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Importance of muscle biopsy to establish pathogenicity of *DMD* missense and splice variants

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Abstract

A precise genetic diagnosis of a dystrophinopathy has far-reaching implications for affected boys and their families. We present three boys with *DMD* single nucleotide variants associated with Becker muscular dystrophy presenting with myalgia, reduced exercise capacity, neurodevelopmental symptoms and elevated creatine kinase. The *DMD* variants were difficult to classify: AIII:1 a synonymous variant in exon 13 c.1602G>A, p.Lys534Lys; BIII:1 an essential splice-site variant in intron 33 c.4674+1G>A, and CII:1 a missense mutation within the cysteine-rich domain, exon 66 c.9619T>C, p.Cys3207Arg. Complementary DNA (cDNA) analysis using muscle-derived mRNA established splice-altering effects of variants for AIII:1 and BIII:1, and normal splicing in CII:1. Western blot analysis demonstrated mildly to moderately reduced dystrophin levels (17.6 – 36.1% the levels of controls), supporting dystrophinopathy as a probable diagnosis. These three cases highlight the diagnostic utility of muscle biopsy for mRNA studies and western blot to investigate *DMD* variants of uncertain pathogenicity, by exploring effects on splicing and dystrophin protein levels.

Keywords: Becker muscular dystrophy; Duchenne muscular dystrophy; mRNA studies; Splice variants; Muscle biopsy; Missense variants.

1. Introduction

Dystrophinopathies (Duchenne and Becker muscular dystrophies and X-linked dilated cardiomyopathy) are disorders of striated muscle in which dystrophin is absent, reduced or dysfunctional. Dystrophin is encoded by the giant *DMD* gene, spanning two megabases of chromosome Xp21 [1].

The causative genetic variant in *DMD* is found in 96% of Duchenne muscular dystrophy (DMD) cases and 82% percent of Becker muscular dystrophy (BMD) cases [2,3]. Around one third of mutations in *DMD* are *de novo* in the affected male proband [4,5]. The most common genetic variants within *DMD* are large deletions (approximately 70%) or duplications (10 - 14%); often encompassing numerous exons [5,6]. The remaining *DMD* cases involve small deletions or insertions of one or more bases causing a frameshift (3 - 4%), nonsense substitutions creating a premature stop

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codon (9 - 10%), or splice site mutations (2 - 3%) [5,6]. Pathogenic missense variants in *DMD* are comparatively rare (< 1%) [7,8], but have been identified within key functional domains, such as the N-terminal actin binding domain (ABD1, exons 1–8) where they are most commonly associated with the milder Becker muscular dystrophy phenotype [9,10], and the conserved ZZ β-dystroglycan binding domain (aa 3307–3354), which typically cause the more severe Duchenne muscular dystrophy phenotype [11]. Most missense variants are of uncertain significance, and assigning pathogenicity is difficult, especially considering that sarcoglycanopathies and other diseases involving the dystrophin-associated glycoprotein complex can cause secondary abnormalities in dystrophin [12].

Genetic diagnosis of a dystrophinopathy can be determined by multiplex ligation-dependent probe amplification (MLPA) or X-chromosome comparative genomic hybridization array (CGH array) to detect deletions or duplications, or, through parallel or targeted sequencing of *DMD* [13]. Massively parallel sequencing can reveal more difficult to interpret genetic variants, such as missense variants, putative splicing variants or structural rearrangements, which are becoming recognized as important rare causes of dystrophinopathy [5,14,15]. In the subset of individuals for whom a rare, segregating *DMD* variant is identified as a variant of uncertain significance, muscle biopsy remains an important diagnostic investigation to establish abnormal levels or size of dystrophin via immunohistochemistry and western blot, and provide *DMD* mRNA for analysis of abnormal pre-mRNA splicing [2,4,16].

Herein we describe three case reports of single nucleotide variants identified in *DMD* in three families with male probands presenting with myalgia and/or muscle weakness with elevated serum creatine kinase (CK). In two cases aberrant splicing was confirmed to result from the synonymous or splice-site variant, through targeted reverse transcription polymerase chain reaction (RT-PCR) of mRNA isolated from skeletal muscle. mRNA transcripts were normal in the third case, indicating that dystrophin levels were reduced by a different mechanism. These simple mRNA studies extend a growing body of evidence indicating a proportion of synonymous (or missense) variants cause splicing abnormalities and are therefore pathogenic more commonly than is currently recognized. If the splicing pattern is normal, other mechanisms for pathogenesis must also be considered, as illustrated by case 3.

2. Results

2.1. Family A

The male proband AIII:1 from Family A presented at age 10 years with a persistently elevated serum creatine kinase (CK) of 19,372 U/L (normal levels < 200 U/L) and myalgia with exercise, but no associated weakness. He had a history of mild speech delay and was diagnosed with autism spectrum disorder at the age of 14 years and major depression at 16 years of age. At 18 years of age, he experiences muscle cramps when exercising for longer than one hour. His-power remains normal. He has mild tendoachilles and hamstring contractures. Electrocardiography and echocardiograms have been normal. Family history revealed that the maternal grandfather (AI:1, Fig. 1A) was diagnosed with Becker muscular dystrophy (with superimposed inflammatory myositis) after presenting at the age of 50 with mild limb-girdle muscle weakness and a modestly elevated CK. AI:1 was noted to have large calves and reported difficulty playing sport in childhood. Muscle biopsy from AI:1 showed patchy dystrophin staining and rimmed vacuoles.

MLPA did not identify any deletions or duplications. Sanger sequencing for AIII:1 identified a variant in *DMD* (GRCh37 chrX:32613874C>T, NM_004006.2:c.1602G>A, p.Lys534Lys); the last base of exon 13, initially reported as, 'a variant of uncertain significance'. Segregation analysis identified the same variant in AI:1 and AII:2. Alamut Visual® v2.9.0 splicing prediction programs MaxEntScan and NNSPLICE predict this synonymous variant abolishes the donor splice site; Human Splicing Finder (HSF) and SpliceSiteFinder-like (SSF) predicted weakening of the donor site (−11.5% and −14.4% respectively). Muscle histopathology showed two focal areas of myofibre destruction associated with histiocytic infiltrate and some myofibre size variation. Immunohistochemistry demonstrated mildly reduced antibody staining against DYS1 (rod domain) and DYS3 (C-terminal), normal staining for DYS2 (N-terminal), and reduced and patchy labeling for gamma-sarcoglycan (Fig. 1B). Reduced levels of dystrophin were confirmed by western blot (Fig. 1C, 31.8 ± 5.2% levels observed in controls, of normal molecular weight). RT-PCR studies of extracted mRNA showed clear evidence of *DMD* splicing abnormalities (Fig. 2A). Primers in exons 11 and 15 amplified a smaller cDNA product for AIII:1 compared to an age-matched control, with Sanger sequencing consistent with in-frame skipping of exon 13 (abnormal exon 13 skipping was also confirmed in amplicons from exons 12–14, *not shown*). Exon 13 skipping causes an in-frame deletion within the central rod domain of the encoded DMD protein; p.Val495_Lys534del. No normal-splicing of exons 12–13–14 was observed in muscle mRNA isolated from AIII:1, using a forward primer bridging exons 12 and 13 (Fig. 2A, this primer will anneal only to normally-spliced cDNA). We did not detect evidence for elevated levels of intron-13 retention in AIII:1 (using intron 13 primers, *not shown*). Upon review of the mRNA studies and western blot analysis, c.1602G>A was re-classified using ACMG criteria as a likely pathogenic variant [17].

2.2. Family B

BIII:1 was found incidentally to have a persistently elevated serum CK of 5397 U/L at 2.5 years of age during investigation for restless sleep. There was no family history of weakness or myalgia. At 13 years of age, he can swim

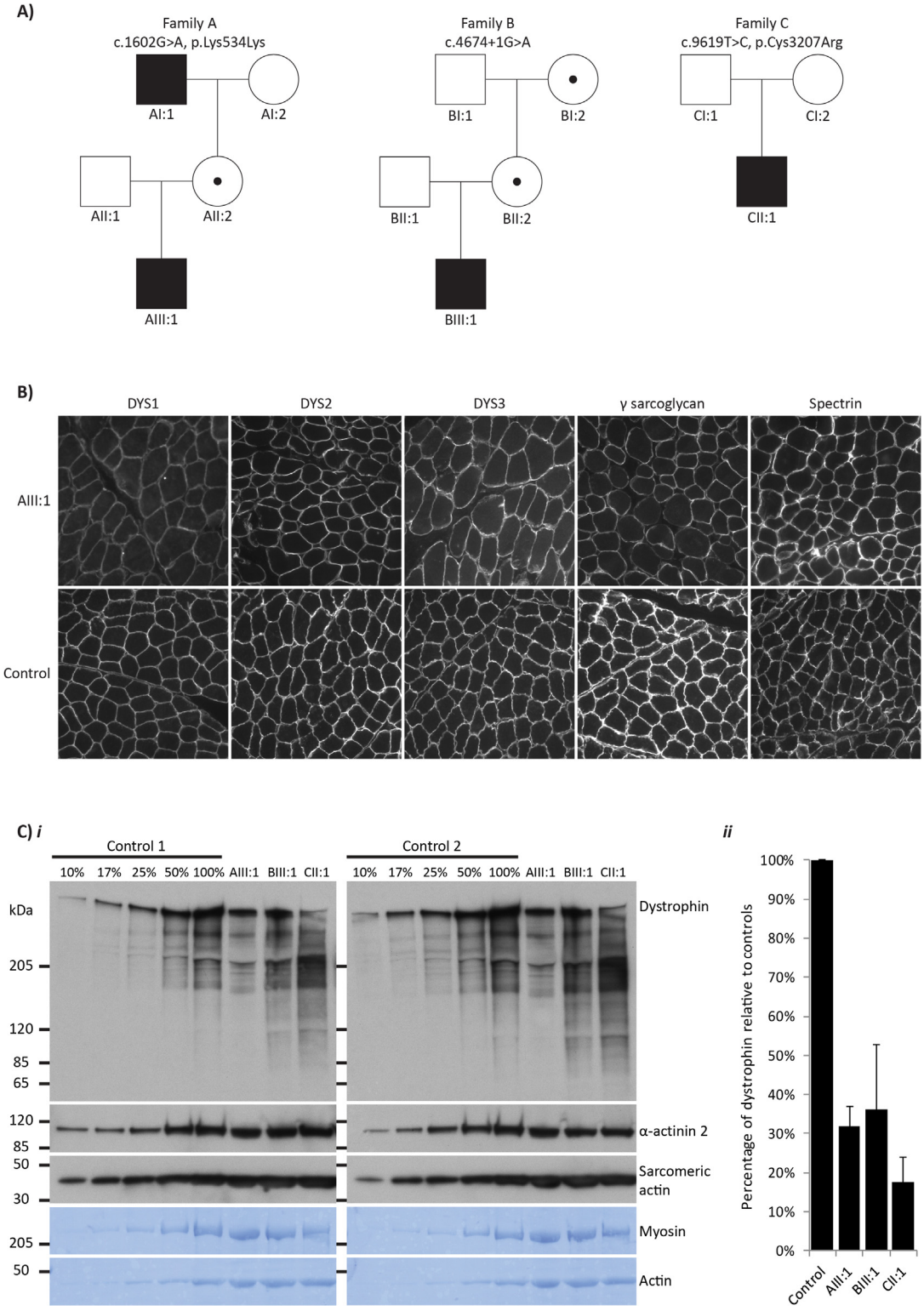

Fig. 1. (**A**) Pedigrees for family A, B and C, with the *DMD* variant numbered as per NM_004006.2. Carriers for the *DMD* variant are denoted with a black dot. Segregation data for family A was unavailable, thus AII:2 is presumed a carrier. (**B**) Immunohistochemical staining with antibodies directed against dystrophin (DYS1, DYS2 and DYS3), γ sarcoglycan and spectrin. Staining with DYS1 and DYS3 was abnormal. Staining with DYS2 was normal. γ sarcoglycan showed secondary patchy staining. (**C**) (**i**) Western blot confirmed a mild-moderate reduction in dystrophin levels in skeletal muscle from the probands (AIII:1, BIII:1, CII:1), consistent with Becker muscular dystrophy. 10 μg total protein was loaded for each proband, alongside a standard curve of 1–10 μg total protein from two skeletal muscle controls (Control 1 - male, 16 years; Control 2 – male, 14 years). Levels of α-actinin-2, sarcomeric actin, and Coomassie staining for myosin and actin, demonstrate protein loading. (**ii**) The levels of dystrophin in patients relative to control standard curves in four replicate gels, with error bars showing the standard deviation between gels. The relative densities of the dystrophin and myosin bands were determined using ImageJ for each gel. The standard curve was used to quantify the levels of dystrophin relative to controls, which were normalised to the myosin loading control.

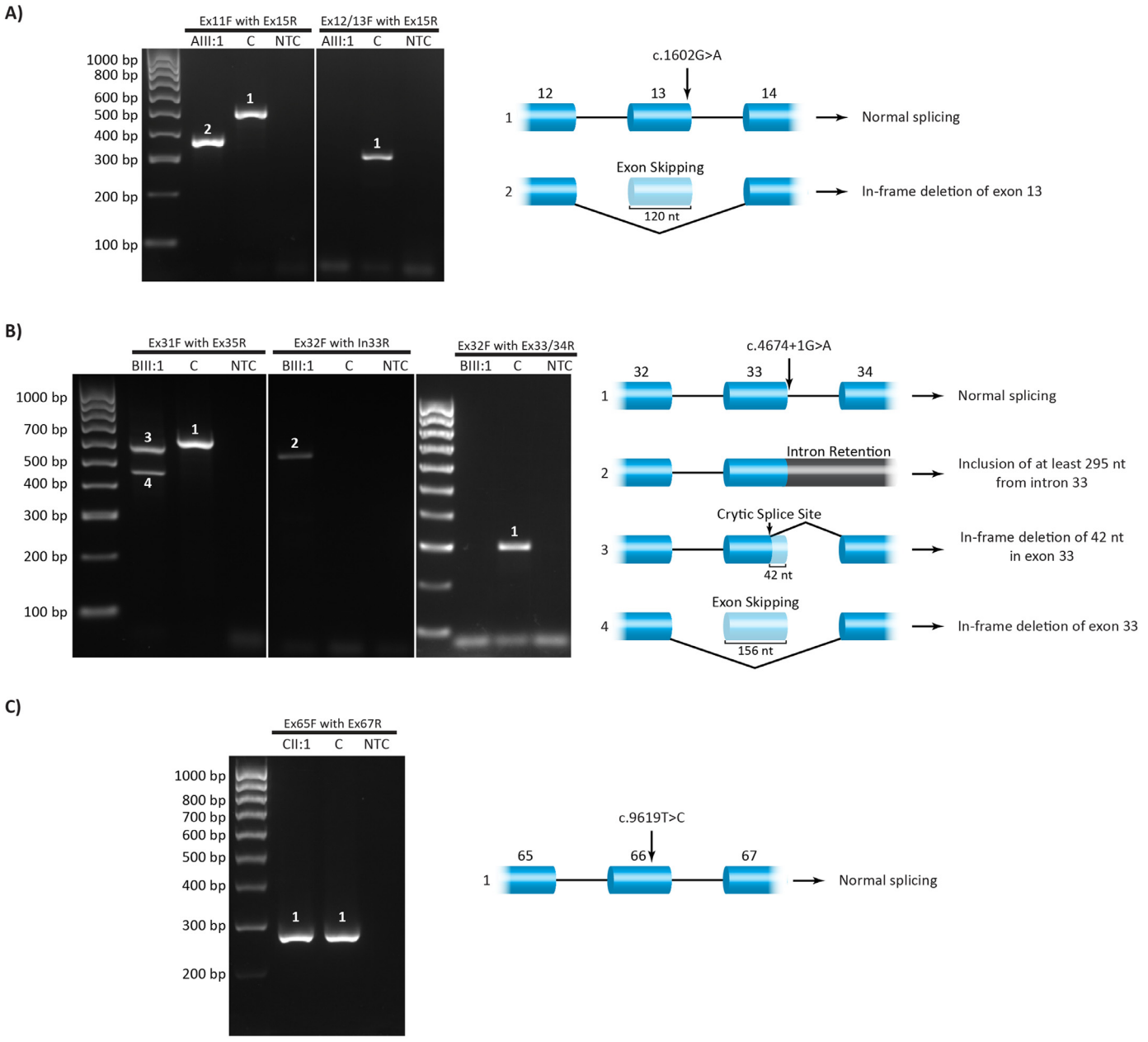

Fig. 2. RT-PCR of mRNA extracted from muscle for probands AIII:1, BIII:1 and CII:1, with *DMD* mRNA consequences illustrated. **A)** AIII:1 - Using primers in exons 11 and 15 of *DMD*, a 482 bp band corresponding to correctly spliced *DMD* mRNA can be seen in the control. In contrast, for AIII:1 the c.1602G>A variant induces exon 13 skipping, resulting in a 120 bp decrease in product size (362 bp). No normal product was detected in AIII:1 using a forward primer bridging exon 12 and exon 13 (to specifically amplify normally-spliced *DMD* mRNA) with a reverse primer in exon 15. **B)** BIII:1 – primers in exons 31 and 35 of *DMD* amplified two products for BIII:1; shorter than the expected 597 bp correctly-spliced *DMD* product seen in the control. Sanger sequencing of the shorter amplified products revealed use of a cryptic splice site in exon 33 resulting in an in-frame deletion of 42 bp (555 bp product), and exon 33 in-frame skipping (441 bp). RT-PCR using primers in exon 32 and intron 33 (to amplify products with intron 33 retention) amplified a 569 bp band for BIII:1, absent from the control, and confirmed by Sanger sequencing to correspond to intron 33 retention which encodes a stop codon. A reverse primer bridging exon 33 and exon 34 (to specifically amplify normally-spliced *DMD* mRNA) amplified a 291 bp product in the control, but not in BIII:I, suggesting negligible levels of normal splicing in BIII:1. (**C**) CII:1 - primers in exons 65 and 67 of *DMD* amplified the same sized products (280 bp) for CII:1 as the control. Sanger sequencing of the amplified products revealed normal splicing of *DMD* pre-mRNA.

for up to 45 min before complaining of myalgia. BIII:1 has some inattentiveness but does not meet diagnostic criteria for attention deficit hyperactivity disorder. Cognition and cardiac investigations are normal. On examination he has 4+/5 power in his proximal upper and lower limbs with a negative Gowers sign.

MLPA did not identify any deletions or duplications. *DMD* Sanger sequencing identified a splice site mutation in *DMD* (GRCh37 chrX:32404426C>T,

NM_004006.2:c.4674+1G>A, intron 33). This variant was initially reported as, 'a splice site mutation, predicted to lead to altered mRNA splicing of dystrophin'. Alamut Visual® v2.9.0 predicted ablation of the 5′ donor splice site of intron 33 using SSF, MaxEntScan, NNSPLICE and HSF. Segregation analysis revealed that his mother (BII:2) and maternal grandmother (BI:2) are both carriers of the same variant. BIII:1 muscle immunocytochemistry reported antibody staining against DYS1, DYS2 and DYS3 as normal. Histopathology was reported to show some variation in fibre size and possible mild increase in internal nuclei (not shown). Western blot demonstrated reduced levels of dystrophin protein to 36.1 ± 16.5% the levels of controls, of normal molecular weight (Fig. 1C). RT-PCR studies showed normal splicing of exons 32–33–34 was not observed in muscle mRNA (Fig. 2B). Three abnormal splicing events were detected: (1) Use of a cryptic splice donor in exon 33, inducing loss of 42 nucleotides from the *DMD* mRNA, and deletion of 13 amino acids from the encoded rod-domain of dystrophin protein; (2) In-frame exon 33 skipping, and deletion of 52 amino acids from the encoded rod-domain of dystrophin; (3) Elevated levels of intron 33 retention within spliced *DMD* mRNA transcripts, resulting in a frameshift p.Thr1560Cysfs*4. Upon review of the mRNA studies and western blot analysis, c.4674+1G>A was re-classified using ACMG (American College of Medical Genetics and Genomics) criteria as a pathogenic variant [17].

2.3. Family C

CII:1 was found to have elevated serum creatine kinase >700 U/L at eight years of age during investigations for learning difficulties and attention deficit hyperactivity disorder. His-developmental milestones were mildly delayed. He walked at 18 months of age, with only single words at age two years. At 25 years, he has moderate intellectual disability and some obsessive-compulsive traits. He walks long distances without myalgia. Mild cardiomyopathy was detected in his early 20′s. On examination there was no calf hypertrophy and power was normal. MLPA testing did not detect any deletions or duplications. *DMD* Sanger sequencing identified a *de novo* missense variant in exon 66 (GRCh37 chrX:31224729A>G, NM_004006.2:c.9619T>C, p.Cys3207Arg). This mutation was not previously reported but was interpreted as, 'very likely to be pathogenic' based on its position within the cysteine-rich domain, in which other mutated cysteine residues have been associated with DMD. *In silico* splicing prediction software did not predict aberrant splicing. Histopathology showed mild Type II atrophy (not shown). Immunoperoxidase studies for Dystrophin 1, 2 and 3 were normal (not shown). Western blot (DYS1) demonstrated reduced dystrophin levels to 17.6 ± 6.4% the levels of the controls (Fig. 1C). RT-PCR studies of mRNA extracted from skeletal muscle showed normal splicing of *DMD*, using primers located in exons 65 and 67 (Fig. 2C). This result was confirmed with a second set of primers in exons 64 and 68 (not shown). We did not detect evidence for elevated levels of intron-65 or intron-66 retention in CII:1 (using intron 65 or 66 primers, *not shown*). Western blot and mRNA studies confirmed the variant c.9619T>C, p.Cys3207Arg was pathogenic through a mechanism other than aberrant splicing, causing reduced dystrophin levels consistent with Becker muscular dystrophy. Upon review of the mRNA studies and western blot analysis, c.9619T>C was re-classified using ACMG criteria as a pathogenic variant [17].

3. Discussion

These three cases illustrate the challenges in diagnosing boys with potential dystrophinopathies due to single nucleotide variants in *DMD* causing missense substitutions or splicing abnormalities, and the importance of muscle biopsy for accurate diagnosis [18]. Interpretation of potential splicing variants is difficult, and affected boys are at risk of remaining undiagnosed [5,6,14]. With the recent explosion in genomic medicine, geneticists commonly turn to *in silico* predictive algorithms, which effectively predict adverse consequences of essential splice site variants (affecting the almost invariant GT and AG at either end of an intron) [19], but have demonstrable weaknesses in their abilities to accurately predict consequences of extended splice site variants and variants creating cryptic splice sites in either exons or introns [20,21]. Existing algorithms such as those offered within Alamut Visual® biosoftware can offer mixed predictions, and it is difficult to derive a clinically meaningful prediction of pathogenicity from a diminution in splice site strength.

While the increased statistical likelihood that a *de novo* missense variant in a phenotypically consistent gene is sufficient in some cases to enable classification as likely pathogenic, missense variants in large muscle genes (*DMD*, *TTN*, *NEB*) are particularly challenging to interpret [22,23]. In the cases described, each at the mild end of the BMD spectrum, evidence from muscle pre-mRNA splicing studies and western blots showing reproducible abnormalities in dystrophin supported their (re)classification as likely/pathogenic variants.

The missense variant p.Cys3207Arg identified in CII:1 lies within the EF hand domain of the cysteine-rich domain, which facilitates interaction between the WW domain of dystrophin and β-dystroglycan [11]. The effect of the missense substitution on dystrophin function is uncertain, though reduced dystrophin levels suggest the mutation leads to protein instability. The synonymous splice variant p.Lys534Lys detected in AIII:1 was impossible to interpret without RNA studies, which confirmed exon 13 skipping with no evidence of normal splicing, as demonstrated by Hagiwara for a different substitution at the same nucleotide [24]. For BIII:1, three abnormal splicing events were detected that evoked different in-frame or out-of-frame consequences for the encoded protein.

In all three cases immunohistochemistry failed to provide compelling evidence for dystrophin abnormalities, but quantitative western blot reproducibly demonstrated a mild-moderate reduction in dystrophin levels, with four repeat

western blots (using standard curves from two controls) confirming the subtle reduction in dystrophin levels.

While RNA sequencing is emerging on the diagnostic horizon, we show that clinically meaningful results can be conferred by established RT-PCR approaches. However, it is important to acknowledge the limitations of amplification-based approaches; you detect only what your primers amplify. Primer design must probe specifically for different abnormal splicing events; exonic primers to probe for exon skipping or use of cryptic splice sites, coupled with intronic primers to probe for intron retention. Technical consideration must be applied to minimize caveats associated with nonsense-mediated decay and PCR amplification bias for shorter (exon-skipping) versus longer (intron-retention) amplicons.

A precise genetic diagnosis of dystrophinopathies has far-reaching implications for carrier testing and genetic counseling, cardiac surveillance, and informing prognosis and treatment [20]. Genetic testing has replaced muscle biopsy analysis for diagnosis of many dystrophinopathies. However, in a small but important proportion of cases, current analysis methods may not detect clinically significant splice variants, complex rearrangements, or reliably infer likely pathogenicity of missense variants and a muscle biopsy is needed. We advocate that a high index of suspicion is maintained for any *DMD* variant identified in boys presenting with myalgia and elevated CK, with or without associated weakness, particularly in the presence of neurocognitive disorders. This group is often difficult to diagnose, but establishing a diagnosis has important clinical implications [25].

Beyond the immediate clinical management, an accurate genetic diagnosis will be increasingly valuable in the era of targeted genetic therapies. Boys with *DMD* splice-altering variants could benefit from personalized-medicine in the form of morpholino-based therapies, to mask an out-of-frame cryptic splice site and/or promote an in-frame abnormal splicing event to reinstate or elevate levels of dystrophin [26,27].

In conclusion, we demonstrate the clinical utility of *DMD* mRNA studies and dystrophin western blot analysis to enable a confirmed genetic diagnosis of a pathogenic *DMD* splice or missense variant in three cases with male probands presenting with myalgia, reduced exercise capacity, neurodevelopmental symptoms and an elevated CK.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2019.09.013.

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