RESEARCH PAPER

Molecular and Histopathological Characterization of Patients Presenting with the Duchenne Muscular Dystrophy Phenotype in a Tertiary Care Center in Southern India

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Objective: To study the histopathological characteristics and mutation spectrum of patients presenting with the Duchenne muscular dystrophy (DMD) phenotype. **Methods:** This was a descriptive study conducted over a period of 8 years. Multiplex ligation-dependent probe amplification (MLPA) was done in patients presenting with the DMD phenotype. If MLPA was negative, patients were offered muscle biopsy for histopathological studies and/or next generation sequencing (NGS) based multigene panel testing for muscular dystrophies. **Results:** Of the 510 patients included, mutation in the *DMD* gene was detected by MLPA in 372 (72.9%), of whom 342 (67.1%) had exonic deletions and 30 (5.9%) had exonic duplications. Exons 45-55 were most commonly involved in large deletions and exons 1-10 were the commonest exons involved in duplications. In the MLPA-negative cohort, 27 proceeded for muscle biopsy. NGS was done in 14 patients, 10 of whom had pathogenic mutations in the *DMD* gene, 3 were non dystrophinopathies and no pathogenic variant could be identified in one patient. **Conclusions:** For patients presenting with the DMD phenotype, MLPA of the *DMD* gene has a high diagnostic rate of about 73%, and non-dystrophinopathies may constitute a small but significant proportion.

Keywords: *Diagnosis, Histopathology, Multiplex ligation-dependent probe amplification, Neuromuscular disorders, Next generation sequencing*.

With an incidence of 1 in 3500, Duchenne muscular dystrophy (DMD) is the commonest genetically inherited primary muscle disease [1]. DMD is an X-linked condition caused by mutation in the *DMD* gene which codes for dystrophin, and usually presents in boys within the first decade of life with rapidly progressive proximal muscle weakness. Limb girdle muscle dystrophies (LGMD) are a group of disorders mostly inherited in an autosomal recessive or dominant fashion, some of which, especially the sarcoglycanopathies, can clinically resemble DMD.

Multiplex PCR (mPCR) was the preferred first-line genetic test for DMD until the advent of Multiplex Ligation-dependent Probe Amplification (MLPA), which in comparison to mPCR, has better sensitivity in detecting deletions and can detect duplications and carrier status as well. However, point mutations and small indels are not detected by MLPA. With the advent of next generation sequencing (NGS), these sequence variants are being more frequently identified. This study describes the diagnostic yield of MLPA and the spectrum of mutations (including point mutations) occurring in DMD patients hailing from Southern India. In this era of rapid technological advancement, it is pertinent that the mutation pattern be known so that specific treatment options could be offered with the likely availability of mutation-specific therapies in the not-so-distant future**.**

Accompanying Editorial: Pages 549-50.

METHODS

This observational study was conducted between March 2009 and June 2017 at a tertiary care hospital in Southern India. After the approval of the Institute Ethics committee, informed consent was obtained from parents of patients with the DMD phenotype. Boys aged 2 to 18 years were considered to have the DMD phenotype if one or more of the following features were present: history of progressive, symmetrical muscle weakness with onset in early childhood; history of motor developmental delay; positive family history suggestive of X-linked inheritance; weakness predominantly in the proximal

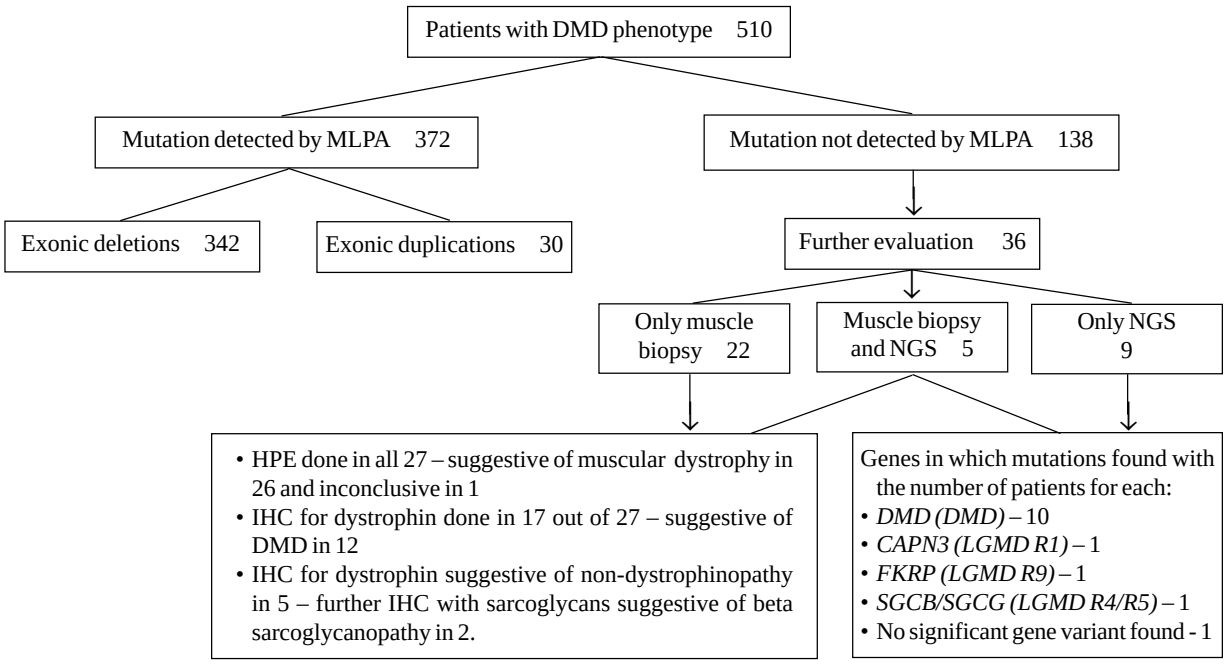
muscles; earlier onset and more severe weakness in the lower limbs compared to the upper limbs with positive Gower's sign; calf muscle hypertrophy; raised serum creatine phosphokinase (CPK) to more than 10 times the normal value; and loss of independent ambulation by the age of 13 years.

Detailed clinical features and the results of investigations like serum creatine phosphokinase (CPK), electromyography (EMG) and muscle biopsy if already performed, were recorded. MLPA (MRC, Holland) was done as per the manufacturer's protocol to detect deletions and duplications of the *DMD* gene. As per the Institute protocol, if the MLPA test results were negative, the patients were offered muscle biopsy for histopathological studies. From the year 2014, NGS-based multigene panel testing for muscular dystrophies was offered as the other option. The patients were counseled regarding the advantages and disadvantages of both the choices. In the first choice after the confirmation of muscular dystrophy on histopathology, immuno-histochemistry (IHC) of the fresh frozen biopsy specimen was offered with staining for dystrophin 1, 2 and 3 (Novocastra – lyophilised mouse monoclonal antibodies by Leica biosystems). If negative, then IHC staining for alpha, beta, gamma and delta sarcoglycans & dysferlin (Leica biosystems) was advised. NGS was outsourced to a commercial diagnostic lab, if the family opted for it.

RESULTS

A total of 510 patients were included in the study. The median (range) age of presentation was 8 (3-10) years. All the patients included in the study had proximal muscle weakness in the lower limbs at the time of presentation. Calf hypertrophy, developmental delay (motor/global) and raised serum transaminase levels were the other reasons for consultation. Family history was positive for DMD in 25% of the cases. Global developmental delay or poor scholastic performance was noted in about 26% of the children. The median (range) serum CPK level was 10,000 (5762-14960) IU. 2D echocardiography showed a mildly dilated left heart with preserved ejection fraction in only one patient aged 10 years, but none of the other patients had features of cardiac insufficiency in the initial cardiac evaluation done at the time of diagnosis.

The results of the histopathological and molecular genetic evaluation are summarized in the flowchart in ***Fig.* 1**. Of the 510 patients, mutation in the *DMD* gene was detected by MLPA in 372 (72.9%). Of the mutations identified, 342 (67.1%) were exonic deletions and the remaining 30 (5.9%) were exonic duplications. Exons 45-55 were most commonly involved in large deletions [245



DMD: Duchenne muscular dystrophy; MLPA: Multiplex ligation-dependent probe amplification; NGS: Next generation sequencing; HPE: Histopathology examination; IHC: Immunohistochemistry; LGMD: Limb-girdle muscular dystrophy

***Fig.* 1** *Flowchart summarizing the histopathological and molecular genetic evaluation of the patients in the present study.*

(71.64%) of the deletion mutations]. Exons 1-10 were the commonest exons involved in duplications [17 (56.67%) of the duplication mutations]. Only a single exon was involved in either deletion or duplication in 72 (19.35%) patients, with exon 45 deletion being the most common single exon deletion. One child with deletion of exon 1 presented with a slightly milder phenotype, probably due to alternative initiation of translation [2]. One patient had a complex deletion involving exon 1 and exons 44-49.

Thirty-six out of the total 138 MLPA-negative patients underwent further evaluation through muscle biopsy [22 (15.9%)] or NGS [9 (6.5%)] or both [5 (3.6%)]. The remaining patients [102 (73.9%)] did not opt to undergo further evaluation due to financial constraints, or were lost to follow-up.

Out of the 27 MLPA-negative patients who opted for muscle biopsy, IHC for dystrophin could be done in only 17 patients. Twelve of them showed absence of dystrophin staining along the sarcolemma, suggestive of dystrophinopathy. The remaining five showed presence of normal dystrophin staining which ruled out dystrophinopathy. Out of these five cases, further IHC with alpha, beta, gamma and delta sarcoglycans showed a pattern suggestive of beta sarcoglycanopathy in two cases and in the other three further characterization could not be done. Representative histopathological pictures are shown in ***Web Fig*** 1. Five of the 12 patients whose dystrophin immunostaining was suggestive of dystrophinopathy opted for further NGS testing also, in order to identify the exact disease-causing mutation, either for the purpose of prenatal diagnosis and/or carrier testing of family members through targeted mutation analysis, or with the intent of ascertaining suitability for mutation-specific therapies such as stop codon read-through therapy.

NGS was done in a total of 14 MLPA-negative patients; following confirmation of dystrophinopathy through muscle biopsy and IHC in 5 and directly after MLPA in 9. Out of them, 10 had sequence variants in the *DMD* gene (transcript id ENST00000357033), with known mutations in 5 (c.6283C>T, c.8692C>T, c.3257dupA, c.10454_10454delT and c.2047G>T) and novel variants predicted to be pathogenic in 5 (c.2168_2168+1delGGinsCT,c.5478 _5490del TCTTTGCAACAAT, c.3154A>T,c. 3037G>T and c.2646_2646delT). Three patients were identified to have non-dystrophinopathic muscular dystrophy through NGS – one with limb girdle muscular dystrophy autosomal recessive 1 (LGMDR1/ LGMD2A/ Calpainopathy; homozygous known mutation c.802-9G>A in *CAPN3* gene); one with LGMD autosomal recessive 9 (LGMDR9/ LGMD2I/ LGMD-Dystro-glycanopathy type C5; homozygous known mutation c.1343C>T in *FKRP* gene); and one with variants of uncertain significance – homozygous c. 799C>T variant in *SGCB* and compound heterozygous variants c.479T>C and c.653T>C in *SGCG* (LGMD R4 or R5/ beta or gamma sarcoglycanopathy). In the last patient, further muscle biopsy and IHC with beta and gamma sarcoglycans was recommended for further functional corroboration and characterization, but the family did not opt for it. NGS did not reveal any significant gene variants in one patient.

DISCUSSION

A large proportion of cases of DMD are caused by large deletions/duplications. Initial Indian studies which were largely based on mPCR, reported a diagnostic rate of about 60-70% for this diagnostic modality [3-6]. Verma, *et al*. [7] found that MLPA had an additional diagnostic yield of 5.6% when compared to mPCR. The diagnostic rate of MLPA in our cohort was around 73%. Majority of the deletion mutations identified in our study involved the hot spot region in the central domain between exons 45-55, which is consistent with what is reported in many previous studies [3-6]. Duplications on the other hand were found to be commoner in the initial 10 exons. These exonic regions are frequently involved in duplication mutations, in various populations across the world [8].

Next generation sequencing-based muscular dystrophy gene panel sequencing was successful in identifying the mutation in all but one case where it could be done. A few recently published studies have demonstrated the successful usage of NGS for detection of large deletions and duplications also and thus this could become the investigation of choice for DMD in the near future [9]. However, as can be seen in our study, NGS was feasible only in 14 patients out of the 138 MLPA-negative cases who needed it. In spite of a significant decrease in the price of NGS in the past few years, the cost still remains a major deterrent for many families from the lower socioeconomic strata.

Muscle biopsy (histopathology and immuno-histochemistry) may not be preferable as the first line investigation in the diagnosis of MLPA-negative patients due to its invasive nature, but is helpful in resolving variants of uncertain significance (VUS) identified by NGS. Skin biopsy has been shown in some studies to be a sensitive and specific, less-invasive testing modality for dystrophinopathies and could be considered as an alternative option for histopathological characterization of MLPA-negative patients [10].

Seventy-two patients had a mutation which is amenable to the recently approved exon 51 skipping

WHAT THIS STUDY ADDS?

- MLPA of the *DMD* gene has a high (73%) diagnostic rate in patients with the DMD phenotype.
- Next generation sequencing, a non-invasive and precise diagnostic modality, has the potential to replace invasive techniques such as muscle biopsy as the preferred investigation for MLPA-negative DMD patients.

therapy (Eteplirsen) and forty-six others had mutations which are potentially amenable to exon 53 skipping. Five patients with nonsense mutations who may potentially benefit with stop codon read-through therapy have also been identified.

Following initial characterisation of the DMD phenotype as per the inclusion criteria of the study, further phenotypic delineation was not done for the patients. Therefore, genotype-phenotype correlation could not be established for the different types of mutations identified, which is one of the limitations of this study. As an extension of this study, further deep phenotyping of the patients is planned with correlation of the type of mutation with the age at onset, severity and/or progression of individual phenotypic parameters.

To conclude, MLPA has a good diagnostic rate for DMD and should be the first line genetic investigation of choice in a child presenting with the DMD phenotype. Non-dystrophinopathies, especially the childhood-onset autosomal recessive limb girdle muscular dystrophies, may constitute a small but significant proportion of patients presenting with the DMD phenotype, who test negative by MLPA. Next generation sequencing-based multigene panel testing for muscular dystrophy-associated genes, because of its non-invasive nature and its power to identify mutations in various DMD mimickers, should be offered to all MLPA-negative cases with the DMD phenotype. Identification of the exact disease-causing mutation(s) would help not just in molecular confirmation of the diagnosis, but also in appropriate counseling and in offering prenatal testing and carrier screening for the families. It would also be of help in identifying patients amenable to the various mutation-specific therapies that are being developed and/or investigated. However, cost is still a deterrent for doing NGS-based molecular studies in our country, especially in resource-poor settings. Thus, there is a need to devise strategies to lower the cost of diagnostic work up in MLPA-negative cases.

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