c.548C>T (het)	c.2039G>A (het)	c.2094G>A (het)	c.2097G>A (het)	c.2184G>A (het)
P4		c.2039G>A (het)	c.2094G>A (het)	c.2097G>A (het)	c.2184G>A (het)
P5		c.2039G>A (het)	c.2094G>A (het)	c.2097G>A (het)	c.2184G>A (het)
P6		c.2039G>A (het)	c.2094G>A (het)	c.2097G>A (het)	c.2184G>A (het)
P7	c.548C>T (het) c.2803G>A (het)				
P8					c.2980G>A (het)

c.2980G>A is only found in our normal control and Ullrich congenital muscular dystrophy patients. The other SNPs above are reported previously.

Fig. 4A) in the C2 domain of the other allele, which was found in his mother. These compound heterozygous mutations were novel. In P5, the glycine (Gly292Asp) mutation was *de novo*. DNA for the parents of P7 was not available. However, the mutation (Arg539Gln), which resides at a Yaa position in a Gly-Xaa-Yaa motif, is a novel missense variant of those occurring at highly conserved amino-acid residues (Fig. 4B) and was not found in 100 control chromosomes, suggesting that this is a pathogenic mutation.

A novel in-frame deletion (p.850del\_His), which removed a histidine residue in the C2 domain (Fig. 4C), was identified in the *COL6A2* of one patient (P3). The mutation was found in her father. A known in-frame deletion (p.2960del\_Ala), which removed an alanine residue in the C3 domain, was identified in the *COL6A3* of P3. It was also found in her non-symptomatic mother.

In addition to the mutations that affected the coding sequences of *COL6A1*, *COL6A2* and *COL6A3*, many single-nucleotide polymorphisms (SNPs) were identified in these genes. Some SNPs of *COL6A2* are shown in Table 3. Thus, the highly polymorphic nature of these genes is emphasized.

In summary, we identified 10 mutations: 1 in *COL6A1*, 8 in *COL6A2* and 1 in *COL6A3*. Five of the mutations were novel: three were identified in THD, including an arginine substitution, an exon-skipping mutation that resulted in a large in-frame deletion, and a nonsense mutation that introduced a premature termination codon; two were identified in the C2 domain, both were deletions of a few amino acids.

## Discussion

We reported here an analysis of 8 UCMD patients residing in China. These patients were classified into three groups based on disease severity. That was, the early-severe group (P2, P4 and P7) had never achieved ambulation; the moderate-progressive group (P1, P5, P6 and P8) could walk initially but showed a progressive course during childhood and a loss of ambulation

at 4-8 years of age; the mild group (P3) maintained ambulation and needed to be followed up.<sup>[8]</sup>

The mutations found in the moderate-progressive group all resided in the THD of the  $\alpha 2(\text{VI})$  chain. THD is the central domain of COLVI, and is composed of repeating Gly-Xaa-Yaa motifs. This domain allows the three  $\alpha$  chains to assemble into the central triple helical structure of a COLVI monomer, which assembles further into disulfide-bonded anti-parallel dimers and then tetramers. The tetramers are secreted and assembled into microfibrils at the border between the interstitial extracellular matrix and the muscle basement membrane.<sup>[10]</sup> THD contains a single cysteine residue at position 89, which is important for dimer assembly. Notably, the mutations we identified in the moderate-progressive group were clustered in a THD region on the N-terminal side of this cysteine residue (Fig. 3), leaving the mutant  $\alpha$  chain competent for the assembly and secretion of dimers (which is a prerequisite for the mutations to exert a dominant-negative effect).<sup>[11]</sup> Two types of mutations were identified in the moderate-progressive group: glycine substitutions within THD and splice-site mutations that led to in-frame skipping of exons encoding part of the THD. Both types of mutations were likely to disrupt the central helical structure of COLVI monomers, thereby acting in a dominant-negative fashion (presumably by disrupting the assembly or stability of COLVI microfibrils at the basement membrane).<sup>[12,13]</sup> This finding was supported by our immunohistochemical analyses on P1 and P6, which showed aberrant accumulation of mutant COLVI in the interstitial and perivascular space rather than at the basement membrane.

Another glycine substitution in THD was identified in the early-severe group (P2). This is a known mutation in *COL6A1*, which is reported to be a frequently reported mutation, producing a severe UCMD phenotype.<sup>[3,8,9]</sup>

THD is flanked by large N- and C-terminal globular domains.<sup>[14]</sup> We identified a novel mutation that resides in the second C-terminal globular (C2) domain in *COL6A2* and a potentially nonpathogenic variant<sup>[6]</sup> that resides in

the third C-terminal globular (C3) domain in *COL6A3* in the mild group (P3): both were found in her parent. These two variants were small in-frame deletions that removed an amino residue. However, the parents, who also had this mutation, did not have muscle weakness. Although the patient with these mutations did not lose ambulation, she shared some UCMD phenotypes,<sup>[15]</sup> presenting with a combination of proximal contractures, distal hyperextensibility and abnormal muscle biopsy, suggesting that the C domain also contributes to the structure, assembly or function of COLVI. This finding was in accordance with a report stating that some patients showed changes in more than one of the *COL6* genes.<sup>[16]</sup> Deletion in the C domain is thought to cause the C domain to misfold. These misfolded chains could be secreted because the cysteine residues are preserved and dimers can be formed, thereby having a moderate dominant-negative effect.<sup>[17,18]</sup>

Besides the C2 and C3 mutations in the mild group, one additional C2 mutation was identified in the early-severe group (P4). This patient was compound heterozygous with a nonsense arginine mutation in THD and an in-frame deletion in C2; both mutations were novel. Notably, the nonsense arginine mutation was identified in the father of P4 and the in-frame deletion was identified in the mother of P4, who did not have UCMD, suggesting that these mutations were recessive. It has been reported that nonsense-mediated mRNA decay can reduce steady-state COLVI mRNA levels.<sup>[16]</sup> Compounded with this nonsense mutation, the in-frame deletion in the C2 domain of the other allele of P4 may have removed a region of the  $\alpha 2$  (VI) chain that is responsible for supermolecular assembly and organization of COLVI.<sup>[19,20]</sup> Thus, the compound mutations could prevent the formation of COLVI dimers, leading to severe UCMD phenotypes.

In the early-severe group, one patient (P7) carried a novel arginine substitution (Arg539Gln) that altered Yaa in a Gly-Xaa-Yaa motif in the THD of the  $\alpha 2$  (VI) chain. Arginine is a strongly positively charged amino acid and is highly conserved at the Yaa position, where it has a key role in stabilizing the triple helical structure of COLVI. Therefore, an arginine substitution at Yaa is likely to be dominant and pathogenic. This possibility was supported by the results of immunohistochemical analyses that showed a marked reduction of COLVI in the muscle biopsy of the patient.

In conclusion, we have provided clinical, immunohistochemical and genetic evidence of the involvement of COLVI in eight unrelated UCMD patients in China. We found five known and five previously non-described mutations in the THD and C-terminal domains of *COL6A1*, *COL6A2* and *COL6A3*. Some of these mutations led to severe UCMD. The novel

COLVI mutations that we identified underscore the importance of THD and the C-terminal domain in the assembly and function of COLVI, thereby providing useful information for the genetic counseling of UCMD families. However, a larger number of cases need to be analyzed over a longer follow-up duration and we also should consider other mutation types such as large genomic deletions.<sup>[21]</sup>

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**Ethical approval:** This study was approved by the Ethic Committee of the First Hospital of Peking University (Beijing, China).

**Competing interest:** The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Contributors:** Zhang YZ and Zhao DH conducted the experiments and wrote the first draft of the manuscript. Yang HP, Hong DJ, Liu AJ, Chang XZ participated in collecting the samples, study design, and critical discussion. Bonnemann C, Yuan Y and Wu XR took part in study design, conduct of the experiment, and critical discussion. Xiong H conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final version of this manuscript.

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