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RNA Mis-Splicing Effects of Noncanonical Splicing Variants in Limb-Girdle Muscular Dystrophy Type R1/2A

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Abstract

Background and Objectives

Biallelic pathogenic variants in the *CAPN3* gene cause limb-girdle muscular dystrophy type R1/2A (LGMDR1/2A). Our study investigated RNA mis-splicing effects of 5 noncanonical intronic variants in patients with LGMDR1/2A.

Methods

Total RNA was obtained from the skeletal muscle samples of patients with LGMDR1/2A. Reverse-transcription PCR, DNA electrophoresis, agarose gel extraction, pMD18-T vector cloning, and sequencing were conducted.

Results

Transcriptional analysis revealed that three of these 5 variants (c.1193 + 30G > A, c.1194-9A > G, and c.1354 + 5G > A) induced CAPN3 pre-mRNA mis-splicing through recognition of cryptic donor or acceptor splice sites. In addition, the c.2185-14T > G variant in the polypyrimidine tract of intron 20 caused the pseudoexonization of the entire intron 20 while the c.946-29T > C variant in the branch point sequence (BPS) of intron 6 led to the retention of the last 390 bp of intron 6 through disruption of original BPS and recognition of cryptic BPS and acceptor splice site. All of these noncanonical splicing variants triggering pre-mRNA mis-splicing were predicted to introduce premature termination codons. Western blotting showed deficiency of full-length (94-kDa) and 60-kDa autolytic fragments of the calpain 3 protein in skeletal muscle samples from 4 probands.

Discussion

Our study broadens the spectrum of aberrant mRNA splicing caused by intronic variants in calpainopathy.

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Supplementary Material

Introduction

The *CAPN3* gene, located at 15q15.1-q21.1 and composing of 24 exons, encodes the calpain 3 protein, a classical calpain primarily expressed in skeletal muscle.[1,2] Calpain 3, formerly known as p94, is a calcium-dependent protease that consists of 821 amino acids and has a molecular mass of 94 kDa.[2] In muscle fibers, calpain 3 is involved in calcium release and uptake, muscle formation, sarcomere assembly, and maintenance of sarcomere structure.[3,4]

Limb-girdle muscular dystrophy (LGMD) is a group of muscular dystrophies characterized by proximal muscle weakness and significant clinical and genetic heterogeneity.[5] Biallelic pathogenic variants in the *CAPN3* gene can lead to LGMD type R1/2A (LGMDR1/2A), the most

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common subtype of autosomal recessive LGMD in many populations.[5] According to the Human Gene Mutation Database, hundreds of pathogenic variants have been identified in patients with LGMDR1/2A, with some studies revealing aberrant splicing of CAPN3 mRNA due to intronic variants in the *CAPN3* gene.[6-11] In this study, we identified 5 families with LGMDR1/2A carrying distinct noncanonical splicing variants. RNA analysis showed that all the 5 variants caused mis-splicing of pre-mRNA. Our study broadens the aberrant splicing spectrum of calpainopathies. In addition, enhancing the understanding of noncanonical splicing variants in genetic diseases can improve the interpretation of genetic test results and increase the positive rate of genetic testing.

Methods

Clinical, Pathologic, and Genetic Characteristics

Five Chinese families affected by LGMDR1/2A were enrolled in our study. All 5 probands underwent muscle biopsies after informed consent was obtained. Routine pathologic staining and immunohistologic staining were conducted. Venous blood samples were collected from the 5 probands and their family members for next-generation sequencing and pedigree verification. Clinical and pathologic characteristics of the 5 probands are summarized in eTable 1. The genetic findings (according to the transcript NM_000070.3) are as follows: proband 1: compound heterozygous variants c.2185-14T > G and c.2305C > T (p.R769W); proband 2: compound heterozygous variants c.1193 + 30G > A and c.2069_2070delAC (p.H690Rfs*9); proband 3: homozygous variant c.1194-9A > G; proband 4: homozygous variant c.1354 + 5G > A; proband 5: compound heterozygous variants c.946-29T > C and c.2289T > G (p.Y763*) (eFigure 1). The c.2185-14T > G variant was located in intron 20. The c.1193 + 30G > A and c.1194-9A > G variants were both located in intron 9. The c.1354 + 5G > A variant was located in intron 10, and the c.946-29T > C variant was located in intron 6.

RNA Analysis

Total RNA was extracted from the skeletal muscle samples of the 5 probands, followed by reverse transcription PCR (RT-PCR). For proband 1, the forward primer was 5′-TCAGGAAAGTGAGGAACAG-3′ and the reverse primer was 5′-TCCAGCCTAACGAAGCAGC-3′. The PCR product length was 435 bp, spanning from exon 17 to exon 22. For proband 2, the forward primer was 5′-ATAGATGGAAGGACTGGAG-3′ and the reverse primer was 5′-AACTTTGTGAAATGGTAGA-3′. The PCR product length was 121 bp, spanning from exon 8 to exon 10. For proband 3, a pair of primers were designed that were the same as those used for proband 2. For proband 4, the forward primer was 5′-AAGATGAGAAGGCCCGTCTG-3′ and the reverse primer was 5′-CGGAGGATGAATTCCCCCTC-3′. The PCR product length was 574 bp, spanning from exon 9 to exon 13. For proband 5, the forward primer was 5′-TCCTACGAAGCTCTGAAAGGT-3′ and the reverse primer was 5′-CACTTTCTCACCTTTGAACGGG-3′. The PCR product length was 411 bp, spanning from exon 5 to exon 8. Two normal controls were used in RNA analysis. For proband 1, proband 3, and proband 4, the normal control was a 45-year-old man, and for proband 2 and proband 5, the normal control was a 24-year-old woman. RNA used was also isolated from the skeletal muscle sample of the two normal controls. DNA agarose gel electrophoresis, gel extraction, and sequencing were applied to determine the sequence of abnormal RT-PCR bands. However, if DNA electrophoresis failed to effectively separate PCR bands of normal and abnormal sizes, the PCR bands would be extracted from the gel, cloned into the pMD18-T vector (Takara), transformed into the *E. coli* DH5α, and sequenced to detect abnormal spliced RT-PCR bands.

Western Blotting

Total protein was extracted from the skeletal muscle sample of the 3 probands using radio immunoprecipitation assay lysis buffer. Full-length and autolytic fragments of the calpain 3 protein were analyzed by Western blotting using the NCL-CALP-12A2 antibody (Novocastra) diluted 1:100.

Ethical Approval and Patient Consents

The study was approved by the Ethics Committee of Qilu Hospital of Shandong University. All family members gave their written informed consent.

Data Availability

The data that support the findings of this study are available from the corresponding author on reasonable request.

Results

RNA Analysis

The c.2185-14T > G variant of proband 1 located in the polypyrimidine tract caused retention of the entire intron 20, forming a pseudoexon (Figure 1, A and B, eFigures, 2A and 3A). The c.1193 + 30G > A variant of proband 2 led to retention of the first 31 bp of intron 9 (Figure 1, C and D, eFigures, 2B and 3B). The pMD18-T cloning and sequencing showed that the c.1194-9A > G variant of proband 3 led to retention of the last 8 bp of intron 9 (7 of 10 sequenced *E. coli* colonies) (Figure 2A, eFigures, 3C and 4A). However, normally spliced transcripts were also detected (3 of 10 sequenced *E. coli* colonies), indicating that the homozygous c.1194-9A > G variant induced a subset of transcripts that were aberrantly spliced (eFigure 2C). The homozygous c.1354 + 5G > A variant of proband 4 led to skipping of the last 40 bp of exon 10 (Figure 2B, eFigures, 3D and 4B). In addition, the presence of normal spliced transcripts indicated that the splicing efficiency of the splicing c.1354 + 5G > A variant was not complete either (eFigure 2D). The c.946-29T > C variant of proband 5 led to retention of the last 390 bp of intron 6 (Figure 2C and D, eFigures, 2E and 3E). The agarose gel electrophoresis results for variants in proband 3 and proband 4 are presented in eFigure 4. Owing to the small size

differences between normal and aberrant transcripts (8 bp for proband 3 and 40 bp for proband 4), distinguishing between them on DNA agarose gel was difficult. Collectively, we confirmed the deleterious transcriptional effects of these 5 noncanonical splicing variants.

In addition, we predicted the translational effects of abnormally spliced mRNA using the ExPASy-Translate tool.[12] For the c.2185-14T > G variant, retention of the entire intron 20 of 91-bp length led to a p.K729Vfs*21 change in the amino acid sequence. For the c.1193 + 30G > A variant, retention of the first 31 bp of intron 9 led to p.R399*. For the c.1194-9A > G variant, retention of the last 8 bp of intron 9 induced p.W398Cfs*39. For the c.1354 + 5G > A variant, skipping of the last 40 bp of exon 10 led to p.V439Ifs*11. For the c.946-29T > C variant, retention of the last 390 bp of intron 6 induced p.T316Efs*2. In conclusion, all of these noncanonical splicing variants triggered RNA mis-splicing and generated premature termination codons. Some of the aberrant transcripts may be degraded by nonsense-mediated mRNA decay while others were predicted to be translated into truncated calpain 3 proteins.

Western Blotting

The full Western blots revealed a deficiency of 60-kDa autolytic fragments of calpain 3 protein in skeletal muscle samples from 5 probands compared with the normal control (Figure 3). An additional band of approximately 33 kDa was detected, likely resulting from nonspecific antibody binding. For proband 3, both calpain 3 and GAPDH protein signals were absent, which can be attributed to complete replacement by adipose tissue in the biopsied muscle sample (Figure 3).

Discussion

In this study, we identified 5 distinct noncanonical splicing variants causing mis-splicing of pre-mRNA in the *CAPN3* gene. The c.2185-14T > G variant caused the entire intron 20 to form a pseudoexon. The single intronic pyrimidine to purine substitution is located in the polypyrimidine tract between the branch point sequence (BPS) and the 3′ acceptor splice site of intron 20.[13] The introduction of purines into the polypyrimidine tract, shortening its length, may be detrimental to spliceosome assembly and reduce splicing efficiency.[14] Similarly, the c.937-11C > G variant in the polypyrimidine tract of intron 5 of the *LMNA* gene induced insertion of 40 bp of intron 5 in a familial dilated cardiomyopathy family.[15] However, pseudoexonization of the entire intron caused by variants in the polypyrimidine tract appears to be rarely documented in genetic diseases.

The c.1193 + 30G > A variant may induce recognition of the cryptic donor splice site at the c.1194 + 32 G and c.1194 + 33A sites of intron 9, leading to the retention of the first 31 bp. Three Tunisian patients were reported to be carrying the homozygous c.1194-9A > G variant, which triggered retention of the last 8 bp of intron 9. This finding is consistent with our results, as the variant created a novel upstream acceptor splice site.[7] In addition, we found that both wild-type and aberrantly spliced transcripts were present in proband 3 carrying the c.1194-9A > G variant, indicating that the variant induced a subset of transcripts that were aberrantly spliced.

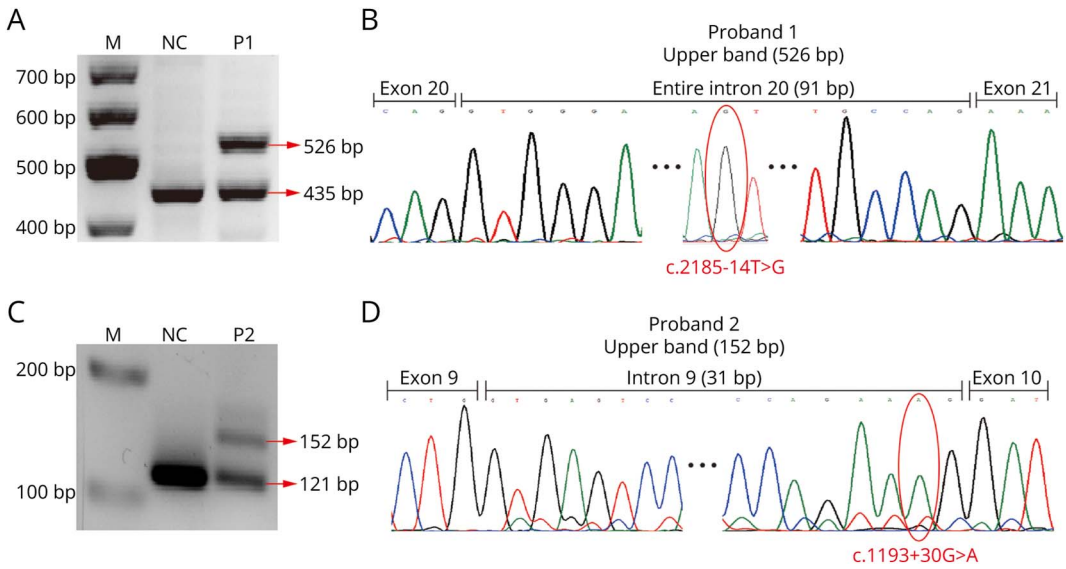
A previous study[16] revealed that the intronic +5 G site was highly conserved among vertebrates and the substitution may be detrimental to the pairing of the 5′ donor splice site with U1 snRNP in the first steps of pre-mRNA splicing. Therefore, we speculated that the c.1354 + 5G > A of proband 4 disrupted the canonical donor splice site, and a cryptic donor splice site 40 bp upstream was activated. For proband 5, the c.946-29T site was located in the BPS of intron 6. In pre-mRNA splicing, the BPS attacks the first intronic 5′ guanine, resulting in the formation of a 2′, 5′-phosphodiester bond and a lariat intermediate.[17] Another previous study[18] showed that 90% of the branch point sequences were located within 19–37 (median 25) nucleotides upstream of the 3′ acceptor splice site in human genome. Thus, the c.946-29T > C variant disrupted the original BPS, and the cryptic BPS was activated 393 bp upstream in intron 6. Consequently, the cryptic acceptor splice site was also activated 390 bp upstream of the original acceptor splice site of intron 6, leading to the retention of the last 390 bp of intron 6.

Splicing variants typically do not achieve 100% efficiency in inducing aberrant splicing. For example, proband 3 and proband 4 carried homozygous noncanonical splicing variants, and both normal and aberrant transcripts were indeed detected. The complete absence of calpain 3 protein on Western blots for 4 probands, despite the presence of some normal transcripts, may be explained by interference of truncated protein with wild-type protein expression and/or function and normal calpain 3 protein levels being too low for Western blot detection. Furthermore, the Western blot for proband 3 showed a nearly complete deficiency of both calpain 3 and GAPDH (data not shown), likely due to complete replacement by adipose tissue in the biopsied muscle.

Noticeably, the deep intronic c.1782+1072G>C variant in intron 14 of the *CAPN3* gene was identified in LGMDR1/2A patients, and RNA analysis revealed that the variant led to pseudoexonization of 100 bp of intron 9 (r.1782_1783ins1783-1070_1783-971).[9] Next-generation sequencing and Sanger sequencing are powerful tools for identifying deletions, duplications, and point variants in exonic regions and exon-intron boundaries but may not identify deep intronic variants. Therefore, in addition to RT-PCR analysis of muscle mRNA, other new technologies, such as array comparative genomic hybridization[19] and RNAseq,[20] have been used to detect deep intronic variants and aberrant RNA splicing.

In treatment strategies, designing antisense oligonucleotides (AONs) targeting the donor splice site, acceptor splice site, and serine/arginine-rich splicing factor 1 induced pseudoexon

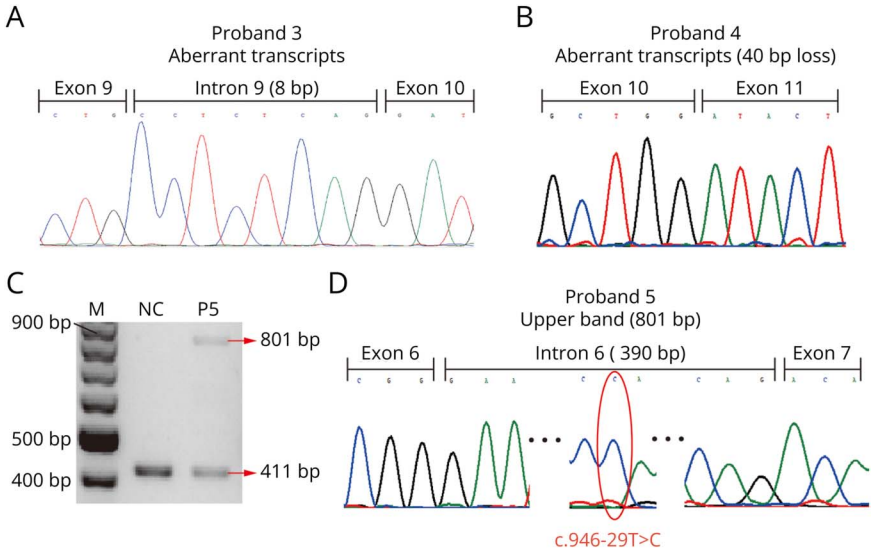
Figure 1 Transcriptional Analysis of the Noncanonical Splicing Variants c.2185-14T > G and c.1193 + 30G > A



(A) DNA electrophoresis presented a 435-bp and a 526-bp band in proband 1 carrying the c.2185-14T > G variant. (B) Sequencing of the upper band (526 bp) revealed retention of the entire intron 20 (91 bp) induced by the c.2185-14T > G variant. (C) DNA electrophoresis presented a 121-bp and a 152-bp band in proband 2 carrying the c.1193 + 30G > A variant. (D) Sequencing of the upper band (152 bp) revealed retention of the first 31 bp of intron 9 caused by the c.1193 + 30G > A variant. M = marker; NC = normal control; P1 = proband 1; P2 = proband 2.

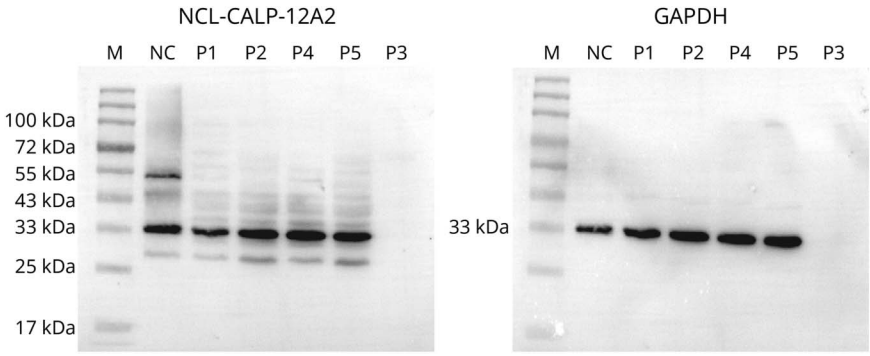
skipping and restored normal splicing.[21] The c.946-29delT variant, which resulted in the retention of 389 bp of intron 6, was prevented by phosphorodiamidate morpholino oligomers blocking the new acceptor splice site.[10] The 2 studies highlight the therapeutic potential of AONs as splice modulators for splicing variant–specific patients with LGMDR1/2A. AONs can correct RNA mis-splicing by blocking cryptic splice sites or directly masking splicing variants. The variants c.1193 + 30G > A, c.1194-9A > G, and c.946-29T > C may be amenable to AON treatment. However, therapeutic challenges exist for the 2 variants: for c.1354 + 5G > A, AONs blocking the cryptic donor splice site in exon 10 would likely trigger skipping of the entire exon 10, and for c.2185-14T > G (located in the polypyrimidine tract of intron 20), AONs directly masking this variant would likely fail to correct the intron 20 pseudoexonization.

Figure 2 Transcriptional Analysis of the Noncanonical Splicing Variants c.1194-9A > G, c.1354 + 5G > A, and c.946-29T > C



(A) The pMD18-T cloning and sequencing revealed aberrant spliced transcripts with retention of the last 8 bp of intron 9 induced by the c.1194-9A > G variant. (B) The pMD18-T cloning and sequencing showed aberrant spliced transcripts without the last 40 bp of exon 10 induced by the c.1354 + 5G > A variant. (C) DNA electrophoresis presented a 411-bp band and an 801-bp band in proband 5. (D) Sequencing of the upper band (801 bp) revealed aberrant spliced transcripts with insertion of the last 390 bp of intron 6 induced by the c.946-29T > C variant. M = marker; NC = normal control; P5 = proband 5.

Figure 3 Full Western Blots Revealed a Deficiency of 60-kDa Autolytic Fragments of Calpain 3 Protein in Skeletal Muscle Samples From 5 Probands Compared With the Normal Control



An additional band of approximately 33 kDa was detected. For proband 3, both calpain 3 and GAPDH protein signals were absent.

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Author Contributions

G. Wang: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data. H. Liu: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data. G. Yang: drafting/revision of the manuscript for content, including medical writing for content; analysis or interpretation of data. S. Gu: drafting/revision of the manuscript for content, including medical writing for content. C. Yan: drafting/revision of the manuscript for content, including medical writing for content. P. Lin: drafting/revision of the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data.

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Disclosure

The authors report no relevant disclosures. Full disclosure form information provided by the authors is available with the full text of this article at Neurology.org/NG.

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