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Clinical phenotypes as predictors of the outcome of skipping around *DMD* exon 45

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Abstract

Objective—Exon-skipping therapies aim to convert Duchenne muscular dystrophy (DMD) into less severe Becker muscular dystrophy (BMD) by altering pre-mRNA splicing to restore an open reading frame, allowing translation of an internally deleted and partially functional dystrophin protein. The most common single exon deletion – exon 45 (Δ45) – may theoretically be treated by skipping of either flanking exon (44 or 46). We sought to predict the impact of these by assessing the clinical severity in dystrophinopathy patients.

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Methods—Phenotypic data including clinical diagnosis, age at wheelchair use, age at loss of ambulation, and presence of cardiomyopathy was analyzed from 41 dystrophinopathy patients containing equivalent in-frame deletions.

Results—As expected, deletions of either exons 45-47 (Δ45–47) or exons 45-48 (Δ45-48) result in BMD in 97% (36/37) of subjects. Unexpectedly, deletion of exons 45-46 (Δ45-46) is associated with the more severe DMD phenotype in 4/4 subjects despite an in-frame transcript. Notably, no patients with a deletion of exons 44-45 (Δ44-45) were found within the UDP database, and this mutation has only been reported twice before, which suggests an ascertainment bias attributable to a very mild phenotype.

Interpretation—The observation that Δ45-46 patients have typical DMD suggests that the conformation of the resultant protein may result in protein instability or altered binding of critical partners. We conclude that in DMD patients with Δ45, skipping of either exon 44 or multi-exon skipping of exons 46 and 47 (or exons 46-48) are better potential therapies than skipping of exon 46 alone.

Keywords

Dystrophinopathy; exon skipping; in-frame mutation; ambulation

BACKGROUND

The progressive muscle diseases Duchenne (DMD) and Becker muscular dystrophy (BMD) both result from mutations in the *DMD* gene, which encodes the dystrophin protein. DMD is associated with mutations that interrupt the messenger RNA (mRNA) open reading frame, resulting in severe muscle weakness and, typically, death before the age of 30[1, 2]. In contrast, BMD is typically associated with mutations that maintain the open reading frame, producing an internally altered but partially functional dystrophin protein, with an intact C terminal domain[1, 3]. BMD patients have variable phenotypes, but are less severely affected, and have a much longer to normal life expectancy[3]. Antisense oligonucleotides (AONs), a promising experimental line of molecular therapy for DMD, aim to restore gene expression by altering splicing at the pre mRNA level. These AONs hybridize to specific target sequence and can lead to skipping of a targeted exon[4], resulting in restoration of an open reading frame, production of an internally truncated dystrophin protein, and conversion of the DMD phenotype to BMD. AONs are currently in phase 2 clinical trials for treatment of deletion mutations by exon 51 skipping[5, 6].

Exon 45 is the most common single exon deletion. In nearly all cases, this mutation is associated with DMD rather than BMD, including in 100% of 13 Δ45 patients in a recent United states series[7]; in 97% (60 of 62) of patients in the UMD-DMD database[7, 8]; in 15 of 18 (83%) of the patients with defined phenotypes in the United Dystrophinopathy Project database [9]; and in 93% (263 of 284) of the patients with defined phenotypes in the Leiden database (http://www.dmd.nl/)[10]. Exon-skipping therapies intended to treat Δ45 patients are currently in development, and multiple strategies to restore the reading frame exist for these patients. In addition to skipping of either exon 44 or 46, multi-exon skipping of 46-47 or 46-48 would restore the reading frame, and recent studies using adeno-associated virus

(AAV) mediated gene delivery of U7 small nuclear ribonucleoproteins (snRNPs) have shown efficient in-vivo skipping of up to three consecutive exons[11]. However, not all internally truncated proteins are expected to be equivalent in function, or equivalent in expression.

To assess the likely outcomes of exon skipping therapies for DMD patients with Δ45, we utilized the United Dystrophinopathy Project (UDP) database of genotype, phenotype, and natural history data, to analyze patients with equivalent in frame deletions of exons 44-45, 45-46, 45-47, or 45-48.

METHODS

Subject Ascertainment

All patients were enrolled in the United Dystrophinopathy Project (UDP), which began enrollment in 2004; the last of these patients enrolled in June 2012. The UDP is a database of genotype, phenotype, and natural history data assembled by a multicenter consortium of neuromuscular physicians. Patient entry into the UDP is described elsewhere[9]. Briefly, patient entry required a dystrophinopathy diagnosis based on clinical features of DMD or BMD along with confirmation by an X-linked family history, muscle biopsy, or *DMD* gene testing. All enrolled patients underwent *DMD* gene testing. Based upon age at loss of ambulation (LOA), patients were classified as DMD (LOA by age 12), intermediate muscular dystrophy (LOA between ages 12 and 15), or BMD (LOA after age 15). Patients who had not yet lost ambulation were classified based upon an expert clinician diagnosis (as described elsewhere) [9], taking into account age at presentation, clinical history in affected family members, steroid treatment, and muscle biopsy results (when available). Although clinical categorization by these criteria may not be perfect, unpublished data from the UDP suggest that revision of the original diagnosis occurs in less than 2% of cases. Dystrophinopathy phenotype analysis included ambulatory status, wheelchair use, cardiomyopathy, use of ventilatory support, steroid use and scoliosis surgery.

Cell Culture, Transdifferentiation, and RNA Analysis

Primary fibroblast cell lines from one patient were established following skin biopsy and immortalized via infection with two lentiviruses either containing human telomerase (hTER) or doxycycline-inducible MyoD construct. Following treatment of cultures with doxycycline (4μg/ml) for three days, transdifferentiation is evident by morphology and expression of myogenic genes, including dystrophin. For analysis of splicing, total RNA was isolated by standard techniques (Trizol, Life Technologies) for reverse transcription (Maxima reverse transcriptase, Thermo Scientific) followed by PCR (PCR Master Mix, Promega) using primer pairs located in exon 42 and exon 49.

RESULTS

We identified 41 male patients enrolled in the UDP database carrying in-frame deletions equivalent to a DMD patient with Δ45 receiving exon skipping therapy (Table 1). These genotypes included Δ45-46 (n=4), Δ45-47 (n=17), and Δ45-48 (n=20). No patients were identified with Δ44-45. All 4 patients with Δ45-46 carried a diagnosis of DMD. In contrast,

all 17 patients with Δ45-47, 19/20 patients with Δ45-48 carried a diagnosis of BMD, and the remaining patient was diagnosed with IMD.

The ages at examination and at loss of ambulation are represented in Figure 1. The mean age at last examination of the four patients with Δ45-46 was 10.5 years. Two had lost ambulation at ages 10 and 11.5 (mean = 10.75 years); the remaining two were ambulant at the last examination at ages 9.8 and 10.1. Among the Δ45-47 patients, the mean age at last examination was 30.8 years (range, 7 to 53.1), and only one patient (6%) had entirely lost ambulation, at the age of 38.5 years. Among the Δ45-48 patients, the mean age at last examination was 31.3 years (range, 4.5 to 65.3). Only 4 of 20 (20%) were non-ambulant, having lost ambulation at the mean age of 49.3 years (range, 34–62).

Patients were evaluated for cardiomyopathy, defined as an ejection fraction of less than 55% or a shortening fraction less than 28%. Echocardiograms were available for two of the four Δ45-46 patients, neither of whom had evidence of cardiomyopathy when echocardiograms were performed at 8.3 or 8.4 years of age. Nine Δ45-47 patients had echocardiograms, two of whom (22%) had been diagnosed with cardiomyopathy at an average age of 26.9 years; the average age of Δ45-47 patients without cardiomyopathy was 22.9 years. Nine of the Δ45-48 patients had echocardiograms, four of whom (44%) were diagnosed with cardiomyopathy at an average age of 48.0 years, whereas the average age of Δ45-48 patients without cardiomyopathy was 29.5 years.

We sought to evaluate exon splicing in Δ45-46 DMD patients. None had archived muscle tissue specimens available, and only one had an archived fibroblast specimen. Following MyoD transformation of the primary fibroblast cell line, RT-PCR analysis of *DMD* mRNA using primers located in exon 42 (forward) and exon 49 (reverse) revealed no evidence of alternatively spliced transcripts, and only the expected in-frame transcript was detected.

DISCUSSION

To deduce the potential therapeutic efficacy of an exon skipping therapy for Δ45 DMD patients, we searched the UDP database for patients with the equivalent in-frame mutations. One such group of patients carried Δ45-46 and all were diagnosed with DMD, representing an exception to the reading frame rule. Such exceptions are relatively common, as only 63% of patients with in-frame, non-truncating mutations carry a diagnosis of BMD or intermediate muscular dystrophy (IMD)[9].

These exceptions have been attributed to mutations located in indispensible areas of the protein, such as the cysteine-rich or actin binding domains, or due to alternative splicing events that create out-of-frame transcripts[10, 12]. At least seven alternatively spliced transcripts involving exons 44-58 have been identified in wild type skeletal muscle, and 5 novel alternative splicing events were activated in skeletal muscle of BMD patients carrying deletions within the rod domain, a mutational hot-spot of the *DMD* gene[12, 13]. UDP patients with Δ45-46 might deviate from the reading frame rule because of alternative splicing of additional exons, resulting in an out-of-frame transcript. For example, an out-of-frame transcript would be generated in Δ45-46 patients by splicing of exon 44, which is easily

skipped and is often spontaneously spliced out when surrounding exons are deleted in experimental systems[14, 15]. Unfortunately, muscle tissue from any of the four UDP patients with Δ45-46 was not available for mRNA or protein studies, limiting our analysis to *DMD* mRNA obtained from MyoD-transformed fibroblasts from one of these subjects; although a single sample presents only limited evidence, this model did not show such alternate splicing.

Another possible explanation for the severe phenotype seen in Δ45-46 patients could be due to involvement of the binding site for neuronal nitric oxide synthase mu (nNOSμ) which binds spectrin-like repeats 16 and 17 and is encoded by exons 42-45 in the *DMD* gene. In BMD patients with in-frame deletions of exons 45-55, the severity of muscle weakness and histopathology was associated with cytosolic mislocalization of nNOSμ[16]. However, muscle from 12 patients with BMD due to Δ45-47, Δ45-48, or Δ45-49 showed levels of sarcolemmal nNOS staining to actually be lower than levels in muscle from 9 DMD patients with mutations treatable by skipping of exons 44 or 45[17]. These results argue strongly against differential nNOS binding as being the likely explanation for the comparable severity of the Δ45-46 phenotype.

We explored whether an alternative explanation might lie in the phasing of the spectrin repeats resulting from the in-frame transcript. We note that although most of the patients in our cohort had not lost ambulation, the mean age at loss of ambulation in the Δ45-48 cohort was comparable to that found in the same genotypic cohort of Nicolas et al (47 years)[18], in which differences in age at LOA and onset of cardiomyopathy among subjects with Δ45-47, Δ45-48, and Δ45-49 mutations were shown to be associated with stability of the resultant peptide. In particular, those mutations resulting in the formation of fractional as opposed to hybrid spectrin repeats were associated with a more severe phenotype. However, the modeling of the proteins resulting from Δ44-45 and Δ45-46 predicts that both should result in comparable stable hybrid repeats (Fig. 2).

These observations have therapeutic implications. For patients with isolated Δ45, skipping of either exon 44 or exon 46 would restore an open reading frame, and AONs to skip either exon are in development. One study tested AON-induced skipping of exon 46 in primary myoblasts from patients with a deletion of exon 45, justifying their strategy by suggesting that “exon (45+46) deletions cause only a mild form of BMD,” although no source was provided for this information[19]. In fact, 11 of 22 (50%) patients reported with deletions of exons 45-46 have been described as DMD[9, 10, 20–27], and consistent with this, protein studies have been reported in a single Δ45-46 patient which revealed less than 5% of normal dystrophin staining on muscle biopsy[22]. Even allowing for possible differences among authors in applying dystrophinopathy classification, the frequency of the DMD phenotype in association with Δ45-46 suggests that skipping of exon 46 is a poor treatment option for patients with exon 45 deletions and – given the presence of some clinical heterogeneity in Δ45 patients – has s the potential to actually worsen a given patient’s outcome.

An alternative strategy for restoring the reading frame in patients with Δ45 is to skip exon 44. The equivalent in-frame mutation, Δ44-45, was not present in the UDP database and has been reported within the literature only twice[10, 21]. One of the patients with Δ44-45 was

labeled as DMD in a review of Leiden database patients carrying mutations that do not follow the reading frame rule[10], but analysis of the original article in which the patient was first reported indicated that no definitive diagnosis was given[28]. The second patient with Δ44-45 was given a diagnosis of DMD, but no diagnostic criteria or clinical data were provided[21]. Furthermore, the patient was genotyped using multiplex PCR which may not characterize the extent of deletions as accurately as multiplex ligation dependent probe amplification (MLPA), as it does not interrogate all exons. Since deletions beginning in intron 43 as well as deletions ending in intron 45 are both common breakpoints found in *DMD* deletions[8–10], a cold spot for deletion formation is an unlikely explanation for the paucity of Δ44-45 patients. Two cases of Δ45 patients with such alternative splicing of exon 44 in muscle have been reported. The first patient carried a diagnosis of DMD and was found to have Δ44-45 transcript expressed at less than 1% of normal levels, hypothesized to be enough to create revertant fibers but insufficient to alter the patient's phenotype[29] A second Δ45 patient remained ambulant until the age of 18 and was found to express a Δ44-45 alternatively spliced transcript at 6% of the total DMD mRNA, suggesting this level of in-frame transcript was sufficient to change the patient's phenotype to a milder form[30]. Interestingly, the Δ44-45 dystrophin protein is very similar to wild type dystrophin with regards to folding thermodynamics and resistance to proteolysis[31]. We interpret the absence of Δ44-45 patients in our UDP cohort, which we expanded to more than 1100 subjects[9] – and the near complete lack of Δ44-45 patients within all dystrophinopathy databases – to suggest that individuals with this mutation may be asymptomatic and do not seek medical attention.

Other treatment strategies include multi-exon skipping of exons 46-47, or 46-48. Although multi-exon skipping with AONs has been challenging[15], efficient skipping of these exact exons has been achieved in mice using AAV-delivered U7 snRNPs[11]. Among published reports, only 3.8% (13 of 342) Δ45-47 patients and 5.18% (13 of 251) Δ45-48 patients were diagnosed as DMD[9, 10, 20–27]. Consistent with these reports, all 17 Δ45-47 and 19 of the 20 Δ45-48 patients within the UDP database had been diagnosed with BMD, and no significant differences in age at loss of ambulation or detection of cardiomyopathy were found for these patients.

The observations in this large cohort should be of interest in the design of future exon skipping approaches. They allow us to predict that in patients with isolated deletions of exon 45, skipping of exon 44 will result in a significantly better outcome than skipping of exon 46. Similarly, our data suggest that multi-exon skipping of either exons 46-47 or 46-48 is likely to result in a better treatment response than skipping of exon 46 alone.

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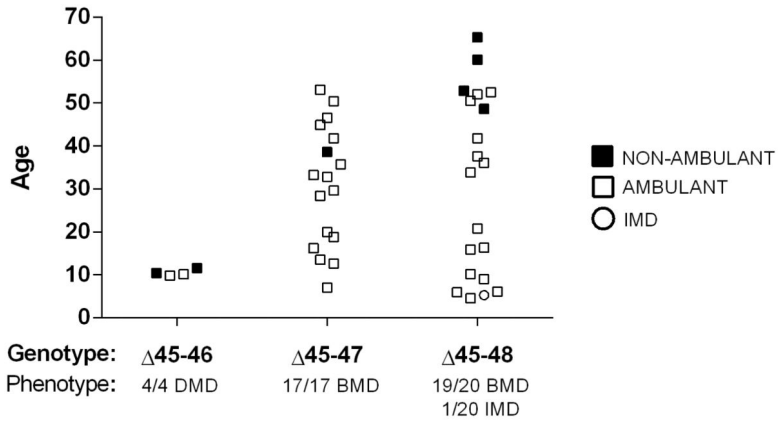

Figure 1. *DMD* genotypes vs. age at last evaluation

The clinical diagnosis of patients is noted below each genotype class. Non-ambulant patients are marked by filled squares.

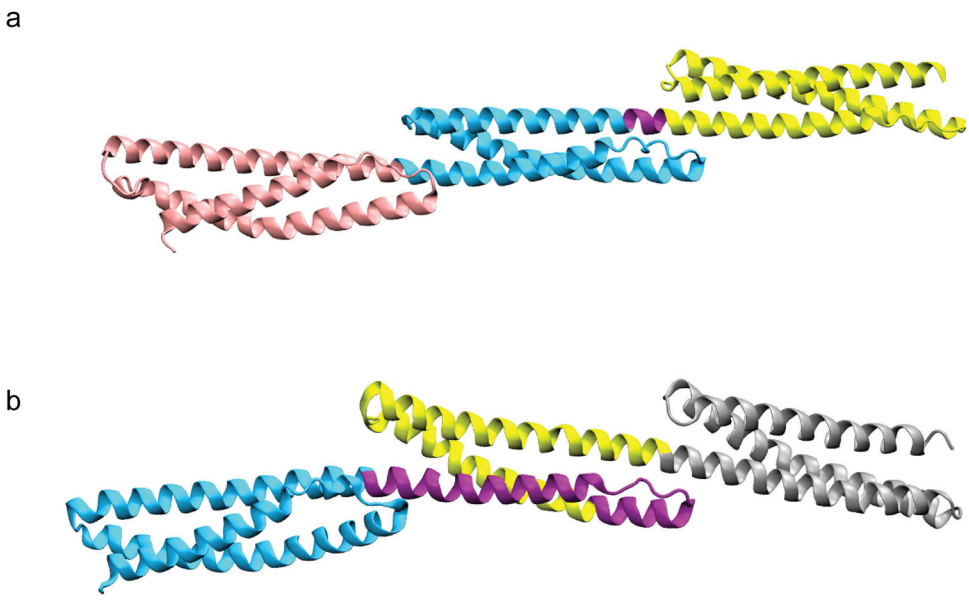

Figure 2. Molecular modeling of dystrophin proteins predicted to result from deletions of (A) exons 44-45 and (B) exons 45-46

Each is predicted to result in a hybrid repeat that contains three helices in a coiled-coil pattern, as opposed to a fractional repeat, which would be predicted to be less stable. **(A)** Δ44-45 is composed of R15 (pink), part of R16 (blue) and part of R17 (purple), R18 (yellow). **(B)** Δ45-46 is composed of R16 (blue), part of R17 (purple) and part of R18 (yellow), R19 (grey).

Table 1

Summary of natural history data for patients included in the study

Age at which full or part-time wheelchair use occurred is noted in parentheses. **ΔDp140**: + marks patients in which a deletion of the Dp140 isoform promoter was detected; for all others, this promoter (found in intron 44) was present. **Clinical diagnosis** is the "expert clinician" diagnosis as discussed in the text. **Age** is the age at last evaluation/data collection. **Ambulation:** use of wheelchair (WC) and age at which used in years. **mMRC**: The mean Medical Research Council (mMRC) consists of the modified MRC score for each muscle examined, converted to a numerical (0–10 point) scale. The standard UDP evaluation grades 34 muscles. These values are summed and divided by the number of muscles actually examined. **LVEF**: Left ventricular ejection fraction (LVEF%) is shown for those patients for whom the data was provided, and the age at which it was obtained is shown in years. For patients with multiple echocardiograms, the LVEF% value and the date provided are for either the *first abnormal*, or if no abnormal results were seen, the *most recent normal* recorded echocardiogram.

Patient	Genotype	ΔDp140	Clinical Diagnosis	Age (y)	Ambulation Status (age at WC use in y)	mMRC	LVEF (%)	Age at LVEF (y)
1	Δ45-46		DMD	9.8	WC part-time (8)			
2			DMD	10.1	WC part-time (7)	8.2	60	8.4
3			DMD	10.4	WC full-time (10)	5.8	61	8.3
4			DMD	11.6	WC full-time (11.5)	4.9		
5	Δ45-47		BMD	7.0	Ambulant	6.6		
6			BMD	12.6	Ambulant	7.0	67	12.2
7			BMD	13.5	Ambulant	7.0	65	13.1
8			BMD	16.2	Ambulant	7.6	69	16.2
9			BMD	18.8	Ambulant	6.3	57	18.8
10			BMD	20.0	Ambulant	7.2	70	20.0
11			BMD	28.4	Ambulant	8.0	20	24.6
12			BMD	29.6	Ambulant	7.0	30	29.2
13			BMD	32.8	Ambulant	8.4		
14			BMD	33.2	ambulant			
15			BMD	35.7	Ambulant	7.4	62	38.5
16		+	BMD	38.6	WC full-time (38)	6.3		
17			BMD	41.8	Ambulant	6.9	57	41.8
18			BMD	44.9	Ambulant	8.1		
19			BMD	46.6	Ambulant	6.7		
20		+	BMD	50.4	Ambulant	7.5		

Patient	Genotype	ΔDp140	Clinical Diagnosis	Age (y)	Ambulation Status (age at WC use in y)	mMRC	LVEF (%)	Age at LVEF (y)
21			BMD	53.1	WC part-time (48)	7.8		
22			BMD	4.5	Ambulant			
23			IMD	5.3	Ambulant	7.0		
24			BMD	5.9	Ambulant	7.6	63	6.6
25			BMD	6.1	Ambulant	6.6		
26			BMD	9.0	Ambulant	7.6		
27			BMD	10.2	Ambulant	6.3	70	10.2
28			BMD	15.9	WC part-time (8)	8.4		
29		+	BMD	16.3	Ambulant	9.3		
30			BMD	20.8	Ambulant	9.4		
31	Δ45-48		BMD	33.8	Ambulant	7.8	59	34.8
32			BMD	36.1	Ambulant			
33			BMD	37.6	Ambulant	7.8	59	36.6
34			BMD	41.8	Ambulant	6.8		
35			BMD	48.6	WC full-time (34)	5.7	48	39.2
36			BMD	50.5	Ambulant			
37			BMD	52.0	WC full-time (48)	4.5	50	47.3
38			BMD	52.5	Ambulant	6.0	22	52.5
39			BMD	52.9	Ambulant	6.1	50	52.9
40			BMD	60.1	WC full-time (53)	5.5	71	59.3
41			BMD	65.3	WC full-time (62)	5.1		60.0