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CASE REPORT

Stepwise Diagnostic Strategy Integrating Long-Read Sequencing for the Interpretation of Phenotype-Genotype Discordance in Dystrophinopathy

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Introduction: Pathogenic variants in the *DMD* gene maintaining the open reading frame typically cause Becker muscular dystrophy. Here, we report a 7.7-year-old boy exhibiting a severe Duchenne muscular dystrophy phenotype, despite an in-frame deletion of *DMD* exons 50–51 identified by initial genetic testing, representing a notable exception to the conventional reading-frame rule.

Methods: To elucidate his phenotype-genotype discordance, muscle biopsy and subsequent dystrophin protein and mRNA analyses were conducted, followed by long-read sequencing of *DMD* gene and splicing analysis.

Results: Muscle biopsy revealed a dystrophic pattern and negative expression of dystrophin-N and dystrophin-C. The *dystrophin* mRNA analysis identified two out-of-frame *DMD* transcripts, which were different from the in-frame deletion of *DMD* exons 50–51 and can explain his severe phenotype. Long-read sequencing uncovered a novel deletion variant (~97kb) in *DMD* gene, which produced the two out-of-frame transcripts through aberrant splicing.

Conclusion: This case underscores the necessity of a stepwise molecular analysis strategy for the interpretation of phenotype-genotype discordance in dystrophinopathy. This stepwise diagnostic approach is essential for accurately characterizing *DMD* variants, guiding patient management, and genetic counseling.

Keywords: dystrophinopathy, phenotype-genotype discordance, reading-frame rule

Introduction

Dystrophinopathy, an X-linked recessive disorder caused by pathogenic variants in the *DMD* gene, covers a phenotypic spectrum that includes X-linked dilated cardiomyopathy, Duchenne muscular dystrophy (DMD), and Becker muscular dystrophy (BMD).[1] Given the complex genetic structure of *DMD* gene, its mutation events manifest in various forms, with intragenic deletions and/or duplications being the most common.[2] The reading-frame rule, proposed by Dr. Monaco and his colleagues,[3] posits that exonic deletions or duplications in *DMD* gene disrupting the open reading frame are typically associated with the severe DMD phenotype, as they result in the formation of unstable *dystrophin* mRNA with a premature stop codon and, therefore, lead to the absence of dystrophin or production of extremely low levels of truncated and non-functional dystrophin. Conversely, pathogenic *DMD* variants that preserve the open reading frame can be transcribed and translated into partially functional dystrophin, which can cause the mild BMD phenotype.[3] Current understanding suggests that there is no straightforward correlation between the size of deleted or duplicated segment of dystrophin and clinical severity in dystrophinopathies. The reading-frame rule is widely used in clinical practice to differentiate DMD from BMD, which has been shown to hold true for the majority of dystrophinopathy patients.

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Nevertheless, exceptions to the reading-frame rule do occur, with approximately 4%~9% of patients showing a genotype-phenotype mismatch.[4] More specifically, the estimated frequencies of such exceptions are about 10% in BMD and 5% in DMD, with a higher prevalence near the 5' end of the gene.[5] This discordance largely results from discrepancies between the predicted functional consequences of genomic variants and the actual alterations observed at the mRNA transcript level.[6] Although standard genetic testing approaches—multiplex ligation-dependent probe amplification (MLPA) for large deletions/duplications followed by short-read sequencing for small coding variants—identify most pathogenic *DMD* variants, an estimated 2–7% of cases remain undiagnosed.[7,8] More importantly, even when variants are detected, conventional methods may fail to accurately predict their transcript-level effects, particularly in cases involving deep intronic variants or complex structural rearrangements that disrupt splicing regulation. Therefore, a stepwise diagnostic strategy is essential for clarifying complex genotype–phenotype relationships, achieving precise molecular diagnosis and resolving apparent contradictions between genetic and clinical findings.

In this case study, we encountered a 7.7-year-old boy with a severe clinical presentation and magnetic resonance imaging (MRI) findings consistent with DMD phenotype. Initial genetic testing, however, identified only an in-frame deletion of *DMD* gene, which is typically associated with a milder BMD phenotype. This apparent genotype-phenotype discordance prompted a comprehensive diagnostic re-evaluation. We employed a stepwise molecular analysis strategy including dystrophin protein analysis, full-length *dystrophin* mRNA analysis, long-read sequencing of genomic *dystrophin*, and splicing prediction analysis to elucidate the underlying molecular mechanism. This report highlights the diagnostic challenge posed by such exceptions and underscores the necessity of a stepwise approach to achieve a precise diagnosis in dystrophinopathy.

Materials and Methods

Collection and Summarization of Phenotypic Information

A 7.7-year-old boy with phenotypic features consistent with a DMD phenotype was included in this study. Detailed neurological examination ascertained his pattern of weakness distribution. Serum creatine kinase was measured to assess muscle damage. Muscle MRI of the thigh muscles followed a standardized protocol.[9]

Routine Genetic Testing

MLPA analysis[7] of *DMD* gene was initiated in this patient due to his DMD phenotype. To rule out the possibility of small pathogenic variants (single-nucleotide variants and small insertions/deletions) in *DMD* and other genes, whole exome sequencing at a mean depth of >100× was also performed (detailed methodology provided in Supplementary Material 1). Copy number variant (CNV) analysis was subsequently conducted using CNVkit.

Muscle Biopsy-Based Dystrophin Protein and mRNA Analyses

Muscle biopsy was collected from the biceps brachii of the patient and an age matched healthy control, and subsequently subjected to histological and molecular analysis. The control muscle sample was obtained from our institutional tissue bank and came from an anonymized individual with no documented neuromuscular pathology who had provided written informed consent for the use of residual tissue in future research. The use of this sample was approved by the ethics committee. Standard procedures[7] were followed for histological and immunohistochemical staining of muscle sections. Dystrophin expression was assessed by immunohistochemical staining, using a panel of monoclonal antibodies (Novocastra Laboratories, Newcastle) against different domains of the dystrophin protein, including dystrophin-N (amino-terminal; DYS3), dystrophin-C (carboxyl-terminal; DYS2), and dystrophin-R (rod-domain; DYS1).

From the remaining biopsy tissue, total muscle RNA was extracted and retrotranscribed into cDNA by reverse transcription polymerase chain reaction (RT-PCR). The primers used for RT-PCR amplification are listed in Supplementary Table 1. Full-length *dystrophin* cDNA was amplified in 22 overlapping fragments and analyzed by agarose gel electrophoresis as described previously.[7] Sequences of aberrant transcripts were determined by Sanger sequencing. Detailed methods are provided in Supplementary Material 1.

Long-Read Dystrophin Sequencing and Splicing Prediction Analyses

Long-read sequencing of genomic *DMD* gene was performed to identify large-scale or structural variants, followed by validation through Sanger sequencing. Detailed experimental protocols can be found in Supplementary Material 1. In silico splicing prediction was carried out using Human Splicing Finder[10] and Maximum Entropy Scan[11] algorithms to evaluate the effect of detected variants on splicing.

Results

Phenotypic Features

The proband presented to our hospital at the age of 7.7 years with progressive lower limb weakness. No consanguinity or family history of neuromuscular disorders was reported for the patient. Neurological examination confirmed his proximal muscle weakness, a positive Gowers' sign, and bilateral tendon contractures. Serum creatine kinase levels were significantly elevated in each test, fluctuating between 8820 and 14132 IU/L (normal range 25–195 IU/L). Muscle MRI showed severe fatty infiltration of the pelvis and thigh muscles, including the gluteus maximus, gluteus medius, gluteus minimus, pectineus, adductor brevis, adductor magnus, vastus lateralis, vastus intermedius, vastus medialis, and the long head of biceps femoris muscles (Figure 1A and B). In addition, a specific fatty infiltration pattern known as the trefoil with single fruit sign was observed on his thigh muscle MRI.[12]

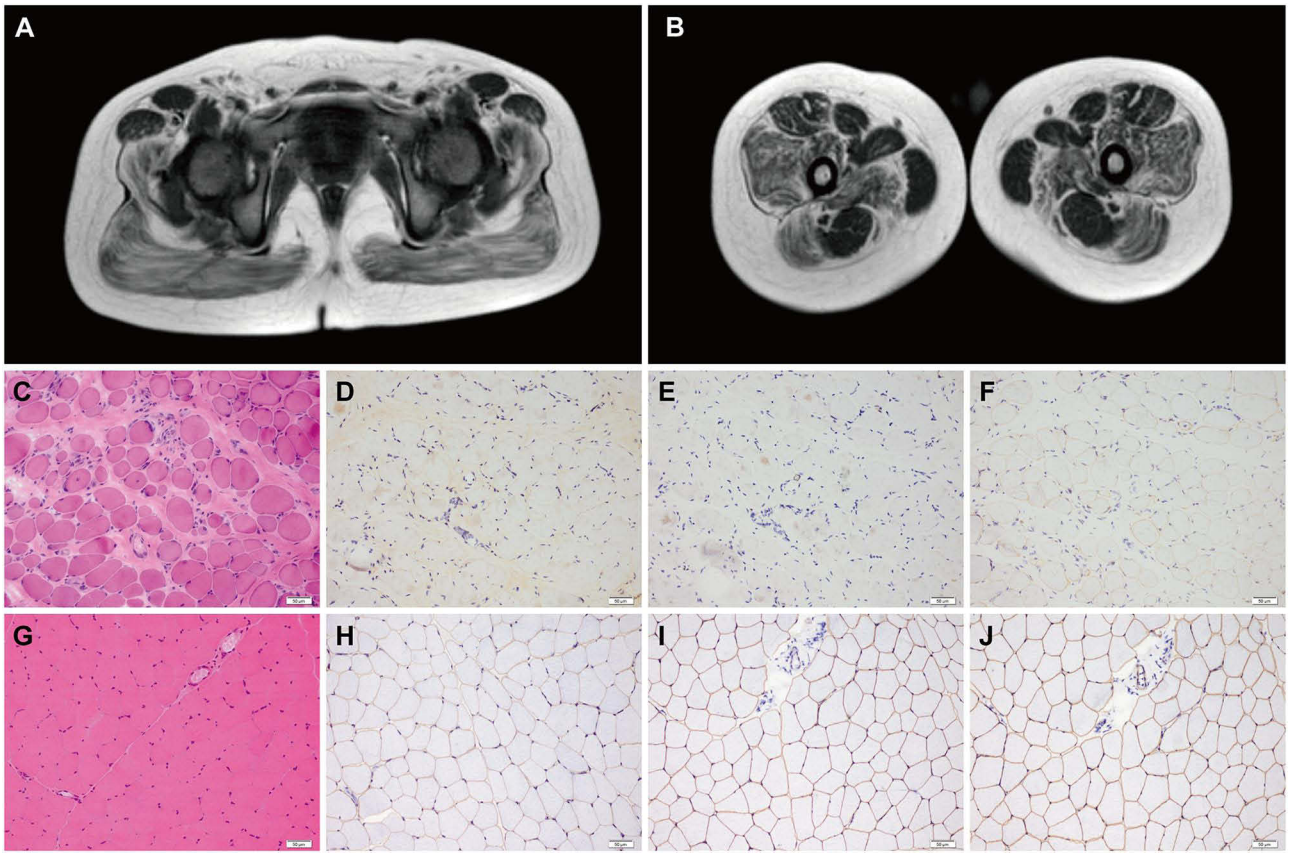

Figure 1 Muscle imaging and pathologic features of the patient. Axial T1-weighted MRI images of the patient at pelvis (**A**) and thigh (**B**) levels. (**C**) Hematoxylin-eosin staining revealing a muscular dystrophic pattern. Immunohistochemical staining showing negative expression of dystrophin-N (**D**) and dystrophin-C (**E**), and a severe reduction of dystrophin-R with a few negative expression myofibers (**F**). (**G–J**) Normal control.
Abbreviation: MRI, magnetic resonance imaging.

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Initial Genetic Testing

Whole exome sequencing revealed no small pathogenic variants in the *DMD* gene or in other genes known to cause neuromuscular disorders. An in-frame deletion of *DMD* exons 50–51 was detected by MLPA analysis and independently confirmed through CNV analysis (Supplementary Figure 1).

Muscle Biopsy-Based Dystrophin Protein and mRNA Analyses

Given the phenotype-genotype discordance that did not align with the reading-frame rule, a stepwise molecular analysis strategy was implemented to elucidate the underlying cause. Histopathological examination of the proband's biopsy revealed a dystrophic pattern, including increased variation in myofiber diameter, connective tissue deposition in the endomysium and perimysium, and signs of muscle degeneration and regeneration (Figure 1C). Dystrophin protein analysis by immunohistochemical staining demonstrated negative dystrophin-N and dystrophin-C expression, along with severe reduction of dystrophin-R (Figure 1D–J). No revertant fibers were observed, confirming the molecular diagnosis of DMD at the protein level.

Agarose gel electrophoresis analysis of the amplified 22 overlapping *dystrophin* cDNA fragments showed two aberrant splicing transcripts that were shorter than the normal band (Figure 2A). Sanger sequencing of the aberrant splicing transcripts revealed two truncated *DMD* transcripts. One truncated transcript was the out-of-frame skipping of *DMD* exons 50–52 (NM_004006.2:r.7201_7660 p.Arg2401Leufs*22; Figure 2B and C), while the other one was the skipping of *DMD* exons 50–51 and a part of *DMD* exon 52 and also a partial intron inclusion of *DMD* intron 49 (NM_004006.2:r.[7200_7201ins7201-7440_7201-7399;7201_7568del]; Figure 2D and E). The other one truncated transcript was also an out-of-frame transcript that encoded a frameshift and premature termination codon occurring 5 codons downstream of exon 49 (p.Arg2401Phefs*5). Both the two truncated and out-of-frame *DMD* transcripts harbored premature termination codon that would be targeted by nonsense-mediated mRNA decay, resulting in the absence or severe reduction of dystrophin-N, -C, and -R observed in the patient, which demonstrated his genetic diagnosis of DMD at the mRNA level.

Long-Read Dystrophin Sequencing and Splicing Prediction Analyses

To further characterize the aberrant *DMD* splicing-causing variant(s) at genomic DNA level, we performed long-read sequencing of genomic *dystrophin*. Long-read sequencing identified a novel large-scale deletion variant (~97kb) in *DMD* gene (NC_000023.10:g.31747840_31845598 NM_004006.2:c.7201–7398_7568del), which was validated by Sanger sequencing (Figure 2F and Supplementary Figure 2). This deletion variant has not been reported in the Database of Genomic Variants and published literature. Apart from this deletion, no other pathogenic structural variants were detected in *DMD* gene.

To clarify how the two truncated *DMD* transcripts were produced by the novel deletion variant, we performed splicing prediction analysis using the Human Splicing Finder[10] and Maximum Entropy Scan[11] algorithms. Splicing prediction analysis revealed that the novel deletion variant created a new acceptor splice site within *DMD* intron 49 (TTCTTTGTTAAG|TT with a Maximum Entropy Scan score of 5.89 and a Human Splicing Finder score of 83.31; Figure 2D), causing the partial intron inclusion of *DMD* intron 49 (46-bp sequence). The novel deletion variant was classified as pathogenic, as it complies with the PVS1, PM2, and PP4 criteria based on the ACMG guidelines,[13] thereby establishing the genetic diagnosis of DMD at the DNA level for the proband.

Discussion

Our integrated analysis provides a definitive molecular explanation for a case of dystrophinopathy with genotype-phenotype discordance. We show that a seemingly in-frame deletion of *DMD* exons 50–51, initially suggestive of a BMD phenotype, in fact results in a severe DMD phenotype because the underlying genomic deletion induces out-of-frame splicing. This case highlights an important diagnostic principle: the reading-frame rule must be applied cautiously when inferred solely from genomic DNA, as clinical severity is mainly dictated by transcriptional and translational outcomes.

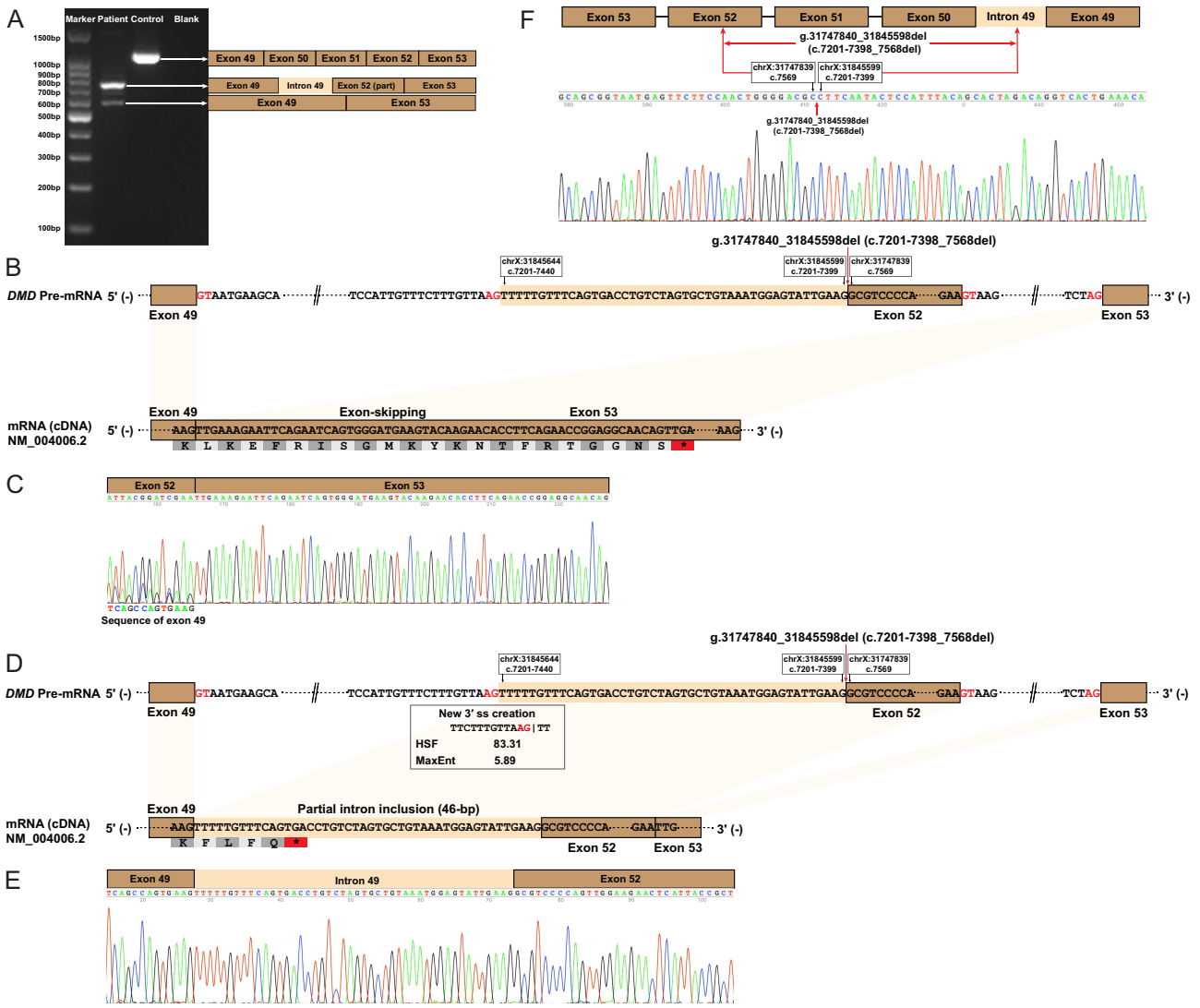

Figure 2 Full length *dystrophin* mRNA analysis and long-read *dystrophin* sequencing of the patient. (**A**) Agarose gel electrophoresis analysis showing two aberrant bands that were shorter than the normal band. Schematics depict the structure of the transcripts corresponding to each band. (**B**–**E**) Graphic representation of the two aberrant *DMD* transcripts caused by the novel deletion variant in *DMD* gene and its corresponding Sanger sequencing validation. Red bases denote splice sites, red arrows indicate the deleted genomic region. (**F**) Schematic of the novel genomic deletion variant in the *DMD* gene, with Sanger sequencing chromatogram validating the deletion junction.
Abbreviations: MaxEnt, Maximum Entropy; HSF, Human Splicing Finder; 5′ ss, donor splice site; 3′ ss, acceptor splice site; *, premature stop codon.

Our findings extend previously reported exceptions to the reading-frame rule. Earlier studies have attributed discordant phenotypes to disruption of critical functional domains (eg, exon 3–28 deletions affecting the actin-binding domain[14]) or to compensatory splicing events that restore the reading frame (eg, in-frame mRNA transcripts resulting from exon 44–45 skipping or exon 3–7 deletions with frame-restoring splicing).[15,16] By contrast, our case is distinguished by the mechanism through which a large genomic deletion produces multiple aberrant, out-of-frame transcripts. Unlike the case reported by Gorokhova et al,[17] where a large in-frame deletion (exons 10–60) was proposed to exert a dominant-negative effect by disrupting utrophin localization, our patient's phenotype is directly explained by the induction of premature termination codons via aberrant splicing—a mechanism more consistent with classic DMD pathophysiology. This contrasts with both domain disruption and frame-preserving mechanisms, adding a novel example to the spectrum of variants that produce exceptional phenotypes through predominant out-of-frame splicing.

This case delineates a clear diagnostic pathway for resolving genotype-phenotype discordances in dystrophinopathy. The process begins when routine genetic testing identifies a variant inconsistent with the clinical presentation. Muscle biopsy with dystrophin immunohistochemistry then provides crucial protein-level confirmation of pathogenicity.

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Subsequent muscle-derived mRNA analysis is essential for detecting the aberrant splicing events underlying the discrepancy. Finally, long-read *DMD* sequencing enables definitive characterization of the complex genomic variant driving the aberrant splicing. This stepwise strategy emphasizes that, despite advances in genetic technologies, muscle biopsy remains indispensable for guiding RNA-based molecular analyses.[7]

Our study has several limitations. As a single-case report, the generalizability of the specific molecular mechanism described here may be limited. Moreover, the proposed diagnostic framework requires access to specialized methodologies such as muscle biopsy and long-read sequencing, which may not be available in all clinical settings. In addition, we proceeded directly to long-read sequencing without prior short-read analysis of the *DMD* gene. While long-read technology offers superior resolution of complex structural variants, bypassing short-read sequencing may pose cost-effectiveness limitations in clinical practice.

In conclusion, by employing the stepwise strategy, we resolved a diagnostically challenging case and identified a novel variant underlying phenotypic exception to the reading-frame rule in dystrophinopathy. The strategy we applied —combining histopathological evaluation, mRNA-level analysis, and advanced genomic technologies including long-read sequencing—provides an effective approach for achieving precise molecular diagnoses.

Data Sharing Statement

The authors confirm that the data supporting the findings of this study are available within the article. Raw sequencing data is available from the corresponding authors upon request.

Ethics Approval and Informed Consent Statements

This study was approved by the Ethics Committee at Peking University First Hospital (2023 109-002) for both the investigation and publication of the case details. Informed consent for publication of the case was obtained from the patient's mother.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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