ANNALS of Clinical and Translational Neurology
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AMERICAN NEUROLOGICAL ASSOCIATION

CASE STUDY

An in-frame pseudoexon activation caused by a novel deep-intronic variant in the *dysferlin* gene

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Received: 6 October 2022; Revised: 23 November 2022; Accepted: 23 November 2022

***Annals of Clinical and Translational Neurology* 2023; 10(2): 292–296**

doi: 10.1002/acn3.51716

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Abstract

The precise detection and interpretation of pathogenic *DYSF* variants are sometimes challenging, largely due to rare deep-intronic splice-altering variants. Here, we report on the genetic diagnosis of a male patient with dysferlinopathy. He remained genetically unsolved after routine exonic detection approaches that only detected a novel heterozygous frameshift variant (c.407dup, p.Thr137Tyrfs*11) in *DYSF* exon 5. Via muscle-derived *DYSF* mRNA studies, we identified a novel deep-intronic *DYSF* variant in the other allele (c.1397 + 649C > T), which causing in-frame alterations in *DYSF* mRNA and protein structure and confirmed his genetic diagnosis of dysferlinopathy. Our study emphasizes the potential role of undetected deep-intronic splice-altering variants in monogenic diseases.

Introduction

Dysferlinopathy is caused by biallelic loss-of-function variants in the *dysferlin* (*DYSF*) gene, resulting in the reduced functioning, decreased expression, or complete absence of dysferlin protein in skeletal muscles.[1] Dysferlinopathy is one of the most common subtypes of autosomal recessive limb-girdle muscular dystrophies[2] and covers a spectrum of muscle diseases with high clinical heterogeneity, including hyper creatine-kinase emia, distal myopathy with anterior tibial onset, Miyoshi muscular dystrophy 1, and limb-girdle muscular dystrophy type 2B (LGMD2B) which is recently renamed as limb-girdle muscular dystrophy recessive type 2.[3] The broad spectrum of pathogenic *DYSF* variants consists of both coding and noncoding variants, ranging from single-nucleotide variants (SNVs) to large-scale copy number variants.[4–6] Pathogenic *DYSF* variants, occurring throughout the entire gene with no apparent hot spots, are predominantly located in exons and/or flanking intronic regions.[4,5] Therefore, most pathogenic *DYSF* variants can be detected by routine exonic detection approaches which comprise of multiplex ligation-dependent probe amplification (MLPA) and next-generation sequencing (NGS) of all exons and flanking intronic sequences of *DYSF*. However, approximately 17% of patients with dysferlinopathy remained without a definite genetic diagnosis after routine approaches, as only one mutant allele was identified in them.[5,7] The undetected pathogenic variants in the other allele probably lie within deep-intronic regions of *DYSF*,[7–10] of which the identification requires mRNA studies and *in silico* bioinformatic analyses.

In this study, a male patient with a suspected dysferlinopathy based on his clinical and pathologic characteristics was enrolled, for whom MLPA analysis of *DYSF* and a diagnostic NGS panel for neuromuscular diseases failed to yield a definite genetic diagnosis. The patient is heterozygous for a novel frameshift variant in *DYSF* exon 5 inherited from his unaffected mother. To identify the potential splice-altering variant in *DYSF* that might be missed by the NGS panel, muscle-derived *DYSF* mRNA studies were performed. A novel deep-intronic *DYSF* variant (c.1397 + 649C > T) inherited from his unaffected father was identified, causing a 66-bp pseudoexon (PE) activation and resulting in an in-frame deletion–insertion of 23 amino acids within the C2C domain of the dysferlin protein. The patient carrying the pathogenic compound heterozygous variants in *DYSF* was eventually diagnosed with dysferlinopathy.

Methods and Results

Clinical, muscle MRI, and pathologic features of the patient

This study was approved by the Ethics Committee at Peking University People's Hospital. The patient enrolled and analyzed in this study was a 40-year-old male patient. The patient is the only son of the non-consanguineous parents. He was presented to our hospital at the age of 21 years because of an incidental finding of mildly elevated transaminases (alanine aminotransferase 86.4 U/L and aspartate aminotransferase 91.7 U/L). Serum creatine kinase was markedly elevated in every test (range: 3245–5004 IU/L; normal: 25–170 IU/ L). He had proximal muscle pain in the bilateral upper extremities for 2 weeks. Currently, he has no obvious symptoms associated with muscle weakness. Physical examination also confirmed that he had no muscle weakness or calf hypertrophy. Muscle biopsy and MRI was performed at his age of 40 years. However, his muscle MRI examination showed some asymmetrical changes regarding muscle fatty infiltration and edema (Fig. 1A–D). Moderate fatty infiltration of the adductor longus, adductor magnus, and semimembranosus muscles was observed in the right thigh, while only mild fatty infiltration of the adductor longus muscle was observed in the left thigh. In addition, mild muscle edema of the adductor magnus muscle was observed in the right thigh, whereas moderate muscle edema of the vastus medialis, adductor magnus, and long head of biceps femoris muscles was observed in the left thigh. No obvious muscle fatty infiltration or muscle edema was observed in the lower leg muscles. He now has no cardiac muscle involvement in terms of clinical manifestations, electrocardiogram, and echocardiography.

His muscle biopsy showed a necrotizing myopathy pattern, including a few blue basophilic regenerating fibers with large nuclei and pale necrotic fibers (Fig. 1M). Immunohistochemical staining showed positive expression of dystrophin-N, dystrophin-C, dystrophin-R, α-sarcoglycan, β-sarcoglycan, and γ-sarcoglycan, and a partial reduction of dysferlin expression (Fig. 1N–T). Hence, the patient was suspected of having a dysferlinopathy based on his clinical and pathologic features.

Routine exonic detection approaches

Based on the suspected diagnosis of dysferlinopathy in this patient, we initiated routine exonic detection approaches, including MLPA-based duplication/deletion analysis of *DYSF* and a diagnostic NGS panel for neuromuscular diseases.[11] However, the MLPA analysis of *DYSF* did not detect any exonic copy number variants. The NGS panel only identified a novel heterozygous frameshift variant in *DYSF* exon 5, NM_003494.3:c.407dup, which was inherited from his unaffected mother. Therefore, the definite genetic diagnosis of dysferlinopathy could not be made in the patient, as the routine approaches did not detect any pathogenic *DYSF* variants in the other allele. The genomic *DYSF* variants, RNA variants, and protein variants identified in the patient were described according to the Human Genome Variation Society nomenclature.[12] The Human Splicing Finder (HSF) and maximum entropy (MaxEnt) algorithms were

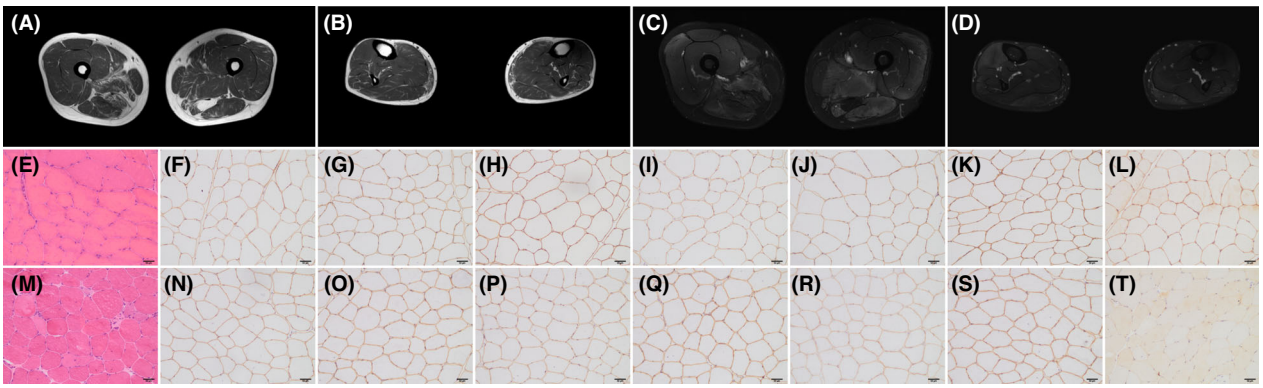

Figure 1. Muscle magnetic resonance imaging findings and pathologic features of the patient. (A and B) Axial muscle T1-weighted images of the patient showing moderate fatty infiltration of the adductor longus, adductor magnus, and semimembranosus muscles in the right thigh, mild fatty infiltration of the adductor longus muscle in the left thigh, and no obvious fatty infiltration of the lower leg muscles. (C and D) Axial muscle short T1 inversion recovery images of the patient showing mild muscle edema of the right thigh muscles, moderate muscle edema of the left thigh muscles, and no obvious muscle edema of the lower leg muscles. (M) Hematoxylin–eosin staining showing a necrotizing myopathy pattern in the patient. Immunohistochemical staining showing positive expression of dystrophin-N (N), dystrophin-C (O), dystrophin-R (P), α-sarcoglycan (Q), β-sarcoglycan (R), and γ-sarcoglycan (S), and a partial reduction of dysferlin expression (T). (E–L) A normal control showing positive expression of dystrophin-N, dystrophin-C, dystrophin-R, α-sarcoglycan, β-sarcoglycan, γ-sarcoglycan, and dysferlin. 20× magnification.

used to predict alterations in splicing signals caused by the detected genomic variants.[13]

Muscle-derived *dysferlin* mRNA studies and genomic Sanger sequencing

To identify the potential splice-altering *DYSF* variant in the other allele that might be missed by the NGS panel, muscle-derived mRNA studies of *DYSF* was performed. We isolated mRNA from the muscle biopsy sample and amplified 12 overlapping cDNA fragments of the entire *DYSF* mRNA (NM_003494.3; Table S1) using reverse transcription-polymerase chain reaction (RT-PCR) amplification. Gel electrophoresis analysis showed that the 12 overlapping cDNA fragments were successfully amplified in a normal control (Fig. 2A) and the patient (Fig. 2B). Direct Sanger sequencing of the 12 cDNA fragments of the patient revealed aberrant *DYSF* transcripts in the first and third cDNA fragments. The aberrant *DYSF* transcript detected in the first cDNA fragment was a single-nucleotide tandem duplication of cytosine base in exon 5 (r.407dup), which was consistent with the genomic variant (c.407dup). The frameshift transcript (r.407dup) was assumed to be targeted for degradation by nonsense-mediated decay, as it created a frameshift and premature termination codon (NP_003485.1:p.Thr137Tyrfs*11). However, direct Sanger sequencing of the third cDNA fragment could not distinguish the overlapping sequences (Fig. 2C). Thus, TA cloning[14] of the third cDNA fragment was performed, which revealed a 66-bp insertion of intron 15 sequence between *DYSF* exons 15 and 16 (PE15 activation; NM_003494.3:r.1397_1398ins1397 + 582_1397 + 647) and the normal splicing of *DYSF* exons 15 to 16 (Fig. 2D,E).

Sanger sequencing of the genomic *DYSF* sequence around the PE15 identified a novel deep-intronic SNV, NM_003494.3:c.1397 + 649C > T (NC_000002.11: g.71763090C > T) inherited from his unaffected father

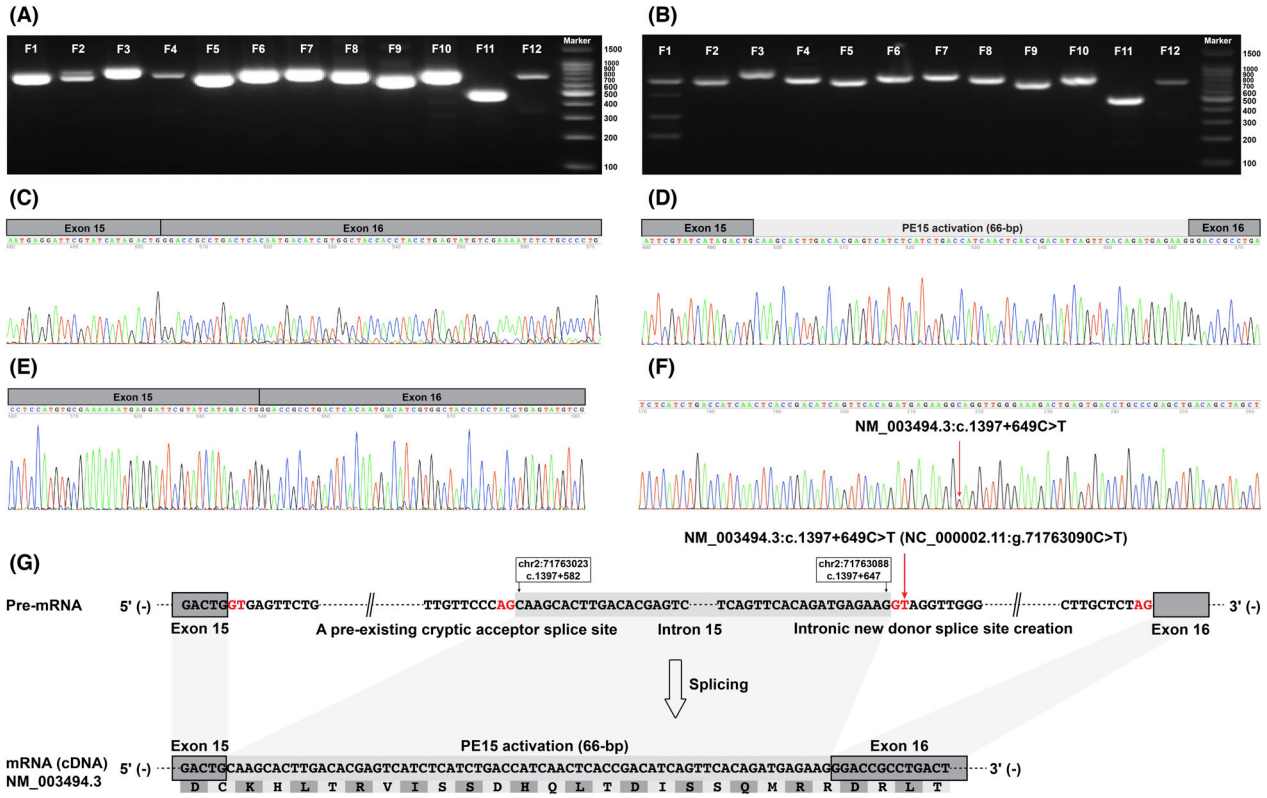

Figure 2. Muscle-derived mRNA studies and genomic *DYSF* sequencing of the patient. The entire *DYSF* mRNA (NM_003494.3) was divided into 12 overlapping cDNA fragments for RT-PCR amplification. Gel electrophoresis analysis showed that the 12 overlapping cDNA fragments were successfully amplified in a normal control (A) and the patient (B). The upper band in F2 might be an alternative transcript of the *DYSF* gene. (C) Direct Sanger sequencing of the third cDNA fragment of the patient revealed aberrant *DYSF* transcripts, but could not recognize the overlapping sequences. (D) and (E) TA cloning of the aberrant *DYSF* transcripts revealed a 66-bp insertion of intron 15 sequence between *DYSF* exons 15 and 16 (PE15 activation) and the normal splicing of *DYSF* exons 15 to 16. (F) Sanger sequencing of the genomic *DYSF* sequence around the PE15 detected a novel single-nucleotide variant, the c.1397 + 649C > T variant. (G) The schematic of the in-frame PE15 activation caused by the novel c.1397 + 649C > T variant in *DYSF*. RT-PCR: reverse transcription-polymerase chain reaction; F: fragment; bp: base pair; PE: pseudoexon.

(Fig. 2F). The c.1397 + 649C > T variant created a new donor splice site (HSF score 92.66; MaxEnt score 10.29) in intron 15 that was paired with a preexisting cryptic acceptor splice site (HSF score 85.28; MaxEnt score 4.31), causing the activation of PE15 (Fig. 2G). The PE15-containing transcript resulted in an in-frame deletion–insertion of 23 amino acids within the C2C domain of the dysferlin protein, which was compatible with the partial reduction of dysferlin expression observed in the patient and described as follows: NP_003485.1:Trp466delinsCysLysHisLeuThrArgValIleSerSerAspHisGlnLeuThrAspIleSerSerGlnMetArgArg. Both the c.407dup and c.1397 + 649C > T variants in *DYSF* were classified as pathogenic variants according to the American College of Medical Genetics guidelines,[15] as they met the criteria for very strong evidence (PVS1), moderate evidence (PM2 and PM3), and supporting evidence (PP1). Therefore, the patient carrying the pathogenic compound heterozygous variants in *DYSF* was finally diagnosed with dysferlinopathy.

Discussion

In this study, a male patient was suspected of having a dysferlinopathy based on his clinical and pathologic features, droving us to perform further muscle-derived *DYSF* mRNA analysis after routine exonic detection approaches that only detected a novel heterozygous frameshift variant in *DYSF*. We identified a novel deep-intronic *DYSF* variant in the other allele which caused in-frame alterations in *DYSF* mRNA and protein structure. This case is an example of how clinical and pathologic data can guide the genetic testing in monogenic diseases. The asymmetric muscle involvement observed on the patient's MRI images are similar to the previous studies.[16,17]

The *DYSF* gene, spanning over 230 kb and consisting of 55 canonical exons and numerous enormous introns, is a large gene annotated in the human genome.[1] More than 400 different pathogenic *DYSF* variants are reported in the Universal Mutation Database for *DYSF* gene (http://www.umd.be/DYSF/),[5] including SNV, small deletions/insertions, and large-scale deletions/duplications. Most pathogenic *DYSF* variants reside within canonical exons and/or flanking intronic regions and can be detected by routine exonic detection approaches. The precise detection and interpretation of pathogenic *DYSF* variants is sometimes challenging, as atypical pathogenic *DYSF* variants including noncanonical splicing site variants[4] and deep-intronic splice-altering variants[7–10] do exist in the *DYSF* gene. The identification and interpretation of atypical pathogenic *DYSF* variants require *DYSF* mRNA analysis and *in silico* bioinformatic analyses in addition to the genomic sequencing. Therefore, after indefinite findings in routine approaches in a patient with a suspected dysferlinopathy, we performed *DYSF* mRNA analysis and relevant bioinformatic analyses. TA cloning was performed as the direct RT-PCR analysis of *DYSF* mRNA failed to identify the overlapping transcripts. We successfully identified a novel deep-intronic *DYSF* variant via the combination of *DYSF* mRNA analysis and genomic Sanger sequencing, which established the genetic diagnosis of dysferlinopathy. The frameshift transcript (r.407dup) created a frameshift and premature termination codon in the patient, which was assumed to be targeted by nonsense-mediated decay. The novel deep-intronic *DYSF* variant caused an in-frame pseudoexon activation and thus was not targeted by nonsense-mediated decay and resulted in the partial expression of dysferlin protein. The C2C domain of the dysferlin protein, which has distinct functions in Ca^{2+} signaling and muscle membrane repair,[18] was altered due to the in-frame pseudoexon-containing transcript.

To the best of our knowledge, only three deep-intronic splice-altering variants have been previously reported in *DYSF*, including c.4886 + 1249G > T in intron 44,[8,9] c.5341-415A > G in intron 48,[10] and c.5668-824C > T in intron 50.[7] The c.1397 + 649C > T in intron 15 identified in our patient is the fourth deep-intronic *DYSF* variant. Different to the c.5668-824C > T[7] and c.5341-415A > G[10] variants that cause premature translation termination, the c.1397 + 649C > T variant leading to in-frame alterations in *DYSF* mRNA and protein structure is similar to the c.4886 + 1249G > T variant which caused in-frame alterations as well.[8,9] There is currently no cure for dysferlinopathy. Hence, prenatal diagnosis that requires accurate detection of pathogenic *DYSF* variants is of great significance for our patient, which can facilitate precise genetic counseling of his family members. Muscle-derived *DYSF* mRNA analysis is significant for dysferlinopathy, as it can detect atypical pathogenic *DYSF* variants missed by the routine approaches. Precise detection and interpretation of deep-intronic *DYSF* variants has implications for potential genetic therapies targeting wild-type expression of dysferlin protein.[7,8]

In conclusion, we identified two novel compound heterozygous variants in *DYSF* in a male patient via various genetic testing techniques, which confirmed his genetic diagnosis of dysferlinopathy. The novel deep-intronic *DYSF* variant identified in this study emphasizes the potential role of undetected deep-intronic splice-altering variants in monogenic diseases.

Acknowledgment

The authors thank the patient and his families for participating in this study.

Author Contributions

Chengyue Sun, Zhiying Xie, Yan Xu, and Zunjing Liu contributed to the study conception and design. Material preparation and data collection were performed by Chengyue Sun, Zhiying Xie, Lu Cong, Yan Xu and Zunjing Liu. Clinical and imaging data analysis was performed by Chengyue Sun, Zhiying Xie, and Lu Cong. Genetic data analysis was performed by Zhiying Xie and Chengyue Sun. The first draft of the manuscript was written by Chengyue Sun and Zhiying Xie. All authors read and approved the final manuscript.

Funding Information

This work was supported by the Research and Development Foundation of Peking University People's Hospital (grant number RDJP2022-34), Peking University People's Hospital Talent Introduction Scientific Research Launch Fund (2022-T-02), and National Natural Science Foundation of China (grant number 82201463).

Conflict of Interest

None.

References

1. Liu J, Aoki M, Illa I, et al. Dysferlin, a novel skeletal muscle gene, is mutated in Miyoshi myopathy and limb girdle muscular dystrophy. Nat Genet. 1998 Sep;20(1):31-36.
2. Liu W, Pajusalu S, Lake NJ, et al. Estimating prevalence for limb-girdle muscular dystrophy based on public sequencing databases. Genet Med. 2019 Nov;21(11):2512-2520.
3. Fernández-Eulate G, Querin G, Moore U, et al. Deep phenotyping of an international series of patients with late-onset dysferlinopathy. Eur J Neurol. 2021 Jun;28(6):2092-2102.
4. Zhong H, Yu M, Lin P, et al. Molecular landscape of DYSF mutations in dysferlinopathy: from a Chinese multicenter analysis to a worldwide perspective. Hum Mutat. 2021 Dec;42(12):1615-1623.
5. Blandin G, Beroud C, Labelle V, et al. UMD-DYSF, a novel locus specific database for the compilation and interactive analysis of mutations in the dysferlin gene. Hum Mutat. 2012 Mar;33(3):E2317-E2331.
6. Alharbi N, Matar R, Cupler E, et al. Clinical, neurophysiological, radiological, pathological, and genetic features of Dysferlinopathy in Saudi Arabia. Front Neurosci. 2022;16:815556.
7. Dominov JA, Uyan Ö, McKenna-Yasek D, et al. Correction of pseudoexon splicing caused by a novel intronic dysferlin mutation. Ann Clin Transl Neurol. 2019 Apr;6(4):642-654.
8. Dominov JA, Uyan O, Sapp PC, et al. A novel dysferlin mutant pseudoexon bypassed with antisense oligonucleotides. Ann Clin Transl Neurol. 2014 Sep;1(9):703-720.
9. Nallamilli BRR, Chakravorty S, Kesari A, et al. Genetic landscape and novel disease mechanisms from a large LGMD cohort of 4656 patients. Ann Clin Transl Neurol. 2018 Dec;5(12):1574-1587.
10. Zhu F, Zhang F, Hu L, Liu H, Li Y. Integrated genome and transcriptome sequencing to solve a neuromuscular puzzle: Miyoshi muscular dystrophy and early onset primary dystonia in siblings of the same family. Front Genet. 2021;12:672906.
11. Xie Z, Hou Y, Yu M, et al. Clinical and genetic spectrum of sarcoglycanopathies in a large cohort of Chinese patients. Orphanet J Rare Dis. 2019;14(1):43.
12. den Dunnen JT, Dalgleish R, Maglott DR, et al. HGVS recommendations for the description of sequence variants: 2016 update. Hum Mutat. 2016;37(6):564-569.
13. Desmet FO, Hamroun D, Lalande M, Collod-Béroud G, Claustres M, Béroud C. Human splicing finder: an online bioinformatics tool to predict splicing signals. Nucleic Acids Res. 2009;37(9):e67.
14. Li F, Liu Y, Liu H, Yang J, Zhang F, Feng H. Phenotype and genotype analyses in seven families with dentinogenesis imperfecta or dentin dysplasia. Oral Dis. 2017;23(3):360-366.
15. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17(5):405-424.
16. Diaz-Manera J, Fernandez-Torron R, LLauger J, et al. Muscle MRI in patients with dysferlinopathy: pattern recognition and implications for clinical trials. J Neurol Neurosurg Psychiatry. 2018;89(10):1071-1081.
17. Reyngoudt H, Smith FE, Caldas de Almeida Araújo E, et al. Three-year quantitative magnetic resonance imaging and phosphorus magnetic resonance spectroscopy study in lower limb muscle in dysferlinopathy. J Cachexia Sarcopenia Muscle. 2022;13(3):1850-1863.
18. Muriel J, Lukyanenko V, Kwiatkowski T, et al. The C2 domains of dysferlin: roles in membrane localization, Ca(2+) signalling and sarcolemmal repair. J Physiol. 2022;600(8):1953-1968.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1

