RESEARCH ARTICLE

A resolved discrepancy between multiplex PCR and multiplex ligation-dependent probe amplification by targeted next-generation sequencing discloses a novel partial exonic deletion in the Duchenne muscular dystrophy gene

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Background: The genetic diagnosis of Duchenne muscular dystrophy (DMD) has been complicated by the large size of the gene and its heterogeneous mutational spectrum. Multiplex PCR and multiplex ligation-dependent probe amplification (MLPA) are two well-established mutation screening methods. Here, we applied targeted next-generation sequencing (NGS) to clarify discrepant results between multiplex PCR and MLPA in a Chinese patient with DMD.

Methods: MLPA was performed to confirm multiplex PCR results obtained previously. Targeted NGS was then used to analyze the full-length *DMD* gene including introns.

Results: Multiplex PCR had previously identified an apparent deletion of exon 43 in the patient with DMD, but current MLPA indicated that exon 43 was present. Targeted NGS to clarify the genetic diagnosis identified a novel mutation, c.6241_c.6290 + 1109del1159insAC, which caused partial deletion of exon 43. This mutation removed the annealing sequence of the exon 43 reverse primer in multiplex PCR but had no influence on the hybridization site of the MLPA probe. Therefore, the discrepancy between the two methods was caused by partial exonic deletion that escaped MLPA detection.

Conclusion: Targeted NGS disclosed a novel partial exonic deletion in the *DMD* gene as the cause of discrepancy between multiplex PCR and MLPA. Targeted NGS could be used to provide a more accurate genetic diagnosis of DMD, particularly in cases of partial exonic deletions, which will be of benefit in patient management and the identification of disease carriers.

KEYWORDS

duchenne muscular dystrophy, multiplex ligation-dependent probe amplification, multiplex PCR, next-generation sequencing, partial exonic deletion

1 | INTRODUCTION

Duchenne muscular dystrophy (DMD; OMIM 310200) is a severe X-linked recessive muscle disease affecting more than 1 in 5000 male births.¹ The major clinical manifestation of DMD is muscle weakness, which is present from childhood and progresses with age. Affected boys typically lose ambulation in their early teens and died from heart or respiratory failure in their 30 s.² DMD is caused by mutations in the *DMD* gene that disrupt the reading frame of the gene transcript. A milder and less common type of muscular dystrophy, Becker muscular dystrophy (OMIM 300376), is caused by mutations in the same gene that preserves the reading frame.³

DMD gene is extremely large, over 2.2 Mb in size, with 79 exons that are interrupted by large introns.⁴ More than 60% of identified *DMD* mutations are large deletions that span one to multiple exons. Duplications of one to multiple exons are less common, only accounting for 5%-10% of all cases. Point mutations and small insertions/deletions are considered the causative mutations for the remaining cases.^{5,6} Although the identification of disease-causing mutations in DMD is complicated by the 79 exons and a heterogeneous mutation spectrum, precise genetic diagnosis is required for the confirmation of clinical diagnosis, genetic counseling, and future treatment with mutation-specific therapy.^{7,8}

Many screening methods are available to detect large deletions, including multiplex PCR that was initially developed to selectively amplify 19 exons at deletion hotspots in two reactions.^{9,10} This simple method identifies more than 95% of deletions, so has been widely used.¹¹ However, additional analysis is required to define the deletion end and obtain a precise deletion diagnosis. Additionally, multiplex PCR is not suitable for checking the female carrier status. Multiplex ligation-dependent probe amplification (MLPA) is a semiquantitative method that has been developed since multiplex PCR.¹² MLPA involves the PCR amplification of probes that hybridize to exonic sequences, allowing the relative quantification of all 79 *DMD* exons in both patients and carriers.¹³ MLPA has become the standard method for detecting not only large deletions but also duplications, which has significantly improved the molecular diagnosis of DMD.^{6,14} More recently, next-generation sequencing (NGS), the high-throughput DNA sequencing technology, has been used for the genetic diagnosis of DMD. Targeted NGS of *DMD* as a single gene or within a gene panel has been developed, offering a single platform to detect a broad mutational spectrum from small mutations to large rearrangements including exon deletions and duplications.¹⁵⁻¹⁷

In a mutation study of a Chinese patient with DMD, we observed a discrepant amplification of *DMD* exon 43 between multiplex PCR and MLPA. Using the benefits of targeted NGS, we were able to identify a novel partial exonic deletion as the cause of the discrepancy.

2 | MATERIALS AND METHODS

2.1 | Patient

The patient was a 14-year-old Chinese boy who was born to healthy parents with no family history of muscular disorders. He started to

walk at the age of 1.5 years, but slowness when climbing the stairs was noted at the age of 4. At 8 years of age, he was clinically diagnosed with DMD based on his waddling gait and elevated serum creatine kinase levels (13760 IU/L, normal: <200 IU/L). Later, genetic testing was performed using multiplex PCR, which identified a deletion of exon 43 in *DMD* gene. He became wheelchair-bound at the age of 11. Recently, he visited our hospital with his older 17-year-old sister to determine her carrier status. Because the extent of his deletion was not previously defined, we reanalyzed his *DMD* gene by MLPA in parallel with that of his sister, after informed consent was obtained. This study was approved by the ethics committees of both Nankai University School of Medicine and Tianjin Children Hospital. All experiments in our study were carried out in accordance with the Declaration of Helsinki.

2.2 | Multiplex PCR

In the patient's previous genetic test, the traditional two sets of multiplex PCR primers were used to amplify 19 *DMD* exons that commonly carry deletions in DMD.^{9,10} The amplified PCR products were separated on a 3% agarose gel and visualized by ethidium bromide staining.

2.3 | MLPA analysis

The SALSA MLPA KIT P034/P035 for *DMD* was purchased from MRC-Holland (Amsterdam, the Netherlands) and used according to the manufacturer's instructions.

2.4 | Targeted NGS analysis

Targeted NGS for *DMD* was conducted by MyGenostics Inc. (Beijing, China). Briefly, a genomic DNA library of the index patient was constructed and hybridized with biotinylated DNA probes to capture nearly 2.1 Mb of the entire genomic region of *DMD* gene (UCSC hg19 corresponding to chrX:31137345-33357726, NC_000023.10, NCBI build 37). The captured DNA was then selected by streptavidin-coated magnetic beads, PCR-amplified, and sequenced on an Illumina HiSeq2000 sequencer (San Diego, CA, USA). Sequenced data were processed using bcl2fastq Conversion Software 2.18.0.12 (Illumina Inc., CA, USA) for base calling and clean data. Low-quality variations were filtered out using a quality score ≥ 20 . Burrows-Wheeler Aligner software was then used to align the clean reads to the reference human genome (hg19), producing a BAM file. Deletions were detected by read depth estimation. The rough breakpoint can be seen in the Integrative Genomics Viewer. The relevant consensus sequence in the SV file was then aligned to hg19 to obtain an accurate sequence. The detailed experimental protocol has been described elsewhere.¹⁸

2.5 | Validation of the breakpoint by PCR amplification and Sanger sequencing

The junction sequence detected by NGS in the index patient was further confirmed by conventional PCR using a

forward primer located in intron 42 (5'-AGAATTGCAAC ACCATTGCTACC-3') and a reverse primer in intron 43 (5'-TCAACGGTCCTTAGGGGATG-3'). PCR products were purified and subjected to Sanger sequencing. The nucleotide position was based on the reference sequence from NCBI for Dp427 m (NM_004006.2), with +1 representing the A of the ATG translational initiation codon.

3 | RESULTS

3.1 | Discrepancy between multiplex PCR and MLPA analysis

The index patient had previously undergone genetic testing. At this time, traditional multiplex PCR failed to detect an amplified product corresponding to *DMD* exon 43, indicating a deletion of this exon

FIGURE 1 *DMD* mutation analysis in the patient. A, Agarose gel electrophoresis of multiplex PCR products. Loss of amplified product of exon 43 (arrow) was detected in the patient (P), but not in the control subject (C). The size marker (M) is shown in the left panel. B, MLPA analysis of *DMD* exons. Peaks corresponding to exon 43 are indicated in both the patient and control. C, Visualization of NGS results by Integrative Genomic Viewer (IGV). Gray tracks represent sequence read coverage. The read bases that mismatch the reference genome are indicated by colored bars. The absence of a matched sequence read was identified between chrX: 32304536-32305695 (UCSD hg19, NCBI build 37), indicating a deletion in the patient genome. The deletion breakpoints are indicated by horizontal arrows

TABLE 1 Next-generation sequencing statistics of the index patient

Sequencing statistics	Index patient
Initial bases on target	2 098 524
Bases covered on target	1 918 096
Coverage of the target region (%)	91.40
Fraction of effective bases on target (%)	51.41
Average sequencing depth on target	237.14
Fraction of target covered with at least 10X (%)	81.09

(Figure 1A). However, because this method does not cover every *DMD* exon, the extent of the deletion had not been fully defined.

To obtain a precise genetic diagnosis for the index patient and to clarify the carrier status of his sister, we performed MLPA to examine the copy number changes of all *DMD* exons. Unexpectedly, MLPA showed that all 79 *DMD* exons, including exon 43, were present in the patient's genome (Figure 1B). No deletions or duplications were identified by MLPA either in the patient or his sister (data not shown). Thus, the apparent deletion of exon 43 detected by multiplex PCR could not be confirmed by MLPA. To exclude the possibility of false-positive multiplex PCR results caused by polymorphisms in the primer binding site, we used different primers for exon 43 but

still failed to amplify this exon (data not shown). The genetic diagnosis of the patient and sister was inconclusive.

3.2 | Targeted NGS disclosed a deletion mutation in the patient with DMD

To clarify the genetic diagnosis, we further examined the patient's *DMD* gene, including the introns, using targeted NGS. The sequence coverage and depth for the patient sample are summarized in Table 1. By carefully analyzing the sequencing data surrounding exon 43 and its flanking introns, the absence of sequence reads between chrX: 32304536-32305695 (NC_000023.10) corresponding to c.6241_c.6290 + 1109 (NM_004006.2) in the *DMD* gene was identified (Figure 1C), indicating the presence of a deletion mutation in the patient.

3.3 | Confirmation of the deletion breakpoint

The deletion junction was further confirmed by conventional PCR using primers flanking the deletion breakpoint suggested by targeted NGS. As expected, PCR yielded a 1461 bp product in the patient, compared with a 2618 bp product in the control individual (Figure 2B). Direct sequencing of the shorter product confirmed a

FIGURE 2 Confirmation of the deletion breakpoint by conventional PCR and Sanger sequencing. A, Schematic of the *DMD* gene structure surrounding exon 43 in the patient and control. Exons and introns are indicated by open boxes and bars; numbers within the box represent the exon number. The MLPA probe binding site is indicated by a horizontal bar, multiplex PCR primer binding sites are shown by arrows, and the reverse primer for amplifying the breakpoint is indicated by an arrowhead. The deleted region is indicated by a bracket. In the patient's *DMD* gene, an AC dinucleotide was inserted at the deletion breakpoint. B, PCR amplification of the deletion junction using a forward primer in intron 42 (arrow in Figure 2A) and a reverse primer in intron 43 (arrowhead in Figure 2A). A smaller PCR product was detected in the patient (P) compared with the control (C). C, Sanger sequencing results of the smaller PCR product. Deletion of the last 50 bp of exon 43 and the first 1109 bp of intron 43, as well as an AC dinucleotide insertion, was revealed in the patient's *DMD* gene. The deletion breakpoint is emphasized by vertical lines

1159-bp deletion covering the last 50 bp of exon 43, which is 173 bp in length, and the first 1109 bp of intron 43. An AC dinucleotide insertion at the deletion breakpoint (c.6241_c.6290 + 1109del1159insAC) was also detected (Figure 2C). Therefore, DMD in the index patient was caused by deletion of part of *DMD* exon 43 rather than the entire exon. Further analysis of the sequence near the breakpoint revealed no sequence homology between exon 43 and intron 43, indicating nonhomologous recombination as the cause of this rearrangement.

When the sequence locations of multiplex PCR primers and the MLPA probe for exon 43 were examined, the multiplex PCR reverse primer binding site (c.6279_c.6290 + 17) was shown to reside within the deleted region (c.6241_c.6290 + 1109), whereas the hybridization site for the MLPA probe (c.6184_6207) was located in the retained part of exon 43. This explained the discrepancy between the two methods (Figure 2A).

To determine the carrier status of the patient's sister, her genomic DNA was examined for the presence of the deletion junction by conventional PCR. Only a PCR product of normal size was obtained, which excluded the possibility of her being a carrier (data not shown).

4 | DISCUSSION

Multiplex PCR and MLPA have been widely used for mutation detection in DMD cases.^{6,19} In these methods, loss of the amplified product of a corresponding exon is commonly interpreted as a deletion of that entire exon. Contradictory amplification results between these two methods may occur if there are nucleotide changes within PCR primer or MLPA probe binding sites,²⁰ especially when the loss of amplification only involves a single exon. Here, we propose an alternative possibility that the discrepancy reflects partial exonic deletion that escapes MLPA detection.

c.6241_c.6290 + 1109del1159insAC is a novel mutation that has not been reported in the Leiden DMD database (www.dmd.nl). This mutation is a 1159 bp deletion spanning the last 50 bp of exon 43 and the first 1109 bp of intron 43, which caused the partial deletion of *DMD* exon 43 in the index patient. An additional AC dinucleotide was inserted at the deletion junction (Figure 2A). The 50 bp deletion at the 3' end of exon 43 is thought to disrupt the reading frame of the *DMD* transcript and to create a premature TGA stop codon in the third codon of exon 44, which is consistent with the DMD phenotype of the index patient. However, because this mutation simultaneously removed the splicing donor site of the downstream intron, the DMD phenotype of the patient most likely reflects an aberrant splicing event, such as skipping of the retained sequence of exon 43 or activation of the cryptic splice site. Further analysis of the *DMD* transcript will be necessary to clarify the splicing outcome.

The precise diagnosis of this mutation by conventional methods is challenging. This partial deletion only removed the multiplex PCR reverse primer binding site, but this hampered exon 43

amplification, leading to its apparent loss (Figure 1A). Conversely, in MLPA testing, the probe recognition sites were retained so exon 43 was amplified, causing the partial deletion to completely escape MLPA detection (Figure 1B). Although MLPA has been accepted as a reliable method for detecting copy number variations, its limited probe coverage on target regions could cause it to miss mutations.²⁰ In fact, MLPA failed to detect some gross rearrangements in the α -galactosidase gene in patients with Fabry disease.²¹ However, little was known about its problems in *DMD* mutation detection, and our results are the first to show that MLPA can miss some partial exonic deletions in DMD.

Although the difference between partial and complete deletions of exon 43 might not influence the clinical manifestation of the disease, discrepancies between the two conventional screening methods might cause misdiagnoses in carrier testing. This could particularly occur if the proband has previously been diagnosed by multiplex PCR, but at-risk female relatives are subsequently examined by MLPA alone. To avoid this problem, single exon deletion should be confirmed by different methods as suggested previously.²² More importantly, accurate molecular diagnosis of the proband is required.

Recently, targeted NGS has been used for genetic diagnosis and has shown notable advantages in identifying the heterogeneous spectrum of mutations in the *DMD* gene.^{15,16} Besides the detection of small mutations, targeted NGS has been reported to successfully validate ambiguous MLPA results and to examine samples from long-preserved, dried umbilical cords that are difficult to analyze by conventional methods.²³⁻²⁵ In our study, we applied this approach to resolve a discrepancy between two conventional methods and to clarify the genetic diagnosis of the index patient. Based on an estimation of single nucleotide sequencing depth, targeted NGS offers the identification of a precise breakpoint position. This enabled us to uncover a partial exonic deletion in the *DMD* gene. In fact, partial deletions were previously identified in four out of 52 MLPA-positive cases by targeted NGS,¹⁶ suggesting that this type of mutation is not rare but rather often improperly diagnosed by conventional screening methods. Together with these results, we showed the efficiency of targeted NGS in the detection of partial deletions.

The identification of a novel partial exon deletion of exon 43 further broadens the *DMD* mutational spectrum. Compared with a previous study that only analyzed *DMD* exons and 10 bp flanking introns,¹⁶ our study performed targeted sequencing of the full-length *DMD* including entire introns. This led to the retrieval of a junction sequence, which provides the first insight into the molecular mechanism of partial exonic deletions in *DMD*.

In conclusion, we report the discrepant amplification of *DMD* exon 43 between multiplex PCR and MLPA in a Chinese patient. Validation by targeted NGS disclosed that the discrepancy was caused by a novel partial exonic deletion that escaped MLPA detection. Our results demonstrate the pitfalls of conventional screening methods in the accurate detection of partial deletions and demonstrate the power of targeted NGS in identifying unexpected DNA changes.

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AUTHOR CONTRIBUTIONS

CL, HD, CY, and XL performed the experiments. YZ, XC, and SL participated in the analysis of sequencing data. HC, XZ, and HL helped with data interpretation. XT and DL were involved in clinical sample collection and drafted the article. ZZ participated in study design and drafted the article.

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