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Lessons Learnt from A Study of Children with Duchenne Muscular Dystrophy: Approach to The Molecular Diagnosis and Its Importance in Emerging Therapies and Preventive Measures

Snehal Mallakmir[1], Ami Shah[2], Rashid Merchant[3]

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Abstract

Currently, there is no curative therapy for Duchenne muscular dystrophy (DMD), but several clinical trials are in progress. It is important to confirm diagnosis using molecular biology techniques. We discuss the molecular approach to diagnosis, limitations of various diagnostic methods and relevance in the light of various emerging therapies.

Methods: We retrospectively studied 35 children, aged 3 to 18 years, with clinical phenotype of DMD and study of the dystrophin gene.

Results and conclusion: 34 of the 35 children had a confirmed genetic diagnosis. One patient with negative genetic analysis had undergone muscle biopsy and immunohistochemistry for confirmation. New emerging therapies and clinical trials in this field demand an accurate diagnosis, especially when therapy is targeted towards specific mutations. In cases with no specific mutations, final diagnosis needs to be confirmed as well as other dystrophies ruled out in order to guide prognosis, preventive strategies, and family counselling.

Keywords: Clinical trials; DMD; Dystrophin gene; Dystrophinopathies; Exon skipping.

Introduction

Duchenne muscular dystrophy (DMD) is an X linked recessive disorder with reported incidence of 1:4000 live male births. DMD is the most common progressive muscle disease presenting as early as 3 years of age with delay in development of motor milestones and calf muscle hypertrophy. The disease slowly progresses to involve skeletal muscles with loss of ambulation as well as cardiac and respiratory muscle weakness leading to early death due to complications. The clinical diagnosis is usually confirmed by analysis of the only gene known to be associated with this condition, the Dystrophin gene, at locus Xp21 encoding dystrophin protein. It is the largest human gene, comprising 79 exons, spanning 2.5 MB of the genomic sequence and accounting for 0.1% of the total human genome. Dystrophin is a rod-shaped protein, responsible for connecting the extracellular matrix to the cytoskeleton of each muscle fiber via a large protein complex containing many subunits. Mutations lead to reduction in the stability of the cell membrane inducing various proinflammatory molecules, ultimately resulting in muscle degeneration and necrosis.[1] Thousands of different mutations have been detected to date.[2] Muscle immunohistochemistry (IHC) uses special stains for various muscle proteins. This is the standard method of testing in genetically negative patients and allows us to differentiate from other forms of muscle dystrophies. New emerging therapies for DMD and rapidly advancing research demand accurate genetic diagnosis especially where the

Author's Affiliation: [1,2]Clinical Geneticist, [3]Senior Consultant Paediatrician, Department of Pediatrics, Nanavati Superspeciality Hospital, Vile parle, Mumbai 400056, India.

Corresponding Author: Snehal Mallakmir, Clinical Geneticist, Department of Pediatrics, Nanavati Superspeciality Hospital, Vile parle, Mumbai 400056, India.

E-mail: drsnehalm@gmail.com

therapy is targeted towards specific mutations. It is also important for prognosis, management strategies and family counseling.

Methods

We retrospectively studied 35 children of ages ranging from 3-18 years with DMD phenotype and high serum creatine kinase levels (CK). Molecular diagnosis was obtained by multiplex ligation dependent probe amplification (MLPA) as the first choice of test followed by Sanger sequencing in case of negative MLPA. Muscle biopsy and IHC were advised if both genetic tests were negative.

Results

Table 1: It shows details of mutations seen in our patients. The most common exon deleted was exon 50 and there was no exon deletion from 63-79 exons. Three patients had more than one exons duplicated. Two patients had point mutation in exonic and intronic region each.

Mutations detected	Number of Patients	%
Exon deletion		
1(18,30,50,51)	4	14.8
2(42-43, 46-47, 49-50)	5	18.5
3(52-54)	1	3.7
4(57-60)	1	3.7
5(3-7, 12-16)	2	7.4
6(45-50)	1	3.7
7(46-52)	2	7.4
8(45-52, 46-53)	3	11.11
>9(8-43,50-62,6-42,45-55,2-21, 8-20,3-28)	8	29.62
Total patients with deletion	27/34	79.4/100
Exon duplication		
1(17)	1	33.3
2(46-47)	1	33.3
4(57-60)	1	33.3
Total patients with duplication	3/34	8.82/100
Point mutation		
Exon 14	1	25
Exon 41	1	25
Intron 21	1	25
Intron 69	1	25
Total patients with point mutation	4/34	11.76/100

Genetic analysis confirmed diagnosis in thirty four children (97%) and IHC was required in one child (3%). Amongst the thirty four patients confirmed by genetic analysis, thirty (88%) were positive on MLPA with exon deletions in twenty seven (79%). Furthermore,duplication was detected in three (9%) patients and four (11%) showed point mutations on gene sequencing. The details of genetic results are as shown in Table 1. The patient who underwent muscle biopsy showed lack of expression of dystrophin 2 and 3 but preserved expression of dystrophin 1, merosin, dysferlin and sarcoglycans on IHC.

Discussion

Molecular diagnosis for DMD is complicated by the large size of the gene and multiple different mutations spread all across the gene[1,2] however, optimum testing strategy and best practice guidelines have been established[3] and recent experimental treatments seem more promising. Emerging clinical trials for DMD demand accurate diagnosis of the disorder, especially where the therapy is targeted towards specific mutations. Affected males suspected to have DMD based on clinical phenotype and high serum CK levels are referred for a molecular confirmation by testing for the presence of a pathogenic variant in the DMD gene.

Absence of pathogenic variation would reduce the possibility of dystrophinopathy, but depends on the sensitivity of the method used. The majority of patients have a deletion (~68%) or duplication (~11%) of one or more exons, and small mutations are found as well (in ~20% of patients). These deletions and duplications can occur anywhere in the gene, but are concentrated between exons 45–55 and exons 2–10 for deletions and duplications, respectively.[2]

The multiplex PCR (mPCR) technique described by Chamberlain et al.[4] and Beggs et al.[5] offered a rapid screening tool for detecting deletions in the central and 5′ end hot spot regions of the gene. This method detects 98% of deletions in these regions as well as larger deletions in about 60−65% patients and has replaced muscle biopsy as the preferred method of diagnosis. Its advantages include relative simplicity and cost but it does not detect duplications which account for 6% of mutations, does not characterise all deletion breakpoints, cannot be used for carrier testing of females and has been superseded due to the availability of MLPA. Developed by Schouten et al.[6], MLPA offers a reliable quantitative method to detect deletions and duplications in all 79 exons of the dystrophin gene and also carrier testing. MLPA adds another 10−15% positive case to mPCR.

In cases with point mutations, gene sequencing is needed. Sequencing can be carried out on either genomic DNA or muscle-derived cDNA. Analysis of genomic DNA has the advantage that it does not require the patient to undergo a muscle biopsy.

Analysis of genomic DNA will not detect mutations in the 2% of cases with complex rearrangements or deep intronic changes. Analysis of muscle RNA has a slightly higher sensitivity; however the requirement for a muscle biopsy and transport is a drawback.[3] More recently, oligonucleotide-based array comparative genomic hybridisation (array-CGH) has been used, analysing copy number variations across the entire gene, including intronic and 3′ and 5′ flanking regions, which has advantages of detecting complex rearrangements and intronic alterations.[7] The high density of probes helps in controlling false positives due to single nucleotide polymorphisms.

It is well known that the underlying mutation is not detectable in at least 4% of cases with available genetic testing, and muscle biopsy with IHC might be needed to establish definite diagnosis. Different stains can be used to rule out muscular dystrophies with overlapping phenotype as studied by Karthik Tallapaka et al.[8] It helps in management, explanation of prognosis, and genetic counselling of family members, as well as prenatal counselling. Confirmation of DMD by IHC indicates the possibility of detecting more unidentified mutations.

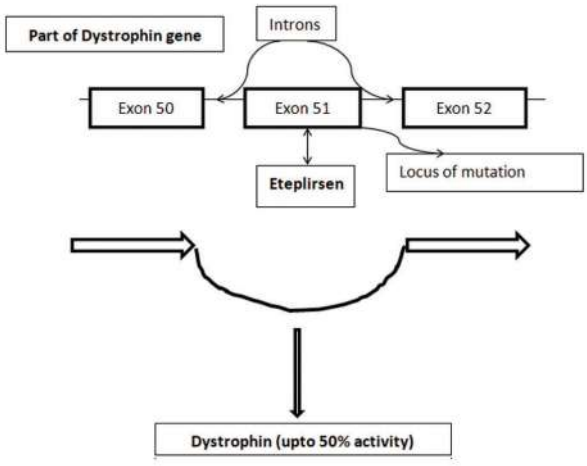

Fig 1: Mechanism of action of Eteplirsen resulting in truncated dystrophin protein.

The size and distribution of mutations across the dystrophin gene have posed challenges for the development of therapies. Current strategies focus on optimizing growth and development, promoting well-balanced diet, participating in physical and recreational activity, and delaying the onset of secondary complications through ongoing medical and psychosocial support. Supportive interventions including corticosteroids, management of heart failure, noninvasive positive pressure ventilation, and effective airway clearance have contributed to prolonged survival of patients. Glucocorticoids, more precisely prednisolone and deflazacort, have been used for over two decades and the benefits are well known. There

Table 2: Patients amenable to exon skipping therapy in present cohort compared with a recent Indian study.

Exon to skip	Amenable Variants	Number of patients (%) benefiting in present study	Number of patients (%) benefiting in recent Indian study by Kohli et al (12)
43	Deletions 44, 44–47, 44–48, 44–49, 44–51, 44–53, 44–57	0	38 (5.9%)
44	Deletions 3–43, 10–43, 14–43, 23–43, 30–43, 31–43, 33–43, 34–43, 35–43, 38–43, 41–43, 42–43, 45–54, 45, 43 Dup 44	2 (6%)	113 (17.5%)
45	Deletions 12–44, 18–44, 44, 46, 46–47,46–48, 46–49, 46–51, 46–53, 46–55 Dup 45	3 (8%)	129 (19.9%)
50	Deletions 51–53, 51, 51–55	1 (3%)	44 (6.8%)
51	Deletions 40–50, 45–50, 47–50, 48–50, 49–50, 50, 52 Dup 51	4 (12%)	211 (32.6%)
52	Deletions 51, 53–55, 53–59, 53–60, 53	1 (3%)	39 (6%)
53	Deletions 43–52, 45–52, 47–52, 48–52, 49–52, 50–52, 52	3 (8 %)	152 (23.5%)

are many clinical trials in progress using various drug molecules.[9,10] One of the more promising therapies for DMD is exon skipping, which uses synthetic antisense oligonucleotide sequences to induce skipping of prespecified exons during pre-messenger RNA splicing of the DMD gene.[11] This results in restoration of the reading frame and production of an internally truncated protein, similar to the dystrophin protein expressed in Becker muscular dystrophy (Fig 1). In the present cohort fourteen (40%) patients would benefit from this therapy (Table 2).

In a recent Indian study, the mutation spectrum was analysed in a large cohort and the result stressed the need for final molecular confirmation.[12] Our study has a smaller sample size as compared to this study (as in Table 2), the mutation spectrum differs, showing genetic heterogeneity in the Indian population. Skipping of different exons –43, 44, 45, 50, 52 and 53 is already in various stages of research. Eteplirsen is a phosphoramidate morpholinooligomer designed specifically for exon 51 skipping, and has received the approval for the treatment from the USA Food and Drug Administration.[9] Ataluren is an orally bioavailable drug designed to overcome premature stop codon mutations. It binds to the ribosomal RNA subunits and impairs the recognition of premature stop codon, thus allowing the translation and production of a modified dystrophin protein.[13,14] Following positive results in earlier studies, it was granted conditional marketing authorization for the treatment of nonsense mutation DMD in ambulatory patients above five years of age. In our group, two (6%) boys are eligible for this drug. Obligatory additional studies since the authorization have reflected a continued positive risk-benefit assessment.

The most recent gene replacement study, using a recombinant adeno associated virus (rAAV) delivered dystrophin transgene, resulted in low level, transient dystrophin expression and cell mediated immune response.[15]

Utrophin modulators increase expression of the utrophin gene, thus increasing utrophin levels in muscles. Ezutromid is a 2-aryl benzoxazole utrophin modulator that increased production of utrophin in mdx mice, improved strength, and reduced fatigue following forced exercise. Cell replacement studies appeared promising in mouse models, but resulted in failure in patients. Apart from these, many drug molecules are being tested by researchers[9], as listed in Table 3.

Suitable strategy for therapy in DMD will require early identification of affected boys to allow implementation of treatment before muscle tissue is irreversibly damaged. This may also include steroid therapy, where there is some evidence that early intervention produces highly beneficial results. Prevention strategies include prenatal counselling and diagnosis. Screening of newborn patients and institution of therapy before damage occurs are currently under investigation. Such clinicopharmaceutical trials hold promise as early therapies for the future.

Table 3: Pharmacological approaches that target the secondary pathology due to dystrophin deficiency (9).

Drug Molecule	Secondary Pathology	Effect
AT-300 (peptide)	Calcium dysregulation, compromise of the intra-cellular Ca2+ homeostasis leading to chronic inflammation	Blocks mechanosensitive Ca2+ channels resulting in modest benefits
Idebenone, (synthetic derivative of Coenzyme Q10)	Oxidative stress	Reduced loss of respiratory function in DMD patients
Alisporovir/Debio-25 (an analogue of cyclosporine shown to inhibit cyclophilin D)	Mitochondrial dysfunction	Prevents formation of large mitochondrial permeability transition poresreducing inflammation and macrophage infiltration
Vamorolone, steroid	Nuclear factor kappa-light-chain-enhancer of acti-vated B cells (NF-kB) pathway	Reduction of inflammation and an increased strength without the immunotoxicity and lesser side effects than other corticosteroids
Givinostat (Histone deacetylase inhibitor)	Deficient S-nitrosylation and constitutive activation of Histone deacetylase, impairment of muscle regeneration and compromise microfibre adaptation to contraction	Positive histological effects on muscle regeneration in clinical trials
Tadalafil and sildenafil (phosphodiesterase-5 inhibitors)	Muscle ischemia	Phosphodiesterase-5 inhibitor (PDE5i) increases the intracellular cGMP level in vascu- lar smooth muscle cells and leads to vasodilation and alleviate muscle ischemia

Conclusion

Our study adds to the approach to molecular diagnosis and the genotype data of DMD patients. In spite of many reports, genotype phenotype corelation in this disorder is lacking and needs further research. There is growing need of awareness among primary physicians, specialists and therapists regarding newer molecular investigations, approach to those investigations for precise diagnosis in muscular dystrophy patients in view of multidisciplinary care needed, emerging new therapies, family counselling and informed choices about future pregnancies.

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