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Application of long-read sequencing in the diagnosis of Duchenne/Becker muscular dystrophy: unveiling complex structural variations and deep intronic mutations

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Abstract

Background Despite the widespread use of Multiplex Ligation-dependent Probe Amplification (MLPA) and Next-Generation Sequencing (NGS) in Duchenne/Becker Muscular Dystrophy (DMD/BMD), these methods have limitations when dealing with complex genetic backgrounds. Long-Read Sequencing (LRS), an emerging technology that provides longer read lengths, is advantageous for uncovering complex gene rearrangements and deep intronic mutations.

Methods This study conducted LRS on 5 children with suspected DMD/BMD. One patient had not underwent genetic testing before, while the others had previously underwent MLPA and WES and were unable to find pathogenic variants. Chromosome analysis and X inactivation analysis were performed on female patient.

Results Among the four patients who had not received a diagnosis through MLPA and NGS, LRS successfully identified translocations and inversions in two patients and deep intronic mutations in the other two. The fifth patient, who underwent LRS, initially showed no apparent mutations. However, muscle biopsy confirmed the disease diagnosis, and RNA sequencing revealed a partial deletion of exon 19 in the mRNA, ultimately pinpointing the causative mutation.

Conclusions The results of this study highlight the advantages of LRS in revealing complex genetic variations, particularly those challenging to detect with conventional methods, such as structural variations and deep intronic regions. Furthermore, combining muscle biopsy and RNA sequencing provides more comprehensive diagnostic information for patients not diagnosed through standard genetic tests. In the future, this technology is expected to complement routine genetic testing, aiding clinicians in achieving precise diagnoses across a broader patient population.

Keywords DMD, Duchenne muscular dystrophy, Long-read sequencing, LRS, X-inactivation

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Introduction

Duchenne Muscular Dystrophy (DMD, OMIM #310200) and Becker Muscular Dystrophy (BMD, OMIM #300376) are severe genetic disorders caused by mutations in the DMD gene (OMIM *300377). These mutations lead to progressive muscle degeneration and weakness. Regarding to the genetic diagnosis of DMD, exon deletion or duplication achieved by multiplex ligation-dependent probe amplification (MLPA) is recommended to use first, which is followed by genetic sequencing to screen for the remaining types of variants (approximately 25–30%), including nonsense variants, missense variants, small deletions, and small duplications or insertions [1].

However, even with the aforementioned techniques, the genetic diagnosis of certain DMD patients remains unclear due to the intrinsic limitations of MLPA and gene sequencing on identifying deep intronic variants and structural variations [2]. Long-read sequencing(LRS), which is able to read the sequence of longer DNA fragments (typically ranging from several thousands to tens thousands of base pairs in length), allows the possibility of explore previously inaccessible regions of the genome, such as telomeres and other highly repetitive regions, as well as complex structural variations [3].

Here, we report 5 patients which variants were identified by LRS and RNA-sequencing. Our research demonstrates the robust capability of LRS in analyzing complex genetic variations and underscores its importance as a complementary tool to existing diagnostic methods.

Materials and methods

Long-read sequencing

Testing is conducted with the consent of the patient and their parents. 5 ml of peripheral venous blood was collected from the proband and her mother. Genomic DNA is extracted using the MagPure Universal DNA Precast Kit (Guangzhou Magen Biotechnology Co., Ltd., Guangzhou, China) according to the manufacturer's protocol. DNA library is constructed using SMRTbell Express Template Kit 2.0. Then, third-generation PacBio Sequel II/IIe high-throughput sequencing was performed. After the evaluation of sequencing data with SMRT Link, data reading and bioinformatics analysis is carried out.

Table 1 Variants of 5 patients

Patient	Variant
1	chrX: g. 32519799_32519868delins32520928-32520965
2(female)	t(x,3)(p21.1,p12.2), chrX:31,800,627-chr3:82376521 and chrX:31,800,635-chr3:82376522
3	chrX: g.31522381_31522386inv
4	NM_004006.3: c.5325 + 1759G > C
5	NM_004006.3: c.1812 + 601 A > G

X-inactivation analysis

Testing was conducted with the consent of the patient and their parents. DNA sample of the patients and her parents were isolated from whole EDTA blood samples according to standard salting-out procedures. X chromosome inactivation (XCI) was assessed using the HUMARA assay, which relies on enzymatic methylation analysis of the CAG repeat within the human androgen receptor gene HUMARA (AR, MIM *313700) employing the methylation-sensitive restriction enzyme HhaI. Fragment analysis was performed on an ABI 3730XL genetic analyser (Thermo Fisher Scientific, Waltham, MA, USA), and data analysis was performed using the software GeneMapper v2.2.0 (AppliedBiosystems, Waltham, MA, USA).

Chromosome analysis

GTG-banded metaphases at a band resolution of 400 were used for the karyotype analysis.

Result

Four males and one female underwent long-read sequencing. Among them, MLPA and NGS had previously been conducted for all but Patient 1, yet no pathogenic variants were identified. Long-read sequencing revealed deep intronic variants in Patient 4 and 2, and a chromosomal inversion in Patient 3. No pathogenic variants were identified in Patient 1 through long-read sequencing, but subsequent transcriptome sequencing indicated a partial exon 19 deletion in the mRNA, pinpointing the pathogenic variant to intron 18 near exon 19. Patient 2, a female with a confirmed translocation through chromosomal analysis, exhibited an X-chromosome inactivation ratio of 98:2. Detailed variant information is presented in Table 1.

Patient 1 At age 4, the patient experienced declining motor abilities, difficulty running, climbing stairs, squatting, and standing, with positive Gowers' sign and bilateral calf hypertrophy. The creatine kinase (CK) level was 8311.4U/L. MLPA tests were negative. At age 10, long-read sequencing was performed at our hospital, but no variants explaining the phenotype were found. Therefore, a muscle biopsy and muscle RNA-seq were performed. Immunohistochemical staining showing severe reduction of dystrophin-N, C, R(Fig. 1).

Muscle RNA-seq indicated the loss of exon 19 in mRNA. Based on the mRNA results, re-analysis of the long-read sequencing data identified a delins mutation in intron 19 (chrX:32519799–32519868), with the inserted sequence originating from chrX:32,520,928–32,520,965, which was identified as multiple SNPs of intron18 before RNA-seq.

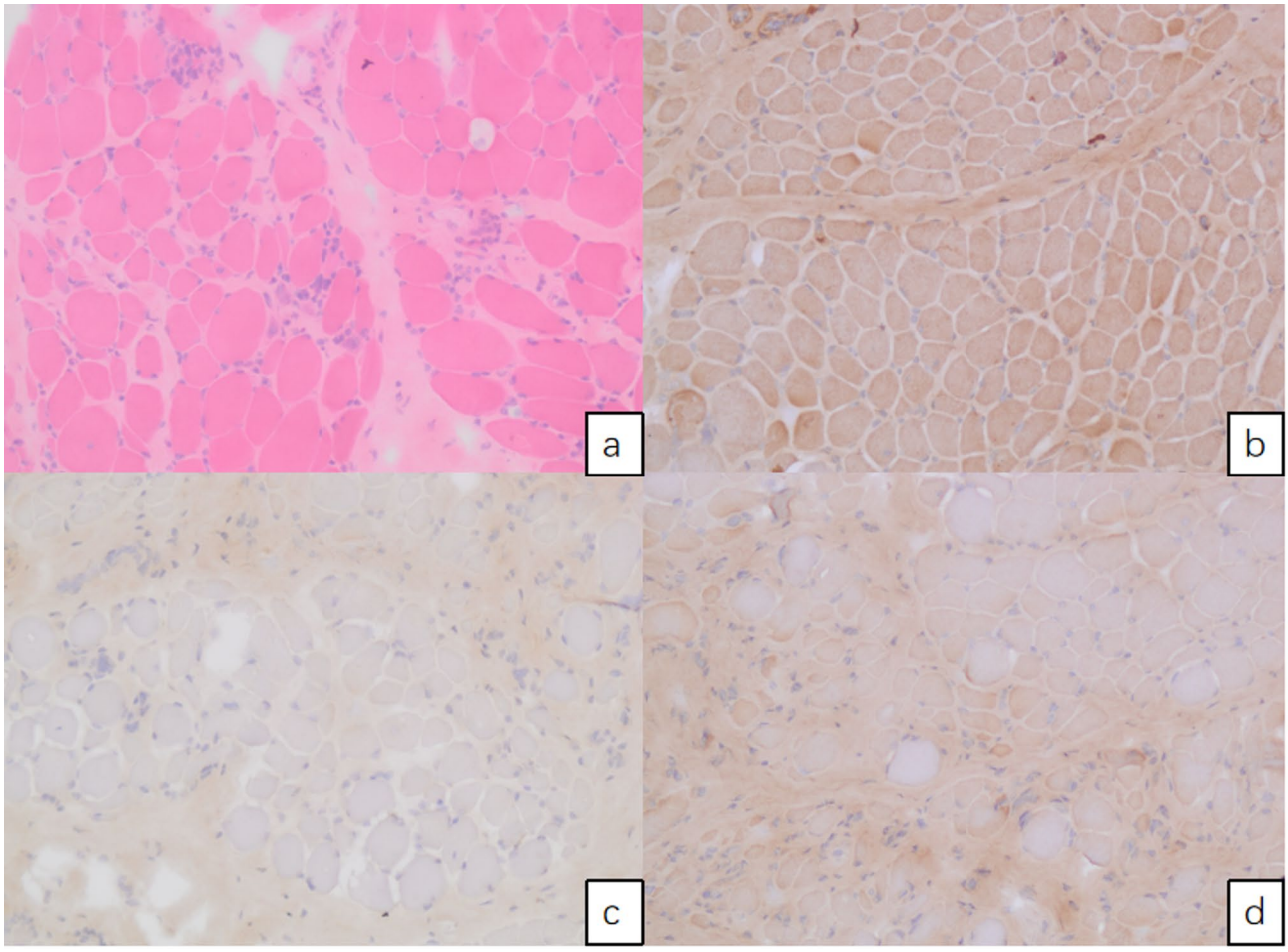

Fig. 1 The muscle pathology of patient 1: **a**. Hematoxylin-eosin staining revealed myopathic changes: areas of fibrosis, fatty infiltrate, and degenerating and regenerating fibers. (**b**-**d**) Immunohistochemical staining showing severe reduction of dystrophin-N, **C**, R

Patient 2 The 25-year-old girl is the second child of *non-carrier and* non-consanguineous parents. She showed motor development delay manifested as inability to walk independently until age 3, and the CK level was around 12000U/L. Then, she manifested progressive inability to walk since she was 5 and completely unable to walk independently when she was 20. At the age of 8, she was admitted to hospital and diagnosed with DMD via muscle biopsy. However, MLPA and WGS failed to identify any pathogenic variants that can explain her phenotype. No family history of neuromuscular diseases or related diseases can be recalled. She is of normal intelligence but with slowed speech.

LRS revealed a chromosome translocation in the proband, which involves chrX:31,800,627-chr3:82376521 and chrX:31,800,635-chr3:82376122 (Fig. 2a), with a 7 bp deletion detected in the chrX:31,800,628–31,800,635 (Fig. 2b). Such translocation or any pathogenic variants was not detected in her mother. Since her father does not show any sign of DMD, we believe that the translocation of Dystrophin gene in the proband is a de novo mutation, instead of inherited from her parents.

Translocation was confirmed by chromosome karyotype. Patient's chromosome analysis using conventional GTG banding showed a balanced translocation between X chromosome and chromosome 3(Fig. 3. The karyotype was reported as 46,X, t(X;3)(p22.2;p14).

XCI analysis showed extremely skewed X chromosome inactivation in the blood cells of the proband (98:2), with more severe inactivation of maternal X chromosome (Fig. 4a and b). This is confirmed by the repeat sizes of the DNA fragment analysis. Fragments from the proband are 240 bp and 254 bp (Fig. 4a), while fragments from father are 240 bp (Fig. 4b) and fragments from mother are 248 bp and 254 bp (Fig. 4c). These results supports that the fragment of 240 bp is paternally inherited, while the fragment of 254 bp is maternally inherited.

By digestion with HhaI, which targets unmethylated DNA, the 240 bp fragment was almost completely digested (Fig. 4b). Consequently, the maternal allele with

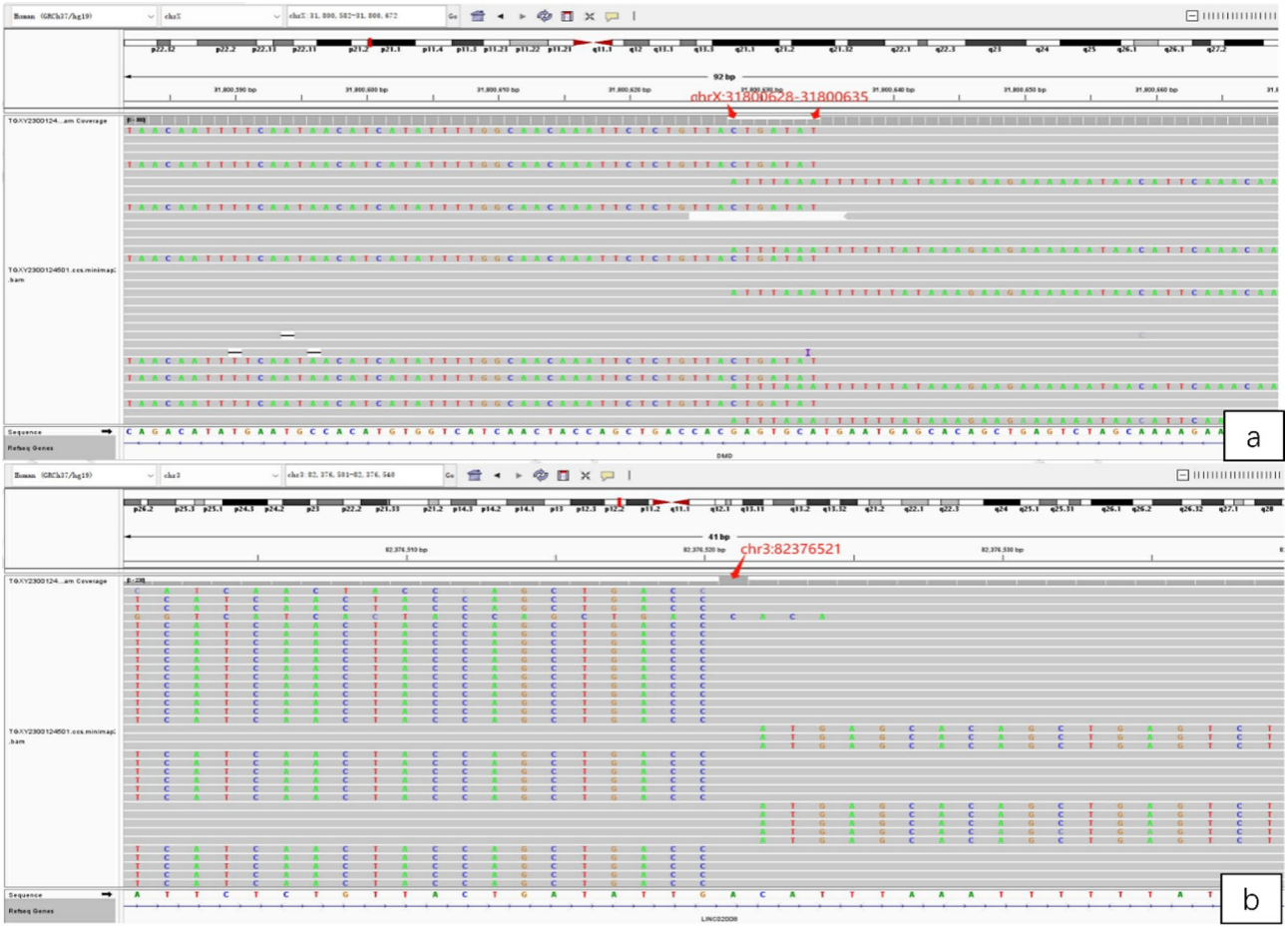

Fig. 2 LRS indicated the translocation:46.XX t(X;3)(p21.1;p12.2), which breakpoint are chrX:31,800,627-chr3:82376521 and chrX: 31,800,635-chr3:82376522,and 7 bp deletion from chrX:31,800,628 to 31,800,635. a and **b**: BAM diagrams

a fragment size of 254 bp is still present and therefore almost completely methylated and inactive.

Patient 3: At 10 months, the patient was found to have elevated muscle enzymes and was suspected of muscular dystrophy. MLPA was negative. NGS identified a missense mutation (c.1318G > A). For further diagnosis, long-read sequencing revealed an inversion mutation covering exon 56 and its flanking intron of the DMD gene (chrX: g.31522381_31522386inv). At age 11, during a follow-up (weight 34 kg), the patient was alert and able to walk independently with a waddling gait and lumbar lordosis. He needed hand support to squat, could not stand without help, could raise both hands above his head, and had calf pseudohypertrophy. Gowers' sign was positive. CK was 6050 U/L.

Patient 4: A 4-year-old boy. After birth, blood tests revealed elevated muscle enzymes, suggesting "progressive muscular dystrophy." MLPA and NGS tests were negative. When he came to our hospital at the age of four, the patient walked on tiptoes, had 4 + muscle strength in the proximal lower limbs, bilateral calf hypertrophy, and slight contracture of the Achilles tendons, more pronounced on the right. He could squat halfway, jump with both feet but not high, and could not hop on one foot. The CK level was around 10000U/L. Long-read sequencing revealed a deep intronic variant (NM_004006.3: c.5325 + 1759G > C).

Patient 5: At age 3, elevated muscle enzymes were detected during a physical examination. He was considered "progressive muscular dystrophy" and come to our hospital for further treatment. MLPA and WES tests did not identify any pathogenic variants. At the last, Long-read sequencing identified a deep intronic variant in intron 15 (NM_004006.3: c.1812 + 601 A > G). At age 7, the patient was re-evaluated (weight 24 kg), showed toe-walking, slight forward bending of the waist, difficulty squatting and standing without hand support, slight jumping with both feet, and bilateral pseudohypertrophy of the calves. The CK level was 18,881 U/L.

Discussion

This study identified two deep intronic variants and three complex structural variants among five patients with suspected DMD/BMD. Traditional molecular techniques

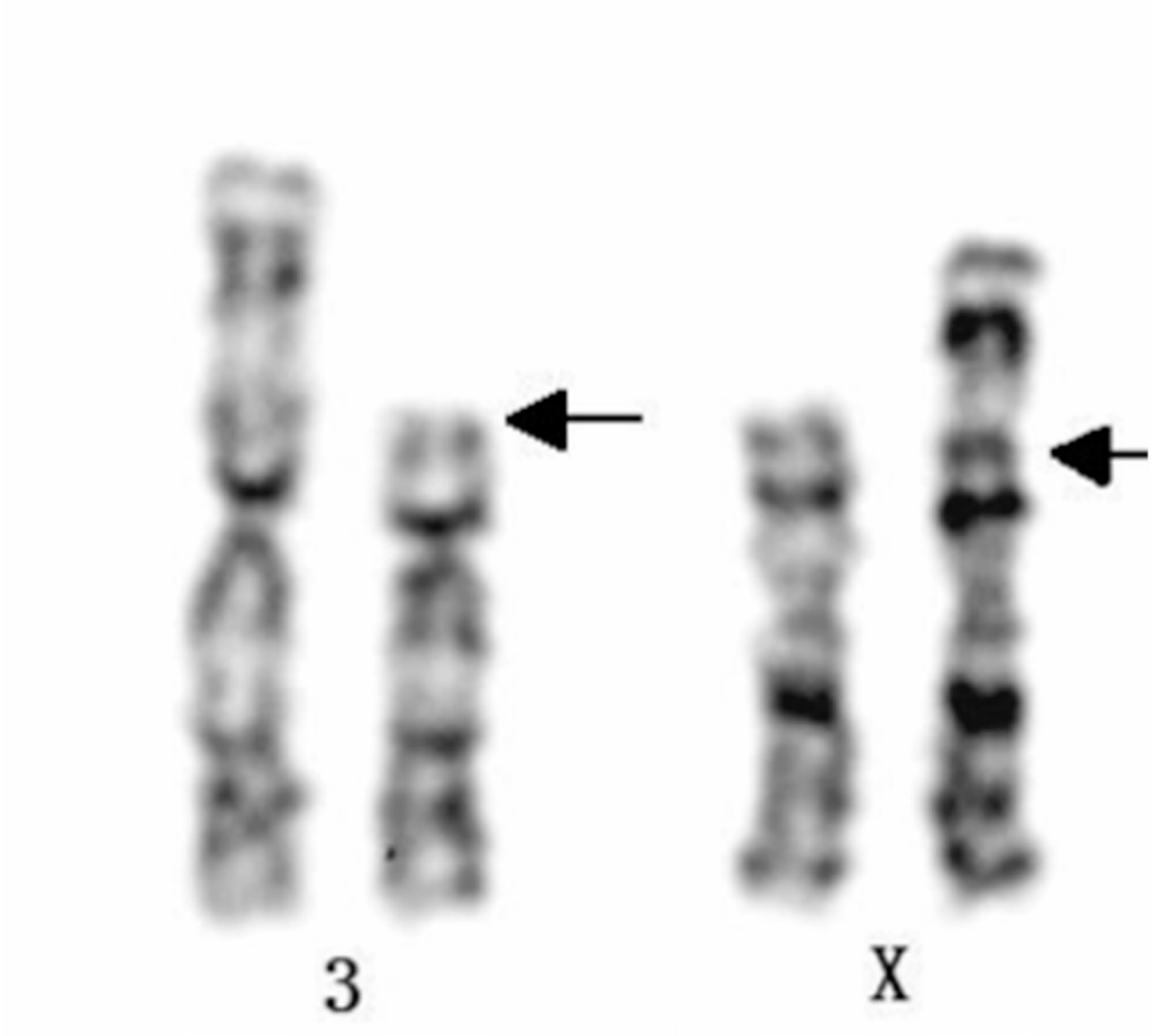

Fig. 3 The G-banding method was used to analyze the karyotypes of 5 cells, all of which showed a balanced translocation between one X chromosome and one chromosome 3

such as MLPA and short-read sequencing are frequently employed in clinical settings [4]. However, they lack the ability to precisely identify the physical locations of breakpoints and structural characteristics of genomic rearrangements [5]. Due to the limitation of short reading length, Long-read sequencing (LRS) offers notable advantages over short-read sequencing methods, including the ability to achieve phased de novo genome assembly, access regions of the genome that were previously difficult to sequence, and identify complex structural variants (SVs) that are often associated with diseases. These capabilities make LRS a powerful tool for comprehensive genomic analysis [6].

The traditional diagnostic workflow for DMD employs a "stepwise escalation" testing strategy—starting with MLPA as the initial screening, moving to NGS if no positive findings are detected, and ultimately relying on muscle biopsy for confirmation if pathogenic variants remain undetermined. Using LRS after MLPA and NGS will bring three benefits to DMD/BMD diagnosis: (a) Reducing the use of invasive procedures. LRS can resolve challenging cases that yield negative results with traditional genetic testing, thereby minimizing the need for traumatic muscle biopsies. (b) Correcting misdiagnoses from traditional methods. Even mutations previously detected by MLPA or NGS may be inaccurate. For example, a literature report described a male individual in whom MLPA

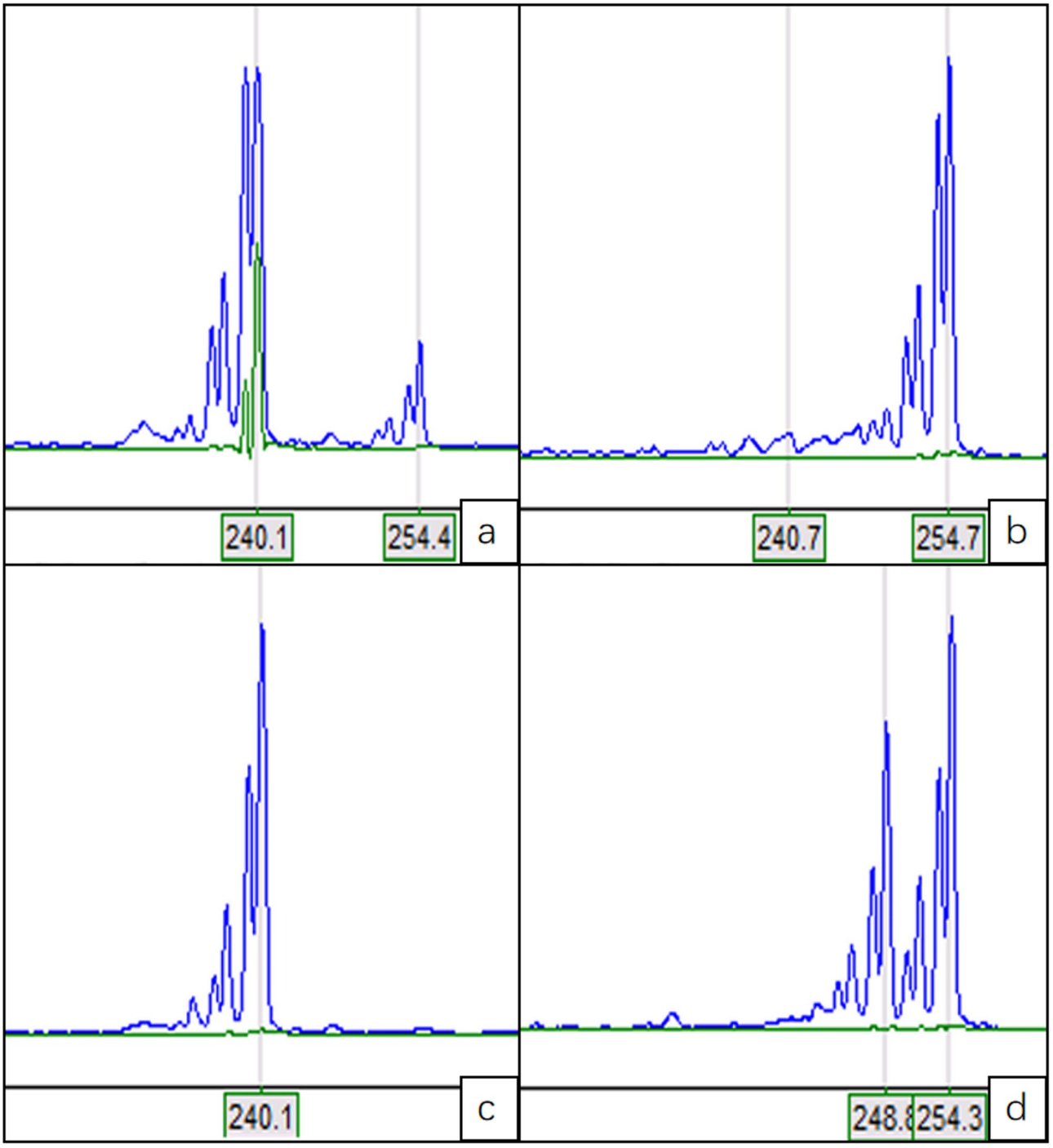

Fig. 4 Fragment analysis of the CAG repeat in the AR gene of patient and her parents, (**a**) undigested (**b**) digested with HhaI. The peak with a size of 240 bp is almost completely lost through digestion. (**c**) Fragment lengths of the undigested AR repeats of the patient's father and (**d**) mother

and WES identified a duplication of exons 56–61 in the DMD gene, yet the patient showed no clinical symptoms [7]. LRS ultimately revealed that this duplication was not a tandem duplication within the DMD gene but rather a copy inserted into the CFAP47 gene on the same X chromosome. This demonstrates LRS's unique ability to clarify the true genomic context of variants misidentified by conventional techniques. (c) Supporting family variant screening and prenatal diagnosis. For DMD patients already confirmed via muscle pathology, traditional muscle biopsy only indicates "dystrophin deficiency" and cannot clarify the specific characteristics of pathogenic variants. This limitation prevents carrier testing and prenatal diagnosis for female relatives (e.g., mothers, sisters).

In contrast, LRS can precisely map pathogenic variants, determine whether female relatives are variant carriers, and provide critical genetic evidence for prenatal diagnosis—ultimately blocking the transmission of pathogenic mutations at the source.

In addition to its unique advantages in detecting complex structural variations and deep intronic variations, LRS has proven capable of identifying exon deletions, exon duplications, point mutations, and micro - mutations [8–10]. These findings indicate that LRS can detect all types of mutations currently found in DMD. Thus, it shows potential as a first - line diagnostic tool for DMD. However, no cohort studies have been conducted so far using LRS as the initial diagnostic tool for DMD. More robust evidence is still needed to support this hypothesis.

In Patient 1, our post-hoc analysis revealed that the data analysis software misclassified the indel mutation (chrX: g.32519799_32519868delins32520928-32520965) as 30 single-nucleotide variants, 6 deletions, and 2 insertions (Supplementary 1). This finding highlights the need to further optimize long-read sequencing workflows, particularly for detecting complex structural variants, which can be addressed through two key approaches: First, researchers should not fully rely on automated software processing when using long-read sequencing to identify potential complex structural variants. For cases with clear phenotypes but no detected complex structural variants via long-read sequencing, manual reanalysis of the data is necessary. Second, the pangenome, by expanding the diversity of reference sequences, can optimize LRS analysis pipelines and reduce false negatives [11, 12]. Although long-read sequencing excels at detecting complex structural variants (e.g., deep intronic variants, large insertions/deletions), traditional analysis relying on a single reference genome may lead to missed detection of certain variants due to the absence of relevant reference sequences (i.e., false negatives). By incorporating more population-specific sequences, the pangenome provides LRS with more comprehensive alignment references, reducing the risk of variant miss-detection caused by incomplete reference sequences. Additionally, the development of pangenome-based alignment tools (e.g., Giraffe) further supports this optimization direction [13].

Extremely skewed X inactivation (98:2) in the patient 2 may explain the DMD-like phenotype of the proband. Theoretically, female carriers of X-linked recessive disorders rarely exhibit clinical symptoms, but it has been reported that approximately 5–22% of females with DMD may manifest related symptoms [14–16], which may ascribe to XCI. XCI refers to the phenomenon that one X chromosome of female become inactivated in early embryonic development. When the extent of inactivation exceeds a certain threshold, it is recognized as XCI skewing, which may lead to the manifestation of muscular dystrophy-related symptoms in female carrying DMD variants [17]. Amos-Landgraf et al. investigated X inactivation patterns in a cohort of over 1,000 phenotypically unaffected females and find a normal distribution of XCI patterns, which only 5% females have extremely skewed XCI [18]. Balanced X-autosome translocations are particularly unique compared to other variants. Studies have demonstrated that XCI can spread to autosomes through X; autosomes translocation, resulting in monosomy of autosomes and subsequently detrimental consequences in cells with inactivated der(X) chromosomes [19]. As a result, the remaining cells are mainly der (X) non inactivated cells, exhibiting severe skewed X- chromosome inactivation. Viggiano, Ergoli et al. compiled data from 43 cases of X-autosome translocations involving the Xp21 locus. Their findings indicate that XCI was highly skewed in 37 out of 43 cases [17].

Muscle biopsy was recommended to patient 4 and patient 5, but was refused. However, for patients with variants with unclear pathogenicity or for whom pathogenic variants have not been found, Western blotting and immunofluorescence analysis can provide a clear diagnosis [4]. Analyzing the RNA obtained from muscle biopsy can further locate potential variations and clarify the pathogenicity of the variations, as demonstrated by the skipping of exon19 in patient 1's RNA.

Despite its promising findings, this study has several limitations. The small sample size of five patients limits the generalizability of the results. Future studies should include larger cohorts to validate these findings and confirm the diagnostic utility of LRS in a broader patient population. Despite the use of long-read sequencing, Patient 1's complex structural variant was not initially identified. Instead, the target mutation was pinpointed with the assistance of mRNA results, highlighting the limitations of long-read sequencing in variant of uncertain significance.

Based on our results, it is recommended that LRS be integrated into the diagnostic workflow for DMD/BMD, especially in cases where traditional methods fail to identify causative mutations. This integration may enhance diagnostic accuracy and inform more personalized treatment plans. Clinicians should consider LRS as a complementary tool to existing genetic tests to ensure comprehensive genomic analysis. And RNA-seq can provide pathogenic evidence for variants of uncertain significance. In conclusion, this study demonstrates the unique advantages of LRS in identifying complex genetic variants in DMD/BMD patients, providing critical insights that conventional methods miss.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13052-025-02053-0.

Supplementary Material 1

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Author contributions
CY conceived and designed the study, collected data, analyzed the data and drafted the initial manuscript, and revised the manuscript. ZC and PZ revised the manuscript. PJ conceived and designed the study, and reviewed the manuscript.

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Data availability
The data are available from the corresponding author on reasonable request.

Declarations

Ethics approvals and consent to participate
The study was conducted in accordance with the Declaration of Helsinki, and approved by the Medical Ethics Committee of Xiangya Hospital Central South University (No: 202310892). Written consent was obtained from the participant's parents or guardians.

Consent for publication
Not applicable.

Competing interests
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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