Unveiling Noncoding *DMD* Variants: Synergizing RNA Sequencing and DNA Sequencing for Enhanced Molecular Diagnosis

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1 Introduction

Pathogenic variants in the *DMD* gene are associated with Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). More than 5,000 pathogenic variants in the *DMD* have been identified in DMD/BMD patients. A wide array of disease-causing alleles exists, comprising intragenic copy number variants that entail deletions or duplications of one or more exons, as well as small deletions or insertions, and single nucleotide variants. Exome sequencing and gene panels have advanced genetic diagnostics, yet some cases remain elusive. Unexplored sources like variants affecting RNA splicing/expression, may lie beyond DNA sequencing. Here, we present cases showing the clinical utility of RNA sequencing (RNAseq) as an effective modality for DMD/BMD diagnosis.

2 Methods

Total RNA sequencing were performed on 15 patients with suspected DMD or BMD and previous DNA sequencing results. RNA was extracted from muscle tissue. Ribosomal RNA (rRNA) was depleted prior to RNA library preparation to focus on analyzing high-value portions of the transcriptome. RNA is fragmented and reverse-transcribed into double-stranded cDNA. The whole transcriptome RNAseq library was generated and pooled for sequencing on the Illumina NovaSeq6000 platform using 150 bp paired-end reads at a mean depth of over 100 million reads. Reverse transcription-PCR (RT-PCR) was utilized to confirm abnormal splicing events identified. Sequence data are analyzed using Dragen Bio-IT Platform. Alignment to the GRCh37 (hg19) is performed and candidate splice junctions are identified throughout the reference genome. Splice junctions are mapped to Ensembl's GRCh37 Gene Transfer Format (GTF) file to provide annotations of the splice junction. Any splice junctions that did not map to the GTF file were marked as unannotated. The absence or presence of *DMD* RNA transcripts meeting quality thresholds is incorporated as evidence towards assessment. Splice aberration events are evaluated and visualized using the sashimi plot in Integrative Genomics Viewers (IGV), NxClinical and Alamut.

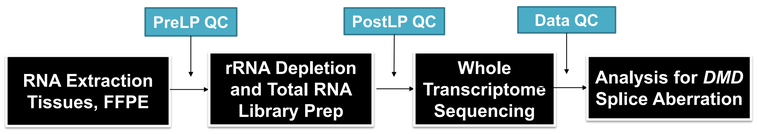

Figure 1. *The RNA sequencing pipeline.*

PreLP/PostLP*, pre/post library preparation;* ***QC,*** *quality check.*

3 Results

Among these 14 patients (excluding the QNS case), previous exome or targeted DNA sequencing (panel or *DMD* gene) and/or multiplex ligation-dependent probe amplification (MLPA) failed to yield a definitive molecular diagnosis for 9 cases including 7 cases with negative results and 2 with variants of uncertain significance (VUS) (**Figure 2**). RNAseq has identified aberrant splicing events across *DMD* gene in 13 cases (**Table 1**). The RNAseq discoveries including splice-altering intronic single nucleotide variants (RNA001, 004, 005, 006, 014), small indel (RNA002), exons deletion (RNA013, 016) or complex rearrangement (RNA007, 016) are aligned with previous or reanalyzed DNA sequencing result. Furthermore, we identified splicing aberration events including intron 55 retention (RNA008), exon 2-79 (RNA010), 68-79 (RNA012), or 45-79 (RNA015) deletions with unclear pathogenicity. Some cases with exon deletions have presented noncanonical transcripts expression. The RNAseq result in different exon regions in most of these cases have corresponded with their immunohistochemical results of weak/partial/no dystrophin staining.

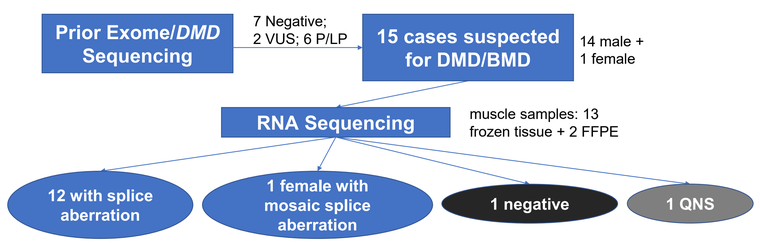

Figure 2. *The RNA sequencing result summary.*

P*, pathogenic;* ***LP****, likely pathogenic;* ***VUS****, variant of uncertain significance;* ***QNS****, quality not sufficient.*

Case No.	Sample	Age	Sex	Prior DNA Test	DNA Test Result	*DMD* RNAseq Result	Muscle Biopsie Result	Symptoms
RNA001	Tissue	16	M	DMDseq	*DMD* c.7309+5G>T-LP	c.7309+5G>T results in exon 50 skipping	NA	Non-ambulatory (lost ~13-14YO); muscle weakness/wasting; elevated AST/ALT, CK
RNA002	Tissue	23	M	DMDseq; Panel	*DMD* c.94-38_94del-VUS; VUS in other gene	c.94-38_94del results in exon 3 skipping	NA	Muscle weakness-proximal, limb girdle muscular dystrophy. The variant was not detected in mother
RNA004	Tissue	5	M	Panel	*DMD* c.3276+1G>A-LP; VUSs in other genes	c.3276+1G>A results in intron 24 retention	NA	Ambulatory; muscle weakness/wasting; Gowers' maneuver; behavioral problems; elevated AST/ALT, CK
RNA005	Tissue	28	F	Exome	*DMD* c.2292+5G>A-LP; VUSs in other genes	c.2292+5G>A results in mosaic exon 18 skipping	NA	Episodes of vertigo, burning sensation of the extremities, intermittent tinnitus, dysphagia, hand weakness, and difficulty walking
RNA006	FFPE	11	M	DMDseq; Panel	Negative for *DMD*; VUSs in other genes	c.3603+820G>T results in an 84-bp pseudoexon inclusion between exon 26-27	DYS-rod, DYS-N, DYS-C weak to no staining	Gait difficulties; non-ambulatory; muscle weakness/wasting; pain/cramp; elevated CK
RNA007	Tissue	6	M	Panel; DMDseq	Negative for *DMD;* VUSs in other genes	Possible aberrant splicing in exon 44-45 likely caused by inversion of 307kb region of chr10p15.1 in 8bp deletion of intron 44	Loss of expression of DYS-1, 3 and partial DYS-2	Gait difficulties; calf hypertrophy; Gowers' maneuver; muscle weakness/wasting; pain/cramp; long bone fractures; elevated AST/ALT, CK
RNA008	Tissue	20	M	2 Panel; DMDseq	Negative for *DMD*; VUS in other gene	Possible intron 55 retention	Absent DYS-3, DYS-2 staining and markedly reduced DYS-1.	Gait difficulties; non-ambulatory; calf hypertrophy; muscle weakness/wasting; weak cough; decreased cardiac function; intellectual disability; developmental delay; gross motor delay; scoliosis; elevated CK
RNA009	Tissue	12	M	2 Panel	*DMD* c.2330T>C-VUS; VUSs in other genes	No splice aberration detected	DYS 1 and 2 is present. DYS 3 and α-dystroglycans weak to absent	Limb girdle muscular dystrophy, myopathy, toe-walker; EMG.
RNA010	FFPE	8	M	Panel; DMDseq; MLPA	Negative	Possible intron 1 retention. Deletion of exon 2-79	Absent DYS rod domain, N and C-termini. Decreased β-dystroglycan and β/Δ/γ sarcoglycans	Gait difficulties; ambulatory; calf hypertrophy; muscle weakness/wasting; Gowers' maneuver; ADHD; Behavior problems; elevated AST/ALT, CK
RNA012	Tissue	12	M	2 DMDseq; MLPA	Negative	Possible deletion of exon 68-79	DYS1, DYS2, DYS3 absent. α/Δ sarcoglycan and α-dystroglycan normal	EEG abnormal since 7YO. EMG abnormal. Severe myopathy. elevated CK (11557, 7YO)
RNA013	Tissue	12	M	Panel	*DMD* exon 51-52 del-LP; VUS in other gene	Possible exon 51-52 skipping event	3 DYS epitopes normal; subtle myofiber abnormalities.	NA. Mother and brother had the same *DMD* variant
RNA014	Tissue	5	M	DMDseq; Panel	*DMD* c.7309+5G>A-LP	c.7309+5G>A result in exon 50 skipping	NA	Gait difficulties; ambulatory; calf hypertrophy; muscle weakness/wasting; Gowers' maneuver; development delay (motor/speech); elevated AST/ALT, CK
RNA015	Tissue	7	M	Panel	*DMD* exon 45-47 del-P; VUS in other gene	Exon 45-47 deletion. No pseudoexon.	Reduced DYS expression. Nearly absence of exon 45-47 and actin binding domain exon 7/8.	Gait difficulties; calf hypertrophy; muscle weakness/wasting; partial Gowers' maneuver; elevated CK
RNA016	Tissue	8	M	DMDseq; MLPA	Negative	Possible exon 44 extension and exon 45-79 deletion likely caused by inversion in intron 44.	Loss of DYS (C and N termini, rod domain)	NA

Table 1. *The RNA sequencing cases in this cohort.*

DMDseq*, DMD gene sequencing;* ***M****, male;* ***F****, female;* ***NA,*** *not available;* **DYS**, dystrophin antibody

4 Representative cases

RNA006

The patient is a 11-year-old male with symptoms including gait difficulties; non-ambulatory; progressively muscle weakness/wasting; pain/cramps; language delays; elevated CK. The immunohistochemical staining showed moderate chronic myopathic changes in muscle biopsy that DYS-rod, DYS-N, DYS-C had very weak to no staining; β/Δ sarcoglycans showed weak circumferential staining. He was clinically diagnosed with DMD and was on steroid treatment since 7-year-old. A limb-girdle neuromuscular DNA sequencing panel was tested and only identified 2 VUSs in 2 autosomal recessive genes separately, which could not explain his clinical features. Then *DMD* gene sequencing was tested and had negative result. The RNAseq identifies an 84-bp pseudoexon inclusion event (ChrX:32471856-32471940), which is predicted to result in the insertion of 8 amino acids followed by a stop codon and is expected to activate nonsense-mediated mRNA decay (**Figure 3**). Further analysis of the original *DMD* gene sequencing data identifies a hemizygous c.3603+820G>T deep intronic variant after exon 26, which is associated with this pseudoexon inclusion event. This variant has been reported as hemizygous in 2 unrelated individuals with dystrophinopathy (PMID: 33977140, 35165973); one of which has been confirmed as *de novo*. Functional studies indicate that this variant increases the strength of the polypyrimidine tract leading to the use of a cryptic acceptor splice site and the subsequent inclusion of an 84-bp pseudoexon into the *DMD* mRNA. Western blotting analysis confirmed undetectable levels of dystrophin compared to controls (PMID: 33977140). Based on the collective evidence, the c.3603+820G>T *DMD* variant is classified as pathogenic.

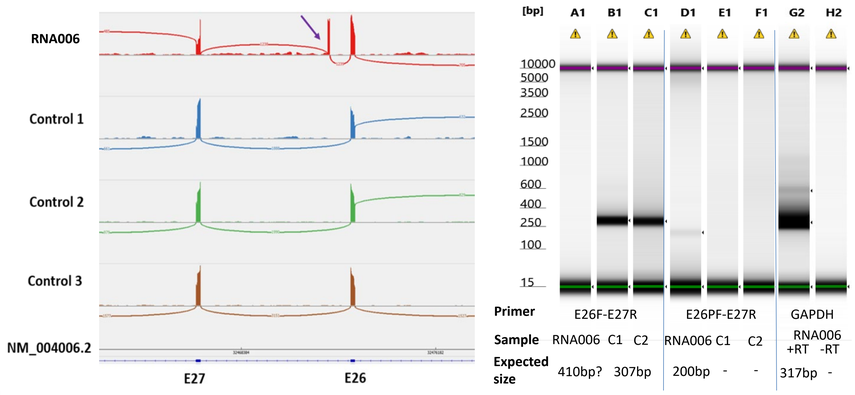

Figure 3. *The aberrant splicing event of RNA006 shown by RNAseq and by RT-PCR.*

RNA007

The patient is a 6-year-old male with symptoms including gait difficulties; Gowers' maneuver; muscle weakness/wasting; pain/cramp; long bone fractures; elevated AST/ALT, CK. The immunohistochemical staining showed loss of expression of α-dystroglycan, DYS-1 (rod domain), DYS-3 (N-terminal) and partial DYS-2 (C-terminal). The previous DNA panel sequencing returned only VUSs in other genes that could not explain his clinical profile. Possible aberrant splicing in exon 44-45 (c.6439-21402_6439-21395del) likely caused by an inversion of 307kb region of chr10p15.1 (chr10:5,914,691-6,221,422) in the 8bp deletion of intron 44 (**Figure 4**). However, quantitative transcriptome analysis or gene expression analysis were not included in this assay. The complex rearrangement event was later confirmed by the *DMD* gene sequencing result. Insertion of sequence from elsewhere in the genome into an intron resulting in aberrant *DMD* transcription has been reported (PMID: 25614876). Therefore, this deletion/inverted insertion variant in the *DMD* gene is classified as likely pathogenic.

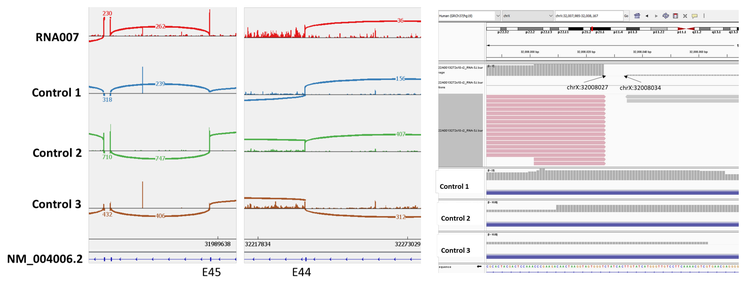

Figure 4. *The aberrant splicing event of RNA007 shown by RNAseq result.*

5 Summary

DNA sequencing leans on RNA studies to provide functional data for *DMD* splice aberration events and splice-altering variants. Our data demonstrate RNA sequencing as a powerful tool, especially when integrating with DNA sequencing, for elucidating the pathogenicity of *DMD* variants, achieving a precise genetic diagnosis, and guiding the potential treatments in patients with clinical and pathological suspicions of DMD/BMD without definitive diagnoses after routine genetic testing.

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