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MLPA Application in Clinical Diagnosis of DMD/BMD in Shanghai

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Background: Duchenne and Becker muscular dystrophy (DMD/BMD) are X-linked recessive disorders caused by mutation in dystrophin gene. We reported 3-year clinic experience from a single hospital in Shanghai using multiplex ligation dependent probe amplification (MLPA) assay to detect *DMD* mutations. *Methods:* Four hundred and fifty-one males and 184 females, who were clinically diagnosed as DMD/BMD patients or carriers at our hospital's outpatient clinic, were collected and performed with MLPA to detect *DMD* gene mutations. *Results:* Seventeen novel mutation points not reported in the Leiden Muscular Dystrophy pages were identified in this study. We found that the most frequent deletion spots ranged from exon45 to exon52, and exon2, exon19 were the two most frequently detected duplication spots. *Conclusion:* The results of our study confirmed MLPA as an efficient clinical method for detecting *DMD* gene mutations in DMD/BMD patients. Single exon mutation detected by MLPA should be verified by other methods, and we should emphasize that only precise clinical molecular diagnosis can lead to the feasibility of prenatal diagnosis. J. Clin. Lab. Anal. 29:405–411, 2015.

Key words: DMD; BMD; MLPA; mutation; clinical diagnosis

INTRODUCTION

Duchenne and Becker muscular dystrophy (DMD/BMD) are X-linked recessive disorders caused by mutation in the *DMD* gene. DMD [MIM 310200] is the most common muscle disorder in children, with a reported incidence of 1 in 3,500 liveborn males (1). BMD [MIM 300376] is caused by mutations in the same gene, but with later onset and mild progression, with a reported incidence of 1 in 18,500 liveborn males (2). Both dystrophies are characterized by progressive symmetric muscular weakness.

The *DMD* is the largest gene among the identified human genes located at Xp21, spanning 2.4 MB of a genomic sequence, and corresponding to about 0.1% of the total human genome (3, 4). The gene contains 79 exons and encoded a 14.6 kb mRNAs, which is expressed in the skeleton, muscles, and brain (5, 6). The majority of identified mutations are deletions, accounting for approximately 60∼65% of DMD and 85% of BMD mutations, and duplications have been observed in 5∼15% mutations (3, 4, 7). The remaining causes are small mutations such as indel, point mutation, or splicing mutation (4).

The diagnosis of DMD or BMD is based on the clinical severity of the patient. DMD refers to the more severe phenotype with delayed walking in early childhood, pseudohypertrophy of muscles, a sky-high serum creatine kinase level, and progressive deterioration in muscle power, and most patients become confined to a wheelchair by the age of 12. BMD is a milder phenotype, most BMD patients remain ambulatory beyond the age of 12 (8). The mutations in the *DMD* gene that disrupt the open reading frame (out of frame) will cause the premature abortion

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of the synthesis of the dystrophin protein, thus leading to the severe DMD phenotype. Some mutations are "in-frame," which conserved the reading frame, a truncated but mostly functional dystrophin is produced, and caused less-severe BMD phenotype. According to the "reading frame" hypothesis, any mutation that does not affect the reading frame should produce a BMD phenotype (9). In many occasions, when the patient came to see a doctor, he was too young to be defined as either DMD or BMD—at that time the reading frame rule is ultra-important—it will have guiding significance on the prediction of life expectancies for the patient.

Many methods are available for detecting deletions and duplications in the *DMD* gene. Southern blot using cDNA probe (10, 11), multiplex PCR (mPCR), quantitative mPCR (12, 13), and high-resolution melting curve analysis (14) was reportedly used in detecting *DMD* mutations. The most commonly used method is mPCR that allows for 90~95% of deletions to be detected in male patients. Since this method is only semiquantitative, neither duplications nor female carriers can be reliably identified (3, 15). Multiplex ligation dependent probe amplification (MLPA) is a technique that had first been reportedly used in *DMD* analysis in 2004 by Schwartz (16). Parallel comparison between mPCR and MLPA had been made and reported from many clinic labs, MLPA was reported more superior (13, 15, 17, 18). Since then MLPA has been increasingly used for deletion/duplication screening of the *DMD* gene in male patients and female carriers worldwide (15, 16, 19).

In this study, we reported genetic analysis of *DMD* gene by using MLPA method. We emphasized on several new mutations found in our patients using MLPA method, and also reported the importance of MLPA, which should be combined with other technologies to establish a reliable clinical diagnosis of *DMD* gene mutations.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

This study included 451 male and 184 female patients seeking help at outpatient clinic of genetic counseling and prenatal diagnosis center—Department of Shanghai Xinhua Hospital—between 2009 and 2013. These 451 male patients were diagnosed with DMD/BMD based on typical clinical presentation, elevated creatine kinase levels (most over 10,000 U/l), or have a family history. Four hundred and fourteen of them were younger than 15 years at the time of diagnosis, and among these 184 female patients, 15 of them were clinically diagnosed as DMD/BMD, the others were asymptomatic females who had given birth to at least one DMD/BMD patient before or had close relatives diagnosed with DMD/BMD.

All subjects were given written informed consent. Genomic DNA was extracted from leukocytes of 2 ml peripheral blood sample using QIAamp DNA blood Mini Kit (QIAGEN, Hilden, Germany). The quality of the DNA was monitored by NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, US), the working density of the DNA was adjusted to 50~70 ng/μl. Workflow of clinical diagnosis of *DMD* gene mutations in our lab is shown in Figure 1.

Multiplex Ligation Dependent Probe Amplification

The MLPA reactions were performed to screen all the exons of *DMD* gene using two SALSA MLPA probe mix sets: P034 and P035 (MRC-Holland, Amsterdam, Netherlands) (20). According to the manufacturer's instruction, 5 μl of the working DNA was used for each sample. At least two normal control samples were included in each reaction, for every six samples there was at least one normal control sample. The carboxyfluorescein (FAM)-labeled MLPA PCR products were separated by capillary electrophoresis on ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, California, US). The sizes of the exon-specific amplified fragments were identified according to their migration relative to the GeneScan Rox-500 size standard (Applied Biosystems) using GeneMapper version 4.0 software. Relative amounts of PCR products were determined using Coffalyser software provided online by the manufacturer (www.mlpa.com).

Confirmation of an Ambiguous MLPA Result by PCR, Q-PCR, and Direct Sequencing

The MLPA results from both male and female patients were initially assessed visually for the detection of deletion and duplication. Absence or duplication of the *DMD*-specific peaks corresponding to two or more contagious exons were regarded as reliable, and no further investigation was performed. The absence/duplication of only one *DMD* peak was further examined by quantitative PCR (Q-PCR) using SYBR® Premix Ex Taq™ (TaKaRa, China) on Applied Biosystems® 7500 Real-Time PCR Systems. If the MLPA result and Q-PCR result are not corroborated, a direct sequencing on ABI 3730xl DNA Analyzer will follow up. Both PCR and Q-PCR primers used for each specific *DMD* exon were according to the published sequences in the Leiden database (http://www.dmd.nl). Sequences were compared against the NCBI reference sequence NG_012232.1.

RESULT

MLPA Mutation Detected in Male Patients

Among the 451 male patients, 245 (54.3%) were found to have exon(s) deletions, 48 (10.6%) were found to have

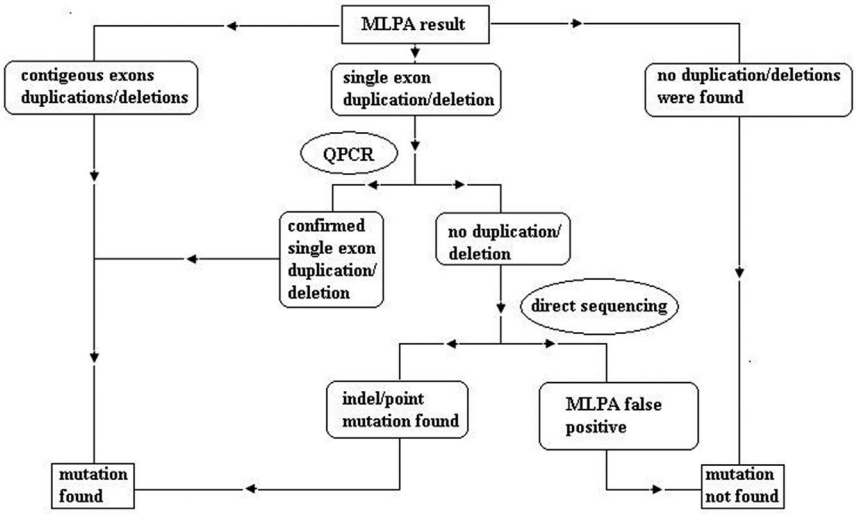

Fig. 1. Workflow for clinical DMD mutation test.

exon(s) duplications, and through single exon PCR and sequencing, we found seven (1.5%) indels. The percentage of deletions and duplications was similar to the commonly quoted percentage in the literature, about 60∼65% and 5∼15%, respectively (3, 4, 7). The total detection rate is [(245 + 48 + 7)/425] 66.5%, which is close to the freshly released literature concerning Chinese population, 70.56% (21).

Within the 245 deletion pattern male patients, single-exon deletions were the most frequent, representing up to (48/245) 19.6%. The most frequent deletion spots ranged from exon45 to exon52, which have been detected over 70 times among these 245 patients; the most frequent deletion exon was exon49, which have been detected 116 times (47%) (Fig. 2).

In the 48 duplication pattern male patients, single-exon duplication was also the most frequent, which accounted for (13/48) 27%. Exon2 and exon19 were the two most frequently detected duplication spots, both detected 13 times among these 48 duplication pattern male patients (27%) (Fig. 2). Seven indels were detected through follow-up sequencing in which single-exon mutations were detected by MLPA.

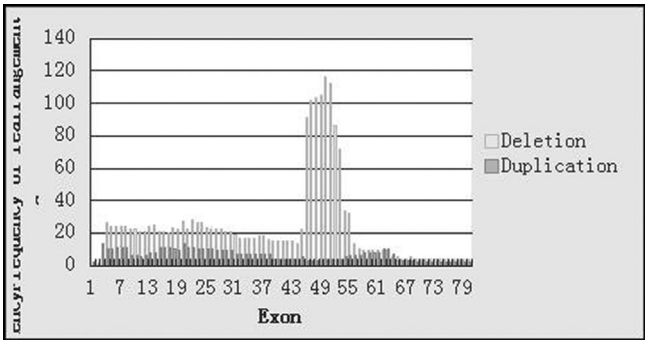

Fig. 2. Deletion and duplication frequency of each exon in the DMD gene.

MLPA Mutation Detected in Female Patients

Among the 184 female patients, 15 were symptomatic female patients, and the rest were potential asymptomatic female carriers of DMD. For the 15 female symptomatic patients, three were detected with heterozygous deletion.

Among the 169 potential female carriers, 152 had history of giving birth to known *DMD* mutation patient or having close relations with known *DMD* mutation, 17 of them had history of giving birth to an untested DMD patient.

Referring to the 152 females who had given birth to identified *DMD* mutation patients, 75 (49.3%) showed the same mutation as the identified patients and 77 (50.7%) showed no mutations through MLPA test. Among the 17 females who had given birth to untested DMD patients, six were found to have deletions in *DMD*, 11 showed no mutation through MLPA test.

Novel Mutations Found Through MLPA Test

Seventeen novel mutation points (not reported in the Leiden Muscular Dystrophy pages, http://www.dmd.nl) were identified in this study, the mutation patterns are listed in Table 1. The MLPA results were reproducible, the repeated MLPA analysis yielded identical findings in all subjects.

TABLE 1. Novel Mutations and Patients Information List

Case no.	Age (years/months)	Sex	Mut pat	Involved exon(s)	CK (U/l)	Clinical information	Clinical diagnosis	Reading frame
1	14 years	M	Del	14~51	11,098	GMP; difficulty in walking, easy to fall down; at age of 13 years, cannot stand up after crouch; Gower+	BMD	IF
2	3 years 4 months	M	Del	14~27	Up	Mild GMP walk, climbing stairs, running, jumping all ok at the time of test	BMD	IF
3	9 years	M	Del	31~45	Up	Lower limbs are faint	DMD	OF
4	4 years	M	Del	8~30	14,046	GMP; difficulty in climbing stairs	DMD	OF
5	3 years	M	Del	14~36	Up	NK	BMD	IF
6	2 years	M	Del	47~64	6,543	NK	BMD/DMD too young	OF
7	10 years	M	Del	5~9	>10,000	GMP; winged shoulders	BMD	IF
8	6 years	M	Del	5~15	NK	NK	BMD	IF
9	3 years 6 months	M	Del	42 + 45~50	Up	GMP; difficulty in climbing stairs; not have much strength from very early age; Gower+	DMD	OF
10	7 months	M	Del	3~39	9,569	GMP; MD	BMD/DMD too young	IF
11	3 years	M	Dup	2~25	NK	NK	DMD	OF
12	5 years	M	Dup	14~37	>10,000	Difficulty in walking, easy to fall down	DMD	IF
13	7 years	M	Dup	14~37	NK	GMP; hypotonic; difficulty in walking, easy to fall down	DMD	IF
14	5 years	M	Dup	Dp427m + Dp427c + 45~55	23,430	GMP; MD; difficulty in climbing stairs; cannot crouch	DMD	NK
15	10 years and 32 years	M&F*	Dup	53~55 + 57~59	NK	Start walking at 26 months, speak at 3+ years; muscle weakness; cannot crouch	DMD/carrier	OF
16	4 years 8 months	M	Indel	50: c.7302_7309del	18,431	GMP	DMD	OF
17	8 years	M	Indel	44: c.6385_6388del	NK	Have difficulty in walking	DMD	OF
18	2 years 7 months and 27 years	M&F*	Indel	34: c.4750_4751del	NK	Family history of DMD	DMD/carrier	OF

Notes: Mut pat, mutation pattern; F*, mother of the male patient; M, male; GMP, gastrocnemius muscle pseudohypertrophy; MD, myogenic damage; IF, in-frame; OF, out of frame; NK, not known; age, age at test; Gower+, a clinical sign for use of arms and hands from a sitting position to a standing position by grasping and pulling on body parts from the knees to hips until they are in an erect position.

Phenotype and Reading Frame Check

All the deletion(s) and duplication(s) we found underwent reading frame check on http://www.dmd.nl. Most of the mutations fitted the reading frame rules (not including those patients who were too young to be classified as DMD or BMD). But in several cases, the reading frame result did not accord to clinical diagnosis. We listed those phenotype and reading frame rule discrepancy patients in Table 2.

DISCUSSION

In this study, we agreed with several other studies that MLPA technology has great advantage in detecting both deletion and duplication mutations of *DMD* gene.

TABLE 2. Phenotype and Reading Frame Rule Discrepancy Patients List

Case no.	Age (years/months)	Sex	Mut pat	Involved exon(s)	CK (U/l)	Clinical information	Clinical phenotype	Reading frame
1	3 years	M	Del	10~13	NK	GMP; difficulty in climbing stairs; difficulty in standing up after crouch	DMD	IF
2	18 years	M	Del	45~54	15,100	GMP; cannot walk	Mild DMD	OF
3	19 years	M	Del	46~52	1,297	Difficulty in walking; at 23 years, on the wheelchair; GMP; MD	Mild DMD	OF
4	3 years 7 months	M	Dup	3~4	9,763	Difficulty in climbing stairs; GMP; MD	DMD	IF
5	4 years 5 months	M	Dup	19~44	958	Difficulty in walking; on wheelchair at age 12; GMP	Mild DMD	IF
6	7 years	M	Dup	45~57	NK	Difficulty in climbing stairs; cannot run; GMP; MD	DMD	IF
7*	5 years	M	Dup	14~37	>10,000	Difficulty in walking, easy to fall down	DMD	IF
8*	7 years	M	Dup	14~37	NK	GMP; hypotonic; difficulty in walking, easy to fall down	DMD	IF

Notes: 7* = Table 1, case 12; 8* = Table 2, case 13.

Our results indicated that the detection rate of MLPA is 66.45%, same as former reported. Through MLPA test, we found many novel mutations in *DMD* gene that caused DMD/BMD. If we used mPCR method to detect the patients, all the duplications and indel mutations could be missed. So, we strongly supported that MLPA should be considered as an initial clinical test for suspected DMD/BMD patients (15, 19, 21).

There were still 151 male patients (33.5%) among whom MLPA did not detect a mutation. For these patients, whole gene sequencing can be further used for *DMD* gene defects detection. Next-generation sequencing technology (NGST) seems to be a very promising technology when their cost could come down to a suitable level for clinical test (22). In our lab, we are working on sequencing these patients using NGST, the result will be published later.

Among those women who had given birth to a mutation-identified patient, 49.3% of them carried the same mutation and 50.7% did not carry that mutation. For those in whom no mutation had been found, there are two probable explanations: either the proband's mutation is de novo or there is a germline mosaic that exists. In our study, we eliminated the percentage of possible germline mosaic, which is estimated about 4.3% among those nonmutation mothers (23,24); we got 46.6% de novo mutation increase in our patients. This rate is much higher than reported human gene de novo mutation rates, which is around one-third (25, 26). So, we assumed that *DMD* might have some other mutation mechanism that makes the de novo mutation rate much higher. This needs to be further explored and more follow-up data collection is required.

In our study we found that the most frequent deletion exon was exon49, it is not the same as Leiden-reported exon47, but exon47 is also a very frequent deletion exon in our study. The most frequent duplication exons were exon2 and exon19. These three kinds of mutations add up to 47% of deletions and 54% of duplications found. As 46.6% of mutations we found in this study were de novo, we thought that these most frequent exons could be a good target point for noninvasive prenatal screening test for *DMD* del/dup mutation screening.

The reading frame rule is quite helpful in clinical evaluation of the patients at an early age. Our result shows that most of the time, the reading frame rule is correct; physicians can give explanation of the disease process to the patient based on it. But sometimes, there will be discrepancy between the clinical phenotype and expectations based on the reading frame rule. We now classify the discrepancy cases in Table 2 into three groups, and give out conceivable reasons on how this might happened. (1) Four different types of "in-frame" duplications with clinical DMD phenotype. Duplication is harder to define than deletion; without sequencing, we are not sure

where exactly do these extra exons insert. We assumed that the duplications might interrupt the translation of the dystrophin. (2) One "in-frame" deletion led to DMD phenotype. This could be due to small mutation not detectable by MLPA, which occurred simultaneously with the deletion. If two jumping deletions can occur in one patient (Table 1, case 9), then this can also happen. (3) Two deletions that we found had "out-of-frame" change and got milder DMD phenotype. This could be caused by a nature-generated frame-restoring exon(s) skipping. The patient "exon 45∼54 deletions" could be restored by skipping exon44. The other "exon 46∼52 deletions" patient, 45 + 51 double skip exon could restore the partial function of dystrophin. This nature self-restore mechanism detected in real patients gave the foundation support of the most promising gene therapy of DMD (27). In conclusion, final classification of DMD or BMD is determined by clinical phenotype, reading frame rule is a useful tool for clinical consultation; discrepancy between these two indicated a potential change in the expression of the mutated *DMD*.

In China, all the physicians know that DMD/BMD is a genetic neuromuscular disease, but not all of them are aware that only precise molecular diagnosis could permit future prenatal diagnosis. Sometimes when patients seeking help at our prenatal diagnosis center followed their local physicians' direction, they have already carried a gestation over 16 weeks. And that will give an extra anxiety to the pregnant women because they have to wait extra time to get precise molecular diagnosis first. And in some cases no prenatal diagnosis is allowed to be processed, and then the pregnant women could be depressed. So here, we strongly suggest that when there are likely DMD/BMD patients, physicians better suggest the patients to take a precise molecular test first.

Although in China, people are more and more aware of the importance of prenatal screening, till now only Down's screening is officially recommended by the government and knowledged by the population. There are so many genetic diseases that could be avoided by prenatal screening when clinical technology is well established. Now for *DMD*, technology is ready—maybe in future in China—DMD/BMD could be prioritized as a prenatal screening project as in other countries (18, 28).

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