WGS and RNA Studies Diagnose Noncoding DMD Variants in Males With High Creatine Kinase

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Abstract

Objective
To describe the diagnostic utility of whole-genome sequencing and RNA studies in boys with suspected dystrophinopathy, for whom multiplex ligation-dependent probe amplification and exonic parallel sequencing failed to yield a genetic diagnosis, and to use remnant normal DMD splicing in 3 families to define critical levels of wild-type dystrophin bridging clinical spectrums of Duchenne to myalgia.

Methods
Exome, genome, and/or muscle RNA sequencing was performed for 7 males with elevated creatine kinase. PCR of muscle-derived complementary DNA (cDNA) studied consequences of Duchenne to myalgia.

Results
Splice-altering intronic single nucleotide variants or structural rearrangements in DMD were identified in all 7 families. Four individuals, with abnormal splicing causing a premature stop codon and nonsense-mediated decay, expressed remnant levels of normally spliced DMD mRNA. Quantitative Western blot enabled correlation of wild-type dystrophin and clinical severity, with 0%–5% dystrophin conferring a Duchenne phenotype, 10% ± 2% a Becker phenotype, and 15% ± 2% dystrophin associated with myalgia without manifesting weakness.

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Conclusions

Whole-genome sequencing relied heavily on RNA studies to identify DMD splice-altering variants. Short-read RNA sequencing was regularly confounded by the effectiveness of nonsense-mediated mRNA decay and low read depth of the giant DMD mRNA. PCR of muscle cDNA provided a simple, yet informative approach. Highly relevant to genetic therapies for dystrophinopathies, our data align strongly with previous studies of mutant dystrophin in Becker muscular dystrophy, with the collective conclusion that a fractional increase in levels of normal dystrophin between 5% and 20% is clinically significant.

Duchenne muscular dystrophy (DMD) is a severe X-linked disorder primarily affecting approximately 1 in 5,000 male births.1-3 DMD shows a relentlessly progressive course, resulting in loss of ambulation in teens, and early mortality due to cardiac or respiratory involvement.4,5 Dystrophinopathies range clinically from the severe DMD to asymptomatic hyperCKemia.5-12 DMD is associated with the absence of dystrophin in muscle due to loss-of-function variants in the DMD gene encoding dystrophin,5,6 whereas Becker muscular dystrophy (BMD) is associated with variants in DMD that result in reduced levels of (mutated) dystrophin.5,6

The DMD gene is the largest gene in the human genome, with numerous enormous introns.13,14 One-third of pathogenic DMD variants are de novo,15,16 with most affected individuals bearing insertions or deletions (indels) of coding exons.15,17 Pathogenic DMD missense variants are rare,6,13,15 and non-coding variants are emerging as an important rare cause of dystrophinopathy.15,17,19,20 Approximately 5% of patients clinically diagnosed with DMD do not have a genetic diagnosis after mutational analysis.5

Herein, we show the diagnostic application of whole-genome sequencing, transcriptomics, and dystrophin protein

Glossary

bp = base pair; CK = creatine kinase; DMD = Duchenne muscular dystrophy; gnomAD = Genome Aggregation Database; GTEx = Genotype-Tissue Expression; IGV = Integrative Genomic Browser; MLPA = multiplex ligation-dependent probe amplification; mRNA = messenger RNA; nt = nucleotide; RNA-seq = RNA sequencing; RT-PCR = reverse transcription PCR; SNV = single nucleotide variant; WB = Western blot; WGA = wheat germ agglutinin; WT = wild type.
biochemistry to secure a genetic diagnosis for 13 affected males from 7 families with elevated creatine kinase (CK) who remained undiagnosed following multiplex ligation-dependent probe amplification (MLPA) and exomic sequencing. Importantly, we identify 3 families with DMD splicing variants who produce varying levels of mis-spliced transcripts that encode a premature stop codon and are targeted by nonsense-mediated decay, though express varying levels of remnant, normally spliced DMD mRNA. Therefore, quantitative Western blot (WB) of muscle biopsy specimens from these 3 dystrophin hypomorphs has uniquely enabled specific correlation of levels of wild-type (WT) dystrophin with clinical severity.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents
This study was approved by the Children’s Hospital at Westmead Human Research Ethics Committee (Biopsiemen Bank_10/CHW/45) with informed, written consent from all participants.

We describe a retrospective cohort of boys diagnosed with DMD variants from genomic and RNA studies, who had elevated CK and dystrophic muscle biopsies, and were undiagnosed after MLPA and exomic parallel sequencing.

Immunohistochemistry and Western Blotting

Immunohistochemistry21 and Western blotting22 were performed as previously described; WB used NuPAGE 3%–8% Tris-Acetate precast gels (Invitrogen by Thermo Fisher Scientific, NSW, Australia). Antibodies: for immunohistochemistry, muscle fiber membranes were stained with anti-dystrophin DYS1, DYS2, DYS3, and anti-spectrin SPEC1 (Leica Biosystems, VIC, Australia); with anti-mouse Alexa Fluor 555 secondary antibody, membranes were counterstained with wheat germ agglutinin-AF488 (WGA), and nuclei were stained with DAPI (Invitrogen Thermo Fisher Scientific). WBs were probed with DYS1 (Leica Biosystems), rabbit polyclonal dystrophin antibody (Rb-DMD; ab15277; Abcam), α-actinin-2 (4A3, gift from A. Beggs, Children’s Hospital Boston, Boston, MA), sarcomeric actin (clone 5C5, Abcam), rabbit polyclonal dystrophin antibody (Rb-DMD; ab15277; Abcam), and the anti-mouse or anti-rabbit IgG chemistry, muscle

Massively Parallel Sequencing

Whole-exome sequencing (probands and AI:1, AI:2, and AI:2), PCR-free whole-genome sequencing (probands from families A and B, D–G), and RNA sequencing (RNA-seq; probands from families A, B, D, E, and G) were performed at the Broad Institute of Harvard and MIT as previously described.20 RNA-seq was performed for CII:2 at PathWest Laboratory Medicine WA as previously described for the fetal samples in reference 24.

Sanger Sequencing and RT-PCR

RNA was extracted, and reverse transcription PCR (RT-PCR) was performed as previously described.25 Primers used for AI:1 have been previously described.20 The remaining primer details are as follows: Ex42F 5'-CAATGCTCTGACCTCTGTG-3'; Ex43/44R 5'-CTGTTCAATCAGCCTTGTGCG-3'; LINC00251Ex3R 5'-CTGAAATTGGGTGAGGATGAAG-3'; LINC00251Ex2F 5'-GATGCCCTCTAAACAGGAC-3'; Ex26F 5'-GATGCACGAAATGGATGAC-3'; Ex27R 5'-TGTGCTACAGGTTGAGCTTG-3'; Ex26/27F 5'-GCAGTTGAAGAGATGAAGAG-3'; Ex29R 5'-GGGTTATCCTCTGAATGTGCG-3'; Ex26F 5'-AAGTCTTGCAAAGATGAGAGAC-3'; Ex49/52F 5'-AAGTCTTGCAAAGATGAGAGAC-3'; Ex54R 5'-ACTGGGCGGAGGCTTTTGAG-3'; Ex49/52F 5'-ACTGGGCGGAGGCTTTTGAG-3'; Ex54R 5'-ACTGGGCGGAGGCTTTTGAG-3'; Ex51F 5'-AGTCTTGAAAGATGAGAGAC-3'; Ex50/52F 5'-CAATCCTCCTGAGCAGGACG-3'; GADPHEx3F 5'-TCACACGGCTCTGTCTCACTG-3'; and GADPHEx6R 5'-GGCAGGATGATGAGCCTTCT-3'. Confirmation and segregation analysis of DMD variants was performed by Sanger sequencing,21 except for family F in which DNA was not available. Primers used for families A, D, E, and G have been previously described.20 The remaining primer details are as follows: family B — In43F 5'-TTTAGGTTTTCCACGACTCCCTGTC-3' with chr8R 5'-TAGCAGGGGGCAAGGTTG-3' and chr8F 5'-TGCTCTTCTGAGGAGGAC-3' with In43R 5'-CGGGGAACATCACACACC-3' to confirm insertion breakpoints; family C — In26F 5'-CAAGGAAACATGCTGGATGAG-3' with In26R 5'-AAAGGCTGATGACAGATAGCC-3' to determine causative variant. PCR conditions were 5 minutes 95°C; 35 cycles—30 seconds 95°C, 30 seconds 58°C, and 1 minute 72°C; 8 minutes 72°C; or as described in reference 20.

Whole-Genome Sequencing Analysis

PCR-Free whole-genome sequencing was performed on an Illumina HiSeq X Ten using 2 × 150 paired end reads at 30× mean coverage. The sequencing reads were aligned to the GRCh37 genome reference and single nucleotide variants (SNVs), small insertions and deletions (indels) were detected using methods previously described in reference 20. A reanalysis of rare (Genome Aggregation Database [gnomAD]) AF < 0.005) SNVs and indels

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revealed no pathogenic DMD variants. The Manta tool from Illumina (PMID: 26647377) was used to identify structural variants or split read abnormalities within the DMD gene. Putative structural variants were manually inspected within Integrative Genomic Browser (IGV) to validate and resolve exact breakpoints of structural rearrangements.

### RNA-seq Analysis

RNA-seq analysis was performed as described in reference 20. Briefly, all samples were jointly processed and aligned with the Genotype-Tissue Expression Consortium (GTEx) to identify spliced reads only seen in patients or groups of patients and missing in controls. In addition, given the nature of the previously suspected diagnosis of a dystrophinopathy, in cases in which this approach did not lead to a diagnosis, exonic read depth was mapped in each patient and compared with controls and sashimi plots of patients were manually inspected using the IGV for the DMD gene. In cases, in which RNA-seq identified a mis-splicing event, patient exome and genomes were manually evaluated, depending on availability.

### Data Availability

Data not published within this article are available by request from any qualified investigator.

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**Table Clinical Presentation, DMD Variants, and Dystrophin Western Blot**

| All:1 | BIV:1 | CII:2 | DII:1 | EII:1 | FII:6 | GII:1 |
|-------|-------|-------|-------|-------|-------|-------|
| **Clinical symptoms** | Muscle pain, fatigue, and myoglobinuria with exercise | Proximal weakness and bilateral calf hypertrophy | Progressive limb-girdle weakness and falling regularly | Proximal weakness, calf hypertrophy, and positive Gowers sign | Muscle weakness and calf hypertrophy | Proximal muscle weakness, positive Gowers sign, and calf hypertrophy | Calf hypertrophy and positive Gowers sign; proximal weakness, elbow contractures, and learning difficulties |
| **Onset** | 15 y | 5 y | 9 y | 3.5 y | 6 y | 3.5 y | 5 y |
| **Family history** | 2 affected brothers reporting myalgia and serum CK levels of 300–14,700 U/L | Four-generation family segregating with Duchenne muscular dystrophy with cardiomyopathy | Nil | Nil | Has a similarly affected brother | The mother (FI:6) also has muscle pain and elevated serum CK levels of ~500 U/L | Nil |
| **Serum CK, U/L** | 1,400–7,500 | 9,964 | 420 | 14,500 | 18,889 | 24,000 | >12,000 |
| **Ambulance** | Remains ambulant | Remains ambulant | Intermittent use of a wheelchair from 13 y | Wheelchair dependent at 13 y | Wheelchair dependent at 9 y | Remains ambulant and toe walking at 9 y | Wheelchair dependent at 7 y |
| **Cardiac and respiratory involvement** | Nil | Normal echocardiogram cardiomyopathy in BII:2 and BII:7 | Nocturnal bilateral positive airway pressure at 28 y; normal cardiac function | Cardiac-reduced contractility (ejection fraction 30%–35%) with normal left ventricle size | Nil | Nil | Borderline increase in heart size at 9 y; died at age 10 y from cardiac complications |
| **DMD variant** | Pseudoexon inclusion in DMD intron 43 NM_004006.2:c.6290+30954C>T | Pseudoexon inclusion in DMD intron 43 NM_004006.2:c.6290+28627_6290+28628ins[TGTGGGCAAAGGC] | Inversion of DMD exon 51 NM_004006.2:c.7310-2629_7542+1338inv | Inversion of DMD exons 1–18 NM_004006.2:c.-10599535_117965534_9085-12259inv;NM_004006.2:6433+112319inv; 4233+10599; 5325+387dup; 6117+6701 6438+112319dup | Inversion of DMD exons 1–44 NM_004006.2;c.6818-10658_NM_004006.2:6438+112319inv; 4233+10599; 5325+387dup; 6117+6701 6438+112319dup | Inversion of DMD exons 1–60 NM_004006.2;c.-117965533_9085-12259inv; 117965534_117965531del; 9085-12258_9085-12196del|
| **Western blot** | 15% ± 2% | 10% ± 2% | 0%–5% | 0%–5% | 0%–5% | 0%–5% | 0%–5% | 0%–5% |

Abbreviations: CK = creatine kinase; DMD = Duchenne muscular dystrophy; DMD = DMD gene or transcript.
Results

Clinical Presentation

Four families have been described previously in reference 20: AII:1 as N33; DII:1 as C3; EII:1 as C4; and GII:1 as C2. Clinical presentation, DMD variants, and dystrophin WB results are summarized in the table. Briefly, AII:1 presented at 15 years with muscle pain, fatigue, and episodes of myoglobinuria with exercise and elevated serum CK (1,400–7,500 U/L, normal range <200 U/L). He has 2 affected brothers with myalgia and elevated serum CK (300–14,700 U/L (figure 1A). Family B is a 4-generation family with an X-linked muscular dystrophy with cardiomyopathy. BIII:2 was diagnosed with dilated cardiomyopathy in his 20s, underwent cardiac transplantation at age 29 years, and died of transplant-related complications at age 31 years. BIII:7 was diagnosed with BMD in his mid-teens. He has no known history of cardiomyopathy and remains ambulant in his 40s (figure 1B). BIV:1 showed elevated serum CK 9,964 U/L at age 6 months. Now age 5 years, he has proximal muscle weakness, bilateral calf hypertrophy, and normal echocardiogram. CII:2 presented at age 9 years with progressive limb-girdle weakness, requiring intermittent use of a wheelchair from age 13 years and nocturnal bilateral positive airway pressure (BiPAP) from age 28 years. He has normal cardiac function with serum CK of 420 U/L at age 31 years (figure 1C). DII:1 presented at age 3.5 years with proximal weakness, calf hypertrophy, positive Gowers sign, and serum CK of 14,500 U/L. He required use of a wheelchair from age 13 years. Echocardiogram at age 17 years showed reduced contractility (ejection fraction 30%–35%) with normal left ventricle size (figure 1D). EII:1 presented at age 6 years with muscle weakness, enlarged calves, and serum CK of 18,889 U/L. He required use of a wheelchair at age 9 years and has no known cardiac or respiratory involvement. EII:1 has a similarly affected brother (figure 1E). FII:6 presented at age 3.5 years with proximal muscle weakness, positive Gowers sign, prominent calves, and serum CK of 24,000 U/L. He remains ambulant, but is toe walking at age 9 years. He has no known cardiac or respiratory involvement. FII:6’s mother (FI:6) reports muscle pain and has elevated serum CK of ~500 U/L (figure 1F). GI:1 presented at age 5 years with waddling gait, calf hypertrophy, positive Gowers sign, and serum CK levels of >12,000 U/L. He required use of a wheelchair from age 7 years. Echocardiogram at age 9 years showed borderline increase in heart size, and he died at age 10 years from cardiac complications (figure 1G).

DMD Diagnostic Genetic Testing

DMD MLPA and Sanger sequencing were performed and reported normal for AII:1, BIV:1, CII:2, DII:1, EII:1, and GII:1. DMD MLPA performed for FII:6 revealed duplications of exons 31–37 and 43–44, which were predicted to be in-frame and therefore considered inconsistent with his severe Duchenne-like phenotype, though with high clinical suspicion of causality. A genetic basis could not be identified via whole-exome sequencing (AII:1, BIV:1, DII:1, EII:1, FII:6, and GII:1, with duplications of exons 31–37 and 43–44 confirmed for FII:6) or massively parallel sequencing of a targeted neuromuscular gene panel (CII:2).

Immunohistochemistry Demonstrates Dystrophin Abnormalities in Skeletal Muscle Biopsies

Skeletal muscle immunohistochemistry for AII:1, BIV:1, DII:1, EII:1, FII:6, and GII:1 confirms abnormalities in dystrophin
Figure 2 Muscle RNA Studies of DMD in Patients

(A) RNA-seq read coverage of DMD exons in muscle RNA from AII:1, BIV:1, DII:1, EII:1, and GII:1 and 2 GTEx controls. Red arrows indicate the reduction in read depth, which corresponds with the location of DMD structural variants for BIV:1, DII:1, EII:1, and GII:1. (B–G) RT-PCR studies of muscle-derived RNA of patients with splicing abnormalities and 3 male controls (C1, quadriceps, 6.5 years; C2, vastus lateralis, 17 years; C3, unknown, 20 years). Primers used are listed at the bottom right of each gel image and are labeled according to their location (exon; Ex, intron; In, pseudoexon; P) and orientation (forward; F, reverse; R). Bridging primers span a splice junction and are denoted by X/Y, where X and Y are exons the primer spans. All results were confirmed by Sanger sequencing.

(B) RT-PCR showing reduced levels of correctly spliced DMD transcript (exons 43 and 44) in AII:1 and BIV:1 compared with controls. AII:1 shows the inclusion of a 128-bp pseudoexon. (C) Primers specific to the 128 bp pseudoexon revealed that the inclusion is specific to AII:1 (Sanger sequencing showed that the faint bands in C1 were non-DMD sequences). Sequencing reveals that faint bands in AII:1 correspond to multiple pseudoexons in DMD incorporated into a minority of DMD transcripts. (D) Various chr8 pseudoexons and LINC00251 exons are included in DMD transcripts as a result of the chr8 insertion in BIV:1. The lowest band detected in all samples in the top gel corresponds to non-DMD sequences. (E) RT-PCR confirms the inclusion of a 84-bp pseudoexon in CII:2 in the majority of DMD transcripts. Normal splicing can only be detected in very low levels in CII:2 by bridging primers. The 92 bp pseudoexon is absent in control samples. (F) RT-PCR of DII:1 confirms that exon 51 is absent from all DMD transcripts. A bridging primer indicates that skipping of both exons 50 and 51 is a low frequency event observed in both controls and DII:1. (G) GAPDH loading controls to indicate that similar concentrations of complementary DNA were used for both control and patient samples. DMD = Duchenne muscular dystrophy; DMD = DMD gene or transcript; RT-PCR = reverse transcription PCR.
Figure 3 Schematics of Variants Identified in Families A–G

(A) Family A: intronic c.6290+30954C>T (black arrow) creates a cryptic donor splice site, leading to inclusion of a 128-bp pseudoexon (red, within DMD intron 43) into the DMD mRNA, causing a frameshift and stop codon (red arrow) encoded by exon 44 (ex44). Gene direction is demonstrated by gray arrows. Reading frame between exons is shown by shape complementarity.

(B) Family B: insertion of 116,284 bp of chr8 (red sequence) into DMD intron 43. The insertion includes LINC00251 exons 1–3 (black outlined exons). A 124-bp sequence of intron 43 of DMD (chrX:32,276,895-32,277,018) is duplicated as part of the structural rearrangement and now flanks the chr8 insertion. In addition, there is an insertion of 13 bp (insGCTTTGCCCACA, shown in green) adjacent to 1 copy of the 124-bp duplication. mRNA studies show evidence for numerous, different abnormal splicing events from DMD exon 43 to various pseudoexons (red exons) and LINC00251 exons (red exons with black outlines) within the chr8 insertion. Low levels of normal DMD splicing (from exons 43 and 44; blue exons) are also observed. Frame of splicing in pseudoexons and LINC00251 exons not shown. (D) Family D: inversion of DMD exon 51 and flanking adjacent intronic sequence. Flanking the structural rearrangement are 2 intronic deletions (orange 3.5 kb and purple 44 bp) and an insertion of CCAATA (green). mRNA studies show exon 51 skipping, causing a frameshift and a premature stop codon (TAG, encoded by exon 52; red arrow).

(C) Family C: intronic c.3603+820G>T (black arrow) increases the strength of the polypyrimidine tract leading to use of a cryptic acceptor splice site (3/5 algorithms within Alamut Visual biosoftware predictions; MaxEntScan, NNSPLICE, and GeneSplicer) leading to inclusion of a 84-bp pseudoexon (red, within DMD intron 26) into the DMD mRNA, encoding a stop codon (red arrow) 39 nucleotides into the pseudoexon. Gene direction is demonstrated by gray arrows. Reading frame between exons is shown by shape complementarity. (D) Family D: inversion of DMD exon 51 and flanking adjacent intronic sequence. Flanking the structural rearrangement are 2 intronic deletions (orange 3.5 kb and purple 44 bp) and an insertion of CCAATA (green). mRNA studies show exon 51 skipping, causing a frameshift and a premature stop codon (TAG, encoded by exon 52; red arrow).

(E) Family E: A 2.6-Mb inversion on the X chromosome between 2 breakpoints; A in intron 45 of CFAP47, 1.9 Mb upstream of exon 1 of DMD (GRCh37:chrX:35,180,364) and B in intron 18 of DMD (GRCh37:chrX:32,521,892, NM_004006.2). This reverses the orientation of exons 1–18 of DMD, which are now joined to CFAP47 sequences upstream of exon. The DMD gene is in blue, exons dark blue, and introns light blue. Intergenic sequence (non-DMD ChrX in green). (F) Family F: A 4.1-Mb inversion on the X chromosome between 2 breakpoints; A is 3.8 Mb upstream of exon 1 of DMD (GRCh37:chrX:36236087) and B in intron 44 of DMD (GRCh37:chrX:32122714). This reverses the orientation of exons 1–44 of DMD, which are now joined to intergenic sequence upstream of exon 1. This is accompanied by duplication of exons 31–37 (orange) and exons 43 and 44 (purple) around the breakpoint. (G) Family G: A 119.8-Mb inversion on the X chromosome between 2 breakpoints; A is an intergenic region on the q arm of the X chromosome, 118 Mb upstream of exon 1 of DMD (GRCh37:chrX:151,194,962), and B in intron 60 of DMD (GRCh37:chrX:31,379,010, NM_004006.2). This reverses the orientation of exons 1–60 of DMD, which are now joined to intergenic sequence upstream of exon 1. In addition, 2 deletions were identified at these breakpoints; an intronic 63 bp deletion (orange, GRCh37:chrX:31,378,947-31,379,009) and an intergenic 18 bp deletion (purple, GRCh37:chrX:151,194,963-151,194,980). X chromosome displayed in unusual orientation with q arm to the left, so the DMD gene is presented with exons in order. DMD = DMD gene or transcript. mRNA = messenger RNA.
(figure e-1, links.lww.com/NXG/A367). Using 3 anti-
dystrophin antibodies, AII:1 and BIV:1 showed reduced
dystrophin staining, whereas DII:1, EIi:1, FIi:6, and GII:1
showed absent staining (figure e-1) absent dystrophin staining
shown only for GII:1). WGA outlines the myofibers and la-
bes the endomysium in patient and control skeletal muscle
samples.

**Correlation of Splicing Analyses With Whole-Genome Sequencing Identifies Pathogenic Intrinsic and Structural Variants Inducing Abnormal DMD Splicing**

Six individuals (AII:1, BIV:1, DII:1, EIi:1, FIi:6, and GII:1) were subject to whole-genome sequencing, 6 individuals were
subject to RNA-seq (AII:1, BIV:1, CII:2, DII:1, EIi:1, and
GII:1), and 4 individuals (AII:1, BIV:1, CII:2, and DII:1) were analyzed by RT-PCR of muscle-derived mRNA. Scrut
iny of DMD transcripts (NM_004006.2, 11,058 nucleotides
[nt] in length) shows typical 3’ bias in read depth (vastly
more reads at the 3’ end compared with the 5’ end of DMD
transcripts). Acknowledging 3’ bias, an abnormal profile of
DMD transcript read depth was apparent for BIV:1, DII:1,
EIi:1, and GII:1 (figure 2A), relative to multiple muscle
controls from the GTEx consortium.26

Standard variant filtering approaches of genomic sequencing
failed to identify most causal variants. RNA-seq identified
abnormal pseudoexon inclusion into DMD transcripts for
families A and C. The remaining pathogenic variants were
identified only through the combination of whole-genome
sequencing, bioinformatics, and RNA analyses.

A genetic diagnosis in AII:1 was identified in a previous
study20 with a deep intrinsic pathogenic variant GRCh37:
ChrX:32274692G>A; c.6290+30954C>T inducing partial
mis-splicing of DMD. The DMD c.6290+30954C>T variant
creates a cryptic donor 5’ splice site resulting in inclusion of a
variant-activated pseudoexon of 128 nt inserted between exon
43 and exon 44, which encodes 59 missense amino acids and
effects a frameshift, resulting in a premature termination co-
don encoded by exon 44 (figure 3A). RT-PCR confirmed
abnormal inclusion of the variant-activated pseudoexon and
residual normal splicing of DMD exons 42-43-44-45 (figure
2, B and C).

RNA-seq for BIV:1 showed low levels of DMD transcripts,
with a distinct drop in reads from exon 44 onward (figure
2A, arrow). Bespoke realignment and analyses of WGS data
identified insertion of ~118,000 nt of chromosome 8 (chr8)
sequences within DMD intron 43, encompassing the
LINC00251 gene locus. RT-PCR showed that the chr8 in-
sertion induced abnormal splicing of the DMD gene (figure
3B). Multiple adverse events were detected that involved
splicing from exon 43 of DMD to various pseudoexons and

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**Figure 4 Western Blot Panel for All Patients**

(A.a) Western blot was performed on skeletal
muscle from index patients from families A and B
(AII:1 and BIV:1) against DYS1 (rod domain epi-
tope) and Rb-DMD (C-terminal epitope) with serial
dilutions (1/2, 1/4, 5/6, and 9/10) human control
skeletal muscle. Muscle lysate derived from an
individual with Duchenne muscular dystrophy
and undetectable levels of dystrophin by Western
blot (DMD control; deltoid, 14-year-old boy,
GRCh37:chrX:32364116G>A, NM_004006.2:
c.6300G>T, p.Arg1844*) were added to diluted
controls to normalize total protein loading in each
lane of the gel. Loading controls: a-actinin-2 and
myosin (coomassie). (C) Western blot analysis on skeletal
muscle from patient CII:2 against DYS1 shows undetectable levels of
dystrophin relative to controls. BIV:1 demonstrates 9.6% ± 1.7% levels of dystrophin
protein relative to control. (B) Western blot analysis on skeletal muscle from patients DI:1, EII:
1, FIII:6, and GII:1 against DYS1 shows very low levels of dystrophin. EII:1 FIII:6, and GII:1 show
undetectable levels of dystrophin. Loading controls: a-actinin-2 and sarcomeric actin. Male
controls used: C1, tibialis anterior, 16 years; C2, unknown, 5.5 years; C3, unknown, 14 years;
C4, quadiceps, 4.5 years. DMD = Duchenne muscular

dystrophy.
LINC00251 exons within the chr8 insertion. Sanger sequencing with bespoke PCR over the breakpoints on gDNA confirmed the chr8 inclusion in intron 43 and provided a diagnostic assay that confirmed segregation of the insertion within the family pedigree. Normal splicing of DMD exons 42-43-44-45 was observed as a low-frequency event (figure 2, B and D).

For CII:2, manual analysis of RNA-seq data identified abnormal inclusion of 84 nt from intron 26 into a majority of DMD transcripts (figure 3C). Sanger sequencing of the genomic region in gDNA from CII:2 identified a deep intronic variant GRCh37: ChrX:32471959C>A, c.3603+820G>T that was absent in gnomAD. The DMD c.3603+820G>T variant in intron 26 disrupts an AG, creating an AG-excision zone between an available consensus lariat branch point and 3’ splice site. Spliceosomal use of a naturally occurring consensus 5’ splice site sequence and this strengthened 3’ splice site result in the inclusion of a variant-activated pseudoexon into a majority of DMD transcripts, encoding 19 missense amino acids followed by a stop codon (figure 3C). RT-PCR confirmed abnormal inclusion of the variant-activated pseudoexon into DMD transcripts and residual, low levels of DMD transcripts with normal splicing of exons 25-26-27 (figure 2E). Sanger sequencing confirmed that the c.3603+820G>T variant was de novo in CII:2.

For DII:1, RNA-seq in a previous study showed low levels of DMD transcripts with exon 51 skipping, inducing a frameshift and premature stop codon encoded by exon 52 (r.7310_7542del, p.Ser2437Cysfs*33, figure 3D). Interrogation of WGS determined presence of a DMD structural rearrangement rendering DMD exon 51 in the reverse orientation and unable to be spliced into the DMD mRNA, confirmed by Sanger sequencing. RT-PCR confirms exon 51 skipping as the predominant mis-splicing event in DII:1, with skipping of exons 50 and 51 a low-frequency, in-frame event observed in both DII:1 and controls (figure 2F). Low levels of exon 50 and 51 skipping are consistent with low levels of dystrophin detected by WB analysis (figure 4C).

RNA-seq showed an abrupt loss of transcripts after exon 18 in EII:1, as previously described in reference 20 (figure 2). WGS showed evidence for an inversion within the DMD gene reversing the orientation of exons 1–18 of DMD, which are now joined to intergenic sequences upstream of exon 1, explaining the presence of abruptly terminating exon 1–18 transcripts transcribed from the DMD promoter (figure 3E). The 1.9 Mb intergenic region included in the inversion contains FAM47A, FAM47B, and TMEM47 genes. Sanger sequencing of genomic DNA over the breakpoints confirmed the inversion.

FII:6 with in-frame duplications of exons 31–37 and 43–44 identified on DMD MLPA, was shown by WGS to have a larger, more complex structural rearrangement (figure 3F), which reverses the orientation of exons 1–44 of DMD which are now joined to intron 45 of CFAP47, upstream of exon 1. Expression of CFAP47 is likely to be disrupted. However, the clinical significance of loss of CFAP47 expression is unknown.

In GII:1, RNA-seq in a previous study showed low read count for DMD transcripts, with evidence for even fewer reads from exon 60. Closer scrutiny of whole-genome sequencing data identified a structural rearrangement reversing the orientation of exons 1–60 of DMD, which are now joined to intergenic sequences upstream of exon 1 (figure 3G). Sanger sequencing of genomic DNA over the breakpoints confirmed the inversion.

**WB Analyses Define the Threshold of WT Dystrophin Conferring Clinical Phenotypes of Duchenne to Myalgia**

Our splicing studies reveal that AII:1, BIV:1, and CII:2 each have residual levels of normally spliced DMD transcripts, with abnormal splicing events apparently targeted for degradation by nonsense-mediated decay (figure 2, B–E). Therefore, these individuals uniquely provide an opportunity to quantify levels of WT dystrophin and correlate with clinical phenotype. Quantitative WB (figure 4) using skeletal muscle biospecimens reveals (1) ~15% ± 2% normal dystrophin levels in AII:1, correlating with a myalgia phenotype without apparent weakness; (2) ~10% ± 2% levels of dystrophin in BIV:1 (figure 4A) with Becker muscular dystrophy, mild weakness, and cardiac phenotype; and (3); 0%–5% levels of dystrophin in affected individuals who present with a severe Becker (CII:2) or Duchenne phenotype (DII:1, EII:1, FII:6, and GII:1) (figure 4, B and C).

**Discussion**

Our study further substantiates DMD splicing variants as an important causal basis for males presenting with symptoms consistent with a dystrophinopathy, for whom exomic sequencing approaches or MLPA return negative findings. A causal splicing variant in DMD was identified in all 7 families within our dystrophinopathy cohort and includes 13 affected males presenting with hyperCKemia with pain and/or muscle weakness and/or cardiac involvement.

Importantly, identification of the causative variant in DMD within this hard-to-diagnose cohort required deployment of WGS, RNA-seq and/or bespoke RT-PCR studies of mRNA isolated from skeletal muscle. For example, for CII:2, RNA-seq was crucial to identify the inclusion of an 84 base pair (bp) pseudoexon encoding a frameshift which prompted Sanger sequencing of this region, which lead to the identification of the casual intron 26 c.3603+820G>T variant, which was undetectable by gene panel testing, Sanger sequencing of the individual exons or MLPA. Although multiple genetic investigations are costly and not available currently to many diagnostic laboratories, costs incurred through muscle biopsy, WGS, or RNA studies are insignificant relative to the cost burden to health services for dystrophinopathy cases, for example, the heart transplantation for family B. A precise genetic diagnosis for an X-linked disorder has important and wide-reaching implications for genetic, prenatal, and prognostic counseling across the wider family unit and can inform
reproductive decision making. In addition, a genetic diagnosis could enable future customizable treatments such as splice-modulating antisense oligonucleotide drugs, which would theoretically be applicable to families A, C, and D.

Although WGS and RNA-seq bring powerful adjunct tests to clinical genomics, shortcomings of short read massively parallel sequencing were clearly observed in this study. As human exons are typically 100–150 bp in length, short-read RNA-seq is limited in that a single read does not effectively bridge multiple exons. Most significantly, RNA-seq for BIV:1, DII:1, EII:1, and GII:1 was confounded by the effectiveness of nonsense-mediated decay, an innate surveillance mechanism that degrades mRNA bearing a premature stop codon. We suspect that the reason we do not see a profound reduction in read depth for AII:1 (figure 2A) is due in part to higher read depth across the transcriptome, including DMD, and in part to the residual normal splicing of a significant proportion of DMD transcripts (27%). Nonsense-mediated decay amplifies inherent challenges associated with RNA-seq of very large mRNAs, where mRNA capture and sequencing library construction result in a characteristic bias in read depth, with vastly more reads at the 3’ end than the 5’ end of a very long mRNA. Notably, common disease genes in neuromuscular disorders are among the largest coding mRNAs in humans, with DMD mRNA ~14,000 nt, NEX mRNA ~50,000 nt, and TTN mRNA ~100,000 nt. Therefore, ribosomal RNA depletion and/or long read RNA-seq approaches, which display reduced 3’ bias, may be more effective for diagnosing neuromuscular disorders.

Regular data filtering approaches of genomic sequencing failed to identify most of the causal variants (excluding families A and C found on RNA-seq). This is likely due to the nature of the variants themselves (noncanonical splice affecting variants or structural variants), small read lengths, and mapping restrictions against the reference sequence. The structural rearrangement within DMD intron 43 of family B took extensive bioinformatic analysis to delineate, even when our RT-PCR (data not shown) and RNA-seq studies had indicated intron 43 as the likely location of the problem. Although (in retrospect) the copy number variation of the duplicated region of chr8 is evident, informatics approaches to map split reads to precisely define the breakpoints were challenging and ultimately required both informatics and Sanger sequencing of PCR amplicons to fully resolve. Of note, the bespoke PCR uniquely identifying the DMD intron 43 structural rearrangement was clinically preferred as the diagnostic test for segregation and carrier testing due to its greater specificity relative to the microarray to detect the chr8 copy number variation. The availability of a validated bespoke PCR also means that carrier females in this family could have prenatal diagnosis of male pregnancies.

Although families B, D, and G have cardiac involvement that is common in dystrophinopathy, families A, C, E, and F do not have reported cardiac symptoms and are being monitored for possible development of cardiac symptoms. The profound cardiac involvement in family B raises suspicion of potential differences in DMD pre-mRNA mis-splicing between cardiac and skeletal muscle activated by the insertion of 118 kb of Chr8 sequences containing the LINC00251 gene. It is plausible that the severe cardiac involvement in family B is due to more fully penetrant DMD mis-splicing in cardiac tissue compared with skeletal muscle. Unfortunately, no stored cardiac specimens were available for mRNA studies from other affected family members who had undergone transplant surgery. It is also possible that levels of inclusion of the frameshifting pseudoexon in family C may differ between skeletal muscle (and potentially between different skeletal muscles) and cardiac muscle.

In conclusion, we highlight DMD splicing variants as an important causal basis in individuals with a suspected dystrophinopathy who remain undiagnosed after exomic sequencing or MLPA approaches. Causative DMD variants identified in AII:1, BIV:1, and CII:2 that induce partial mis-splicing of DMD mRNA provided us with a unique opportunity; each affected individual produced varying levels of remnant, normally spliced DMD mRNA, with all mis-spliced transcripts encoding a premature stop codon and targeted by nonsense-mediated decay. Therefore, we were able to use quantitative WB to correlate levels of WT dystrophin with clinical severity. We establish a steep therapeutic range of WT dystrophin protein levels (figure 4A); with ~15% WT dystrophin associated with myalgia without apparent weakness, ~10% levels of WT dystrophin associated with Becker muscular dystrophy, mild weakness, and cardiac phenotype, and <5% WT dystrophin associated with a severe Becker or Duchenne-like phenotype. Our findings broadly concur with previous studies correlating levels of mutated dystrophin in BMD with clinical severity, supporting the notion of a functional redundancy within the spectrin-like repeats of the dystrophin rod domain. Of great relevance to international efforts to develop genetic therapies in DMD, our data provide compelling evidence that with early intervention, only fractional increases in levels of dystrophin are likely to result in clinical improvement.

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Disclosure
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Appendix (continued)

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WGS and RNA Studies Diagnose Noncoding \textit{DMD} Variants in Males With High
Creatine Kinase

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