Received: 8 July 2019 | Revised: 12 November 2019 | Accepted: 17 November 2019

DOI: 10.1002/jcla.23142

CASE REPORT

WILEY

Identification of two novel insertion abnormal transcripts in two Chinese families affected with Dystrophinopathy

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Funding information
This study was supported by National Key R&D Program (Grant No. 2016YFC1000703) and the first Affiliated Hospital of AFMU (Grant No. XJZT18MJ53).

Abstract

Background: Duchenne muscular dystrophy (DMD) is an X-linked recessive inheritance muscle dystrophy disease, associated with pathogenic variants in the *DMD* gene. MLPA, DHPLC and *DMD* sequence studies fail to found the causative alteration in two cases. This study intends to evaluate the disease-causing mutations and explains the correlation genotype-phenotype.

Methods: The mRNA analysis and Long-range PCR with sequencing were used for molecular diagnosis.

Results: In case one, an insertion of 78 nucleotides between exons 40 and 41 (r.5739_5740insMN602429:r415_492) was identified in case one. The insertion sequences were highly homologous to the intron 40 (NG_012232.1:g.1001760_g.1001837). Long-range PCR with sequencing analysis showed that a novel deep intronic *DMD* mutation (NG_012232.1:g.1001838A>G) was identified, generating a premature stop codon and terminating protein translation. The likely pathogenic mutation was detected in fetal sample. In case two, an insertion of 74 nucleotides which located inside the consensus sequence AG/GT was detected between exons 2 and 3 (r.93_94insMN584887:r61_134), which resulted in a premature stop codon. The insertion sequences were traceable in the intron 2 of *DMD* gene (NG_012232.1:g.415926_g.415999). We did not perform prenatal *DMD* gene diagnosis for case two due to lack of sufficient genetic information.

Conclusion: These findings clarify importance of proceeding to the mRNA analysis when no causative mutations were found neither by MLPA/DHPLC nor gene sequencing so as to reach the molecular confirmation of DMD and carry out an accurate genetic assessment/ carrier status testing.

KEYWORDS

cDNA analysis, duchenne muscular dystrophy, fetal muscle biopsy, prenatal diagnosis, splicing abnormalities

Xu and Song are contributed equally to this work.

1 | INTRODUCTION

Duchenne muscular dystrophy (DMD) is a serious type of primary skeletal muscle necrotizing disease with skeletal muscle atrophy and weakness.[1] Almost all patients are boys and girls are extremely rare due to one single copy of the defective gene. Most patients die of respiratory failure, lung infection and heart failure around the age of 20.[2] The disease is an X-linked recessive monogenic disease, which caused by mutation of the dystrophin gene (*DMD* gene) located on chromosome Xp21.1.[3] About 60% of the mutations reported are deletions, about 10% are duplications, and the remaining 30% are small mutations, including point mutations, small deletions, or small insertions.[4] The molecular characterization of deleterious mutations has been considered an endpoint for genetic diagnosis of genetic diseases.[5] We previously identified 237 Chinese families with DMD history using DHPLC to detect deletions and duplications of patients and Sanger sequencing of the coding region and splice sites to detect point mutations.[6] However, approximately 1%-2% of the dystrophinopathy patients are principally caused by deep intronic alterations or other regulatory alterations. These mutations must be altering the normal processing of the mRNA. Therefore, cDNA sequencing is recommended according to the best guidance for Duchenne genetic testing.[7] In our clinical practice, we were unable to identify disease-causing mutations in two DMD patients by previous MLPA, DHPLC and DMD sequence studies. Therefore, we genetically analyzed the cDNA for muscle RNA in two Chinese families with DMD history and detected two novel pathogenic mutations, which can explain the correlation genotype-phenotype.

2 | MATERIALS AND METHODS

2.1 | Study subjects and fetal sample collection

Two families with DMD history were involved in prenatal diagnostic center of Xi Jing Hospital. Informed consent was obtained from all the participants in the study. All procedures were approved by the Chinese People Liberation Army General Hospital medical ethics committee (Approval no. S2016-120-02).

Clinical information and peripheral blood samples from the proband and their relatives were collected into EDTA anticoagulant tubes. Amniotic fluid sample was collected by following the standard procedures as described by our previous report.[6] And fetal muscle biopsy was carried out using a double needle 18 gauge for the guide, 20 gauges for the second needle (Modena, PNM0418-1) under transabdominal ultrasonographic guidance. The needle must be heparinized to avoid clots and located directly over the fetal thigh muscle. The specimens were then frozen in liquid nitrogen for transport to the laboratory. After washing twice by normal saline, the specimen was cut into pieces by surgical scissors to further genetic analysis.

2.2 | Pathogenic mutations detection

Genomic DNA was extracted from blood and amniotic fluid as described before.[6] The previous MLPA/DHPLC and *DMD* sequence studies but no mutations were identified. Total RNA was extracted from patients, related relatives, and control amniotic fluid or muscle samples using the TianGen RNA simple total RNA extraction kit (TianGen, DP419) according to the manufacturer's instructions. Total RNA was then converted to cDNA using PrimeScript™ RT Master Mix (Takara Dalian, RR036A). *DMD* transcripts were amplified by PCR covering the entire coding region, and Sanger sequencing using primer sets was performed by Sangon Company (Table S1). These sequences were analyzed by preliminary comparison with sequences in the GenBank database and software Vector NTI. The variant description was assessed using the *DMD* reference sequence (NG_012232.1). The fully informative markers (STRMP, STR44, and STR49, in combination with AMEL) were applied to rule out maternal contamination.

3 | RESULTS

3.1 | Case 1

A 36-year-old gravid woman (II-4), Gravida 2, para1, was referred for genetic counseling at 24 menstrual weeks for the prenatal diagnosis of DMD. She had an asymptomatic daughter (III-2). Her brother (II-1) had died age 20 from DMD, and genetic material was unavailable. Her sister's son (III-1) was diagnosed with DMD at five years of age according to clinical features, elevated serum creatine kinase (CK) (11 765 U/L normal:37-174U/L). The family pedigree is shown in Figure 1A. Initially, previous MLPA, DHPLC, and Sanger sequencing studies failed to identify pathogenic variants in patient (III-1). The *DMD* cDNA analysis of patient's muscle (III-1) was carried out. An insertion of 78 nucleotides was identified between exons 40 and 41 ((r.5739_5740insMN602429:r415_492), Figure 1B). The mutated sequences were analyzed by preliminary comparison with sequences in the GenBank database, and the results indicated that the insertion of 78 base pairs had highly homology with the sequence of the intron 40 (NG_012232.1:g.1001760_g.1001837). We designed primers in the upper and lower 200 bp ranges of the inserted sequences and reanalyzed patient's genomic DNA to characterize the variant at the nucleotide level. A deep intronic mutation (NG_012232.1:g.1001838A>G) was found, predictably generating a new splice site (GT/AG rule). We deduced the change affected RNA coding process, yielding an extra “exon” inserted at exons 40 and 41 (r.5739_5740insMN602429:r415_492). The insertion from intron 40 shifted the reading frame (ORF) of exons 40 and 41, generating a premature stop codon (UAA) and leading to protein translation to end.

The pregnant woman and fetus (II-4 and III-3) all carried this deep intronic mutation in *DMD* DNA level (Figure 1C). The pregnant woman showed depression and strongly requested to perform the fetal muscle biopsy. The cDNA analysis of the fetal muscle specimen

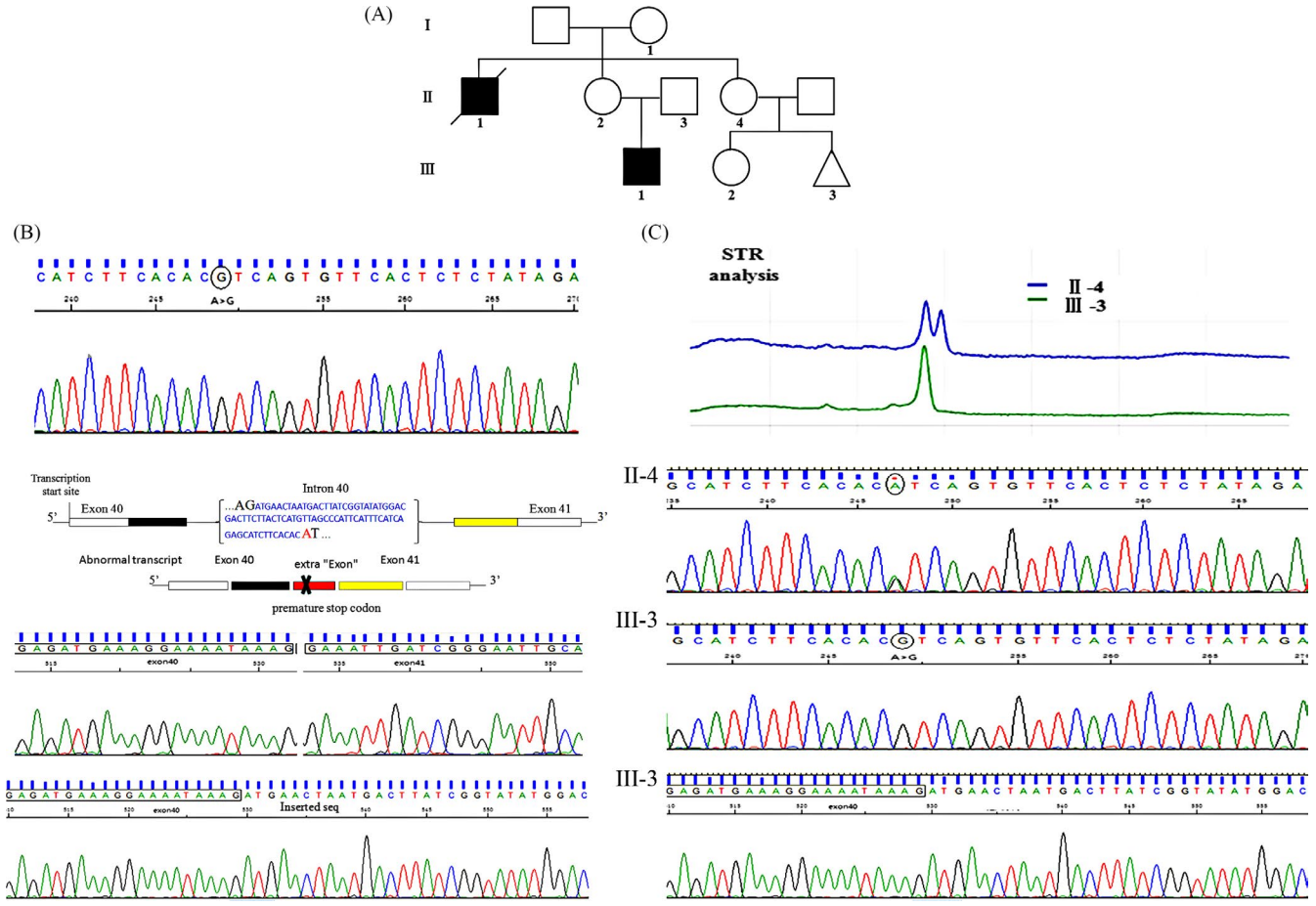

FIGURE 1 Representative abnormal mutations detected in case 1. A, The family pedigree of the case 1 is shown. B, Long-range PCR analysis of DMD patient of mutation at the nucleotide level(top); Schematic of the mutation and its consequences (middle); mRNA analysis of DMD patient muscle and overlapping primer sets that span multiple DMD exons (bottom). C, Prenatal genetic diagnosis results

showed the same insertion mutation in exons 40 and 41 (r.5739_5740insMN602429:r415_492). We have fully informed pregnant woman and her spouse about the pathogenic sites, process, possible complications, and risk evaluation. The family chose the termination of pregnancy.

3.2 | Case 2

A 32-year-old gravid woman (II-3), Gravida 2, para1, was referred to our department at 10 menstrual weeks for the prenatal diagnosis of DMD. She had a 2-year-old son (III-2) in whom DMD had recently been discovered. Her son had an abnormal CK value of 2900 U/L, the family pedigree is presented in Figure 2A. The mutations were also not found by MPLA, DHPLC, and *DMD* sequencing genetic studies. The cDNA analysis indicated that an insertion of 74 nucleotides was found between exons 2 and 3 (r.93_94 insMNMN584887:r61_134) Figure 2B). The BLAST analysis indicated that the insertion of 74 nucleotides was traceable in the intron 2 of *DMD* gene (NG_012232.1:g.415926_g.415999). The emerge of the insertion from intron 2 shifted the reading frame (ORF) of exons 2 and 3, generating a premature stop codon (UAA) and avoiding protein translation (Figure 2B). The future DNA-based testing showed only two SNPs (NG_012232.1:g.415932C>G and NG_012232.1:g.415954T>G) were detected at the DNA level. The other relatives also carried the two SNPs (Figure 2C). Although the pathogenicity of two different SNP mutations was not clear to explain the abnormal transcript, the 74bp insertion which located inside the consensus sequence AG/GT was detected between exons 2 and 3 of dystrophin in the child. We highly suspect a cryptic splicing site, yielding an extra "exon" and arising premature stop codon (Figure 2B). We did not perform the prenatal diagnosis for case 2 because the fetus was female of being less affected or a carrier. At term, the baby girl was found to have normal serum creatine kinase levels, and was therefore unaffected with DMD.

4 | DISCUSSION

DMD severely affects the survival and life quality of the patients, and currently there is no effective treatment. Given the severity of the disease, molecular genetic analysis of DMD patients is critical for final diagnosis, guidance for appropriate clinical management, and provision of further prenatal diagnosis and genetic counseling.[8]

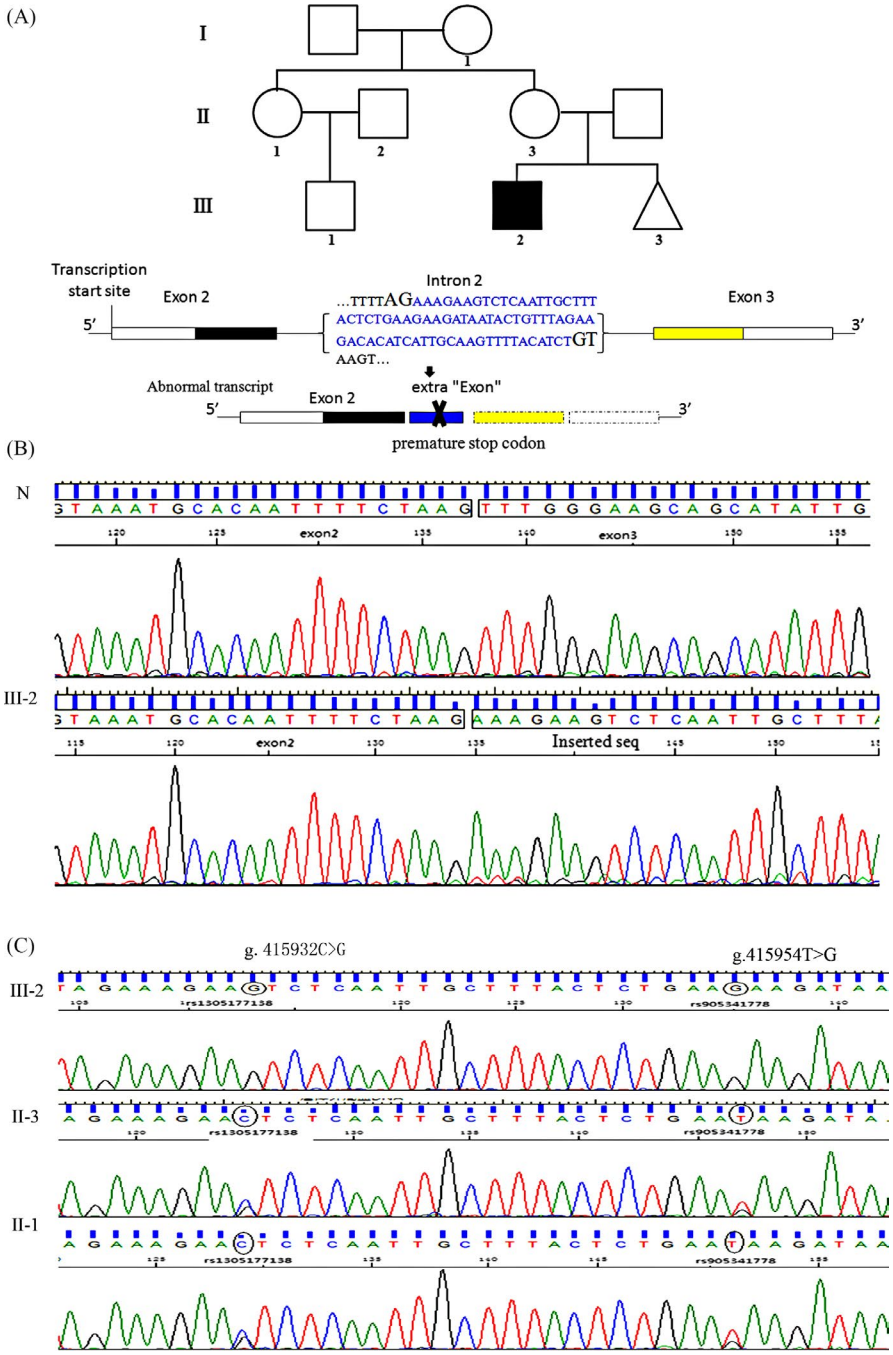

FIGURE 2 Representative abnormal mutations detected in case 2. A, The family pedigree of the case 2 is shown. B, Schematic of the mutation and its consequences (top); mRNA analysis of DMD patient muscle and overlapping primer sets that span multiple DMD exons (bottom). C, Long-range PCR analysis of the proband's relatives

Currently, the most commonly used methods for clinical gene diagnosis of DMD include MLPA analysis, DHPLC, next-generation sequencing technology, and direct sequencing methods, which can detect pathogenic mutations in most patients.[9-12] We performed MLPA, DHPLC, and Sanger sequencing analysis on two DMD families to detect the presence of exon deletions/duplication or point mutations. But no causative alterations were found. Past studies have suggested that deep intronic mutation or splicing mutation with a gene could have deleterious effect, causing human genetic diseases.[13-16] The further related tests have to be performed in order to identify the pathogenic mutations. Interestingly, when we carried out mRNA analysis two insertion abnormal transcripts were identified in two Chinese families affected with dystrophinopathy. In case one, the 78bp de novo nucleotides which give rise to an extra "exon" were inserted in exons 40 and 41 of *DMD* in patient muscle. When a pathogenic variant is identified in cDNA, it should be checked by sequencing of the appropriate region(s) in genomic DNA to confirm the result.[7] A deep intronic *DMD* mutation (NG_012232.1:g.1001838A > G) was recognized by the Long-range PCR with sequencing methods. This mutation generated the donor splice site GT (5′splice site) of intron 40. This extra "exon" generated premature stop codon in the *DMD* coding sequence. We concluded that this mutation is causative for disease in case one.

In case two, we found an insertion of 74 nucleotides between exons 2 and 3 (r.93_94 insMNMN584887:r61_134), which shifted the reading frame of exons 2 and 3, resulted in a premature stop

codon (UAA). Further test showed the 74bp insertion which located inside the consensus sequence AG/GT within intron 40. The *DMD* gene, which spans approximate to 2.4 MB containing 79 exons and the intron-exon junctions follow the GT-AG rule.[17] We speculated this activation of cryptic splicing sites results in exons with altered length.

DMD is a single-gene genetic disease with the characteristics of genetic transmission from parents to offspring. For women who have previously given children with DMD or pregnant women carriers of mutations in the *DMD* gene carriers, the risk of gestational fetus developing DMD is 25%. High-risk families have extremely strong willingness to conduct prenatal diagnosis of DMD.[8] Currently, for the prenatal diagnosis of DMD, a variety of sampling methods including villi tissue, amniotic fluid, umbilical cord blood and a variety of molecular detection methods including MPLA, real-time PCR, DHPLC, and DNA sequencing have been adopted.[18-20] Gonorazky et al reported that RNA analysis would be a critical required element for establishing pathogenicity of noncoding mutations for a significant fraction of the currently "unsolved" cohort of patients with DMD.[21] *DMD* transcript mutations can unable to identify though chorionic villus or amniocentesis techniques. Fetal muscle biopsy technique was developed to perform immunohistochemical diagnosis of Duchenne and Becker muscular dystrophies.[22-25] Our studies have shown the effectiveness of fetal muscle biopsy. Moreover, analysis of fetal transcript mutation evidenced that the pathogenic mutation was detected in fetal muscular tissue.

Our study identified two novel insertion mutations in two Chinese families affected with dystrophinopathy at the *DMD* transcriptional level. The limitation of this study was that our evidences does not completely explain how generate the 74bp insertion from intron 2 in case two.

In conclusion, this study provides importance of proceeding to the mRNA analysis for "unsolved" cohort of patients with DMD. This finding expands the noncoding mutations and proves accurate genetic assessment/ carrier status testing in families with DMD.

ACKNOWLEDGMENTS

We thank the patients and their families for participating in the study. We also are grateful for the valuable discussion with Xu Zhang, Beijing GH Epigen Medical Institute, Beijing 100089, China.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

INFORMED CONSENT

Two cases signed the individual informed consent to participate in the study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Xu Y, Song T, Li Y, et al. Identification of two novel insertion abnormal transcripts in two Chinese families affected with Dystrophinopathy. *J Clin Lab Anal*. 2020;34:e23142. https://doi.org/10.1002/jcla.23142