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CASE REPORT

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Long-read sequencing identifies complex structural variants in DMD patients

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Abstract

Background Duchenne muscular dystrophy (DMD) is an X-linked disorder caused by mutations in the *DMD* gene. Reports of DMD resulting from complex structural variants involving the *DMD* gene are rare, partly because such variants are often undetectable by standard diagnostic approaches such as multiplex ligation-dependent probe amplification (MLPA) and short-read whole-exome sequencing (WES).

Case presentation Using long-read sequencing, we identified two unrelated pedigrees with complex structural rearrangements affecting the *DMD* locus. Case 1 carried an inversion encompassing exon 2 of *DMD*. Case 2 harbored a large-scale inversion of the *DMD* gene accompanied by two segmental duplications arising as a direct consequence of the inversion; the entire complex allele was maternally inherited. In both instances, simple tandem repeats were present at most of the breakpoints.

Conclusions Our findings demonstrate that long-read sequencing is a powerful tool for resolving complex structural variants. The simple tandem repeats identified at the inversion breakpoints in DMD patients enhance our understanding of the mutational mechanisms underlying structural variation, which in turn aids in developing potential therapeutic strategies.

Keywords Long-read sequencing, Duchenne muscular dystrophy, *DMD* gene, Inversion, Sanger sequencing

Background

Duchenne muscular dystrophy (DMD, OMIM #310200) is an X-linked recessive genetic disease characterized by progressive muscle degeneration and weakness [1]. It is caused by variants in the *DMD* gene (OMIM #300377) [2], including deletions (the most common forms [3, 4]), duplications, small rearrangements, and single nucleotide variants (SNVs). Among these mutations detectable by traditional detection methods, intragenic deletions affecting one or more exons are the most commonly reported, accounting for approximately 65% of all the mutations [5, 6], while duplications are less common, occurring in 6% to 8% of cases. Complex rearrangements, such as balanced translocations or inversions, are rarely reported [7, 8]. However, recent advances in long-read sequencing technologies have led to an increasing number of reports identifying complex structural variants in *DMD* gene [5, 9, 10].

The incidence rate of DMD in live born male infants is approximately 1/4700 [11]. Around 400–500 children with DMD are born each year in China, which is one of the countries with the largest number of DMD patients in the world, and about 70,000 people have been diagnosed with DMD. Accurate molecular genetic diagnosis

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holds significant importance for the clinical diagnosis and treatment, multidisciplinary management, genetic counseling, prenatal diagnosis, and selection of gene therapy in patients with DMD. Currently, the conventional diagnostic approaches for *DMD* gene mutations include multiplex ligation-dependent probe amplification (MLPA) [12] for the detection of deletions and duplications, and short-read whole exome sequencing (WES) for the detection of the coding region of the *DMD* gene [4, 13, 14]. Nevertheless, due to the presence of extremely rare and complex structural variations, some patients still fail to obtain a genetic diagnosis even after the application of these techniques [15, 16].

Targeted long-read sequencing (LRS) can effectively detect a range of *DMD* gene mutations, spanning from SNVs to structural variants (SVs), and accurately identify the positions of breakpoints [5, 9]. Long-read sequencing platforms, exemplified by Oxford Nanopore Technologies (ONT) and Pacific Biosciences (PB) single-molecule real-time (SMRT) sequencing, leverage their extended read length capabilities to achieve more precise localization of breakpoints for complex structural variations in the *DMD* gene, consequently enabling comprehensive decoding of sequences within rearranged *DMD* regions. In this study, by employing the PB SMRT platform of LRS, we identified inversions within chromosomes involved in the *DMD* gene and complex structural variants including inversions and duplications in two unrelated families, respectively. In the samples from these two families, repeat sequences were observed at the breakpoints of the structural variants.

Case presentation

Patient 1

The first patient analyzed in this study was a full-term boy born to a gravida 2, para 2 (G2P2) mother. Notably, other male relatives of the mother, including her father, uncle, nephew, and her eldest son, did not exhibit muscle weakness or atrophy (Fig. 1A). The boy was referred to our hospital due to elevated serum levels of creatine kinase (CK, 9880 U/L), alanine transaminase (ALT, 481 U/L) and aspartate aminotransferase (AST, 152 U/L) detected during a medical examination for kindergarten enrollment at the age of 3 years old. His mother reported delayed motor milestones and difficulties with running, jumping, squatting and standing. Physical examination revealed calf hypertrophy, wadding gait and a positive Gowers' sign. His electrocardiogram, liver ultrasound, and cardiac ultrasound were all within normal limits.

One year following the genetic diagnosis, when the boy was 4 years old, he experienced frequent falls and required assistance when climbing stairs. Prednisone was prescribed to help preserve motor function at his age of 4 years and 4 months.

A 271.25 kb inversion (INV) on the X chromosome was detected in the male patient from Family 1 (Fig. 1A) by PB SMRT targeted sequencing. INV occurred in exon 2 and flanking intron sequence of *DMD* gene (Fig. 2A). IGV software showed that the breakpoint chrX:32,883,536 was located in the intron 2 region of *DMD* gene, and the breakpoints at chrX:32,883,536 and chrX:33,154,787 were located in intron 2 and intron 1 of the *DMD* gene (transcript NM_004006.2), respectively (Table 1; Fig. 2B). Sequence analysis of the breakpoint-flanking regions indicated that these segments originated from the repeat sequences overlapping long interspersed nuclear element (LINEs, L2 family) and long terminal repeat elements (LTR, ERVL - MaLR family) (Table 1).

Patient 2

The second patient (Fig. 1B) presented to our hospital at the age of 7 years with an abnormal gait, frequent falls, and difficulties in climbing stairs, running, and jumping. The pregnancy was uneventful, and there was no definite family history. On physical examination, findings included calf hypertrophy, lumbar lordosis while standing, a duck-like gait, and a positive Gowers' sign. Biochemical analysis revealed markedly elevated serum creatine kinase levels (27,339 U/L). Electrocardiogram and cardiac ultrasound results were within normal limits. Following diagnosis, the patient promptly initiated prednisone treatment at the age of 7 years and 11 months.

PB SMRT targeted sequencing revealed complex SVs on chromosome X in this patient (Fig. 1B), comprising a 1001.02 kb INV and two duplications (DUP) of 259.68 and 534.22 kb (Fig. 3A). The INV spanned the 3'UTR of *TAB3* (chrX:30,848,997) to intron 49 of *DMD* (chrX:31,850,016). The DUP involved intron 55

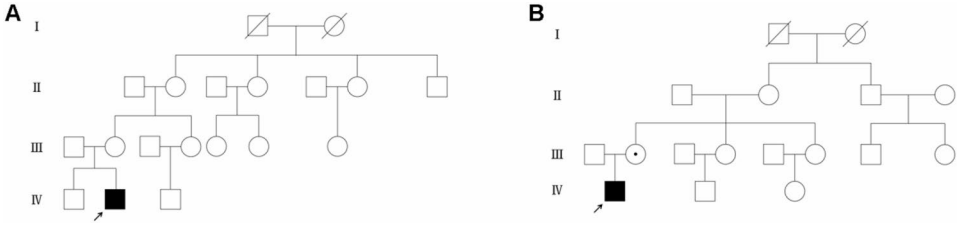

Fig. 1 The family pedigree of the patient 1 (**A**) and patient 2 (**B**)

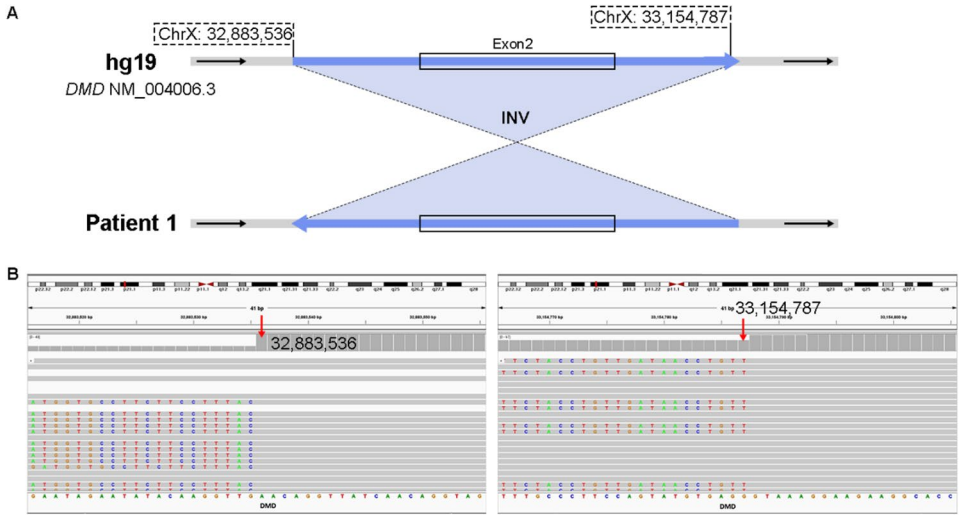

Fig. 2 Schematic representation of the inversion and IGV plot of the sequence near the breakpoint in patient 1. **A** Schematic representation of the reference genome and Patient 1 genome sequences showing inversion variants including exon 2 of the *DMD* gene. **B** The overview of the breakpoints after analyzing the original BAM file of patient 1 by IGV

Table 1 Molecular signatures at breakpoint sequence

Family	patient	Breakpoint (chrX)	Confirmation methods	Repeat elements		
				SINE	LINE	LTR
1	1	32,883,536	SMRT target sequencing	-	L2c	-
1	1	33,154,787	SMRT target sequencing	-	-	MLT1A0
2	2	31,383,213	Sanger, SMRT target sequencing	AluSc8	-	-
2	2	31,850,016	Sanger, SMRT target sequencing	AluY	-	-
2	2	30,848,997	Sanger, SMRT target sequencing	-	-	-
2	2	31,590,339	Sanger, SMRT target sequencing	-	-	-

Repeat elements were defined according to Repeat Masker

SINE Short interspersed nuclear element, *LINE* Long interspersed nuclear element, *LTR* Long terminal repeat elements

(chrX:31590339) / intron 49 (chrX:31850016) of *DMD* and 3'UTR of *TAB3* (chrX:30,848,997) / intron 60 of *DMD* (chrX:31383213), respectively (Fig. 3B).

Sanger sequencing confirmed the breakpoints at chrX:31,590,339/chrX:30,848,997 and chrX:31,850,016/chrX:31,383,213 (Fig. 3C) and verified maternal inheritance. Breakpoint-flanking regions (chrX:31383213 and chrX:31850016) are considered to be derived from repeat elements overlapped with short interspersed nuclear elements (SINEs, Alu family) (Table 1).

Discussion and conclusions

Despite the identification of over 7000 pathogenic variants in the *DMD* gene through conventional genetic testing over the past three decades [17], novel mutations continue to be uncovered [5, 18]. Inversions disrupting the *DMD* gene represent an uncommon etiology of Duchenne muscular dystrophy, with intrachromosomal inversions being particularly rare [8, 19]. To date, only a few X-chromosomal inversions involving the *DMD* gene have been reported [9]. In this study, we describe two novel inversion variants of the *DMD* gene identified by long-read sequencing, which have not been previously reported in affected individuals, both of which result in structural disruption of the gene. Patient 1 carried a pericentric inversion of approximately 270 kb on the X chromosome. Patient 2 harbored a complex structural rearrangement involving a ~1 Mb inversion accompanied by noncontinuous duplications of 259.68 kb and 534.22 kb.

Inversions may lead to disruption of the reading frame within the gene or abnormal splicing [20], thereby affecting the normal synthesis of dystrophin. A recent study suggested that breakpoints of inversion residing within introns could activate cryptic splice sites or alter the conformation of splicing enhancers/silencers, resulting in exon skipping, partial exon deletion, or "pseudo-exon" insertion, and ultimately producing a truncated and non-functional protein [21]. In our previous study [5], we characterized an inversion with a breakpoint within intron 2, in which RNA sequencing demonstrated complete skipping of exons 3–55. In the current study, Patient 1 harbored an inversion with a breakpoint at chrX:33,154,787 within intron 1, whereas

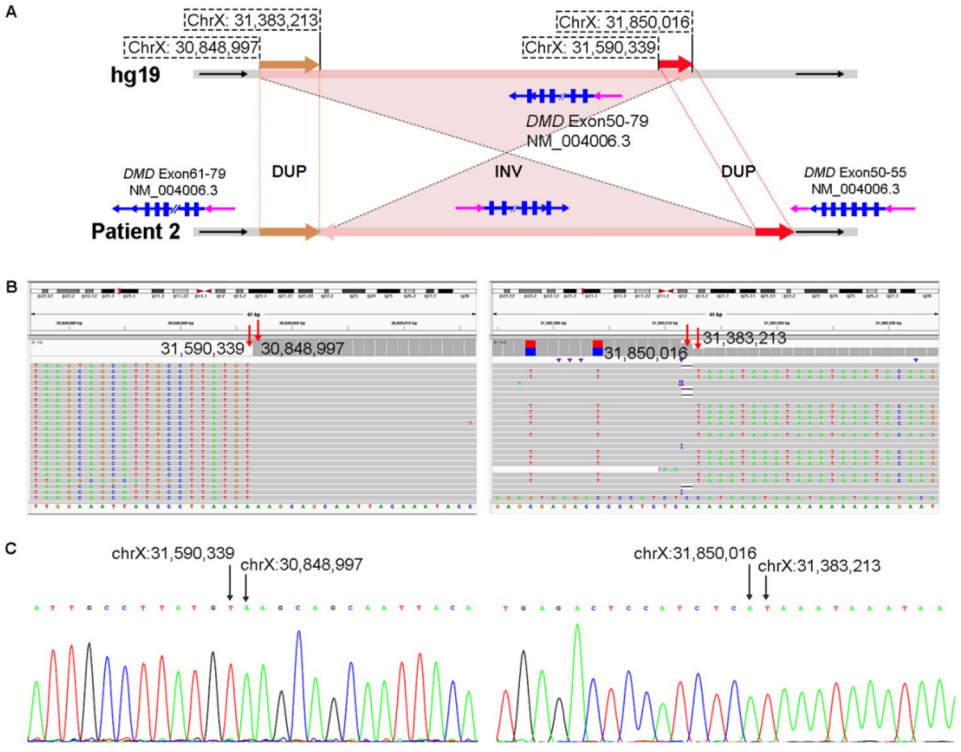

Fig. 3 Schematic of the complex structural variant in patient 2 and the IGV schematic of the variant breakpoints and Sanger sequencing results. **A** Schematic representation of the reference genome and Patient 2 genome sequences showing inversion variants including the *DMD* gene and two noncontinuous duplications including the *DMD* gene. **B** The overview of the breakpoints after analyzing the original BAM file of patient 2 by IGV. **C** The resulting sequence was verified by Sanger sequencing of the variant breakpoint in patient 2

Patient 2 carried a breakpoint at chrX:31,850,016 located within intron 49. We therefore hypothesized that these intronic breakpoints may trigger comparable exon skipping events. RNA analysis derived from muscle biopsy specimens could provide definitive evidence to further elucidate the pathogenicity of these variants. Regrettably, both patients declined the proposed muscle biopsy procedures. The severity of disease in patients with DMD varied depending on the specific variant. In general, inversions that disrupt the translational reading frame are associated with a severe DMD phenotype [5, 17]. In some DMD patients, inversions may coexist with other genetic variants, such as repeat expansion, further influencing the clinical presentation of the disease [22]. Notably, breakpoints mapping downstream of the distal promoter (e.g., within introns 42–47) have been reported to preferentially disrupt the brain-enriched Dp140/Dp71 isoforms, leading to cognitive impairment or learning difficulties in addition to skeletal muscle weakness [9].

The molecular mechanisms underlying inversion formation remain to be fully elucidated. Non-allelic homologous recombination (NAHR), non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), and microhomology-mediated break-induced replication (MMBIR) have been proposed to contribute to the generation of inversions [23, 24]. Repeat sequences have been reported to be frequently involved in the formation of pathogenic inversions [25]. In the present study, LINE and LTR sequences were detected at the breakpoints in Patient 1, whereas only SINE sequences were identified at the breakpoints in Patient 2. It is well established that a variety of repeat sequences are known to drive non-homologous genomic rearrangements, thereby driving the formation of various SVs, including inversion events [26–28].

The detection of complex structural variants, including inversions, adds to the complexity of diagnosing DMD. Traditional diagnostic methods, such as MLPA and WES, may fail to accurately identify these variants due to their inherent technical limitations, resulting in some patients not receiving a definitive genetic diagnosis. In contrast, LRS has proven to be an efficient and crucial tool in the diagnostic process [5, 9, 29]. LRS identified a complex structural variation in Case 2 comprising one inversion and two duplications, with definitive breakpoints subsequently validated by Sanger sequencing. In contrast, Case 1 harbored only a single inversion variant, representing a relatively simple structural rearrangement. Due to the high precision of breakpoint detection by long-read sequencing, we did not perform Sanger validation for this case. LRS provides markedly higher methodological rigor and consistency in resolving complex variants compared

with conventional methods such as Sanger sequencing or WES.

In summary, identification of complex structural variants in *DMD* gene is crucial for accurate diagnosis, genetic counseling, and potential future therapeutic interventions. Such investigations deepen our understanding of the genetic complexity underlying DMD and may facilitate the optimization of diagnostic and therapeutic approaches. Characterization of the sequence features at breakpoint regions contributes to the elucidation of mechanisms driving structural variation, thereby supporting the development of targeted interventions. Furthermore, long-read sequencing enabled direct and efficient detection of complex structural variants including inversions in both patients, yielding precise breakpoint mapping. Our findings highlight the considerable diagnostic value of LRS in the molecular evaluation of DMD.

Abbreviations

DMD	Duchenne muscular dystrophy
CK	Creatine kinase
MLPA	Multiplex ligation-dependent probe amplification
WES	Whole-exome sequencing
LRS	Long-read sequencing
SMRT	Single Molecule Real Time
SVs	Structural variations
SNVs	Single nucleotide variants
NAHR	Non-allelic homologous recombination
NHEJ	Non-homologous end joining
MMEJ	Microhomology-mediated end joining
MMBIR	Microhomology-mediated break-induced replication
LINE	Long interspersed nuclear elements
LTR	Long terminal repeat elements
SINE	Short interspersed nuclear element

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Authors' contributions
YX and LB contributed equally to this work. YX and LB drafted the manuscript and prepared the figures. YL did the follow-up with the patient. LB and YL edited the manuscript. YT and XY edited the figures. All authors contributed to the article and approved the submitted version.

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Data availability
The datasets generated during the current study are available in the Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra/) repository, with the accession number: PRJNA1395292.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. All study procedures were conducted in accordance with the tenets of the Declaration of Helsinki. All the participants and parents of the minor participants provided written informed consent to participate in this study.

Consent for publication
Written informed consent to publish this case was obtained from all the participants and parents of the minor participants, including case description and medical data.

Competing interests
The authors declare no competing interests.

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