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Duchenne Muscular Dystrophy and Becker Muscular Dystrophy Confirmed by Multiplex Ligation-Dependent Probe Amplification: Genotype-Phenotype Correlation in a Large Cohort

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Background and Purpose Studies of cases of Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) confirmed by multiplex ligation-dependent probe amplification (MLPA) have determined the clinical characteristics, genotype, and relations between the reading frame and phenotype for different countries. This is the first such study from India.

Methods A retrospective genotype-phenotype analysis of 317 MLPA-confirmed patients with DMD or BMD who visited the neuromuscular clinic of a quaternary referral center in southern India.

Results The 317 patients comprised 279 cases of DMD (88%), 32 of BMD (10.1%), and 6 of intermediate phenotype (1.9%). Deletions accounted for 91.8% of cases, with duplications causing the remaining 8.2%. There were 254 cases of DMD (91%) with deletions and 25 (9%) due to duplications, and 31 cases (96.8%) of BMD with deletions and 1 (3.2%) due to duplication. All six cases of intermediate type were due to deletions. The most-common mutation was a single-exon deletion. Deletions of six or fewer exons constituted 68.8% of cases. The deletion of exon 50 was the most common. The reading-frame rule held in 90% of DMD and 94% of BMD cases. A tendency toward a lower IQ and earlier wheelchair dependence was observed with distal exon deletions, though a significant correlation was not found.

Conclusions The reading-frame rule held in 90% to 94% of children, which is consistent with reports from other parts of the world. However, testing by MLPA is a limitation, and advanced sequencing methods including analysis of the structure of mutant dystrophin is needed for more-accurate assessments of the genotype-phenotype correlation.

Key Words Duchenne muscular dystrophy, Becker muscular dystrophy, genotype, phenotype.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe, progressive X-linked disease affecting 1 in 3,600–6,000 live male births.[1] Becker muscular dystrophy (BMD), which has a milder phenotype, has an incidence of 1 in 18,450 live male births,[2] with the patients experiencing a later onset and longer survival. In the intermediate phenotype (which represents outliers), patients may continue to walk until they are 16 years of age. All three patterns are caused by mutations in the Xp[21]DMD gene, which encodes a 427-kDa cytoskeletal protein called dystrophin.[3-6]

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The DMD gene is the largest human gene, spanning 79 exons. The clinical severity of DMD depends on the disruption or maintenance of reading frames. Out-of-frame deletions usually cause DMD with a complete absence or very low level of dystrophin protein,[7] while deletions maintaining reading frames produce a protein of abnormal low molecular weight that results in the BMD phenotype,[8,9] though there are exceptions to this rule. The reading-frame rule was found to hold for 90% of cases.[10]

Deletions account for 60−65% of cases of BMD and DMD, while duplications cause another 10−15% and the remainder may be due to point mutations.[6] Most of the deletions occur in hot-spot exons 45−53, while the duplications occur mainly in minor hot-spot exons 2−20.[10] Various diagnostic techniques are available, with the main one being multiplex ligation-dependent probe amplification (MLPA). The small mutations that might not be identified by MLPA include small insertions/deletions within an exon, missense/nonsense mutations, and splice-region variants.[6]

Studies of mutation characteristics and the genotype-phenotype correlation have been performed in various countries.[11] The present study is the first large-scale one aimed at identifying the MLPA-based genotype-phenotype correlation in an Indian population.

METHODS

This retrospective study involved 317 patients with MLPA-confirmed DMD or BMD who visited the neuromuscular clinic of a quaternary hospital in southern India between 2013 and 2016. Clinical data on the age at onset, presentation, loss of ambulation, and family history were collected. Data on IQ (as assessed using the Binet Kamat scale) and cardiac evaluations were also obtained. The institutional ethics committee approved the study.

The MLPA reaction was carried out to screen the exons of the dystrophin gene using SALSA MLPA P034 and P035 probe sets (MRC Holland, Amsterdam, The Netherlands). The procedure was performed according to the manufacturer's instructions. Amplified products were separated using a genetic analyzer (ABI 3500 XL, MRC Holland) and data were analyzed using Coffalyser software (MRC Holland). Normal healthy individuals were used as controls and included in every run.

RESULTS

Demographic and clinical profile of patients

The 317 patients comprised 279 cases of DMD (88%), 32 cases of BMD (10.1%), and 6 cases of intermediate phenotype (1.9%). Most of the boys exhibited delays in attaining motor milestones. Around 50.8% of them had achieved independent walking after 1.5 years of age, with 59% of them starting to walk after 2 years of age. The age at the onset of illness for the children with DMD was 1.5 to 8 years, and their age at evaluation ranged from 3 to 14 years. Around 9.1% of DMD cases were wheelchair-dependent at evaluation, with this state appearing at a mean age of 9.5 years (range 7 to 12 years). The age range at the evaluation for BMD patients was 16 to 46 years.

Deletions accounted for 91.8% of cases, while duplications caused the remaining 8.2%. The mutation pattern among the 279 cases of DMD comprised 91% deletions and 9% duplications, while the 32 cases of BMD comprised deletions in 96.8% and duplications in 3.2%. The six cases of intermediate type had deletions. Thus, among 291 probands with deletions, 254 had DMD, 31 had BMD, and 6 had the intermediate phenotype, while among the 26 probands with duplications, 25 had DMD and 1 had BMD.

Deletion pattern in the DMD gene

The deletions among the 317 probands were as follows: those in a single exon was the most frequent (24.2%), followed by 3 (15.1%), 2 (9.7%), 5 (8.2%), 6 (7.2%), and 4 (4.1%) exons. Thus, deletions of six or fewer exons constituted 68.8% of cases. Deletions of more than 10 exons comprised only 8.2% of cases. Among the BMD cases, the most frequent deletion was that of exons 45−47 (37.5%), followed by exons 45−48 (18.7%). The largest deletion found in BMD was that of exons 14−42.

Single-exon deletions constituted in 26.4% of all 291 deletions: exon 45 (29/77) and exon 51 (10/77). Among the 291 cases, exon 50 was most frequently deleted (118/291), followed in order by deletions of exons 48, 49, 46, 47, and 45. Of the 214 cases with multiexon deletions, the 8-exon deletion involving exons 45−52 was the most common (n=19), followed by the 3-exon deletion involving exons 45−47 (n=18) and a 6-exon deletion involving exons 45−50 (n=12). The most-common proximal deletion was from exons 10−17.

Distal deletions constituted 81.7% of all deletions, proximal deletions constituted 15.4%, and proximodistal deletions constituted 2.9%. The largest deletion involved exons 8−47. None of the deletions involved exons 58−63 or 65−79. The frequencies of the deletions are shown in Fig. 1, while Table 1 summarizes the clinical features of common multiexon deletions.

Duplications in the DMD gene

Duplications were identified in 26 children, with the most common involving exons 8, 9, and 60−66 (n=2 for each).

Table 1. Summary of clinical features of the prominent multi exonic deletions

Exons deleted	Number	Mean age at onset (years)	Mean age at presentation (years)	Children with low IQ (*n*)	Families with affected sibling (s)	Number of children who lost ambulation (mean age at loss of ambulation)
45–52	19	4.13	8.9	2	2	6 (9 years)
45–50	12	4.9	7	1	2	-
45–54	8	4.8	8.7	-	-	1 (10 years)
46–51	7	4.4	5.7	1	-	-
10–17	3	2.8	6	-	2	-
10–43	2	3.2	8	-	-	-
48–52	8	5	9.5	-	4	1 (10 years)
8–47	1	2	8	-	4	-

Only one patient with BMD showed the involvement of exons 4–6 and 61–69. One case of DMD also showed the involvement of exons 45–55 and 63. Single-exon involvement occurred in 23.1% of cases, while six or fewer exons were involved in 50%. Large duplications involving 10 or more exons were found in eight cases (30.7%). Exons 8 and 9 were the most commonly affected (*n*=8 for each, 30.7%). The largest duplication involved exons 3–25. Proximal duplications were seen in 46.1% of cases. None of the duplications involved exons 1, 30–42, and 75–79. The child with the largest duplication of exons 3–25 (an in-frame duplication) exhibited symptom onset at 7 years of age and had presented at 8 years of age. Another large duplication was that of exons 8–29 in a child with symptom onset at 4 years of age. Two other cases of proximal in-frame duplications (involving exons 3–12 and 3–18) experienced symptom onset at 7 and 2 years, respectively. Two patients with duplications had lost ambulation: 1) in-frame duplication of exons 50–55 with symptom onset at 3 years of age and loss of ambulation at 7 years of age, and 2) out-of-frame duplication of exons 18–21 with symptom onset at 3 years of age and loss of ambulation by 12 years of age. The frequencies of duplications are shown in Fig. 2.

Association with reading frames

Among the 279 cases of DMD, 31 cases (11.1%) were due to in-frame deletions or duplications. Of the 291 deletions in the entire cohort, 254 resulted in DMD, 228 were out-of-frame deletions, and 25 (8.9%) were in-frame deletions, of which 12 were proximal deletions: 2 pairs of siblings had deletions of exons 21–23 and 3–34. Two children with DMD also had deletions of exons 45–49, with symptom onset at 3 years of age and presentation for evaluation at 5 years of age. The larger in-frame deletions involved exons 11–31 (onset at 4 years of age), 11–41 (onset at 5 years of age), 3–34 (siblings with onset at 3 years of age), and 21–39 (onset at 3 years of age). One DMD child showed deletion of exons 1 to 29,

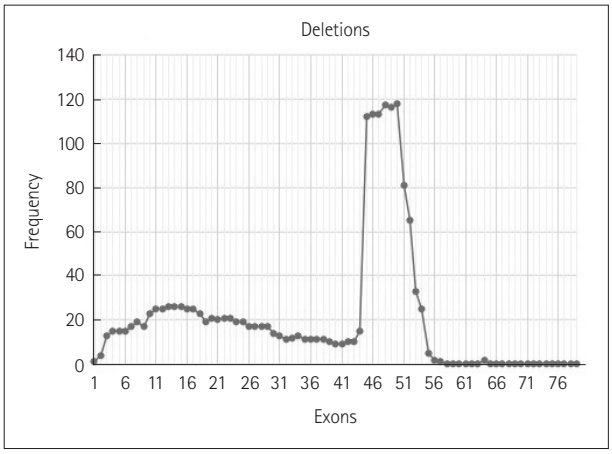

Fig. 1. Frequencies of deletions of exons in DMD and BMD. BMD: Becker muscular dystrophy, DMD: Duchenne muscular dystrophy.

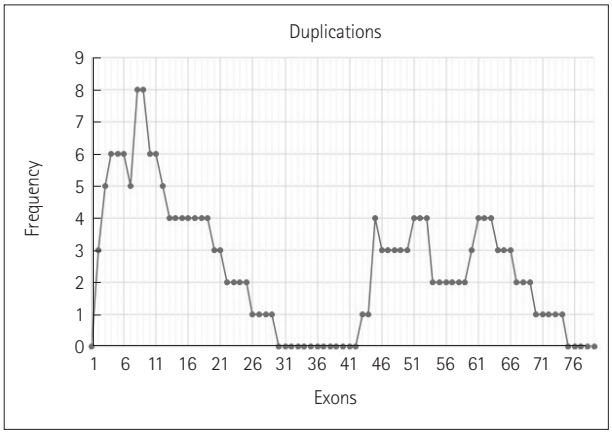

Fig. 2. Frequencies of duplications of exons in DMD and BMD. BMD: Becker muscular dystrophy, DMD: Duchenne muscular dystrophy.

whose effect may be difficult to predict using the Leiden reading-frame checker. This child experienced symptom onset at 2 years of age and was evaluated at 7.5 years of age.

In-frame deletions contradicting the reading frame were almost equally distributed between the 5' and 3' ends of the DMD gene. Two patients with BMD had out-of-frame dele-

tions: one with deletion of exons 42 and 43, symptom onset at 10 years of age, and presentation at 23 years of age, and the other with deletion of exons 45–50, symptom onset at 5 years of age, and presentation at 29 years of age.

There were 26 duplications, of which 25 resulted in DMD: 19 were out of frame and 6 were in frame. These duplications were also equally distributed toward the 5' and 3' ends of the dystrophic gene. Out of 6 cases of intermediate dystrophinopathy, 2 had out-of-frame deletions of exons 51–55 and exons 42 and 43, and the remaining 4 were due to in-frame deletions.

Thirty of the 32 cases of BMD were due to in-frame deletions or duplications, with the other 2 comprising out-of-frame deletions of exons 45–50 and exon 50.

Other clinical parameters

IQ tests performed in 29 DMD children showed average intelligence (90–109) in 48.3%, below-average intelligence in 17.2%, borderline (70–79) intelligence in 13.8%, mild mental retardation in 10.3%, and moderate mental retardation in 3.4%, while 6.8% had above-average intelligence. The children with below-average intelligence had deletions toward the distal part of the gene.

Cardiac abnormalities such as dilated cardiomyopathy and left ventricular dysfunction were found in five of the BMD patients. Their age ranged from 22 to 30 years, two of them had deletions of exons 45–48, while one each had deletion of exons 45–49, exon 50, and exons 10–18. Two of the DMD children had congenital heart disease: one had a bicuspid aortic valve and the other had an atrial septal defect with right ventricular volume overload and trivial aortic regurgitation. Two DMD patients also had mild mitral regurgitation and tricuspid regurgitation.

At the time of the last evaluation, 29 children with DMD were wheelchair-dependent, with a mean age at loss of ambulation of 9.6 years. Twenty-seven of them had deletions, most of which were of exons 44–55; the other two had deletions of exons 20–29 and 34–54. Two patients had duplications of exons 50–55 and 18–21. Twenty-one of the 29 DMD patients were not receiving steroids prior to becoming wheelchair-dependent. Six patients had lost ambulation before 8 years of age, two patients had deletions of exons 45–52, one each had deletions of exons 46 and 47, exons 48–50, and exon 50, and one had duplication of exons 50–55.

DISCUSSION

MLPA is a simple, rapid, and reliable screening tool that analyzes all 79 exons of the DMD gene, and can reportedly be used to identify deletions and duplications with a sensitivity ranging from 63% to 79.5%.[11-13] The present study included MLPA-positive cases in the analysis and correlated the findings with various phenotypic presentations/outcomes in dystrophinopathies. More than 50% of our patients exhibited delays in the acquisition of milestones. A study involving cases from eastern India found that 46.9% experienced delays in attaining milestones,[14] which is similar to the present proportion. The mean ages at onset and presentation in our study were 3.4 and 9.5 years, respectively; these ages are very similar to the findings of a previous study from the same institute and also those of other studies from eastern and western India.[14-17]

A family history of DMD or BMD was present in 18.5% of our cohort, which is also comparable to previous findings for the prevalence of family histories of 20–27%.[11,14-16] Despite having a positive family history, in many cases medical attention was sought late,[11] with some of the DMD children already having lost ambulation by the time they visited a neurologist.

The most-common phenotype resulting from mutations in the dystrophin gene is DMD. In our study also, 88% of the rearrangements in the dystrophin gene caused DMD. Studies conducted in other parts of India and China have showed similar proportions of around 92%.[11,16]

In our entire cohort, 91% of the cases resulted from deletions. Previous studies from China have found similar rates (84–90%) among genetically confirmed cases, with an overall positivity of 64–67% among all probands suspected of having DMD.[11,18] The reported deletion rate for MLPA-confirmed cases of DMD or BMD has varied from 80% in an Argentinian population[12] to 89% in a Korean population;[19] these proportions are very close to that for our cohort. In contrast, only 59% of MLPA-confirmed children with DMD in a Taiwanese cohort[20] and 67.4% in the Universal Mutation Database-Duchenne muscular dystrophy (UMD-DMD) showed deletions.[21]

In the current cohort, single-exon deletions were most common (26%), and deletions of six or fewer exons constituted 68.7% of cases. Similarly, Magri et al.[22] found that single-exon deletions in the DMD gene constituted 24% of cases. Exon 45 was the most frequently deleted single exon in the present study, while exon 50 was most commonly affected overall. Among multiexon deletions in our study, exons 45–52 were the most commonly involved. Similarly, Yang et al.[11] found that the most-prevalent single-exons deletions involved exons 45 and 51 in a Chinese population. Exons 45–50 were predominantly involved in multiexon deletions, although those of exons 45–52 (as found in our study) were also common. The deletion of exons 45–52 has also been the most-common finding in other studies, with single-exon deletions

being less common.[11,22]

Most of the deletions were centered in a distal hot-spot region, constituting 81.8% of our cohort. Distal deletions constituted 74% of the deletions in the UMD-DMD,[21] and three patients in that database exhibited deletion of all exons, with one case showing spread of the deletions outside the DMD gene to also involve the contiguous glycerol kinase gene. The largest deletion found in our study extended from exons 8 to 47.

Most of the duplications also resulted in DMD. The duplications of six or fewer exons constituted more than 50% of the present cases. The most-common duplication found in the DMD gene in Western studies is that of exon 2,[21-23] in contrast to our finding of duplications involving exons 8 and 9 being the most frequent. Similar to us, Yang et al.[11] found exons 8 and 9 to be affected in duplications. This suggests that there is a preferential pattern of the involvement of exons 8 and 9 among Asians relative to Caucasians. Single-exon duplications were found in 23% of duplications in our study, while other studies have found prevalence rates of around 20%[11] to 36%.[22] Moreover, multiexon duplications were reported to be frequent in the UMD-DMD, also with involvement of proximal hot spots in 64%.[21] Duplications are said to occur due to unequal crossing over of sister chromatids, interchromosomal events, or tandem duplications due to nonhomologous end joining.[23-25] We found one case each of DMD (exons 45−55 and 63) and BMD (exons 4−6 and 61−69) due to two nontandem duplications. Two cases of noncontiguous duplications were found in the UMD-DMD: one of noncontiguous deletion and one of exon triplication. Such duplications involving exons in two separate fragments of the gene may be due to two tandem duplications occurring in the same patient.[25]

No deletions were found in exons 58−63 and 65−79 in our cohort. A Chinese study[11] and the UMD-DMD[21] include cases involving the deletion of all exons. Exons 1, 30−42, and 75−79 were not involved in duplications in our cohort. Similarly, exons 72−79 were not involved in a study from China.[11]

The reading-frame rule held for 89% of DMD and 93.7% of BMD patients in our study. Previous studies found that 87.6%, 89.6%, and 96% of DMD patients had out-of-frame deletions or duplications.[11,18,21] There are known exceptions to the reading-frame rule, and there are case reports of relatively benign cases of dystrophinopathy with absent dystrophin expression in muscle fibers.[26] Exon-48 deletions in asymptomatic male patients with ages ranging from 8−58 years in a single family have also been reported.[27] Deletions of exons 3−7, which are predicted to shift the reading frame, are the most-common exceptions to the reading-frame rule and are associated with a wide range in clinical severities from DMD to BMD. Winnard et al.[28] postulated that some amount of dystrophin production could be due to the new initiation of ATG at exon 8 or to a second somatic mutation. Other proposed mechanisms include ribosomal frame shift, cryptic promoter, alternate splicing, and RNA editing, which helps in maintaining the reading frame and a certain amount of dystrophin production.[29,30]

The maintenance of the triple coil structure of wild-type dystrophin requires the constituent amino acids to have a heptad pattern. In-frame deletions that respect the heptad pattern permit for formation of hybrid repeats, and triple coiling may occur. The alpha helix may form in fractional repeats, but triple coiling does not occur due to the absence of a heptad pattern.[31] These differences in structure of mutant truncated dystrophin might account for the variability in the phenotypes of BMD and DMD. A study that analyzed deletions of exons 45−48, 45−51, 45−49, and 45−47 found that proteins corresponding to deletions 45−48 and 45−51 had a similar structure of hybrid-repeat-like wild-type dystrophin compared to fractional repeats with an unrelated structure in another two deletions.[32] That study found that dystrophin levels were moderately reduced in all patients. Fractional repeats have a higher molecular surface hydrophobicity and slower refolding dynamics, which is reflected clinically in the early development of cardiomyopathy and earlier wheelchair dependence in patients with deletions of exons 45−47. We found that two of the five BMD patients with cardiomyopathy had deletions of exons 45−48, while one had deletions of exons 45−49. The presence of the hybrid repeat alone does not ensure better dystrophin function—this may also depend upon mRNA stability and protein-protein interactions of mutant dystrophin protein. However, the large size of dystrophin makes it difficult to investigate the consequences of all mutations.[32]

Cardiac involvement in DMD and BMD is attributable to replacement of the myocardium by connective tissue or fat at the left ventricular posterobasal part or lateral walls.[33] Cardiac involvement and a low ejection fraction were found in five of our patients with BMD who were aged 22−30 years. Magri et al.[22] reported cardiac dysfunction in DMD patients aged around 15 years. None of those in our DMD cohort had overt cardiac involvement. Further, only a few patients with BMD had evidence of cardiomyopathy, and we could not identify a definite correlation between the mutation type and cardiac involvement. Previous studies have suggested that early cardiac involvement is associated with proximal deletions[22] and deletions of exons 48 and 49.[34] Exon 48 was involved in three of our patients (two with deletions of exons 45−48 and one with deletions of exons 45−49), and one had a proximal deletion. Cardiac involvement is known to have

more severe in BMD patients with deletions than in those with duplications,[22] which is consistent with all of our BMD patients with cardiac involvement having deletions.

The IQ as assessed in 29 of our patients indicated average or below-average intelligence in 93% of them, with a low IQ being more pronounced in those with distal deletions. This finding is consistent with the findings of Magri et al.[22] In contrast, a study from Kuwait and Egypt did not find any correlation between IQ and the site or frequency of deletions, although a slightly lower IQ was identified in the presence of deletions of exons 12, 45, and 48.[35]

Loss of ambulation in our patients occurred at a mean of about 9.5 years, which is broadly consistent with previous findings of a mean age of around 10.3 years.[22] Most of our patients who lost ambulation exhibited distal hot-spot region involvement. However, most of them presented late and were not on steroids, and hence a definitive correlation could not be determined. Another study found no definite associations between age at onset, age at wheelchair dependence, and deletion mutations in the DMD gene.[22]

In conclusion, the results in our study are comparable with those obtained in other countries. The reading-frame rule held in more than 90% of the present cases. Though a general trend toward early wheelchair dependence and low IQ was observed in the presence of distal deletions, a definite correlation was not identified. Further studies including analyses of the structure of mutant dystrophin and the definite functional role of other dystrophin isoforms may be needed to identify a definite genotype-phenotype correlation. We acknowledge the limitation that only MLPA-confirmed cases of DMD or BMD were included in this study, and hence future studies that include cases of point mutations will further enhance our understanding of the genotype-phenotype correlation in dystrophinopathies.

Conflicts of Interest

The authors have no financial conflicts of interest.

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