

Clinical and genetic characteristics of female dystrophinopathy carriers

JINGZI ZHONG^{1*}, YANSHU XIE^{1*}, VIDATA BHANDARI¹, GANG CHEN²,
YIWU DANG², HAIXIA LIAO¹, JIAPENG ZHANG¹ and DAN LAN^{1,3}

Departments of ¹Pediatrics and ²Pathology; ³Team for Major Disease Prevention and Control, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi 530021, P.R. China

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Abstract. The present study aimed to determine the genetic status of manifesting carriers (MCs) of Duchenne muscular dystrophy (DMD)/Becker muscular dystrophy (BMD) and asymptomatic carriers with a family history of DMD/BMD, and identify potential simple and reliable methods for screening dystrophinopathy carriers. Clinical data from probable carriers and MCs were collected and analyzed. MCs underwent multiplex ligation-dependent probe amplification (MLPA) for dystrophin gene exons combined with muscle disease panel test based on a next-generation sequencing (NGS) platform. In addition, the status of probable carriers was determined by MLPA or Sanger sequencing, according to the mutations of probands. A total of 154 female were enrolled, among which 78 cases were found to be carriers, including 4 MCs and 74 asymptomatic female carriers. The 4 MCs exhibited duplication mutations. Among the 74 asymptomatic carriers, 41.89% harbored deletion mutations, including 2 cases with suspected germline mosaicism and no mutation in the dystrophin gene, while 44.59% harbored point mutations in exons and only 10 cases (13.51%) carried duplication mutations. The area under the receiver operating characteristic

(ROC) curve of creatine kinase (CK) was 0.822, with a sensitivity of 65.38% and specificity of 92.1%. In addition, DMD was positively correlated with the CK, alanine transaminase and aspartate transaminase levels of the carriers. MLPA for exons of the dystrophin gene, along with NGS and Sanger sequencing, was effective for the diagnosis of MCs and for determining the status of probable carriers. The ROC curve analysis also demonstrated that CK level was an excellent predictor for distinguishing DMD/BMD carriers.

Introduction

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are the most common childhood muscular dystrophies, and are caused by mutations in the dystrophin gene, which encodes dystrophin protein at the Xp-21 locus. DMD and BMD occur with a birth prevalence rate of 15.9-19.5 cases per 100,000 live male births and 1 case per 6,000-8,000 live male births, respectively (1,2). Approximately 60-65% of mutations are deletions, 5-15% are duplications and 30% are point mutations (3). There are two hotspots in the dystrophin gene. The most important hotspots are located in exon 45-55 and exon 2-19 (4). However, DMD and BMD are X-linked recessive diseases, and thus females are the carriers of the mutation.

The majority of DMD/BMD carriers remain asymptomatic, while only 2.5-10% of carriers are symptomatic and classified as manifesting carriers (MCs) (5). The symptoms of MCs range from mild muscle weakness to severe abnormal gait with frequent falls, and difficulty in rising from the floor or walking on tiptoes. Certain patients even have rapidly progressive muscular dystrophy similar to male dystrophinopathy. Diagnosis of DMD/BMD carriers is based on clinical symptoms, family history, biochemistry markers, echocardiography, pathology, molecular genetic analysis and linkage-testing. Identification of DMD/BMD carriers may present challenges, particularly in the absence of a family history of DMD/BMD. Genetic analysis of the dystrophin gene is typically required for diagnosis, particularly for genetic consulting and prenatal diagnosis.

In the present study, clinical data were collected and analyzed from 154 probable female carriers. The creatine kinase (CK) level of the subjects, and its correlation with

Correspondence to: Dr Dan Lan, Department of Pediatrics, The First Affiliated Hospital of Guangxi Medical University, 6 Shuangyong Road, Nanning, Guangxi 530021, P.R. China
E-mail: land6785@163.com

*Contributed equally

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; BMD, Becker muscular dystrophy; CK, creatine kinase; DMD, Duchenne muscular dystrophy; InDels, insertions or deletions; LGMD, limb-girdle muscular dystrophy; NGS, next-generation sequencing; MCs, manifesting carriers; MLPA, multiplex ligation-dependent probe amplification; ROC, receiver operating characteristic; SNP, single-nucleotide polymorphism

Key words: Duchenne muscular dystrophy, Becker muscular dystrophy, manifesting carriers, asymptomatic carriers, genetic analysis, germline mosaicism

alanine transaminase (ALT) and aspartate transaminase (AST) levels were analyzed. Multiplex ligation-dependent probe amplification (MLPA) for exons of the dystrophin gene, combined with a muscle disease panel based on next-generation sequencing (NGS), was applied to detect the MCs. Two of the MCs were confirmed by muscle pathological examination. The status of probable carriers was determined by MLPA and Sanger sequencing according to the mutations of probands. The present study presents a non-invasive, easy screening for MCs of DMD/BMD, and describes an effective, accurate method for the detection of deletions/duplications and small mutations in DMD/BMD carriers.

Materials and methods

Participants. The study participants were selected between January 2013 and October 2017 at the First Affiliated Hospital of Guangxi Medical University (Nanning, China). The subjects included male DMD patients (proband), symptomatic females and their relatives, including mothers, maternal grandmothers, maternal aunts and sisters of probands with confirmed DMD dystrophin gene mutations. The present study was conducted in strict accordance with the recommendations in the Guidelines of the Centers for Disease Control and Prevention (1). The study was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University. Informed consent was obtained from all participants included in the study.

Inclusion and exclusion criteria. Subjects included in the present study fulfilled one of the following criteria: i) Exhibited clinical manifestations, such as muscle weakness, walking with frequent falling, abnormal gait, and difficulty running, jumping and going upstairs; ii) were maternal female relatives of probands with confirmed diagnosis of DMD/BMD; and iii) exhibited a persistent CK level that was >2 times greater than the upper limit of the normal range (CK, <178 U/l). Symptomatic females who were confirmed by genetic testing to suffer from another muscular dystrophy disease, such as limb-girdle muscular dystrophy (LGMD), or females with incomplete clinical information were excluded from the present study.

Clinical analysis. The clinical data (including the chief complaint, history of present illness, growth and development history, past medical history and family history) of all subjects were collected and reviewed retrospectively. Blood samples were collected and levels of serum enzymes CK, liver function and cardiac enzymes concentrations were measured using a Hitachi 7600-020 analyzer (Hitachi, Ltd., Tokyo, Japan). All assays were conducted according to the manufacturer's instructions using the same batch reagent. Echocardiography findings and muscle biopsies were analyzed.

Genetic analysis. All probands and MCs underwent MLPA for dystrophin gene exons. If no mutation was identified by MLPA, NGS-based muscle disease panel tests (containing 169 known muscle disease-associated genes, including for LGMD and facioscapulohumeral muscular dystrophy) was performed. When large deletions/duplications were observed by MLPA

analysis, MLPA was also used to classify the female relatives. If a point mutation was identified by NGS in probands, The mutation site was amplified by PCR (the primers were designed by the website Primer Z(<http://genepipe.ncgm.sinica.edu.tw/primerz/primerz4.do>), and then sequenced by Sanger sequencing for probands and their female relatives.

MLPA analysis. DNA samples were extracted from peripheral blood obtained from the subjects according to standard procedures [FlexiGene DNA kit (cat. no. 51206; Qiagen GmbH, Hilden, Germany)]. All 79 exons of the *dystrophin* gene were screened by MLPA. Two sets of reagents (SALSA MLPA probe sets P034 and P035) were used to perform the MLPA reaction according to the manufacturer's instructions (MRC-Holland BV, Amsterdam, The Netherlands). Genomic DNA was denatured, hybridized, ligated, and amplified. Amplified products were analyzed on an ABI model 3500XL capillary sequencer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The initial data were analyzed using GeneMapper software (version 140701.0000), and the peak area of each fragment was compared with that of 3 control samples.

Target enrichment of genomic DNA and sequencing. A minimum of 3 µg DNA was used to construct the indexed Illumina libraries according to the manufacturer's instructions. A final library size of 200-300 bp, including adapter sequences, was selected. All exons of the *dystrophin* gene were target-enriched using the *dystrophin* Exon Enrichment kit (MyGenostics, Inc., Baltimore, MD, USA). The muscle diseases panel was a complete kit designed by the Zhongguancun Huakang Gene Institute (Beijing, China) and synthesized using the Agilent SureSelect Target Enrichment technique (Agilent Technologies, Inc., Santa Clara, CA, USA). The capture experiment was conducted according to the manufacturer's instructions. The enrichment libraries were sequenced on an Illumina HiSeq 2000 sequencer (Illumina, Inc., San Diego, CA, USA) for paired-read 100 bp sequencing.

Bioinformatics analysis. Following HiSeq 2000 sequencing (Illumina, Inc.), high-quality reads were retrieved from raw reads by filtering out the low-quality reads and adaptor sequences using the Solexa QA package (sourceforge.net/projects/solexaqa/files/) and the cutadapt program (hpc.nih.gov/apps/cutadapt.html), respectively. The SOAPaligner program (soap.genomics.org.cn/) was then used to align the clean read sequences to the human reference genome (hg19). To detect exon duplications and deletions, the coverage of each position was plotted by base position. Higher coverage of a region indicated duplication, whereas regions that were not covered corresponded to deletions. Identical sequences were produced by polymerase chain reaction (PCR) duplication to obtain cluster formation (human genomic DNA template). After the PCR duplicates were removed using Picard software (<http://broadinstitute.github.io/picard/>; version 2.6.0-SNAPSHOT), single-nucleotide polymorphisms (SNPs) were identified using the SOAPsnp program (<http://soap.genomics.org.cn/soapsnp.html>). Subsequently, the reads were realigned to the reference genome using BWA, and insertions or deletions (InDels) were

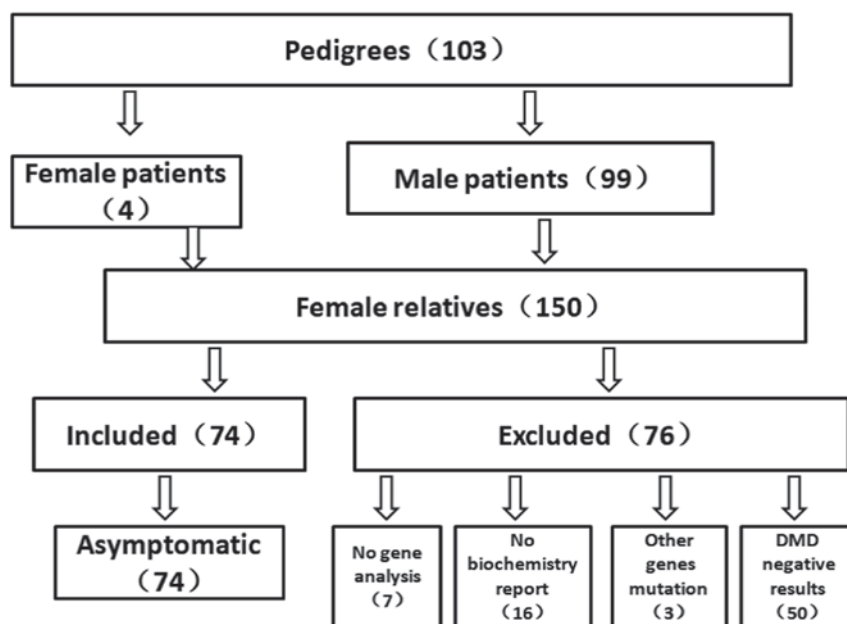


Figure 1. Flow diagram of the study.

identified using the GATK program (www.broadinstitute.org/gsa/wiki/index.php/Home_Page). The identified SNPs and InDels were annotated using the Exome-assistant program (<http://122.228.158.106/exomeassistant>).

Muscle pathology. Biopsy of the right gastrocnemius was performed in 2 female patients (both MCs) and 1 male DMD patient, and non-muscular dystrophy muscle tissue was utilized as a control. Each patient underwent open muscle biopsy from the right gastrocnemius under local anesthesia. Fresh specimens were fixed in 10% neutral buffered formalin and further processed into paraffin-embedded blocks. The morphology was observed under a microscope following hematoxylin and eosin staining. In addition, immunohistochemistry was used to evaluate dystrophin protein expression. Briefly, sections were incubated at 4°C overnight with a rabbit polyclonal antibody targeting dystrophin (cat. no. RB-9024; 1:100; Thermo Fisher Scientific, Inc.), and then incubated at room temperature for 30 min with Supervision TM Universal (6) (Anti-Mouse/Rabbit) Detection Reagent (HRP) (cat. no. D-3004; Lab Vision Corporation, Fremont, CA, USA) conjugated to peroxidase in Tris-HCl buffer containing carrier protein and anti-microbial agent. The experiment was performed according to the manufacturer's protocol.

Statistical analysis. Statistical analysis was conducted using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). Independent-samples t-test was used to compare the difference in CK level between Group 1 (MCs) and Group 2 (asymptomatic female carriers with high CK levels). Pearson's correlation coefficient (r) was used to identify the probable correlation of CK level with the AST and ALT levels in female carriers. Receiver operating characteristic (ROC) curve analysis was used to distinguish the predicted value of CK in DMD/BMD carriers. $P < 0.05$ was considered to denote differences that were statistically significant.

Results

Participant characteristics. A total of 103 families from the south of China were recruited in the present study, including 4 MCs and 99 male DMD patients. Furthermore, 150 female relatives were collected. Of these 150 participants, 23 participants were excluded due to incomplete clinical information, and 3 participants were excluded due to testing positive for a mutation in other genes that resulted in muscular dystrophy disease, confirmed as LGMD by muscle disease panel analysis. A homozygous mutation c.583G>A in the SGCA gene (LGMD2D), compound heterozygous mutations c.620T>C and c.823C>T in the FKRP gene (LGMD2I), and compound heterozygous mutations c.77848C>T and c.97099C>T in the TTN gene (LGMD2J) were detected in these 3 cases. A total of 50 participants were also excluded as their DMD genetic analysis results were negative. Ultimately, 78 females were enrolled into the present study. Among these, 4 cases were MCs and 74 cases were asymptomatic female carriers, including 2 cases who were carriers of suspected germline mosaicism with no mutation in the dystrophin gene (Fig. 1).

Clinical symptoms. The present study included 4 symptomatic female carriers. Two of these MCs were admitted to the hospital due to elevated CK levels. All exhibited a variety of symptoms, including falls, waddling gait and tiredness (Table I).

Laboratory examination

Variation of CK level. The 4 MCs exhibited an increased CK level (100%), and the mean CK level was 4,584.75 U/l. Among the 74 asymptomatic carriers, 47 cases had high CK levels, including 45 first-degree relatives and 2 sec-degree relatives of DMD/BMD probands (Fig. 2), with a mean CK level of 987.8 U/l (range, 180-7,012 U/l). The ROC curve analysis revealed that the CK level was an excellent predictor for distinguishing DMD/BMD carriers. The area under the

Table I. Clinical and genetic characteristic of 4 MCs.

MC patient	Age at onset	Family history	Severity	Clinical symptoms	CK (U/l)	AST (U/l)	ALT (U/l)	Biopsy	Mutation position
1	2 years	Positive	DMD-like	Mild motor developmental delay, easy fatigue, slow gait, Gowers sign (+), calf pseudohypertrophy, muscle weakness of upper limbs, lower limbs 4(+)	6,162	107	154	Small amount of the proliferation of connective tissue in the muscle fibers, few stromal small vessels with small amount of granulocytes Immunohistochemical examination for dystrophin antibody exhibited a mosaic reduction of the protein expression	Duplication at exons 45-55
2	3 years	Negative	DMD-like	Easy fatigue, Gowers sign (+)	5,306	171	97	Not performed	Duplication at exons 9-10 and 69-70
3	Early childhood	Positive	Mild BMD-like	Slow gait, myalgia, easy fatigue, muscle weakness of lower and upper limb 4 (+)	4,713	83	101	Not performed	Duplication at exons 45-55
4	4 years	Unknown (adopted)	Mild, BMD-like	Myalgia, muscle weakness of lower and upper limb 5	2,158	91	60	Some muscle atrophy, fuzzy muscle fibers Immunohistochemical examination for dystrophin antibody exhibited a mosaic reduction of the protein expression	Duplication at exons 49-52

Normal CK level, <178 U/l; normal AST level, <45 U/l; normal ALT level, <60 U/l. MC, manifesting carrier; CK, creatine kinase; AST, aspartate transaminase; ALT, alanine transaminase; DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; 4 (+), movement against external resistance; 5, normal muscle strength and movement.

Table II. CK level of all female carriers included in the study.

Group	No. (n=78)	CK level (U/l)
Group 1	4	6162, 5306, 4713, 2158
Group 2	47	7012, 4142, 2724, 2078, 2065, 1647, 1516, 1454, 1419, 1390, 1233, 1183, 1169, 1095, 967, 944, 920, 855, 794, 759, 758, 655, 633, 582, 550, 525, 515, 500, 494, 484, 460, 434, 431, 415, 372, 355, 335, 325, 322, 322, 318, 288, 242, 197, 186, 183, 180
Group 3	27	174, 167, 157, 154, 147, 135, 128, 128, 128, 118, 110, 106, 102, 97, 96, 90, 84, 83, 81, 76, 73, 72, 71, 69, 66, 61, 24

Group 1 represents the manifesting carriers; Group 2 represents asymptomatic female carriers with a high CK level; and Group 3 represents asymptomatic female carriers with a normal CK level. CK, creatine kinase.

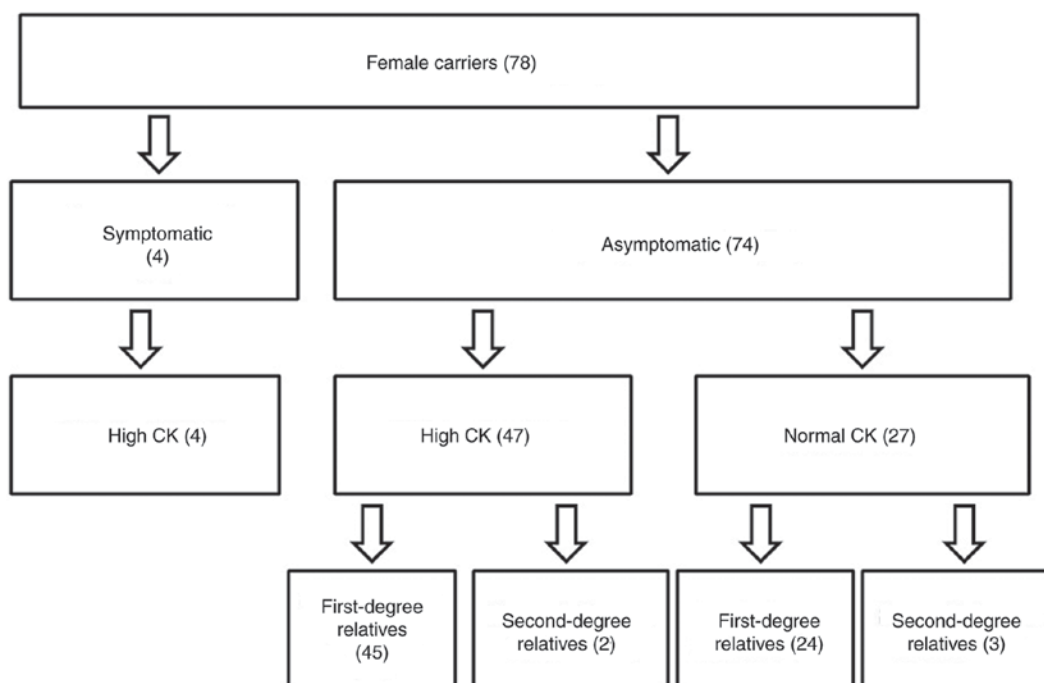


Figure 2. Population distribution and consanguinity of female carriers with different level of CK level. CK, creatine kinase; DMD, Duchenne muscular dystrophy; NGS, next-generation sequencing; MLPA, multiplex ligation-dependent probe amplification.

ROC curve was 0.822 ($P < 0.001$), the sensitivity was 65.38% and the specificity was 92.1% (Fig. 3). Furthermore, a total of 27 asymptomatic female carriers had normal CK levels, accounting for 24 first-degree relatives and 3 sec-degree relatives (Fig. 2). The mean CK level of these subjects was 103.59 U/l (range, 24-174 U/l; Table II). Female carriers were then divided into 3 groups according to their CK level: Group 1, MCs; Group 2, asymptomatic female carriers with high CK levels; and Group 3, asymptomatic female carriers with normal CK levels (Table II). Independent-samples t-test was used to compare the difference in CK level between Group 1 and Group 2, indicating that the CK level of MCs was significant higher in comparison with that of asymptomatic female carriers with high CK ($P < 0.001$).

Correlation of CK with AST and ALT levels. Among the 4 symptomatic and 74 asymptomatic female carriers, 61 cases exhibited normal ALT and AST levels, whereas 17 cases

had elevated ALT and AST levels. The mean ALT level was 33.56 U/l, with a minimum of 9 U/l and a maximum of 154 U/l. Similarly, the mean AST level was 38.20 U/l, with a minimum of 14 U/l and a maximum of 171 U/l. Further analysis indicated that the variation in ALT and CK levels exhibited a positive correlation ($r = 0.753$, $P < 0.05$; Fig. 4), while a positive correlation was also observed between AST and CK levels ($r = 0.791$, $P < 0.05$; Fig. 5). AST exhibited a stronger correlation with CK levels as compared with that of ALT and CK levels.

Molecular characteristics of female carriers. In the present study, 4 MCs were detected by MLPA analysis of the dystrophin gene exons. All of the MCs exhibited duplication mutations, and duplications of exons 9-10, 69-70, 49-52 and 45-55 were identified. The other 74 asymptomatic carriers were analyzed by MLPA combined with Sanger sequencing. Among these, 31 (41.89%) cases carried deletion mutations, 33 (44.59%) cases exhibited point mutations, and only 10 cases (13.51%)

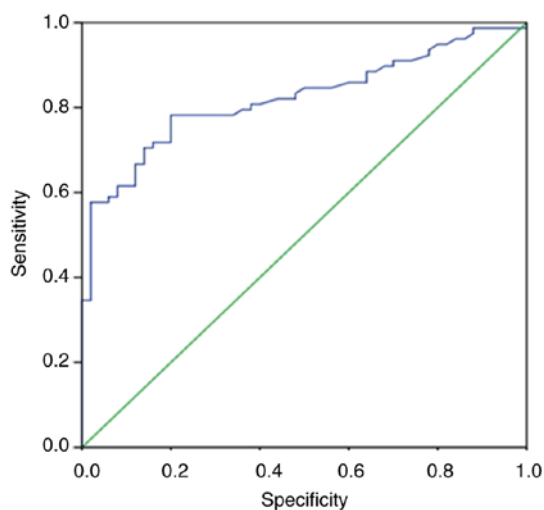


Figure 3. Receiver operating characteristic curve for CK level to classify Duchenne/Becker muscular dystrophy carriers. The area under the curve was 0.822 (ideal value, 1.00). This indicated an excellent diagnostic accuracy, which suggests that CK level has high specificity and sensitivity for the diagnosis of female carriers. CK, creatine kinase.

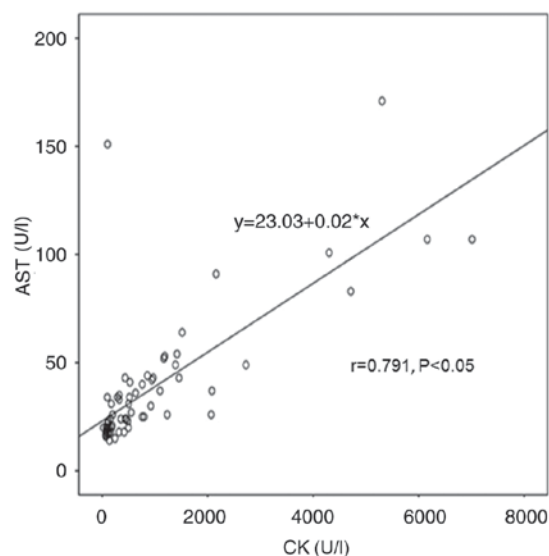


Figure 5. Serum AST and CK levels exhibit a linear correlation ($r=0.791$, $P<0.05$) in female carriers. AST, aspartate transaminase; CK, creatine kinase.

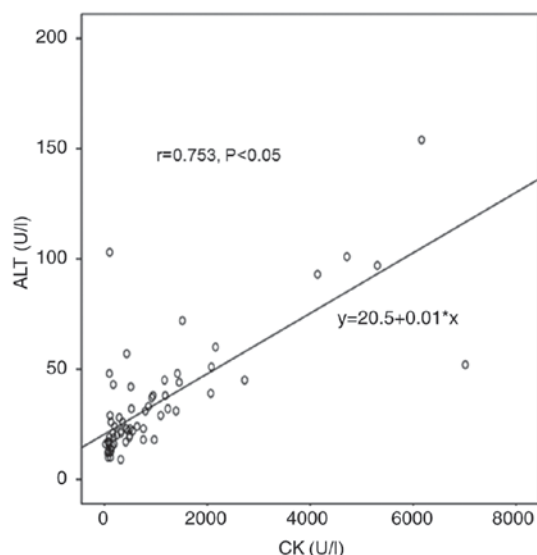


Figure 4. Serum ALT and CK levels exhibit a linear correlation ($r=0.753$, $P<0.05$) in female carriers. ALT, alanine transaminase; CK, creatine kinase.

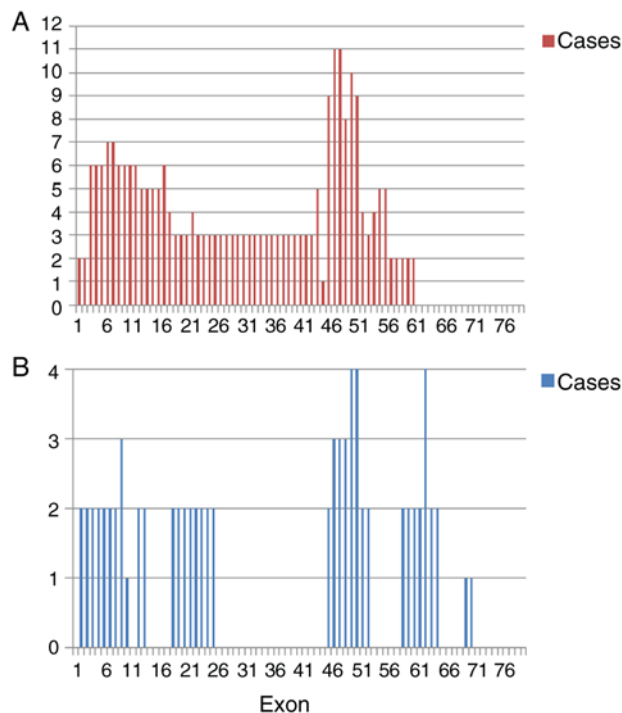


Figure 6. Frequency of deletion and duplication for all 79 exons of the dystrophin gene was analyzed by multiplex ligation-dependent probe amplification. In total, 31 cases carried deletion mutations, while 10 carried duplication mutations. (A) Deletion mutations were detected in the majority of exons, with the exception of exons 61-79, and the deletion hotspot regions were exons 45-55, followed by exons 3-16. (B) Duplication mutations were clustered at the 3' end of the dystrophin gene.

exhibited duplication mutations. In addition, 2 of the asymptomatic females were carriers of suspected germline mosaicism without mutations in dystrophin gene, although their daughters had the same mutations as the probands. Overall, 34 different mutations were characterized in the present study. The deletion breakpoints were mainly clustered at exons 45-55, followed by exons 3-16 (Fig. 6A), whereas duplication breakpoints were clustered at the 3' end of the dystrophin gene (Fig. 6B). The point mutation detection rate and distribution of mutations according to type, including 22 nonsense, 4 frame shift and 7 splice site mutations, are shown in Table III.

In addition, it was observed that 31 (36%) mothers were negative for dystrophin gene analysis, and the associated probands had possible *de novo* mutations. A total of 6 female relatives exhibited negative genetic results by MLPA and NGS,

however, their CK levels were abnormally high (Table IV). These cases were first degree relatives of the probands, and a muscle disease diagnosis could not be made by muscle panel analysis. Therefore, the possibility that these subjects were DMD carriers cannot be excluded, particularly given the possibility of somatic mosaicism. Certainly, other factors that would cause high CK levels should be considered.

Table III. Point mutations of female carriers, analyzed by Sanger sequencing.

Patient	Base change	Effect	Exon ID	Mutation type
1	c.2605C>T	P.Gln869Ter	Exon 20	Nonsense
2	c.6318G>A	p.Trp2106Ter	Exon 44	Nonsense
3	c.1860_1861delGT	p.Leu620LeufsTer14	Exon 16	Frameshift
4	c.8027+1G>A	-	Intron 54	Splicing
5	c.8087 delT	p.Leu2696ArgfsTer30	Exon 55	Frameshift
6	c.6292C>T	p.Arg2098Ter	Exon 44	Nonsense
7	c.5488A>T	p.Arg1830Ter	Exon 39	Nonsense
8	c.1332-9A>G	-	Intron 11	Splicing
9	c.7657C>T	p.Arg2553Ter	Exon 52	Nonsense
10	c.133C>T	p.Gln45Ter	Exon 3	Nonsense
11	c.8010G>A	p.Trp2670Ter	Exon 54	Nonsense
12	c.10498_10499delAG	p.Ser3500Ter	Exon 74	Frameshift
13	c.10108C>T	p.Arg3370Ter	Exon 70	Nonsense
14	c.3151C>T	p.Arg1051Ter	Exon 23	Nonsense
15	c.10223+1G>A	-	Intron 69	Splicing
16	c.5488A>T	pArg1830Ter	Exon 39	Nonsense
17	c.3982C>T	p.Gln1328Ter	Exon 29	Nonsense
18	c.490A>T	p.K164X	Exon 6	Nonsense
19	c.10108C>T	p.Arg3370Ter	Exon 70	Nonsense
20	c.3982C>T	p.Gln1328Ter	Exon 29	Nonsense
21	c.3721G>T	p.Glu1241Ter	Exon 27	Nonsense
22	c.10171C>T	p.Arg3391Ter	Exon 70	Nonsense
23	c.10171C>T	p.Arg3391Ter	Exon 70	Nonsense
24	c.1332-8A>G	-	Intron 11	Splicing
25	c.3603+1G>A	-	Intron 26	Splicing
26	c.1800-1G>A	-	Intron 14	Splicing
27	c.462+1G>T	-	Intron 6	Splicing
28	c.133C>T	p.Gln45Ter	Exon 3	Nonsense
29	c.691T>A	p.Tyr231Asn	Exon 8	Frameshift
30	c.5488A>T	pArg1830Ter	Exon 39	Nonsense
31	c.5488A>T	pArg1830Ter	Exon 39	Nonsense
32	c.10171C>T	p.Arg3391Ter	Exon 70	Nonsense
33	c.3151C>T	p.Arg1051Ter	Exon 23	Nonsense

Table IV. CK level of female relatives with high CK level and negative genetic results.

Female relative	CK (U/l)	AST (U/l)	ALT (U/l)	Mutation of proband
1	297	24	18	DEL EX46-59
2	194	14	19	DEL EX46-48
3	632	19	18	DEL EX45-55
4	257	20	16	DEL EX50-54
5	191	13	19	DEL EX04-11
6	311	38	43	DEL EX51

Normal CK level, <178 U/l; normal AST level, <45 U/l; normal ALT level, <60 U. CK, creatine kinase; AST, aspartate transaminase; ALT, alanine transaminase.

Muscle biopsy results. Among the 4 symptomatic carriers, 2 patients were subjected to muscle biopsy (Fig. 7A and B).

The proliferation of connective tissue in the muscle fibers was low, and individual interstitial small vessels cut through

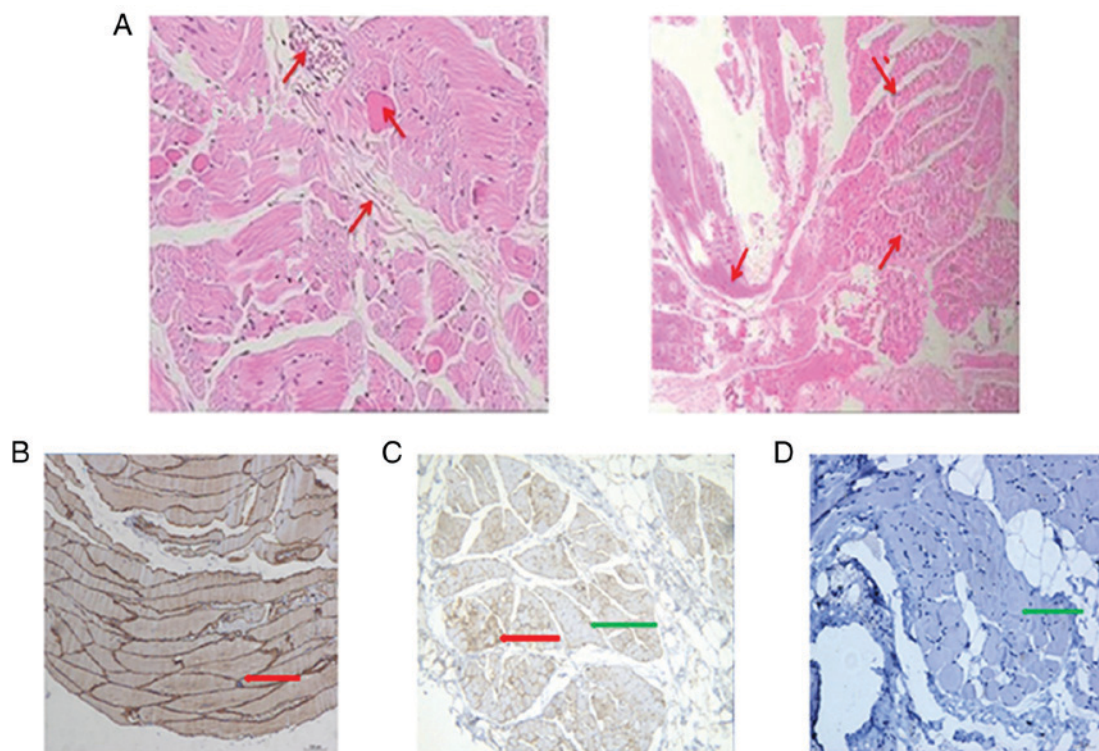


Figure 7. Sections with HE staining and immunolabeling with dystrophin antibodies of patient 1 (magnification, x100). (A) HE staining of muscle biopsies of patient 1, exhibiting a small amount of connective tissue proliferation in the muscle fibers, few stromal small vessels with a small amount of granulocytes and lymphocytes infiltration. Dystrophin expression in the biopsies obtained from a (B) control, (C) female MC (patient 1) and (D) male DMD patient. The control section presented a normal expression of dystrophin, with a consistent and uniform brown muscle fiber membrane. The MC (patient 1) exhibited random presence of normal and dystrophin-deficient fibers, indicating a mosaic expression of dystrophin. By contrast, the male DMD patient exhibited almost no expression of dystrophin in the muscle fiber membrane. The red arrow indicates expression of dystrophin in brown muscle fiber membrane, and the green arrow indicates absence of dystrophin expression. HE, hematoxylin-eosin; MC, manifesting carrier; DMD, Duchenne muscular dystrophy.

only a few granulocytes and lymphocytes. ‘Muscle fiber necrosis was observed and regeneration was not evident, except when combined with clinical myositis. The immune positivity of spectrin in all biopsies indicated well-preserved sarcolemmal integrity, ensuring that false negative results for other sarcolemmal membrane-associated proteins could be ruled out. Furthermore, immunohistochemical examination with monoclonal antibodies against dystrophin indicated a mosaic reduction of the protein expression in these MCs. The control biopsy presented a normal expression of dystrophin, with a consistent and uniform brown muscle fiber membrane. By contrast, in male DMD patients, almost no dystrophin expression was observed in the muscle fiber membrane (Fig. 7C-E).

Discussion

Dystrophinopathy is the most common muscular dystrophy and involves X-linked recessive inheritance, while MCs are rare. In the present study, 4 MC females (4/78 participants; 4.49%) were reported, who presented with muscle weakness and elevated CK levels. Duplication mutations in dystrophin gene were detected in all of the MCs. It has previously been reported that 2.5-10% carriers experience symptoms ranging from mild muscle weakness to rapidly progressive DMD-like muscular dystrophy (5), which is consistent with the results of the present study. Several disease-causing mechanisms

have been implicated in DMD/BMD MCs; however, the most frequently reported mechanism to provoke symptoms in DMD/BMD carriers is skewed X-inactivation (7). In X-linked dominant diseases, X inactivation can influence the severity of the phenotype or the survival of females with heterozygous lethal mutations. Our next step will be relevant to the X inactivation analysis of these carriers to clarify its pathogenesis.

The phenotypes of MCs of DMD/BMD are similar to LGMD and are, therefore, likely to be misdiagnosed with LGMD. MCs with motor weaknesses should be distinguished from other myodystrophies. As many as 17% of dystrophinopathies are misdiagnosed as LGMD (8). In the present study, 3 cases among the 78 cases exhibited progressive motor weaknesses similar to DMD and elevated CK levels. A subsequent muscle disease panel analysis revealed their diagnosis as LGMD, which was confirmed by muscle biopsy and immunostaining. In addition to weakness symptoms, an MC with motor developmental delay was detected, but without any other intellectual or cognitive disorders or mental illnesses. These findings are in accordance with those reported by Seeman *et al* (9) in 2010. More recently, the incidence of mental retardation in MCs was found to be as high as 7% (10). However, all 4 female symptomatic carriers in the present study did not exhibit evidence of mental retardation.

As CK is a component of the muscle fiber cytosol, its serum activity is considered to be positively correlated with

progressing muscle damage. An elevated CK level is one of the characteristic features of DMD/BMD, particularly when screening neonatal carriers. To the best of our knowledge, the present study is the first to use a ROC curve to analyze the diagnostic sensitivity of CK level in DMD/BMD, and the results suggested that the CK level has high specificity and sensitivity for the diagnosis of female carriers. For female relatives with negative genetic tests, particularly those with CK values up to 1.5-3 times the normal upper limit, muscle biopsy and immunohistochemical analyses are recommended to evaluate dystrophin protein expression to confirm the status of probable carriers. As previous studies have suggested, chronic persistent CK elevation indicates the possibility of neuromuscular disease (11). Wang *et al* (12) reported that among a group of 201 female relatives, 97 of the 98 first-degree relatives with high levels of CK were DMD or BMD carriers, while 31 of the remaining 103 subjects exhibiting normal CK levels were diagnosed as carriers. Therefore, the possibility that female relatives with normal levels of CK could be carriers cannot be excluded. In the present study, it was also observed that CK levels were positively correlated with the levels of ALT and AST. Elevated ALT/AST may be the presenting sign of muscle disease in children, which provides an opportunity for early diagnosis. For patients with unexplained long-lasting hypertransaminasemia, serum CK activity should be measured and muscle biopsy should be conducted at an early stage for correct diagnosis of muscular dystrophy. The sensitivity of ALT and AST was observed to be 21.79% in the present study, which is significantly lower compared with that for the CK level. Therefore, the CK level is a better predictor of DMD/BMD carriers, and female relatives with elevated ALT/AST should also be screened for serum CK activity to identify potential carriers. While a total of 6 female relatives in the present study had a high CK level, they did not present any mutations in the *dystrophin* gene. Since these patients did not agree to undergo muscle biopsy, they cannot be excluded as possible carriers.

The limitations of MLPA and NGS analyses can lead to false negatives, while germline mosaicism carriers would also show a negative result in genetic tests. Luce *et al* (13) reported three recombination events and suggested that germline mosaicism had occurred in these families. In addition, high CK levels may be due to other non-neuromuscular factors, such as physical activity or muscle trauma (14), and further study would help to exclude the possibility of myopathy. Currently, muscle biopsy immunostaining has become important for the diagnosis of myopathy. In the present study, the muscle biopsy immunostaining results of two symptomatic carriers exhibited mosaic distribution. Approximately 20% of carriers had abnormal dystrophin immunostaining on muscle biopsy, with a mosaic pattern of dystrophin-positive and dystrophin-negative fibers present (15). When the gene mutation analysis is negative, muscle biopsy with immunohistochemical staining would be useful to assist in the further diagnosis of carriers.

In the present study, 74 asymptomatic carriers were detected by MLPA combined with Sanger sequencing. Among these, 41.89% of cases involved deletion mutations, 44.59% of cases exhibited point mutations in exons and 13.51% exhibited duplication mutations. These results are similar to those of a

previous study (16). Additionally, all of 4 MCs carried frameshift and duplication mutations. By contrast, point mutations and deletions were more common in female asymptomatic carriers, as previously reported in the literature (17). More frameshift and duplication mutations were observed in MCs in the present study, which may be due to the small sample size. Therefore, larger samples should be used in future studies. Large segmental deletions and duplications in female carriers of dystrophin gene mutations involved the majority of the exons. Deletion breakpoints were mainly clustered at exons 45-55, followed by exons 3-16, which was similar to the results previously reported in the literature (4). However, in the present study, duplications were concentrated in the 3' end of the DMD gene, in contrast with previously reported DMD gene mutation hot spots at the 5' end (17). An earlier study analyzed 92 suspected DMD/BMD male patients, and reported that the deletion and duplication hot spots in the dystrophin gene were the same as those of female carriers in the present study (18). The discrepancy between the results of the present and previous studies may be due to the small sample we collected.

According to the literature, approximately two-thirds of the genetic mutations in male DMD/BMD patients were inherited from their carrier mothers, while more than one-third were *de novo* mutations (19). In the present study, 31 (36%) mothers did not exhibit a mutation in the dystrophin gene, as determined by MLPA and Sanger sequencing analysis. This indicated that these probands may present *de novo* mutations. Furthermore, 2 cases of germline mutation female carriers who were the mothers of the 2 probands were identified. They did not exhibit a mutation in the dystrophin gene by MLPA and NGS analyses, and had a normal CK level. However, their daughters presented the same mutation as the probands, although this mutation was absent in the fathers of these cases. This evidence suggests that these mothers are highly likely to have germline mosaicism. Thus, mothers with a DMD/BMD child should be aware of the possibility that they are DMD/BMD carriers, particularly those who show an elevated CK level, even when no mutation in the dystrophin gene is detected by genetic analysis. A number of researchers recommend that mothers who have a DMD child should obtain prenatal diagnosis again when pregnant. In order to prevent DMD/BMD, attention must be paid to dystrophinopathy carrier screening, genetic counseling and prenatal diagnosis.

In conclusion, measurement of the CK level is a non-invasive, easy and excellent screening method for DMD/BMD carriers, and this level was observed to be strongly correlated with ALT and AST. The present study reported that MLPA for exons of the dystrophin gene, along with a muscle disease panel analysis, was effective for the diagnosis of MCs, while MLPA combined with Sanger sequencing determined carrier status. However, a number of carriers remained that could not be confirmed by this strategy. DMD/BMD female relatives with negative genetic testing results, particularly those with high CK levels, are recommended to undergo prenatal diagnosis. Furthermore, female relatives with negative genetic tests and persistent high CK levels should be recommended for muscle biopsy and immunohistochemical analysis to evaluate dystrophin protein expression in order to confirm the status of probable carriers.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DL conducted the study and analyzed the genetic test results. JZho and YX summarized the clinical information, analyzed the genetic test results and drafted the manuscript. VB helped to summarize the clinical information and analyze the genetic test results. GC analyzed the pathological biopsy results. YD performed the pathological biopsy. HL and JZha obtained the clinical information. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and national research committee. Informed consent was obtained from all participants included in the study.

Patient consent for publication

Consent for publication was obtained from all participants included in the study.

Competing interests

The authors declare that they have no competing interests.

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