

Variants of CAPN3 cause limb-girdle muscular dystrophy type 2A in two Chinese families

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Received February 17, 2020; Accepted October 26, 2020

DOI: 10.3892/etm.2020.9536

Abstract. Limb-girdle muscular dystrophies (LGMDs) are a group of neuromuscular diseases that are characterized by progressive muscle weakness. LGMD type 2A (LGMD2A), caused by variants in the calpain-3 (CAPN3) gene, is the most prevalent type. The present study aimed to analyze pathogenic CAPN3 gene variants in two pedigrees affected by LGMD2A. Each family contains three patients who are siblings and sought genetic counseling. Genomic DNA was extracted from the peripheral blood samples collected from the probands and family members and whole-exome sequencing (WES) was used to detect the pathogenic genes in the probands. Suspected variants were subsequently validated by Sanger sequencing. In family 1, WES revealed that the proband carried the compound heterogeneous variants c.1194-9A>G and c.1437C>T (p.Ser479=) in CAPN3 (NM_000070.2). In family 2, WES identified that the proband carried the compound heterogeneous variants c.632+4A>G and c.1468C>T (p.Arg490Trp) in CAPN3 (NM_000070.2). In conclusion, the present study indicated that the compound heterogeneous variants of the

CAPN3 gene were most likely responsible for LGMD2A in the two Chinese families.

Introduction

Limb-girdle muscular dystrophies (LGMDs) are a group of autosomal hereditary diseases characterized by progressive muscle weakness in the scapular and pelvic girdle and trunk muscles. LGMDs were firstly classified into autosomal dominant and autosomal-recessive types by the European Neuromuscular Center in 1995 (1). The autosomal-recessive form of LGMD type 2A [LGMD2A; Online Mendelian Inheritance in Man (OMIM) ID, 253600], which is also referred to as limb girdle muscular dystrophy type R1 (LGMDR1) and caused by variant in the calpain-3 (CAPN3) gene (OMIM ID, 114240), is the most common type of LGMDs, accounting for ~30% of all cases of LGMD (2). However, a 21-bp deletion in the CAPN3 gene was also identified to cause the autosomal-dominant type of LGMD2A (3). According to the updated 2018 classification for LGMD, there are 5 autosomal-dominant and 24 autosomal-recessive subtypes, most of which are due to genetic defects associated with severe congenital muscular dystrophy (4). The incidence of LGMD2A is ~1 in 100,000 individuals and the average age of onset is 17.9 years (5). The clinical characteristics of LGMD2A are highly heterogeneous and not only is there variation in the age of onset (from early childhood to adulthood) and the degree of muscular weakness, but also in the high prevalence of variability among gene variants. Clinical features include difficulties in walking and running, a waddling gait, scapular winging and respiratory failure in the advanced stages of the disease; however, the facial and neck muscles are not affected (6). In addition, cases involving cardiac muscles have been reported at a low frequency (7,8).

The CAPN3 gene consists of 24 exons and encodes the CAPN3 enzyme. CAPN3, a Ca²⁺-dependent protease, is composed of 821 amino acids and is involved in the breakdown and cleavage of a variety of key skeletal myoproteins, particularly those related to the skeletal structure of myofibrils (9). CAPN3 contains four domains: The protease core subdomain 1, protease core subdomain 2, C2 domain-like domain and penta-EF-hand domain (10). Upon Ca²⁺ binding to the protease core subdomains 1 and 2, they fold into a

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Abbreviations: LGMD2A, limb-girdle muscular dystrophy type 2A; WES, whole-exome sequencing; CK, creatine kinase; BMD, Becker muscular dystrophy; ACMG, American College of Medical Genetics and Genomics

Key words: limb-girdle muscular dystrophy type 2A, calpain-3 gene, variant, compound heterozygous variants, whole-exome sequencing

structurally active 'CysPc' domain, which is a CAPN3-like cysteine protease sequence motif as defined in the conserved domain database of the National Center for Biotechnology Information (11). CAPN3 has an important role in LGMD due to the physiological aberrations caused by its defect, such as muscular weakness, as a result of the lack of ryanodine receptor regulation by CAPN3 (9).

The present study reported on two Chinese pedigrees, including six patients affected with LGMD2A caused by CAPN3 gene variants. Whole-exome sequencing (WES) was performed to make an accurate diagnosis, which provided a basis for the genetic counseling of the two families. Furthermore, by analyzing the genetic patterns of these two families, the pathogenic classification of the variants in the present study was further clarified to enhance the current knowledge on the pathology, facilitating future clinical management.

Materials and methods

Patients and DNA extraction. From family 1 (F1), three patients, their mother and three healthy siblings were recruited in Tianjin Children's Hospital (Tianjin, China) in September 2018. From F2, 15 family members recruited in Tianjin Children's Hospital were studied in total in June 2019. Genomic DNA was extracted from the peripheral blood using a Blood Genomic DNA Mini kit (cat. no. CW0541; CoWin Biosciences) according to the manufacturer's protocol. The ratio of the absorbance at 260 and 280 nm (A₂₆₀/A₂₈₀ ratio) were evaluated with 1 μ l of DNA extraction using the NanoDrop[®] 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). A total of 100 μ l DNA solution (≥ 10 ng/ μ l) was obtained, which was stored at -20°C. Written informed consent was obtained from all family members and the study was approved by the ethics committee of Tianjin Children's Hospital (Tianjin, China).

WES and bioinformatics analysis. WES for patient 1 (F1-III1) and patient 4 (F2-III1) was performed by BGI Group. Paired-end sequencing was performed with read lengths of 150 bp and an average coverage depth of 100-fold in >95% of the target regions, including all coding regions and exon-intron boundaries. Sequencing data were aligned to the human reference genome hg19 using the Burrows-Wheeler Aligner software. Genome Analysis Toolkit software was used to analyze the insertion, deletion and single nucleotide polymorphism sites. Variant annotations were made using the ANNOVAR tool (V20180118; <https://doc-openbio.readthedocs.io/projects/annovar/en/latest/>), 1,000 genomes (<https://www.1000genomes.org/>), dbSNP (<https://www.ncbi.nlm.nih.gov/snp/?term=>) and OMIM (<https://omim.org/>) databases. The effect of the variants on the structure and function of the proteins was predicted using Polymorphism Phenotyping v2 software (<http://genetics.bwh.harvard.edu/pph2/index.shtml>) and Sorts Intolerant From Tolerant software (V1.1; <http://sift.jcvi.org.>). In addition, Human Splicing Finder (V3.1; <http://www.ummd.be/HSF/>) was used to predict the splice sites in the gene.

Variant screening and Sanger sequencing. PCR and further Sanger sequencing were performed on all patients and

other available family members to confirm the candidate variants and analyze the co-segregation pattern. The relative exons and flanking intron regions were amplified using primers designed by Oligo7 (12) (version 7.60; exon 3, 5'-CCCCAAACACAAAATAGGATG-3' and 3'-CACATA TGCACGTATAGAGG-5'; exon 10, 5'-GCCACCCTCTTT TCATCCTCC-3' and 3'-TGTTCCCACAGTTTCCTGCTT C-5'; exon 11, 5'-TGTAGGGAATAGAAATAAATGG-3' and 3'-CCAGGAGCTCTGTGGGTCA-5'; and another pair of primers for exon 11, 5'-AGAATGAAAGCCCAG AGAGGA-3' and 3'-TGTGGGTCCTGGGTATTGA-5'). Amplification was performed in a final volume of 50 μ l, containing 25 μ l 2X GC buffer I, 20 mM deoxynucleoside triphosphates mixture, 100-200 ng DNA, 0.5 μ M forward and reverse primers and 2.5 U LA Taq polymerase (cat. no. RR02AG; Takara Biotechnology Co., Ltd.). The following thermocycling conditions were used for the PCR: Initial denaturation for 2 min at 94°C, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 40 sec; and a final extension step at 72°C for 5 min. The PCR products were separated by 1.5% agarose gel electrophoresis and the proper DNA was purified from agarose gel using a Gel Extraction kit (CoWin Biosciences) and sent to Genewiz (Beijing, China) for Sanger sequencing. Chromas software (version 1.62; Technelysium Pty Ltd.) was used to compare the sequencing data with the reference sequences (NM_000070.2) in GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/NC_000015.10?report=genbank&from=42359501&to=42412317) to determine the variants.

Results

Patients. F1 comprised three affected family members born from non-consanguineous Chinese parents (Fig. 1). Patient 1 (F1-III1) was a 58-year-old male who experienced difficulties running and jumping from the age of 9 years. At the age of 19 years, F1-III1 frequently fell down and required help to walk upstairs. Gradually, the patient was no longer able to raise his arms and relied on a wheelchair from the age of 46 years. Approximately 30 years previously, the patient underwent general laboratory tests and imaging examinations; the serum creatine kinase (CK) levels were 560 U/l (normal, <308 U/l) and needle electromyography revealed fibrillation potentials in the scapular girdle muscles. However, the electrocardiogram was normal. Subsequently, F1-III1 was misdiagnosed with Becker muscular dystrophy (BMD). Patient 2 (F1-II7) is a 60-year-old woman, the elder sister of patient 1 who is unable to walk since she was 20 years old and currently relies on a wheelchair for mobility. Patient 3 (F1-II5) is 62 years old, the elder sister of patients 1 and 2 who uses a wheelchair but is able to walk on crutches. The only daughter (F1-III7) of patient 1, who is healthy, wished to have a healthy child, and therefore attended Tianjin Children's Hospital (Tianjin, China) for genetic counseling and pedigree gene screening.

In F2, there were three affected patients (Fig. 1). Patient 4 (F2-III1) was a 37-year-old male; the age of disease onset was 30 years, when the patient first experienced limb muscle weakness and difficulties running, jumping and walking up stairs. At the age of 35 years, F2-III1 was unable to walk independently and a laboratory examination revealed high levels of serum CK

Table I. Clinical and genetic characteristics of patients.

Patient	Sex	AOO (years)	DOWD (years)	SW	CPH	Gene	CK level (U/l)
F1-II ₁₁	M	9	37	No	NA	CAPN3	560
F1-II ₇	F	12	15	No	NA	CAPN3	NA
F1-II ₅	F	20	35	No	NA	CAPN3	NA
F2-II ₁₁	M	30	5	Yes	NA	CAPN3	1609
F2-II ₃	M	26	^a	Yes	NA	CAPN3	1544
F2-II ₁	F	20	20	No	NA	CAPN3	NA

^aProband 5 was still able to walk; AOO, age of onset; DOWD, duration from onset to wheelchair-dependent; SW, scapular winging; CPH, calf pseudo-hypertrophy; CK, creatine kinase; M, male; F, female; NA, not available; CAPN3, calpain-3.

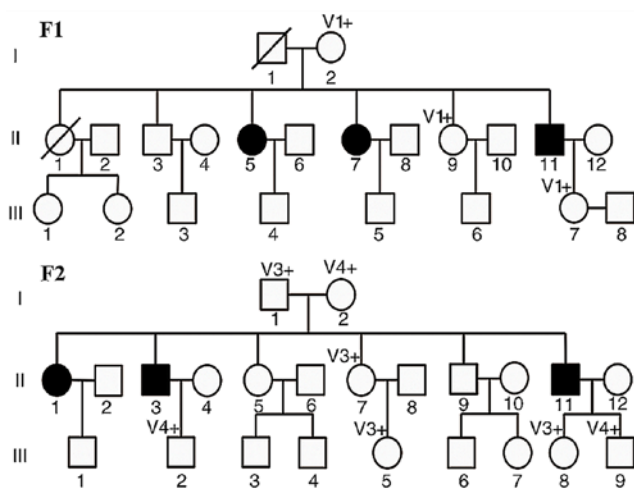


Figure 1. Pedigree charts indicating the segregation of heterozygous variants in family 1 (upper) and family 2 (lower). In family 1, the family members I2, II9 and III7 carried the c.1194-9A>G variant (V1). In family 2, the family members II, II7, III5 and III8 carried the c.632+4A>G variant (V3), and II, III2 and III9 carried the c.1468C>T (p.Arg490Trp) variant (V4). F1, family 1; F2, family 2.

(1,609 U/l). The patient's symptoms were progressively aggravating; he presented with winged scapulae and is dependent on a wheelchair (Fig. 2A and B). Patient 5 (F2-II3) is 45 years old, the elder brother of patient 4, who first experienced muscle weakness at the age of 26 years. After three years, F2-II3 was unable to walk, stand up, stoop or hold objects with his hands. F2-II3 also presented with winged scapulae (Fig. 2C and D). Electromyography also revealed that the muscle tension was low and the serum CK levels were elevated to 1,544 U/l. Patient 6 (F2-III1), a 50-year-old female, is the elder sister of patients 4 and 5. Patient 6 was 20 years old when symptoms of limb weakness first appeared. However, she was misdiagnosed with rheumatoid arthritis. After 20 years, patient 6 was unable to walk and became dependent on a wheelchair. The wife of patient 4 wished to know about her children's health, so she opted for genetic counseling and pedigree gene screening.

CAPN3 gene variants.

F1. The spectrum of clinicopathological and genetic features is presented in Table I. F1 comprised a three-generation

pedigree containing three patients and four unaffected controls. The WES results revealed that the compound heterozygous variants of the CAPN3 gene (NM_000070.2) were responsible for LGMD2A in F1. The heterozygous variant of an A-to-G transition located in -9 upstream of exon 10 of CAPN3 (c.1194-9 A>G) was identified, which has been reported previously and classified as 'pathogenic' in the ClinVar database. Another heterozygous variant was identified in exon 11 [c.1437C>T (p.Ser479=)], which caused the 479th genetic codon change from AGC to AGT; however, the amino acid serine did not change. To the best of our knowledge, this synonymous variant has not been reported in the previous literature. According to the 2015 American College of Medical Genetics and Genomics (ACMG) variant classification guidance (13), the c.1437C>T (p.Ser479=) variation may be classified as 'likely pathogenic'.

F2. The WES results revealed the presence of compound heterozygous variants of the CAPN3 gene in F2. The c.1468C>T (p.Arg490Trp) variant in exon 11 was listed in the ClinVar database and it is regarded as a 'pathogenic/likely pathogenic' variant. The c.632+4A>G variant was identified in the donor site of intron 4, which is regarded as a variant of 'uncertain significance' in the ClinVar database.

No other variations associated with muscular dystrophy were observed from the results of the WES analysis in the two families. Although the two families had no intention to undergo further muscle biopsy, as they only sought genetic counseling and pedigree gene screening, the diagnosis of LGMD2A was possible according to the results of WES (all patients carried the compound heterozygous variants of the CAPN3 gene) combined with the clinical manifestations in the patients in the two families.

Sanger sequencing analysis.

F1. According to the result of the WES, the DNA of three patients and another four healthy family members was amplified. The results of the Sanger sequencing were consistent with those of WES (Fig. 3). Sanger sequencing demonstrated that the variant c.1194-9A>G was inherited from the mother (F1-I2), whereas the c.1437C>T (p.Ser479=) variant was not present in the maternal sequencing results. The genotype of the patients' father could not be verified

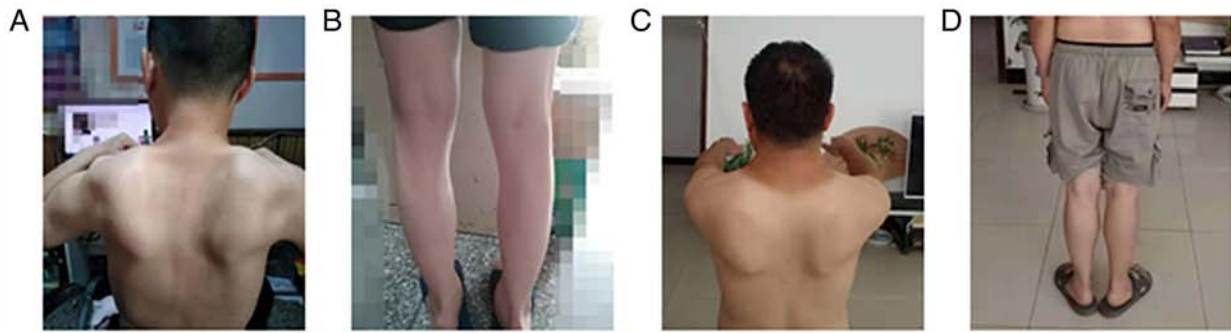


Figure 2. Anatomical features of patients 4 (F2-II 11) and patient 5 (F2-II3). (A) Upper back and (B) back view of the legs of patient 4. (C) Upper back and (D) back view of the legs of patient 5. Winged scapulae were not associated with calf pseudo-hypertrophy.

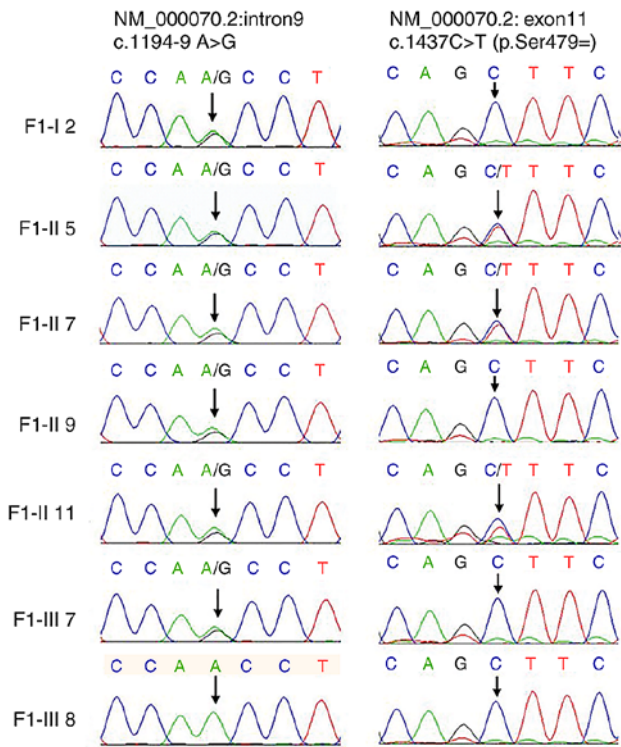


Figure 3. Sanger sequencing of the genomic DNAs from three patients and four relatives in family 1.

due to his prior death. Patients 2 and 3 were both in the same compound heterozygous state. The sister (F1-II9) and daughter (F1-III7) of patient 1 carried the c.1194-9 A>G variant. In the son-in-law of proband 1, the aforementioned variants were not detected.

F2. The DNA of patients 4, 5 and 6 and another 12 family members was amplified. The Sanger sequencing results revealed that the c.632+4A>G variant was inherited from the proband's father (F2-I1) and the c.1468C>T (p.Arg490Trp) variant was inherited from the mother (F2-I2). It also identified that patients 4, 5 and 6 were all carriers of the compound heterozygous variants. F2-II7, F2-III5 and F2-III8 carried the c.632+4 A>G variant, while F2-III2 and F2-III9 carried the c.1468C>T (p.Arg490Trp) variant. The remaining five family members did not carry either of the variants (Fig. 4).

Discussion

LGMDs are a heterogeneous group of neuromuscular diseases associated with weakness in the proximal muscles, which vary in terms of age of onset, clinical severity, genotype, phenotype and duration of disease prior to requiring wheelchair assistance. LGMD2A is the most common subtype of LGMD; it may affect individuals at any age and exhibits variable clinical features, ranging from mild to severe forms. While it may occur at any age from 2 to 55 years, the mean age of onset is 17.9 years (5). In addition, the mean age of patients relying on wheelchair usage is 35.2 years and the average duration from disease onset to wheelchair dependence is 15.2 years, but it ranges from 4 to 29 years (5). In the present study, the minimum age of onset was 9 years (patient 1) and the maximum was 30 years (patient 4). In addition, the longest duration from disease onset to becoming wheelchair-dependent was 37 years in patient 1, while the shortest was 5 years in patient 4. Thus, these results revealed a wide variation in disease progression. The mean period was 22.4 years in the present study; at the early stage, muscle weakness may occur in the pelvic and shoulder girdle. Individuals with LGMD2A may have an unusual walking gait and may experience difficulty walking, running and raising the arms. Toe-walking may also be present in early childhood before muscle weakness is detected (14). As the disease progresses, patients may lose the ability to walk and therefore, eventually become dependent on wheelchair use. In general, LGMD2A predominantly affects the proximal muscles and not the brain. However, additional features such as respiratory failure and severe cardiomyopathy have been reported in specific cases (6-8).

In the first pedigree examined in the present study, patient 1 was misdiagnosed with BMD previously. According to the pedigree study, 2 cases appeared in females in generation II, but none of the males in generation III was affected. BMD is an X-linked dystrophinopathy, of which the age of onset is usually late and the disease progress is slow; the majority of the patients are still able to walk at the age of 30 years (15). Therefore, it is easy to dismiss the previous diagnosis according to the family inheritance pattern and the clinical features. WES revealed that the heterozygous variant of c.1194-9A>G was present in the 9th intron, which was classified as 'pathogenic' in the ClinVar database. This substitution leads to a new splice site in position 9 upstream

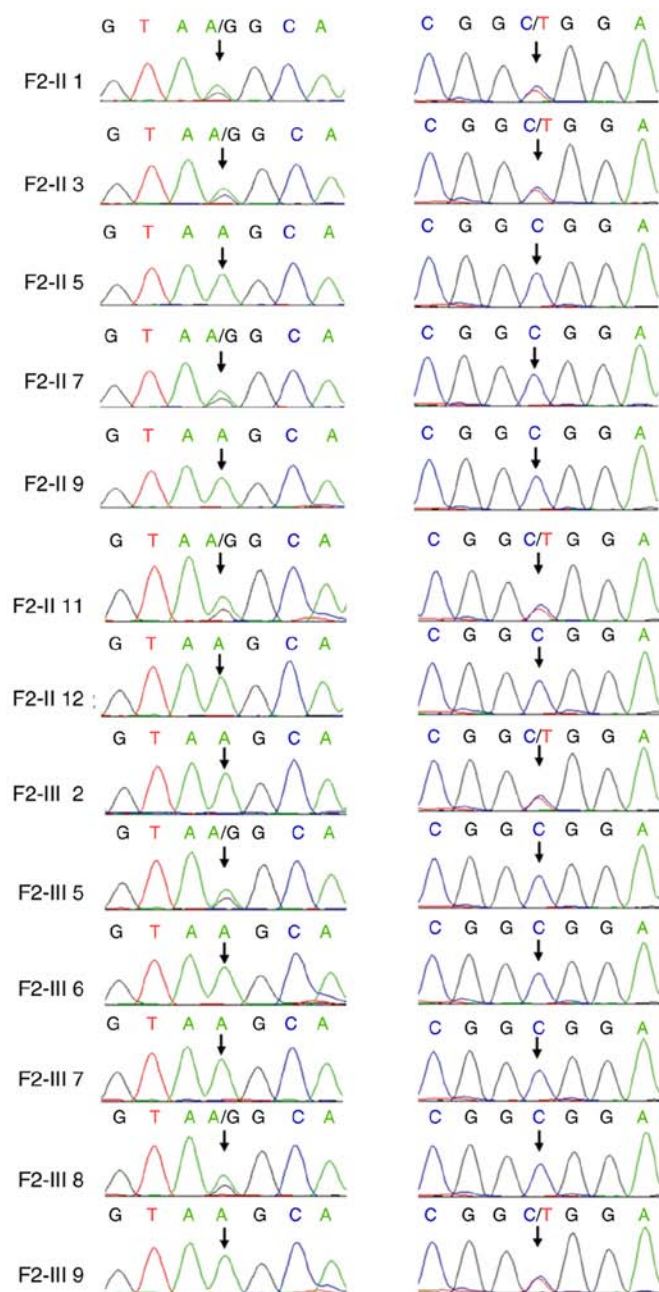


Figure 4. Sanger sequencing of the genomic DNAs from three patients and twelve relatives in family 2.

of exon 10, creating a new AG acceptor splice site and the retention of the eight last nucleotides of intron 9 (16). Another heterozygous variant, c.1437C>T (p.Ser479=), was identified as a synonymous variant in exon 5, which caused the genetic codon to change, but not the amino acid from serine. In the 1000 Genomes and gnomAD databases, the frequency of this synonymous variant in the East Asian population, which did not belong to a single nucleotide polymorphism site, was recorded as 3 and 2.5%, respectively, and the frequency in the whole population was 6 per 10,000 and 1 per 10,000, respectively. It was classified as a variant of 'uncertain significance' in the ClinVar database. Subsequently, the online software Human Splicing Finder (<http://www.ummd.be/HSF/>) was used to predict gene splice sites. The results revealed that the score of the variant was 81.49 above the threshold; thus, it may

be considered to be a splice site acceptor. According to the ACMG variant classification guidance (13), the c.1437C>T (p.Ser479=) variant was consistent with the pathogenic evidence of PM2 [absent from controls (or at extremely low frequency if recessive) in the Exome Sequencing Project, 1000 Genomes Project or Exome Aggregation Consortium], PM3 (for recessive disorders, detected in trans with a pathogenic variant), PP1 (co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease), PP3 (multiple lines of computational evidence support a deleterious effect on the gene or gene product) and PP4 (patient's phenotype or family history is highly specific for a disease with a single genetic etiology); thus, the synonymous variant was classified as 'likely pathogenic'. Previously, it was thought that synonymous variants had no functional effect; however, it has been reported that synonymous variants may affect pre-mRNA splicing by generating new splice sites or interfering with the original site and affecting the efficiency of translation owing to the alteration of the mRNA secondary structure (17,18).

In the second pedigree, the heterogeneous p.A490T variant in exon 11 was determined to be a 'pathogenic/likely pathogenic' variant in the ClinVar database. The other heterogeneous variant was in the donor site of intron 4, which was classified as being a variant of 'uncertain significance' in the ClinVar database. This splice site variant was reported in 2008; Blazquez *et al* (19) amplified CAPN3 complementary DNA and discovered that the A to G change promoted the skipping of exon 4 in the mRNA. According to the ACMG variant classification criteria, the c.632+4A>G variant may be classified as 'likely pathogenic'. Based on the WES analysis along with Sanger sequencing, which confirmed the co-segregation among the family members, it may be concluded that the compound heterogeneous variants were the cause of LGMD2A in the two pedigrees. In fact, there are significant differences in ACMG classifications determined by different laboratories and clinicians; it has been reported that clinicians tended to be more conservative when determining the cardiovascular variant classification compared with laboratories (20). Therefore, further experiments are required to verify the pathogenic mechanisms of the variants.

In the present study, it was attempted to amplify the genomic DNA fragments using PCR and RT-PCR analysis of mRNA was performed to verify the effect of c.1437C>T (p.Ser479=) and c.632+4 A>G variants. As only the peripheral blood of the patients and was obtained the expression levels of CAPN3 in the blood were low, the amplification was not successful. In addition, the disease progression of all patients was studied and it was attempted to obtain their previous medical records. However, due to disrepair and poor preservation, the results are not fully available.

The updated criteria for the diagnosis of LGMD include clinical features of progressive muscle weakness, elevated serum CK levels, the presence of degenerative changes during muscle imaging, the presence of dystrophic changes in a muscle biopsy and genetic inheritance (4). In the clinic, MRI and muscle biopsies are frequently used to diagnose muscle dystrophy; however, numerous types of LGMD do not exhibit specific pathological diagnostic markers or typical clinical features in the early stages of the disease. Furthermore, western

blot analysis of muscle biopsies indicated that ~20% of patients had normal CAPN3 protein expression levels (21). In fact, the autocatalytic function of the proteins was lost. In addition, the European Neuromuscular Center proposed a new LGMD classification in 2018, which highlighted the mode of inheritance. The new proposed nomenclature of LGMD2A is 'LGMD R1 calpain3-related', in which the letter 'R' means recessive and the number '1' means the order of discovery (4). Therefore, genetic testing may become important in preventing invasive testing. In the present study, next-generation sequencing served a critical role in the diagnosis of LGMD2A. The six patients all carried complex heterozygous mutations in the *CAPN3* gene and there were no patients in the next generation, which was consistent with the autosomal recessive inheritance pattern. WES is a widely used strategy to detect variants in coding regions and classic splice sites, and to identify new genes and variants. Considering the clinical and genetic heterogeneity of LGMD, WES represents a rapid, cost-effective and accurate method for the diagnosis of the disease and makes it possible to diagnose both common and rare cases. It has been reported that the use of WES successfully corrected the misdiagnosis from non-4q facioscapulohumeral muscular dystrophy to LGMD2A (22). In fact, with the use of WES, the variant detection rate has increased from 35 to 45% (23). As the pathogenesis of LGMD2A remains unclear, effective treatments currently do not exist. At present, the major treatments are rehabilitation treatment, drug therapy and the prevention of complications, which are aimed at improving the patients' quality of life (14). It is with regret that none of the patients in this study received good treatment due to the ambiguous diagnosis prior. In the future, high-throughput technologies will not only provide us with an accurate diagnosis but also help to determine the pathological mechanisms of muscle weakness and novel targets for treatment.

In conclusion, the present study reported four variants, including two known 'pathogenic' and two 'likely pathogenic' variants of CAPN3. According to the analysis of the genetic patterns in two large Chinese families, clinical evidence was provided for the classification of the variants. In addition, the present study emphasized the importance of WES in the diagnosis and determining the association between the phenotype and genotype in LGMD2A.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81771589), the Key Project of Tianjin Health Care Professionals (grant no. 16KKG166) and the Program of Tianjin Science and Technology Plan (grant no. 18ZXDBSY00170).

Availability of data and materials

The variant c.1437C>T (p.Ser479=) was submitted to the ClinVar database (submission ID: SUB8078779). The datasets used and/or analyzed during the current study

are available from the corresponding author on reasonable request.

Authors' contributions

JZ and XX participated in the conception of the study and writing of the manuscript. JZ, XX, XZ and XW performed the experiments. XW, JS and CC analyzed the experimental results. CC revised the manuscript and submitted the manuscript. All of the authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all family members and the study was approved by the ethics committee of Tianjin Children's Hospital (Tianjin, China).

Patient consent for publication

Written informed consent of the patients were obtained for publication.

Competing interests

The authors declare that they have no competing interests.

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