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Clinical and Genetic Characterization of Manifesting Carriers of *DMD* Mutations

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Abstract

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Participating Study Sites in the United Dystrophinopathy Project consortium: The Participating study sites are University of Utah (Salt Lake City, Utah), Nationwide Children's Hospital (Columbus, Ohio), Washington University (St. Louis, Missouri), Children's Hospital of Philadelphia (Philadelphia, Pennsylvania), Children's Hospital of Cincinnati (Cincinnati, Ohio), University of Iowa (Iowa City, Iowa), and University of Minnesota (Minneapolis, Minnesota).

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Manifesting carriers of *DMD* gene mutations may present diagnostic challenges, particularly in the absence of a family history of dystrophinopathy. We review the clinical and genetic features in fifteen manifesting carriers identified among 860 subjects within the United Dystrophinopathy Project, a large clinical dystrophinopathy cohort whose members undergo comprehensive *DMD* mutation analysis. We defined manifesting carriers as females with significant weakness, excluding those with only myalgias/cramps. DNA extracted from peripheral blood was used to study X chromosome inactivation patterns. Among these manifesting carriers, age at symptom onset ranged from 2 to 47 years. Seven had no family history and eight had male relatives with Duchene muscular dystrophy (DMD). Clinical severity among the manifesting carriers varied from a DMD-like progression to a very mild Becker muscular dystrophy-like phenotype. Eight had exonic deletions or duplications and six had point mutations. One patient had two mutations (an exonic deletion and a splice site mutation), consistent with a heterozygous compound state. The X chromosome inactivation pattern was skewed toward nonrandom in four out of seven informative deletions or duplications but was random in all cases with nonsense mutations. We present the results of *DMD* mutation analysis in this manifesting carrier cohort, including the first example of a presumably compound heterozygous *DMD* mutation. Our results demonstrate that improved molecular diagnostic methods facilitate the identification of *DMD* mutations in manifesting carriers, and confirm the heterogeneity of mutational mechanisms as well as the wide spectrum of phenotypes.

Keywords

manifesting carriers; dystrophinopathy; *DMD*; dystrophin; X-chromosome inactivation; Duchenne muscular dystrophy; Becker muscular dystrophy

Introduction

Mutations in the *DMD* gene, which encodes dystrophin, result in Duchenne muscular dystrophy (DMD), the milder Becker muscular dystrophy (BMD), or X-linked dilated cardiomyopathy (XLDC). Most heterozygous female carriers of *DMD* mutations are asymptomatic; however, between 2.5 and 7.8% of these carriers are manifesting carriers (MCs) who develop symptoms ranging from mild muscle weakness to a rapidly progressive DMD-like muscular dystrophy[1–3]. Dystrophin labeling of muscle biopsies from MCs shows a mosaic pattern, with some fibers having continuous membrane immunostaining, other fibers uniformly unstained, and some fibers with discontinuous or partial dystrophin staining; these abnormalities are often seen in a nonuniform distribution, with groups of normal and abnormal fibers[4].

The diagnosis of a dystrophinopathy carrier state may be considered on clinical grounds alone in the setting of a clear X-linked family history of muscular dystrophy. However, mutational analysis of the *DMD* gene is typically required for diagnosis, particularly in the absence of a family history. Use of modern molecular diagnostic methods facilitates the identification of MCs without a positive family history of dystrophinopathy (“isolated” MCs), who may account for around 10% of women with hyperCKemia and a myopathic muscle biopsy[5], and some of whom may have in the past been diagnosed as having autosomal recessive limb-girdle muscular dystrophy or unknown myopathy[6].

Here we describe a series of genetically confirmed MCs identified from subjects enrolled in the United Dystrophinopathy Project (UDP). Our data expand the mutational spectrum associated with manifesting carriers (including the first example of a presumably compound heterozygous *DMD* mutation in an isolated MC), demonstrate that skewed XCI is present in 38% of cases, and highlight the need for a high index of clinical suspicion among neuromuscular clinicians.

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Methods

Patient selection/clinical data ascertainment

We reviewed all records in the United Dystrophinopathy Project (UDP) database to identify only subjects with an identified mutation in the *DMD* gene. The UDP is a seven-center consortium to prospectively study the mutational mechanisms, clinical features and genotype/phenotype relationships in dystrophinopathies. Enrollment requires a clinical history compatible with dystrophinopathy, and at least one of the following criteria: an X-linked family history of muscular dystrophy; a muscle biopsy that shows absent or altered expression of dystrophin by immunohistochemical, immunofluorescent, or immunoblot analysis; or a previous mutation analysis report showing a *DMD* mutation. All females were enrolled in order to obtain mutation analysis because they were symptomatic. Details regarding the phenotypic evaluation are included in Supplemental Table 1.

We defined patients as MCs if they had muscle weakness in at least one muscle group by manual muscle testing, or dilated cardiomyopathy. Our definition of MCs is similar to previously published criteria, although in contrast to previous studies we did not use dynamometry results in isolation to establish inclusion criteria[7, 8]. Asymptomatic carriers or carriers with daily muscle cramps/myalgias but no muscle weakness were excluded from our analysis. The severity of phenotype was first categorized according to clinical course. We designated subjects as “DMD-like” if the clinical progression was similar to that of DMD in males. Subjects who began using a wheelchair by age 30 were designated “severe BMD-like” and those still walking independently after 30 years of age were grouped as “mild BMD-like”. If the phenotype was milder than DMD but the patient was too young to be assigned to either mild or severe categories, only the term “BMD-like” was used. In addition to designating the clinical course, we defined the current degree of muscle weakness, independent of patient age, at the most recent examination. We relied on functional descriptions (eg., wheelchair use) or the description of expert clinicians to label the current degree of weakness as “mild”, “moderate”, or “severe”, as noted in table 1.

Mutation analysis

Under an Institutional Review Board approved protocol, DNA extracted from peripheral blood underwent mutational analysis using a combination of either single condition amplification/internal primer (SCAIP) direct sequencing analysis, MLPA analysis, or both, using conditions as previously described[9]. MLPA was performed using Salsa MLPA kit (MRC-Holland, Inc; Amsterdam). For selected patients, archived muscle specimens were obtained, and messenger RNA was extracted for reverse-transcription PCR and sequencing of the *DMD* cDNA using published primers[10]. Nucleotide positions were determined according to the standard *DMD* reference sequence (GenBank accession number NM_004006).

X chromosome inactivation (XCI) analysis

Methylation of the highly polymorphic *Hpa*II restriction endonuclease site in the androgen-receptor (*AR*) locus correlates with XCI. We used *Hpa*II digestion followed by PCR to determine the methylation status of both the maternal and paternal X chromosomes in lymphocyte-derived DNA[11, 12]. In this method, parental alleles are distinguished based on the difference between the numbers of CpGs in the CpG island within the *AR* locus. Alleles that are active will be digested while the inactive alleles are not. The ratio of undigested parental alleles gives the pattern of inactivation. If the assay does not differentiate the maternal and paternal alleles, the result is reported as “uninformative”. As per published criteria, XCI ratios of less than or equal to 80:20 were considered “random” pattern, ratios greater than 80:20 but less than or equal to 90:10 were considered “moderately skewed” pattern and ratios greater than 90:10 were considered “highly skewed” pattern[13].

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Results

We identified fifteen manifesting carrier females among 860 UDP subjects with an identified *DMD* mutation. Clinical and genetic data for each MC subject are shown in table 1. Eight patients had a relative with the diagnosis of DMD and seven had no family history of dystrophinopathy (isolated MCs). Age at onset of symptoms varied from 2 to 47 years (median: 8; mean: 14.9). Muscle weakness was the most common presenting symptom (reported in 80% of the cases), followed by myalgia and/or muscle cramps (reported in 60%). One subject (#8) had a phenotype as severe as a typical DMD boy, while other patients showed a mild to severe BMD-like phenotype.

Clinically notable asymmetry in muscle weakness was seen in three subjects in whom there were at least two muscle groups with left-right differences of at least 2 grades in mMRC scale (one grade in MRC scale): subject #4, in which left versus right differences for knee extensors, shoulder abductors, shoulder external rotators, elbow flexors, elbow extensors, and thumb abductors were ≥2 grades in the mMRC scale (≥1 grade in MRC scale); subject #11, who showed ≥2 mMRC grade differences in her elbow flexors and wrist flexors; and subject #14, with ≥2 mMRC grade difference in hip flexion and ankle dorsiflexion. Based on mean intra-individual right-left difference in mMRC scores for each muscle group, muscle weakness in the lower extremity tends to be more symmetric than in the upper extremity (Supplemental Table 1).

Dilated cardiomyopathy was seen in five subjects, two with severe BMD-like phenotypes (subjects #4 and #6) and three with mild BMD-like symptoms (#5, #12, and #14). One severe BMD-like subject (#4) had a rapid decline in cardiac function over one year, with an ejection fraction that dropped from 56% at the age of 28 to 41% at 29 years old (with a fractional shortening of 13%). One mild BMD-like subject (#12) had a postpartum cardiomyopathy that improved with treatment over 18 months (with a rise in ejection fraction from 45% to 65%). A sixth subject (#11; mild BMD-like) had cardiac symptoms but not a dilated cardiomyopathy: she had syncopal episodes due to a drug-resistant bradytachycardia syndrome and required a cardiac pacemaker implantation at the age of 62 years, but cardiac catheterization and echocardiography were within normal limits at age 68 years.

Muscle biopsy was performed in all seven isolated MC subjects, while it was done in only three of eight patients with an affected DMD relative. Clinical biopsy reports are summarized in Supplemental Table 2; archived tissue was not available for most subjects, making standardized analysis impossible. Commonly reported histopathologic findings included variation in fiber size, increased internal nuclei, necrosis and regeneration, and endomysial fibrosis. Inflammatory changes were reported in three isolated MCs, in two of whom the diagnosis of an inflammatory myopathy was initially considered. Dystrophin immunohistochemistry studies usually revealed a mosaic pattern with some fibers lacking or partially expressing dystrophin and others showing normal dystrophin labeling; severely diminished dystrophin expression was seen in two patients. Biopsy of one subject with a severe BMD-like phenotype (#6) was reported to be entirely negative for staining using amino-terminal, carboxy-terminal, and rod-domain antibodies to dystrophin. Another subject (#8) was reported to have a mosaic pattern on immunohistochemical analysis, with the majority of fibers absent of staining but others showing a various degree of staining (Figure).

By definition, all subjects had mutations detected. The results of the mutation analysis and XCI studies are presented in table 1. XCI studies were informative in 14/15 subjects (93%), one of whom had a compound heterozygous mutation; skewed XCI was found in 5 of the remaining 13 (38%). None of the four subjects with nonsense mutations (all of whom had a mild BMD-like phenotype) had skewed XCI, and subjects with *DMD* point mutations (including nonsense,

splice, and subexonic mutations) appeared more likely to have a random XCI pattern compared to those with *DMD* exon deletions/duplications; however, this correlation did not reach statistical significance (p value = .266 by Fisher's Exact test). Both subjects with a 100:0 XCI pattern had normal karyotypes. One subject (#6) had two disease alleles identified: an out-of-frame deletion of exons 8–13, and a splice site mutation in intron 69, predicted to affect the splice donor site of exon 69. A DNA sample from her mother was obtained and tested using the same methods but revealed no mutations; no sample was available for analysis from her father.

Discussion

Our data demonstrate the utility of modern diagnostic methods in the clinical characterization of manifesting carriers; among 15 subjects, seven had point mutations that were detected by direct sequencing methods. We also demonstrate that in the absence of a family history of dystrophinopathy, muscle biopsy remains the common method of diagnosis, and suggest that in the presence of a family history it is appropriate for the clinician to consider genetic testing prior to biopsy. In addition, in the absence of a family history other findings associated with dystrophinopathy may be more likely to be misinterpreted; for example, after an incidental detection of elevated transaminases, subject #7 underwent liver biopsy at the age of 9 years before myopathic symptoms and the detection of an elevated creatine kinase (CK) level led to her muscle disease work-up. Carriers with a BMD relative are less frequently and less severely affected than carriers with a DMD relative[2, 3, 8, 14], which may explain why even though the UDP enrolls probands affected by either DMD or BMD, the proband had DMD for all eight of the MCs with a family history.

Consistent with previous reports[7, 14], the spectrum of clinical presentations in MCs was quite wide, ranging from a rapidly disabling DMD-like phenotype (subject #8) to a very mild late-onset presentation (subject #11). Later onset of symptoms suggests less severe disease; four patients (subjects #11, 12, 14, and 15) developed symptoms after age 25, and all were categorized as mild BMD-like severity at examination 5 to 34 years after symptom onset (table 1). However, earlier age at symptom onset was not necessarily associated with a more severe phenotype; subjects #2 and #9, for example, had onset of symptoms by the age of 8, but were still classified as a mild BMD-like phenotype into their fourth or fifth decade. Most MCs presented initially with mild proximal lower extremity weakness and/or myalgia/muscle cramps. Muscle weakness was usually expressed as having difficulty climbing stairs or running. On manual muscle testing (MMT), weakness might be subtle and initially detectable only in one or two proximal lower extremity muscle groups, and none of our subjects showed weakness only in the arms, in contrast to a previous report[8]. Asymmetry in muscle weakness has previously been described in between 15% and 81% of MCs[5, 8]. The higher number[8] may reflect the use of hand-held dynamometry to compare right-left muscle groups. Asymmetry can also be detected by skeletal muscle MRI imaging[15], and may be related to somatic mosaicism[16]. Among our subjects, asymmetric muscle weakness was clinically notable in only 3/9 (33%) of patients with available detailed muscle strength data. Among the remaining 6 subjects, with limited or no detailed muscle examination data, asymmetric features were not reported.

As in DMD and BMD, cardiomyopathy should always be considered in manifesting carriers. Consistent with a previous report that up to 36% of MCs may have echocardiographic evidence of cardiac dysfunction[17], we found that 5 out of the 13 (38%) subjects who had available echo reports showed evidence of impaired systolic function (shortening fraction <28% or ejection fraction <55%). A decline of cardiac function in carriers may be relatively acute, or related to pregnancy: subject #4 showed a 15% drop in her ejection fraction in one year (28–29 years old), and subject #12 developed a postpartum cardiomyopathy that improved with medical

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treatment. Although not seen in isolation among the cohort we report, cardiomyopathy may be the only clinical manifestation in *DMD* carriers; therefore, dystrophinopathy should be considered in the differential diagnosis of female patients with idiopathic cardiomyopathy[18, 19].

Immunolabeling of muscle biopsies with anti-dystrophin antibodies was reported to show scattered or patchy presence of fibers with reduced or absent dystrophin in all biopsied subjects. No association is expected between the degree of altered dystrophin expression and clinical variables including strength and serum CK[7] or clinical course[14, 20], and dystrophin expression can vary in different muscle groups of *DMD* carriers[20, 21]. Nevertheless, we note that the two subjects reported to have either no appreciable dystrophin expression (subject #6) or a majority of fibers unstained (#8) were in the more severe clinical phenotype category (severe BMD-like and DMD-like).

We report what is to our knowledge the first example of a manifesting carrier with presumably compound heterozygous *DMD* mutations: a deletion of exons 8–13 and an intron 69 splice site mutation (c.10086+2T>C). The out-of-frame exon 8–13 deletion has been reported in association with both DMD and BMD phenotypes[3], whereas the c.10086+2T>C has been included in the Leiden database (www.dmd.nl) as DMD. Genetic analysis of the subject's mother did not reveal either mutation, and despite repeated efforts we could not obtain a blood or tissue sample from her father. No further archived muscle tissue was available for mRNA analysis, and the family declined repeated requests for a skin biopsy. Allele-specific genotyping was not feasible due to unavailability of an archived muscle biopsy sample, and the long genomic distance between the two identified mutations. Given her random XCI pattern, her severe BMD-like phenotype, and the absence of appreciable dystrophin staining on her biopsy, we assume that her two *DMD* mutations lie in *trans* rather than in *cis*. In the absence of a family history, and given her father's apparently normal phenotype, they presumably occurred as *de novo* germ line mutations. Our genetic results otherwise confirm that *DMD* mutational mechanisms in MCs are heterogeneous. Our database is enriched for point mutations[9], so we presume that the actual relative frequency of MCs with point mutations is lower in the general population than in our data set, and equal to the distribution of *DMD* mutations in non-referral cohorts[22].

Nonrandom X-chromosome inactivation (XCI) pattern has been proposed as an explanation for the development of symptoms in manifesting carriers (MCs) without chromosomal translocations[14, 20, 23]. In peripheral blood, most women in the population have a random XCI pattern; only 8% have a skewed pattern of >80:20[13]. The ratio of peripheral blood XCI is significantly correlated with other tissues[24, 25], including muscle[24], although cases exist in which muscle and peripheral blood XCI results diverge[23]. Because skewed XCI can explain the development of symptoms in many instances[20, 23, 26], we expected to find skewed XCI in some portion of our subjects. Indeed, one subject (#8) had 100% skewed XCI, in accordance with her severe DMD-like phenotype and the absence of dystrophin in the majority of muscle fibers by immunohistochemistry (Figure). However, excluding the single subject with an apparent compound heterozygous mutation, we observed nonrandom XCI in 5/13 informative subjects (38%). Our results are consistent with a previous study that showed both highly skewed and completely random XCI patterns in carriers[23]. Unfortunately, insufficient archived muscle tissue was available with which to determine inactivation in muscle at the AR locus.

The lack of a clear correlation between phenotype and the XCI pattern as detected in peripheral blood might be attributed to several factors. First, the assumption that the methylation status of the androgen receptor (AR) locus reflects that of the *DMD* locus on the X chromosomes in muscle tissue might not be correct; furthermore, the phase between the *DMD* and androgen receptor loci has not been established in these patients, such that we cannot formally know

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which *DMD* allele has been inactivated. Second, the pattern of XCI as ascertained from lymphocyte DNA may not reflect the XCI pattern in muscle in all patients[23]. Third, the phenotype may be influenced by early clonal expansion of a skewed XCI precursor cell population in muscle. Finally, myotube formation requires interactions among multiple myoblasts, and muscle is made up of multinucleated cells. The functionality of a muscle fiber may depend in part upon the percentage of non-random XCI nuclei in a fiber, or the distribution pattern of these nuclei within it.

Our data raise the question of a correlation between mutation class and XCI pattern in symptomatic carriers. For example, all four subjects with nonsense DMD mutations (all of whom showed a relatively mild BMD-like phenotype) had random XCI patterns. In contrast, among seven informative subjects with deletions or duplications, only three had a random XCI pattern. We note that the definition of non-random XCI is arbitrary, and that some studies define XCI that equals 80:20 as non-random (rather than a value greater than 80:20)[27, 28]. If our two subjects with a ratio of 80:20 are categorized as non-random, the correlation between mutation category (duplications/deletions versus point mutations) and XCI reaches statistical significance among the informative subjects (p value = 0.029 by Fisher's Exact Test). However, if we make the assumption that the uninformative subject #5 has random XCI, the significance of the correlation disappears. In summary, our data fail to demonstrate a significant association, and we are unaware of a model in which a given mutation class on a disease-causing X chromosome would have the effect of inducing inactivation at an X-chromosome locus in *trans.* Whether an actual correlation exists will need to be addressed in future studies with larger numbers of subjects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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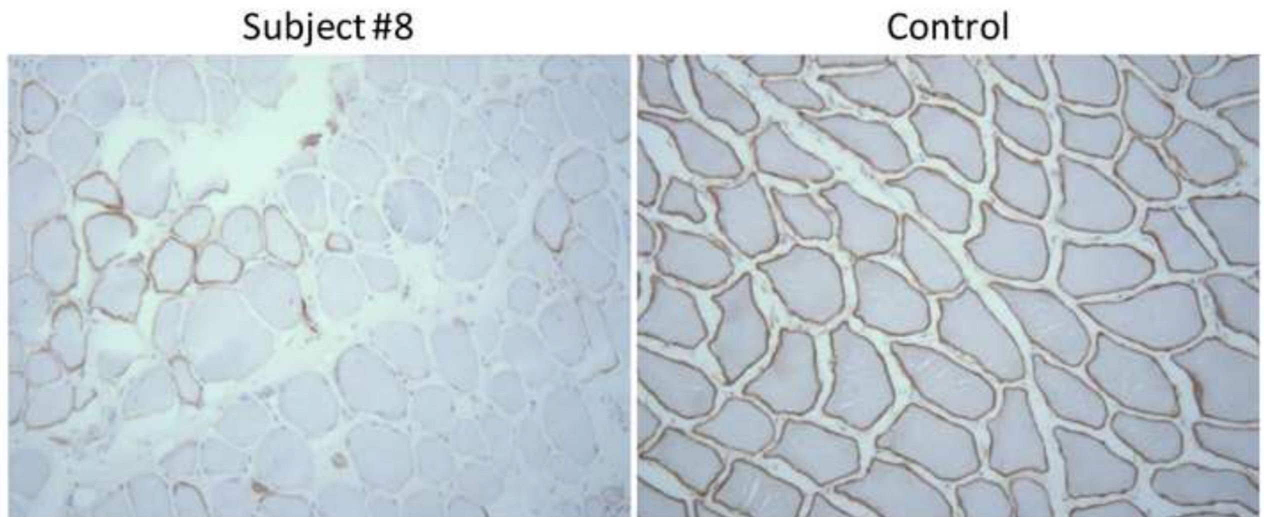

Figure.

Representative fields from clinical muscle biopsy (from 2002) of the quadriceps muscle in a DMD manifesting carrier with 100% skewing of X chromosome inactivation (subject #8), and a control skeletal muscle. Immunohistochemical staining was performed under a clinical protocol using as a primary antibody Mab1645 (Chemicon), raised against a recombinant full-length protein. There is a mosaic pattern of dystrophin expression, but the majority of fibers do not express dystrophin.

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Table 1

Clinical and genetic features of 15 manifesting carriers of *DMD* mutations. **Cardiac status**: the age for either the most recent normal, or first known abnormal echocardiogram are given. Left ventricular cardiac function is reported by clinical laboratories using either fractional shortening (FS%; normal ≥28%) or ejection fraction (EF%; normal ≥55%); the abnormal value reported by the clinical lab is included in the table. **X-inactivation**: Non-random (skewed) X-chromosome inactivation results are in bold; highly skewed results are in bold italics. **n/a** = not applicable; **n.d.** = not done.

Patient (ID#)	Severity	Related proband phenotype	Presenting symptoms/ signs (age at onset in years)	Age (y)/ambulation status at most recent evaluation	Weakness severity (average mMRC)at most recent exam	Cardiac status	Karyotype	X-inactivation pattern	DMD Mutation
1 (DR43784)	BMD-like	n/a	weakness; myalgia (16)	17/walking unaided	moderate	-	n.d.	***90:10***	deletion exons 45–52 (c. 6439-?_7660+?del; out of frame)
2 (DR43600)	Mild BMD-like	DMD	weakness, myalgia/ cramping (5)	44/walking unaided	mild (9.53)	-	n.d.	**86:14**	deletion exon 50 (c.7201-?_7309+?del; out of frame)
3 (DR43940)	too young to be determined	DMD	weakness (2)	6.5/walking unaided	Mild (8.66)	normal echo at age 6.5	normal	***100:0***	deletion exons 46–49 (c. 6615-?_7200+?del; out of frame)
4 (DR43412)	Severe BMD-like	n/a	weakness, myalgia/cramping, pseudohypertrophy (3)	31/part-time wheelchair use since age 28	severe (5.85)	cardiomyopathy diagnosed at age 26 (echo results unavailable)	n.d.	73:27	deletion exons 51–57 (c. 7310-?_8547+?; out of frame)
5 (DR43779)	Mild BMD-like	n/a	developmental delay, weakness (childhood[<5])	31/walking unaided	mild	cardiomyopathy (FS=21% at age 32)	normal	Uninformative	deletion exons 61–79 (c. 9085-?_*2691+?)
6 (DC0117)	Severe BMD-like	n/a	weakness (3)	15/part-time wheelchair use	severe	cardiomyopathy (FS=25% at age 15)	normal	52:48	(i) deletion exons 8–13 (c. 650-?_1602+?del; out of frame) (ii) splice intron 69 (c.10086+2T>C)
7 (DC0242)	BMD-like	n/a	weakness, myalgia (9)	15/walking unaided	mild	normal echo at age 16	n.d.	**80:20**	duplication exon 43 (c. 6118-?_6290+?dup; out of frame)
8 (51455)	DMD-like	n/a	weakness, pseudohypertrophy (7)	12/nonambulant since age 10	severe (4.68)	normal echo at age 13	normal	***100:0***	duplication exons 17–18 (c.l993-?_2292+?dup; predicted out of frame)
9 (DR43334)	Mild BMD-like	DMD	weakness, myalgia/ cramping (8)	38/walking unaided	mild (9.85)	normal echo at age 35	n.d.	**80:20**	duplication exons 45–59 (c.6439-?_8937+?dup; predicted out of frame)
10 (DR43107)	BMD-like	n/a	myalgia/cramping (6)	10/walking unaided	mild	normal echo at age 8	n.d.	**85:15**	splice insertion intron 26 (c.3603+2dupT; r. [spl?])
11 (DR43680)	Mild BMD-like	DMD	weakness (47)	68/walking unaided	mild (8.35)	drug-resistant bradytachycardia syndrome, on pacemaker at age 62	n.d.	77:23	stop exon 14 (c.1615C>T; p.Arg539X)

Patient (ID#)	Severity	Related proband phenotype	Presenting symptoms/ signs (age at onset in years)	Age (y)/ambulation status at most recent evaluation	Weakness severity (average mMRC)at most recent exam	Cardiac status	Karyotype	X-inactivation pattern	DMD Mutation
12 (DC0036)	Mild BMD-like	DMD	weakness, myalgia/cramping (27)	34/walking unaided	mild (8.85)	postpartum cardiomyopathy (EF=45%) improved (EF=65%) after 18 months of therapy	n.d.	52:48	stop exon 15 (c.1783G>T; p.Glu595X)
13 (DR43352)	Mild BMD-like	DMD	myalgia/cramping, pseudohypertrophy (19)	47/walking unaided	mild (9.85)	normal echo at age 45	n.d.	51:49	stop exon 47 (c.6906G>A; p.Trp2302X)
14 (DC0307)	Mild BMD-like	DMD	weakness, myalgia/cramping (40)	47/walking unaided	mild (7.35)	cardiomyopathy (EF=51%at age 45)	n.d.	54:46	stop exon 61 (c. 9100OC>T; p.Arg3034X)
15 (42359)	Mild BMD-like	DMD	weakness (30)	64/nonambulant since 57 yo	severe	normal echo at age 57	n.d.	70:30	subexonic insertion exon 8 (c.782_783insT; p.Lys261Xfs)

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