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Identifying inversions with breakpoints in the *Dystrophin* gene through long-read sequencing: report of two cases

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Abstract

Background Duchenne Muscular Dystrophy (DMD) is an X-linked disorder caused by mutations in the *DMD* gene, with large deletions being the most common type of mutation. Inversions involving the *DMD* gene are a less frequent cause of the disorder, largely because they often evade detection by standard diagnostic methods such as multiplex ligation probe amplification (MLPA) and whole exome sequencing (WES).

Case presentation : Our research identified two intrachromosomal inversions involving the *dystrophin* gene in two unrelated families through Long-read sequencing (LRS). These variants were subsequently confirmed via Sanger sequencing. The first case involved a pericentric inversion extending from *DMD* intron 47 to Xq27.3. The second case featured a paracentric inversion between *DMD* intron 42 and Xp21.1, inherited from the mother. In both cases, simple repeat sequences (SRS) were present at the breakpoints of these inversions.

Conclusions Our findings demonstrate that LRS is an effective tool for detecting atypical mutations. The identification of SRS at the breakpoints in DMD patients enhances our understanding of the mechanisms underlying structural variations, thereby facilitating the exploration of potential treatments.

Keywords DMD, Inversion, Long-read sequencing, Simple repeated sequences, Case report

Background

The *dystrophin* gene (*DMD*; MIM #300377), located within a 2.4 Mb region on the X chromosome at the Xp21.2 locus, exhibits significant complexity by producing multiple isoforms across various tissues. Mutations in this gene can result in the absence of functional dystrophin protein, leading primarily to progressive degradation of muscle tissue, including skeletal, cardiac, and smooth muscle. This degradation can also result in orthopedic and respiratory complications [1]. Loss-of-function mutations in the *dystrophin* gene are responsible for Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD) and X-linked dilated cardiomyopathy. These mutations encompass a broad spectrum of alterations, including deletions, duplications, single nucleotide variants (SNVs), insertions and complex rearrangements [2]. Among these, intragenic deletions affecting one or more exons are the most common, accounting for approximately 60% of all *dystrophin* mutations, with duplications are less frequent, occurring in 6 to 8% of cases [3]. Complex rearrangements, such as balanced translocations or inversions, are exceptionally rare.

In the investigating of causative mutations in DMD/BMD patients, various methods have been employed.

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Multiplex ligation-dependent probe amplification (MLPA) is the conventional method used in genetic testing to detect deletions and duplications. When this analysis does not identify a pathogenic mutation, the next step is to perform whole exome sequencing (WES) to examine the complete coding region of the *DMD* gene [4]. The diagnostic rate can exceed 95% [5]. The mutations that remain undetected are primarily deep intronic mutations [6], which can be identified through RNA analysis of muscle tissue using RT-PCR or short-read sequencing. However, a subset of patients remains genetically undiagnosed, even after applying these techniques, due to the presence of exceptionally rare and complex structural variants.

A previous study demonstrated that the captured long-read sequencing (LRS) panel effectively detected a spectrum of *DMD* mutations, ranging from SNVs to structural variations [7]. LRS, represented by platforms such as Oxford Nanopore Technologies (ONT) and Pacific Biosciences (PB) single-molecule real-time sequencing (SMRT), enabled more precise identification of breakpoint positions for structural variations in the *DMD* gene. This enhanced precision in breakpoints detection allows for the decoding of the entire sequence within the *DMD* rearrangement region. Notably, the sequencing data from PB exhibited more consistent errors compared to ONT, making it easier to identify the correct sequence using PB. In this study, we identified two intrachromosomal inversions involving the *dystrophin* gene in two unrelated families using LRS, facilitated by the PB SMRT platform. In both cases, simple repeat sequences were observed at the breakpoints of these inversions.

Case presentation

Patient 1

A ten-year-old boy was admitted to our hospital with complaints of muscle weakness. He is the elder of two brothers, born to healthy, non-consanguineous Chinese parents, with no significant personal or family medical history. The patient began walking independently at fourteen months of age. However, by the age of five, he started experiencing a gradual decline in his walking abilities, becoming prone to falls. At ten years old, he encountered difficulties with jumping and rising from a squatting position. Additionally, he exhibited intellectual disability and faced challenges in school, leading to lower academic performance, which first became evident at the age of six. Physical examination revealed hypertrophy of the gastrocnemius muscle, a positive Gowers' sign and a waddling gait. His head circumference measured 50 cm and his tendon reflexes were diminished. Based on these clinical findings, a preliminary diagnosis of dystrophinopathy was made. Serum creatine kinase levels were significantly elevated at 10,961 U/L (normal ≤ 190 U/L). The Wechsler Child Intelligence Scale assessed his overall IQ as 65. While electrocardiography results were normal, color Doppler echocardiography revealed left ventricular enlargement.

Following the clinical diagnosis of dystrophinopathy, the patient began standard steroid treatment with prednisone (0.75 mg/kg/d) and received regular followed-up care. Genetic testing was initiated shortly thereafter. Initially, MLPA was performed, which did not reveal any deletions or duplications. WES was subsequently conducted to identify SNVs or splicing mutations across all 79 exons of the DMD gene. Unfortunately, no causative genetic mutations were identified through these methods. Based on previous reports, the patient then underwent LRS directly [2, 7].

A pericentric inversion spanning 110.81 Mb, mapping between chrXp21.1 and chrXq27.3, was identified through LRS. The inversion breakpoints were precisely located at the genomic positions chrX:31,927,491 and chrX:142,742,006. Notably, the breakpoint at chrX:31,927,491 is located within intron 47 of the *DMD* gene. Additionally, the patient's DNA revealed a 9 bp deletion spanning chrX:31,927,482 – 31,927,490 (CTTTGGGAA) and a 313 bp duplication within chrX:142,741,694 – 142,742,006 (Fig. 1).

The mutation identified through LRS was subsequently confirmed by PCR/Sanger sequencing, a widely used technology to validate and accurately determine the breakpoint location in inversion mutations. Two primers were designed based on the LRS results, with specific details provided in Fig. 2. Sanger sequencing accurately pinpointed the inversion breakpoints at chrX:31,927,481/142,742,828 and chrX:31,927,491/142,741,748, confirming an inversion between these genomic coordinates on the X chromosome. Additionally, a 9 bp deletion at chrX:31,927,482 – 31,927,490 and an 81 bp duplication at chrX:142,741,748 – 142,741,828 were identified at these breakpoints, as shown in Fig. 1. Detailed analysis of the breakpoints revealed the presence of LTR sequences at chrX:31,927,411 – 31,927,629 (219 bp) and (TA)n sequences at chrX:142,741,289 – 142,741,461 (173 bp), chrX142,741,503 – 142,741,649 (147 bp), chrX142,741,665 – 142,741,824 (160 bp) and chrX:142,741,855 – 142,742,166 (312 bp). These simple repetitive sequences surround both breakpoints, leading to duplicate alignments during the sequencing process. Consequently, this can result in differences between the LRS and Sanger sequencing results.

Patient 2

The second case involves a 6-year-old boy who was admitted to our hospital with an abnormal gait. A

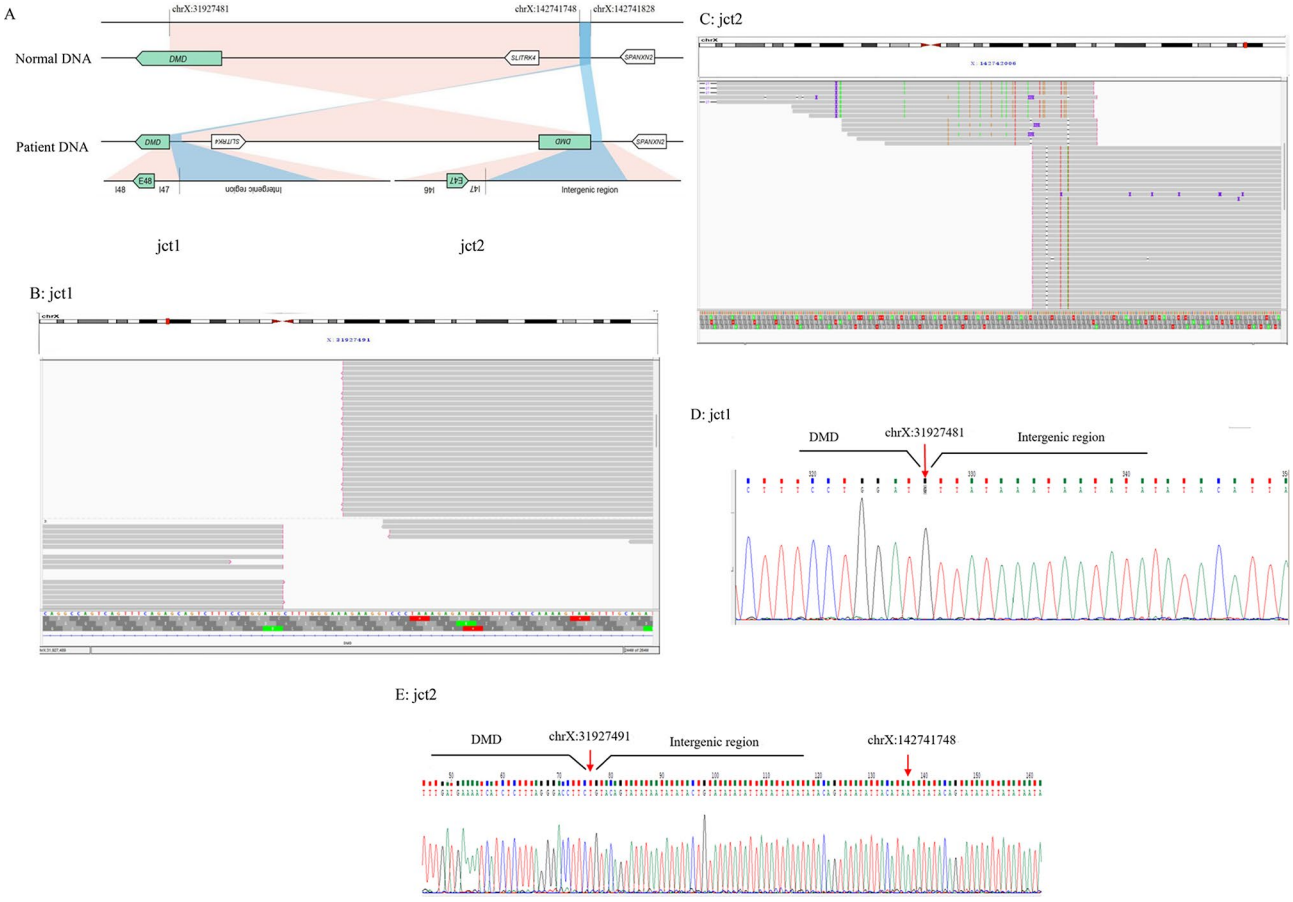
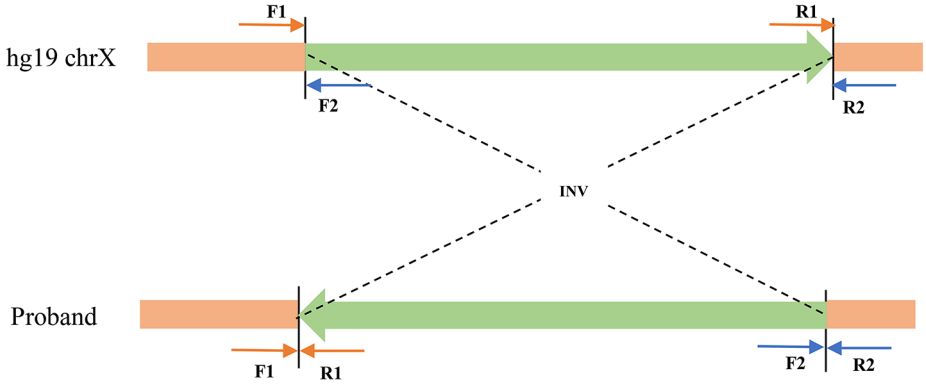


Fig. 1 Diagrammatic representation of the gene rearrangement in the patient 1. This inversion spans a vast distance of 110.81 Mb, stretching from chrX:31,927,491 (Xp21.1) to chrX:142,742,006 (Xq27.3), crossing the centromere on the X chromosome. Additionally, a 9 bp deletion spanning chrX:31,927,482 – 31,927,490 and a 313 bp duplication within chrX:142,741,694 – 142,742,006 in the patient's DNA were identified. **A**: Patient's gene showing the inverted and duplicated segments and mutation breakpoints. **B**: Schematic representation of the IGV at jct1. **C**: Schematic representation of the IGV at jct2. **D**: The proximal mutation breakpoint sequence. **E**: The distal mutation breakpoint sequence

confirmed family history revealed that similar conditions had afflicted his maternal uncle and cousin, both of whom exhibited comparable symptoms and, unfortunately, passed away after reaching the age of 10 (Fig. 3). The boy took his first steps at 18 months. At the age of 3, he was hospitalized for a fever at another institution, where tests revealed an abnormally high serum creatine kinase level (CK = 17215U/L, normal range: 24-204U/L). Despite these findings, he did not exhibit any significant motor deficits at that time. To establish a definitive diagnosis, MLPA and WES were conducted to identify common deletions and duplications within the *DMD* gene. However, these tests did not detect any mutations. Subsequently, he began experiencing walking difficulties, frequent falls, and difficulty climbing stairs. At the age of 6, he presented to our hospital. His academic performance at school was average. Physical examination revealed hypertrophy of the calf and forearm muscles, along with weakness in the pelvic-girdle muscles. He also exhibited a positive Gowers' sign and a wadding gait. A repeat serum creatine kinase (CK) assay revealed an elevated level of 6,981U/L (normal: ≤190U/L). MRI scans of the bilateral hips and thigh muscles showed widespread long T2 signals and signs of myofascial edema. Meanwhile, electrocardiogram results indicated a normal sinus rhythm, and echocardiography did not reveal any cardiac abnormalities. The Wechsler Child Intelligence Scale assessed his overall IQ at 90. Based on these clinical findings, a tentative diagnosis of dystrophinopathy was made. The patient was started on standard steroid treatment (prednisone 0,75 mg/kg/d) and received regular followed-up care. To confirm this diagnosis, we recommended LRS as the next step in our investigation.

LRS identified a 6.31 Mb paracentric inversion between chromosomal regions chrXp21.3 and chrXp21.1. The inversion breakpoints were accurately mapped to genomic coordinates chrX:26,046,702 and chrX:32,353,866. Specifically, the breakpoint at chrX:26,046,702 is located within the intergenic region between the *RANBP1BP1* and *MAGEB18* genes, while



For the first family:

Primers: F1: 5'-GATCTCCACAACCTGCCTACA-3'

R1: 5'-GTGAAACACAGATTCTGGCCC-3'

F2: 5'-AGCAGCTTCACCTGACTTCCC-3'

R2: 5'-GCCAGCAGATTTCAAACAGAA-3'

For the second family:

Primers: F1: 5'-AATGCAGTGCCATGGTGATA-3'

R1: 5'-AAGGTGGTGGCAAACAGTTC-3'

F2: 5'-CAGTGACCCCCTTCACCTTA-3'

R2: 5'-AATCCCAGCTCTTTGTGAGG-3'

Fig. 2 Primers in the sanger sequencing

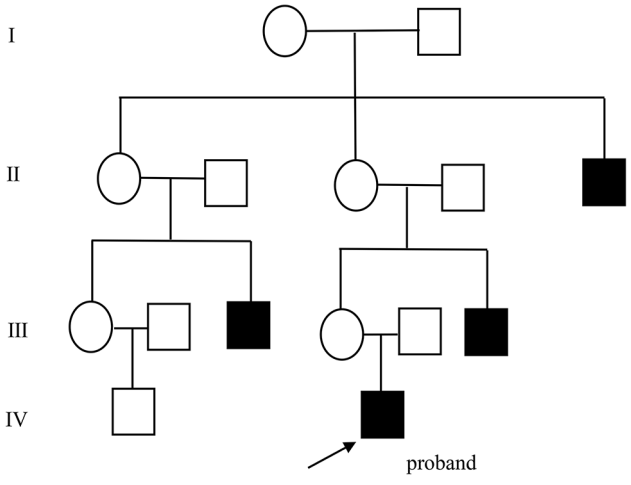


Fig. 3 The family pedigree of the patient

the breakpoint at chrX:32,353,86 lies within intron 41 of the *DMD* gene. Additionally, sequencing revealed a 1 bp deletion at chrX:26,046,701 and a 37 bp deletion spanning chrX:32,353,828−32,353,865 in the patient's genome (Fig. 4). The patient's mother was also found to carry this inversion on one of her X chromosomes.

PCR/Sanger sequencing was conducted to confirm the results obtained from LRS. Primers were specifically designed based on the LRS data (Fig. 1). Sanger sequencing precisely identified the inversion breakpoints at chrX:26,046,700/chrX:32,353,827 and chrX:26,046,702/chrX:32,353,866, indicating an inversion within these genomic coordinates on the X chromosome. Additionally, two deletions at chrX:26,046,701 and chrX:32,353,828−32,353,865 were confirmed in the patient's DNA (Fig. 4). An in-depth analysis of the breakpoints revealed the presence of simple repeats, denoted as (CATATA)n, at chrX:32,353,741−32,353,885. These simple repetitive sequences may ccause duplicate alignments during the genomic alignment process.

Discussion and conclusions

Inversions affecting the *DMD* gene are an uncommon cause of DMD, with intrachromosomal inversions being particularly rare. To date, only 14 cases of X-chromosomal inversions disrupting the *DMD gene* have been reported [2, 8–18]. Our research identified two previously undocumented intrachromosomal inversion variants that disrupt the *DMD* gene in patients. The first case involves a DMD patient with intellectual disability who was found to have an approximately 110 Mb pericentric inversion on the X chromosome. The second case concerns another DMD patient who was identified with an approximately 6.31 Mb paracentric inversion on the X chromosome. Traditional diagnostic methods, such as

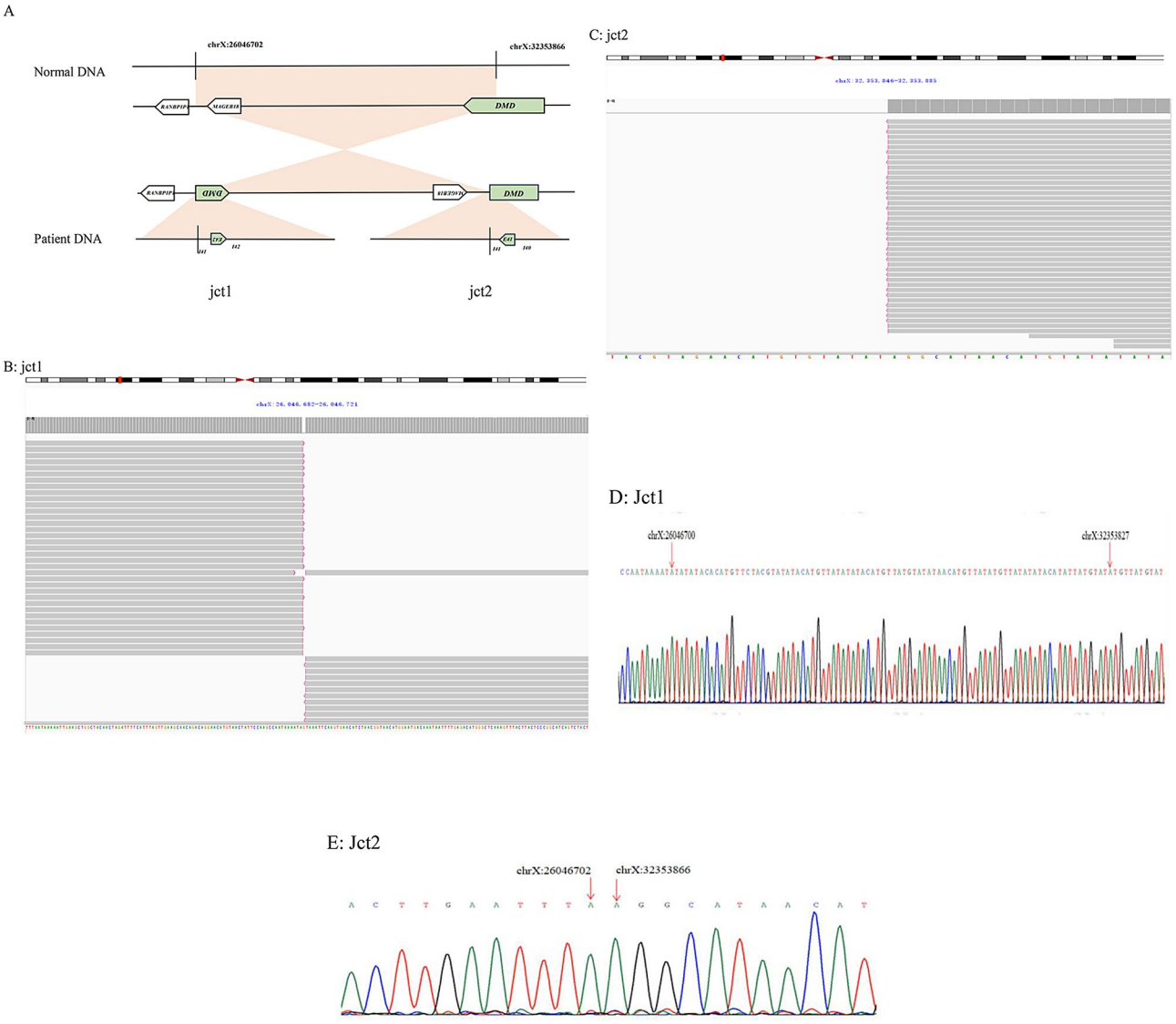


Fig. 4 Diagrammatic representation of the gene rearrangement in the patient 2. This paracentric inversion spans a distance of 6.31 Mb, stretching from chrX:26,046,702 (Xp21.3) to chrX:32,353,866 (Xp21.1). Additionally, a 1 bp deletion on the chrX:26,046,701 and a 37 bp deletion spanning chrX:32,353,828 – 32,353,865 in the patient's DNA were identified. **A**: Patient's gene showing the inverted segments and mutation breakpoints. **B**: The sanger sequence of the proximal mutation breakpoint sequence of the patient 2. **C**: Schematic representation of the IGV at jct1. **D**: Schematic representation of the IGV at jct2. **E**: The sanger sequence of the distal mutation breakpoint sequence of the patient 2

MLPA and WES, were unsuccessful in detecting these mutations, while LRS proved to be highly effective and instrumental in the diagnostic process.

To date, intrachromosomal inversions associated with intellectual disability have been reported in 8 patients with DMD [11–13, 15–18]. In 5 of these cases, an additional gene was implicated at the second breakpoint, potentially explaining the cognitive impairment [11, 13, 15, 18]. In the first case we present, no known genes were identified at the second breakpoint. Previous studies suggest that cognitive and behavioral challenges are common in individuals with DMD and BMD. Approximately 30% of individuals with DMD have been reported to exhibit cognitive impairments, and around 40% experience difficulties with reading [12, 19]. Some reports indicate that cognitive impairment in DMD may be linked to mutations that disrupt the production of the distal Dp140 and Dp71 dystrophin isoforms [20–22]. These isoforms, Dp71 and Dp140, are highly expressed in the brain, suggesting their potential role in the cognitive deficits observed in DMD. Dp71 synthesis begins between exons 62 and 63, while Dp140 synthesis starts upstream of exon 45, with its initial methionine codon located in exon 51 [23]. In the first case from our study, the breakpoint located at chrX:31,927,491 falls within intron 47 of the *DMD* gene, disrupting its sequence. This disruption may impact the

Dp140 isoform, potentially leading to compromised dystrophin-associated brain function. However, the possibility of other contributing factors cannot be ruled out.

Repetitive DNA sequences play a crucial role in generating genetic variation [24]. Various repetitive sequences have been identified as drivers of aberrant recombination events within the genome, leading to the formation of structural variants, including inversions [25, 26]. Traditionally, repetitive DNA sequences have been categorized as either interspersed or tandem repeats, distinguished by their positions and the mechanisms driving their expansion [27]. In previous reports concerning intrachromosomal inversions in DMD patients, the breakpoints were predominantly located within interspersed repeats [2, 12, 14]. Simple repeated sequences (SRS) are defined as tandem repeats of microsatellite-sized (≤9 bp units), minisatellite-sized (10–60 bp units), or satellite-sized (>60 bp units) DNA sequences [27]. In our first case, (TA)n repeat sequences were located at the breakpoint, while in the second case, (CATATA)n repeat sequences were present at the breakpoint. Both (TA)n and (CATATA)n repeat sequences are classified as microsatellite-sized SRS. To the best of our knowledge, this represents the first documentation of intrachromosomal inversions involving SRS in DMD patients. SRS exhibit remarkable instability in terms of length, sequence composition, and copy number, with mutation rates typically 10 to 100,000 times higher than in other parts of the genome [28]. SRS may also play a role in regulating gene expression by providing binding sites for regulatory factors or by acting as targets for epigenetic modifications [24].

Over the past thirty years, more than 7,000 mutations have been identified in DMD using conventional genetic testing methods [29]. However, accurately diagnosing pathogenic variants in DMD patients remains challenging. While short-read-based WES and WGS are effective for detecting point mutations and small insertions or deletions within intergenic and intronic regions [30], they struggle to accurately map duplicated or structurally complex regions of the genome, especially those with highly repetitive sequences [2]. Additionally, PCR-based and short-read sequencing approaches are unable to detect genetic variants in genomic regions resistant to PCR amplification or sequencing. Although current guidelines recommend muscle biopsy and RNA/cDNA sequencing for patients in whom standard genetic tests, such as MLPA or Next-generation sequencing (NGS), fail to identify mutations [4], the invasive and painful nature of biopsies often causes reluctance, particularly in children. LRS offers a novel and effective approach for accurately detecting balanced translocations and inversions, while providing information on the orientation and order of structural variants (SVs). Furthermore, LRS achieves a high mapping ratio in both exonic and intronic regions with repetitive sequences. As a result, LRS may provide a less invasive and more effective alternative for identifying rare mutations, including inversions. The identification of SRS at breakpoints in DMD patients aids in gaining a deeper understanding of the mechanisms involved in structural variations, thereby facilitating the exploration of potential treatments.

Abbreviations

DMD	Duchenne Muscular Dystrophy
MLPA	Multiplex ligation probe amplification
WES	Whole exome sequencing
NGS	Next-generation sequencing
LRS	Long-read sequencing
BMD	Becker Muscular Dystrophy
SNVs	Single nucleotide variants
SMART	Single-molecule real-time sequencing
IQ	Intelligence quotient
CK	Creatine kinase
SRS	Simple repeated sequences

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Author contributions

LC drafted the manuscript and prepared the figures. YL did the follow-up with the patient. XL and YL edited the manuscript. YT and HW edited the figures. All authors contributed to the article and approved the submitted version.

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Data availability

Sequence data that support the findings of this study have been deposited in the ClinVAR with the primary accession code PRJNA1098061.

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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