

A *De novo* Mutation in Dystrophin Causing Muscular Dystrophy in a Female Patient

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Abstract

Background: Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are X-linked recessive neuromuscular diseases resulting from dystrophin (*DMD*) gene mutations. It has been known that the carrier of *DMD* mutations may also have symptoms of the disease. While *de novo* mutation is quite common in BMD/DMD patients, it is rarely reported in the female carriers.

Methods: Two sporadic Chinese patients with progressive muscular dystrophy and their familial members were recruited. The targeted next-generation sequencing (NGS) and the multiplex ligation-dependent probe analysis (MLPA) were performed in the proband. Blood tests, electrocardiography, echocardiography, and electromyography were also evaluated.

Results: Two novel mutations of *DMD* gene were identified, c.7318C>T (p.Q2440*) in the male proband and c.4983dupA (p.A1662Sfs*24) in the female carrier. The MLPA analysis did not detect any large rearrangements. The haplotype analysis indicated that the two mutations were derived from *de novo* mutagenesis.

Conclusions: We identified two novel *de novo* mutations of *DMD* gene in two Chinese pedigrees, one of which caused a female patient with muscular dystrophy. The mutational analysis is important for DMD patients and carriers in the absence of a family history. The NGS can help detect the mutations in MLPA-negative patients.

Key words: Carrier; *De novo*; Duchenne Muscular Dystrophy; Dystrophin

INTRODUCTION

Mutations in the dystrophin (Duchenne muscular dystrophy [*DMD*]) gene, which encodes a protein connecting the cytoskeleton of muscle fibers, result in X-linked recessive dystrophinopathy, including DMD and Becker muscular dystrophy (BMD).^[1] DMD is the most common type of muscular dystrophy, affects 1:3500 to 6000 live male births, and is characterized by weakness of pelvic and shoulder muscles starting in early childhood.^[2] DMD is thought to be caused by the mutations causing totally nonfunctional dystrophin protein.^[3] In comparison, a reduced amount or shortened dystrophin was thought to lead in BMD, which has a milder clinical manifestation and better prognosis. The first symptoms of BMD start with a mean age at 11 years, and the average clinical course can be more than 45 years.^[4]

In general, the heterozygous female carriers of *DMD* mutations are asymptomatic, as long as the gene function

is compensated by the other normal allele. However, 2.5–22.0% of these carriers can develop symptoms which varied from mild muscular weakness to severe clinical complications, which are defined as manifesting or symptomatic carriers.^[5-7]

Although the *de novo* mutation is quite common in BMD/DMD patients,^[8] it is rarely reported in the female carriers.^[9,10] In this study, we identified two novel *de novo* *DMD* mutations in two Chinese pedigrees, including one in a manifesting female carrier. To our knowledge, this is

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the first report of a *de novo* DMD mutation in a Chinese female carrier.

METHODS

Ethical approval

This study was approved by the Ethics Committee for Human Research in Second Affiliated Hospital, Zhejiang University School of Medicine. Informed consents were obtained from all participants before enrollment in the study.

Subjects and ethics statement

Two sporadic Chinese patients with progressive muscular dystrophy and their familial members were recruited. Before the mutation analysis, both patients performed the routine blood tests, electrocardiography, and electromyography. The blood test included a full blood count, liver and renal function, electrolytes, thyroid function, serum cortisol, glucose, lactate, myocardial enzymes, erythrocyte sedimentation rate, antinuclear antibody, antinuclear cytoplasmic antibody, and tumor markers. The clinical diagnosis was based on progressive muscular weakness, muscle strength, elevated levels of creatinine kinase (CK), and myogenic changes on electromyography. Two hundred individuals without a history of muscular dystrophy were recruited as controls for mutation analysis.

Targeted next-generation sequencing

Genomic DNA was extracted from peripheral blood by QIAamp Blood Genomic Extraction Kits (Qiagen, Hilden, Germany). Targeted capture, library preparation, and sequence amplification of 43 related genes of muscular dystrophy were then performed [Table 1]. The sequencing was performed on the Illumina HiSeq2000 platform (Genengy Biotechnology Co. Ltd., Shanghai, China), and the detailed information of targeted next-generation sequencing (NGS) can be found in our previously reported studies.^[11,12]

Sanger sequencing

Sanger sequencing was performed on ABI 3500xL Dx DNA Genetic Analyzer (Thermo Fisher Scientific, USA) with a procedure described previously.^[13] All familial members and 200 controls were sequenced to confirm the identified mutations. The results were mapped and analyzed according to the standard DMD reference sequence (GenBank transcript ID: NM_004006.2).

Multiplex ligation-dependent probe analysis

Multiplex ligation-dependent probe analysis (MLPA) was used to detect the large rearrangements in the DMD gene.^[8] MLPA was performed using the SALSA MLPA kit P034/P035 DMD (MRC-Holland, The Netherlands) according to the manufacturer's protocol. The cutoff values for duplication and deletion were set as >1.2 and <0.7, respectively.

Haplotype analysis

To verify that the family members were genetically related, we performed haplotype analysis based on

Table 1: List of genes responsible for related muscular dystrophy

| Number | Disease | Gene | OMIM gene | Locus |
|--------|-----------------|-----------------|-----------|--------------|
| 1 | DMD, BMD | <i>DMD</i> | 300377 | Xp21.2-p21.1 |
| 2 | EDMD1 | <i>EMD</i> | 300384 | Xq28 |
| 3 | EDMD4 | <i>SYNE1</i> | 608441 | 6q25.1-q25.2 |
| 4 | EDMD5 | <i>SYNE2</i> | 608442 | 14q23.2 |
| 5 | EDMD6 | <i>FHL1</i> | 300163 | Xq26.3 |
| 6 | EDMD7 | <i>TMEM43</i> | 612048 | 3p25.1 |
| 7 | LGMD1A, MFM3 | <i>MYOT</i> | 604103 | 5q31.2 |
| 8 | LGMD1B, EDMD2/3 | <i>LMNA</i> | 150330 | 1q22 |
| 9 | LGMD1C | <i>CAV3</i> | 601253 | 3p25.3 |
| 10 | LGMD1E | <i>DNAJB6</i> | 611332 | 7q36.3 |
| 11 | LGMD1F | <i>TNPO3</i> | 610032 | 7q32.1 |
| 12 | LGMD1G | <i>HNRPDL</i> | 607137 | 4q21 |
| 13 | LGMD2A | <i>CAPN3</i> | 114240 | 15q15.1 |
| 14 | LGMD2B, MMD1 | <i>DYSF</i> | 603009 | 2p13.2 |
| 15 | LGMD2C | <i>SGCG</i> | 608896 | 13q12.12 |
| 16 | LGMD2C1/2K | <i>POMT1</i> | 607423 | 9q34.13 |
| 17 | LGMD2C14/2T | <i>GMPPB</i> | 615320 | 3p21.31 |
| 18 | LGMD2C2/2N | <i>POMT2</i> | 607439 | 14q24.3 |
| 19 | LGMD2C3/2O | <i>POMGNT1</i> | 606822 | 1p34.1 |
| 20 | LGMD2C4/2M | <i>FKTN</i> | 607440 | 9q31.2 |
| 21 | LGMD2C5/2I | <i>FKRP</i> | 606596 | 19q13.32 |
| 22 | LGMD2C7/2U | <i>ISPD</i> | 614631 | 7p21.2 |
| 23 | LGMD2C9/2P | <i>DAG1</i> | 128239 | 3p21.31 |
| 24 | LGMD2D | <i>SGCA</i> | 600119 | 17q21.33 |
| 25 | LGMD2E | <i>SGCB</i> | 600900 | 4q12 |
| 26 | LGMD2F | <i>SGCD</i> | 601411 | 5q33.2-q33.3 |
| 27 | LGMD2G | <i>TCAP</i> | 604488 | 17q12 |
| 28 | LGMD2H | <i>TRIM32</i> | 602290 | 9q33.1 |
| 29 | LGMD2J | <i>TTN</i> | 188840 | 2q31.2 |
| 30 | LGMD2L, MMD3 | <i>ANOS</i> | 608662 | 11p14.3 |
| 31 | LGMD2Q | <i>PLEC</i> | 601282 | 8q24.3 |
| 32 | LGMD2S | <i>TRAPPC11</i> | 614138 | 4q35.1 |
| 33 | LGMD2V | <i>GAA</i> | 606800 | 17q25.3 |
| 34 | LGMD2W | <i>LIMS2</i> | 607908 | 2q14 |
| 35 | MFM1, LGMD2R | <i>DES</i> | 125660 | 2q35 |
| 36 | MFM2 | <i>CRYAB</i> | 123590 | 11q23.1 |
| 37 | MFM4 | <i>LDB3</i> | 605906 | 10q23.2 |
| 38 | MFM5 | <i>FLNC</i> | 102565 | 7q32.1 |
| 39 | MFM6 | <i>BAG3</i> | 603883 | 10q26.11 |
| 40 | UCMD1, BTHLM1 | <i>COL6A1</i> | 120220 | 21q22.3 |
| 41 | UCMD1, BTHLM1 | <i>COL6A2</i> | 120240 | 21q22.3 |
| 42 | UCMD1, BTHLM1 | <i>COL6A3</i> | 120250 | 2q37.3 |
| 43 | UCMD2, BTHLM2 | <i>COL12A1</i> | 120320 | 6q13-q14 |

DMD: Duchenne muscular dystrophy; BMD: Becker muscular dystrophy; EDMD: Emery-Dreifussmuscular dystrophy; LGMD: Limb-girdle muscular dystrophy; MFM: Myofibrillar myopathy; MMD: Multi-minicore disease; UCMD: Ullrich congenital muscular dystrophy; BTHLM: Bethlem myopathy.

Table 2: Haplotype analysis based on 15 SNPs

| Number | SNP | Position | Gene | Alleles | AF (1) | Family 1 | | | Family 2 | | | |
|--------|-------------|-----------|----------------|-----------|--------|----------|-------|----------|----------|-------|-------|----------|
| | | | | | | G_i | G_m | L_{CP} | G_i | G_m | G_p | L_{CP} |
| 1 | rs3815049 | 13753457 | <i>OFD1</i> | 1(A)/2(G) | 0.4479 | 1 | 1/2 | 0.91 | 1/2 | 1/1 | 2 | 2.02 |
| 2 | rs2229137 | 19357664 | <i>PDHA1</i> | 1(A)/2(C) | 0.2537 | 1 | 1/1 | 1.34 | 1/2 | 1/2 | 2 | 1.32 |
| 3 | rs1800280 | 31478233 | <i>DMD</i> | 1(G)/2(A) | 0.9234 | 1 | 1/1 | 13.05 | 2/2 | 2/2 | 2 | 1.17 |
| 4 | rs2270672 | 31657979 | <i>DMD</i> | 1(C)/2(T) | 0.3047 | 1 | 1/1 | 1.44 | 1/2 | 1/1 | 2 | 2.36 |
| 5 | rs2222852 | 32035545 | <i>DMD</i> | 1(C)/2(G) | 0.3523 | 1 | 1/1 | 1.54 | 1/2 | 1/2 | 1 | 1.10 |
| 6 | rs7691 | 40600788 | <i>ATP6AP2</i> | 1(T)/2(C) | 0.1773 | 1 | 1/1 | 1.22 | 2/2 | 1/2 | 2 | 15.91 |
| 7 | rs145478020 | 47846285 | <i>ZNF81</i> | 1(C)/2(T) | 0.1685 | 1 | 1/1 | 1.20 | 1/2 | 1/2 | 1 | 1.78 |
| 8 | rs41305391 | 70330248 | <i>KIF4A</i> | 1(T)/2(C) | 0.1480 | 1 | 1/1 | 1.17 | 2/2 | 1/2 | 2 | 22.83 |
| 9 | rs72630048 | 72495283 | <i>HDAC8</i> | 1(C)/2(T) | 0.7711 | 2 | 2/2 | 1.30 | 2/2 | 2/2 | 2 | 1.68 |
| 10 | rs4148837 | 75114852 | <i>ABCB7</i> | 1(A)/2(G) | 0.9297 | 1 | 1/2 | 7.11 | 2/2 | 2/2 | 2 | 1.16 |
| 11 | rs2227291 | 78013005 | <i>ATP7A</i> | 1(G)/2(C) | 0.2759 | 1 | 1/1 | 1.38 | 1/2 | 1/2 | 1 | 1.25 |
| 12 | rs1126707 | 103787953 | <i>PLP1</i> | 1(T)/2(G) | 0.2500 | 2 | 2/2 | 4.00 | 2/2 | 1/2 | 2 | 8.00 |
| 13 | rs5973851 | 108174500 | <i>COL4A6</i> | 1(C)/2(T) | 0.6116 | 1 | 1/1 | 2.57 | 2/2 | 2/2 | 2 | 2.67 |
| 14 | rs5977104 | 129545053 | <i>OCRL</i> | 1(C)/2(G) | 0.7680 | 2 | 2/2 | 1.30 | 1/2 | 1/2 | 2 | 1.40 |
| 15 | rs2071134 | 153957384 | <i>HCFC1</i> | 1(C)/2(G) | 0.7493 | 1 | 1/2 | 1.99 | 2/2 | 1/2 | 2 | 0.89 |

AF: Allele frequency; G: Genotypes for the tested individual (G_i), putative maternal (G_m), and paternal (G_p) parents; L_{CP} : Likelihood ratio for coparentage; SNPs: Single-nucleotide polymorphisms.

15 single-nucleotide polymorphisms (SNPs), including the SNPs within the *DMD* and the regions flanked *DMD* [Table 2]. The SNPs which had a linkage disequilibrium with $r^2 > 0.8$ were rule out using Haploview 4.2 software (Broad Institute, MA, USA).^[14] The allele frequency was referred to the frequency of correspondent SNPs in Eastern Asian from Exome Aggregation Consortium (ExAC) variants database. The likelihood ratio for coparentage was calculated for each SNP and multiplied together to acquire a cumulative coparentage index (CPI).^[15] The probability of the proband being the biological child of the alleged parents was calculated as $P = CPI / (1 + CPI)$.

RESULTS

Clinical manifestation

Patient 1 was an 18-year-old boy with progressive weakness of both legs for 8 years. His parents denied any family history. The neurological examination revealed a significantly weakened power in the neck flexors (2+/5). The power in the proximal upper extremities was 4+/5 (deltoids) and lower extremities was 4/5 (bilateral hip flexor, hip extensor), and the other muscles were within a relatively normal range. Muscle reflexes were decreased with no abnormalities in the sensation examination. The Babinski sign was negative. The Gower sign was not obvious, but pseudohypertrophy could be seen in the right calf.

The blood tests were all grossly normal apart from elevated muscle enzymes, including CK (7866 U/L, normal reference: <145 U/L), CK isoenzyme (CK-MB) (117 U/L, normal reference: <24 U/L), alanine transaminase (166 U/L, normal reference: <45 U/L), aspartate transaminase (AST) (102 U/L, normal reference: <34 U/L), and lactate dehydrogenase (LDH) (566 U/L, normal reference: <248 U/L). Electrocardiography showed a sinus arrhythmia with high voltage of left ventricle. The echocardiography showed an

enlarged left ventricle with decreased function (ejection fraction: 48.7%). An electromyography study revealed a myogenic damage with short duration and low-amplitude polyphasic potentials in voluntary contraction. The patient had been initially considered as BMD clinically; however, the screening of *DMD* gene by MLPA failed to detect any duplication/deletion.

Patient 2 was a 45-year-old woman with 10 years of progressive weakness of the muscles in legs. On direct questioning, she reported no disturbance of bladder or bowel function or of sensation, no voice changing, choking or swallowing difficulty, no double vision, skin rash or dryness of the mouth, and no fatigable element to the weakness. She denied any family history and had one son without similar symptoms. On the neurological examination, the cranial nerves were normal. The power in the proximal lower extremities was 4/5 (bilateral hip flexor, hip extensor, knee flexor, and knee extensor), and the other muscles were within a relatively normal range. Muscle tone and reflexes were normal and symmetrical, and there were no abnormalities in sensation tests. The Babinski sign was negative. The Gower sign, calf pseudohypertrophy, or fasciculation was not observed, and there was no tenderness or discomfort on palpation.

The CK was mildly elevated to 1826 U/L. The CK-MB, AST, and LDH were 34 U/L, 39 U/L, and 307 U/L, respectively. Other blood tests were normal. Electrocardiography showed mild T wave changes and suspicious Q waves in the lateral wall leads (I, aVL, V5, V6-). The echocardiography showed a decreased diastolic function of the left ventricular. An electromyography study revealed a mild myogenic damage with short-duration polyphasic potentials in voluntary contraction. The magnetic resonance imaging of thighs revealed the muscle atrophy and fatty infiltration. As limb-girdle muscular dystrophy was taken into the clinical consideration first, the targeted NGS was performed for the further diagnosis.

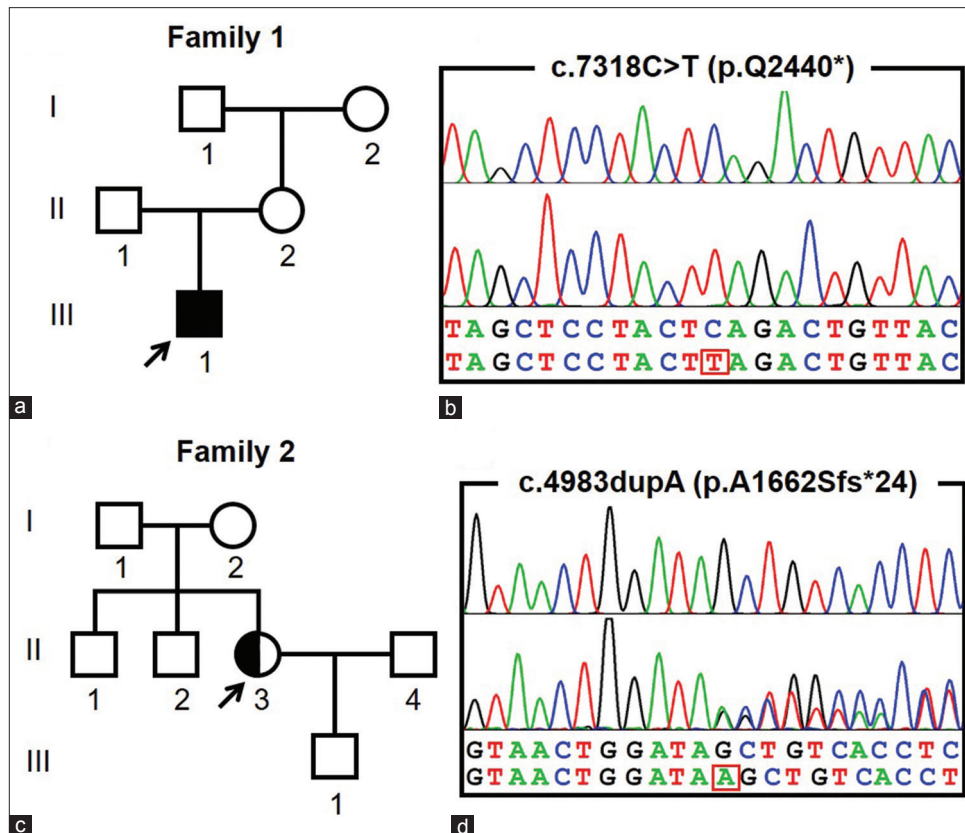


Figure 1: Left: Pedigree of the family 1 (a) and family 2 (c) with mutations in Duchenne muscular dystrophy gene. The empty symbols indicate clinically unaffected individuals. The black symbol filled indicates an affected patient. The symbol filled only half way with black color indicates a manifesting carrier. Right: Chromatogram of the Duchenne muscular dystrophy mutations in family 1 (b) and family 2 (d). The upper panel is a normal sequence, whereas the lower panel represents the mutated one.

Mutation analysis

After sequencing of two sporadic cases, two novel *DMD* variants were identified [Figure 1]. Patient 1 carried a novel nonsense variant, NM_004006.2: c.7318C>T (p.Q2440*), resulting in the substitution of a new stop codon termination for the glutamine. This variant was not found in 200 controls, 1000 Genomes Project (1000G), and ExAC database. According to the American College of Medical Genetics and Genomics (ACMG) Standards and Guidelines,^[16] the variant was classified as “pathogenic (Ia)” (PVS1 + PS2 + PM2). After the mutation was identified, the patient was finally diagnosed with BMD.

Patient 2 carried a novel frameshift variant, NM_004006.2: c.4983dupA (p.A1662Sfs*24), resulting in the substitution of serine for the previous alanine and the addition of 23 additional new amino acid residues prior to the stop codon termination within the new reading frame. Similarly, this heterozygous variant was not found in 200 controls, 1000G, and ExAC database. According to the ACMG Standards and Guidelines,^[16] this variant was classified as “pathogenic (Ia)” (PVS1+PS2+PM2). In addition, the MLPA did not detect any large duplication/deletion in the *DMD* gene. Therefore, the female patient was diagnosed as a manifesting DMD carrier.

Haplotype analysis

Surprisingly, the mutations of two patients were not found

in their family members. Therefore, we performed the haplotype analysis in two families based on 15 SNPs within and flanking the *DMD* gene [Table 2 and Figure 2]. Using reverse parentage testing, we acquired a CPI value of 20,562 in family 1 and a value of 341,303 in family 2. The calculated probability that the proband was the biological child of the alleged parents was >99.99% both in family 1 and family 2, which indicated that the two novel mutations were all derived from *de novo* mutagenesis in the pedigrees.

DISCUSSION

Large rearrangements (large deletions/duplications) count for 77.7% of all *DMD* mutations.^[17] Therefore, the MLPA turned to be a more preferable and faster method for mutation screening in *DMD*.^[8] However, in our patients, the mutations were all small lesions, including nonsense and frameshift mutations, which could count for 8.9% and 7.1% of *DMD* mutations, respectively.^[17] Therefore, the full-length sequencing is essential for diagnosis of dystrophinopathy as well as other myopathies when the MLPA result is negative.^[18,19]

The severity of the clinical manifestation in DMD/BMD patients generally depends on the occurrence of translation reading frame disruption and premature termination of protein synthesis.^[20] In DMD patients, most mutations are null mutations which predict a truncated protein or mRNA

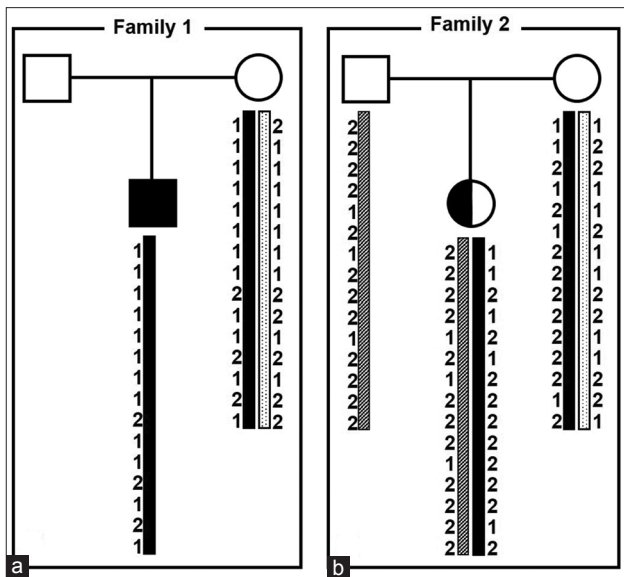


Figure 2: Haplotype analysis of probands and the alleged parents in family 1 (a) and family 2 (b) based on 15 single-nucleotide polymorphisms. Numbers beside the symbols represent the genotypes of the 15 single-nucleotide polymorphisms for each individual (1 and 2 denote the specific allele for each single-nucleotide polymorphism).

with a premature stop codon. Nonsense and frameshift mutations account for up to 48% and 32% of all small lesions in DMD patients, while nonsense and frameshift mutations represent only 24% and 16% of them in BMD patients, respectively.^[17] This reading frame rule holds true, respectively, for 96% and 93% of the mutations in DMD and BMD patients. As an exception, the milder phenotype in our patient carrying a nonsense mutation (exon 51) indicated some other modified factors in the genotype–phenotype correlation in DMD/BMD patients.

Although the clinical manifestation of female carriers vary significantly, the muscle weakness is usually mild and proximally distributed.^[5] The pelvic girdle is more frequently and earlier affected than the shoulder girdle. Age of onset is also variable, ranging from the first to the fourth decade. The serum CK is an important marker for screening the patients. A CK levels >1000 U/L in isolated female cases of myopathy should put consideration of an underlying dystrophinopathy in the front burner. About 10% of the isolated cases of myopathy with hyperCKemia were proven to have a dystrophinopathy as the cause of their disease (manifesting DMD carriers).^[21]

In DMD, BMD, and manifesting carriers, cardiomyopathy should always be considered. It is the leading cause of death in DMD and the main clinical complication in BMD and carriers of *DMD* mutations, which may not be accompanied with muscle weakness.^[3] Up to 40% female carriers can exhibit cardiac involvement, including left ventricle dilatation, global or segmental wall motion abnormality, and dilated cardiomyopathy.^[7] In our patients, the cardiac evaluation also detected the electrophysiological abnormality

and decreased cardiac function at varying degrees. It was recently reported that the addition of eplerenone to background angiotensin-converting enzyme inhibitor (ACEI) or angiotensin receptor blocker therapy attenuates the progressive decline in the left ventricular systolic function in DMD patients with preserved ejection fraction.^[22]

In summary, the female manifesting carrier of *DMD* mutation is a challenging condition for diagnosis which often relies on a clear X-linked family history of dystrophinopathy. Therefore, mutational analysis of the *DMD* gene is typically required for the suspected patients, particularly in the absence of a family history. Using the targeted NGS, we identified two novel mutations of *DMD* gene in two Chinese pedigrees, which broaden its mutation spectrum. Early diagnosis can motivate early treatment such as ACEIs or β -blockers to delay serious complications.

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Conflicts of interest

There are no conflicts of interest.

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