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Duchenne muscular dystrophy: A immunohistochemical profile and deletion pattern in dystrophin gene in North Indian population

Rachna Agarwal[1], Sujata Chaturvedi[2], Neelam Chhillar[3], Ishita Pant[4], Anuradha Sharma[5]

[1,3]Associate Professor, [5]Technician, Department of Neurochemistry, and [2]Professor, [4]Assistant Professor, Department of Pathology, Institute of Human Behaviour and Allied Sciences, Jhilmil, Dilshad Garden, Delhi, India

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ABSTRACT

Background: Duchenne muscular dystrophy (DMD), one of the most common X linked muscular disorder, affecting 1 in 3500 male births and is caused by mutation in dystrophin gene. 65% of DMD cases are caused by large deletion of dystrophin gene, followed by duplication (5-10%) or point mutation (25-30%). There is wide mutation spectrum of the mutations in dystrophin gene. Hence, population specific information is needed on mutation spectrum and frequency of common mutations occurring in that particular population for appropriate counseling, prenatal diagnosis and for developing genetic therapy in future. **Aims and Objectives:** To find out the frequency and distribution of deletion in dystrophin gene in DMD patients along with contribution of pathology and genetic testing in diagnosis of DMD and Becker muscular dystrophy (BMD) in North Indian population. **Materials and Methods:** Dystrophin gene was screened for deletion by multiplex polymerase chain reaction (PCR). Out of 41 patients, 09 patients underwent muscle biopsy, on which immunohistochemistry was performed for dystrophin, sarcoglycan, dysferlin and merosin. **Results:** Majority of the deletions were located in distal hotspot region (26/39 ~66.66%) which includes the exons 45-55 and 15.38% of deletions were located at the proximal hotspot region (2- 19 exons). **Conclusion**: In the present study, 34% patients only showed deletion. Hence complete work up of any muscular dystrophy requires immnohistochemical analysis to see the expression of muscle proteins along with multipleplex PCR test to detect any exon deletion, multiplex ligation-dependent probe amplification (MLPA) to detect point mutation and duplication and western blotting to quantify the dystrophin protein.

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Key words: Dystrophin, DMD, BMD, Multiplex PCR, Sarcoglycan, Dysferlin, Merosin.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is one of the most common inherited X linked muscular disorder; affecting 1 in 3500 male births and is caused by mutation in dystrophin gene. Dystrophin is the largest gene known in human genome spanning 2.6 Mb on X_{p21},[1] with 79 exons. Almost 65% of DMD cases are caused by large deletion of dystrophin gene found at 3'- hotspot and 5'- hotspot. Rest of the cases are caused by duplication (5-10%) or point mutation (25-30%).[2] There is wide mutation spectrum of the mutations in dystrophin gene, making it difficult to detect mutation in it. It is further complicated by the fact that approximately one third of the DMD patients occur by new mutations, either inherited through mother who are carrier or that arise from germ line mosaicism.[3]

Address for correspondence:
Dr. Rachna Agarwal, Associate Professor, Department of Neurochemistry, Institute of Human Behaviour and Allied Sciences, Post Box No 9520, Jhilmil, Dilshad Garden Delhi-110095, India
E-mail: rachna1000@hotmail.com; **Phone:** +91-9868396820.

In DMD patients, there is complete absence of dystrophin protein as compared to BMD patients which have 10 to 40% amount of normal dystrophin or partly functional dystrophin with an altered size[4] which can be seen on immunohistoanalysis of muscle. In addition to this, patients having sarcoglycanopathies, on immunohistochemical analysis, can have absence or reduction of sarcoglycan protein affecting the stability of the entire sarcoglycan complex. This may be associated with reduction of dystrophin level sometimes. The sarcoglycan proteins and dystrophin forms a part of large dystrophin-associated glycoproteins (DAGs) complex, which is necessary for preserving the integrity of the muscle cell membrane by maintaining the link between the cystoskeleton and the extracellular matrix.[5]

As population specific information is needed on mutation spectrum and frequency of common mutations occurring in that particular population for appropriate counseling, prenatal diagnosis and for developing genetic therapy in future, an attempt has been made in the present study to find out the frequency and distribution of deletion in dystrophin gene in DMD patients along with contribution of pathology and genetic testing in diagnosis of DMD and BMD in North Indian population.

MATERIALS AND METHODS

Patients

In the present study, all 41 male patients, attending Neurology clinic in the Institute of Human Behavior and Allied Sciences in last 5 years and tested for DMD by multiplex PCR were included. All the male patients were diagnosed for DMD/BMD on the clinical findings of progressive proximal muscle weakness, calf pseudohypertrophy, positive Gower's sign, electromyography (EMG), and an elevated serum creatine kinase (CK) level.

Immunostaining

Out of 41 patients, 09 patients underwent muscle biopsy, on which immunohistochemistry was performed with mouse monoclonal primary antibodies to dystrophin rod domain, carboxy terminal and NH_2 terminal (dys 1, dys 2, dys 3); α, β, γ and δ sarcoglycan (SGα, SGβ, SGγ, SGδ) and dysferlin (Novocastra) and peroxidase conjugated secondary antibody.

DNA mutation analysis

Peripheral venous samples of all these 41 subjects were taken. From blood leukocytes separated from it, DNA was isolated using standard phenol/chloroform procedures. Dystrophin gene was screened for deletion by PCR multiplex according to Chamberlain et al.[6] and Beggs et al.[7] using four multiplex PCR assays with primers for 25 exons (53, 47, 42, 60, 45, 48, 49, 43, 44, Pm (Dp427 exon 1)), 19, 3, 8, 13, 51, 50, 6, 21, 55, 17, 4, 46, 34, 52, 12). After amplification, the PCR product was separated on 3% Nusieve agarose + 2% agarose gel with added ethidium bromide for fluorescence of DNA and detected in UV light using Alpha Innotech. Deletion was diagnosed when one of the bands present in the amplified control DNA was absent from the patient DNA (Figures 1 & 2).

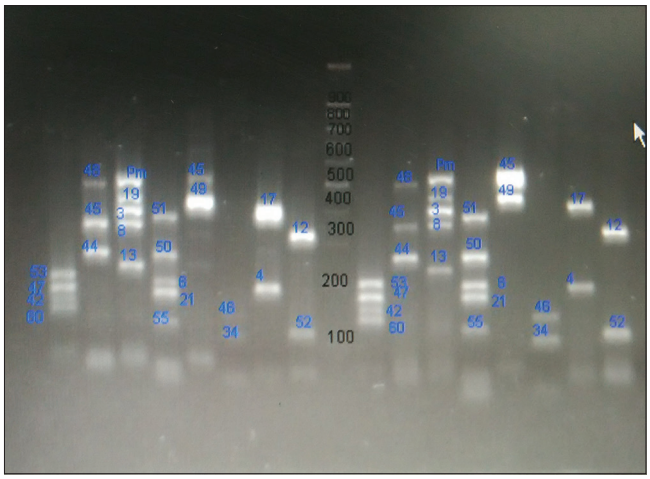

Figure 1: DNA mutation analysis of dystrophin gene by multiplex PCR. The numbers indicate the amplified exon.

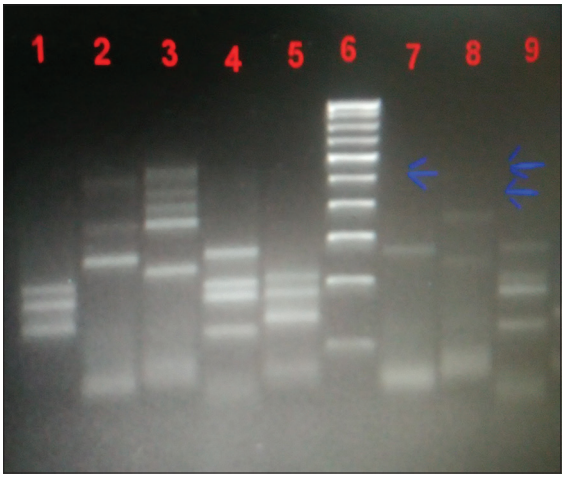

Figure 2: DNA mutation analysis of dystrophin gene by multiplex PCR of patient no. 7 in lane 5,7,8 & 9 and patient no. 27 in lane 1-4. Patient no. 7 shows deletion of exon 48 in lane 7 and Pm, exon 19 in lane 8 as shown by arrows, whereas patient no. 27 shows no deletion as indicated by intact amplified bands for all exons.

RESULTS

The age range of patients included in the study was from 4 years to 36 years. Out of 41 patients, 06 subjects presented with calf muscle hypertrophy, 07 subjects had difficulty in getting up or lower limb weakness. History of delayed milestones was present in 05 cases. In rest of the cases, no history was provided.

Frequency of deletion of exons

DNA samples from 41 patients were analysed by multiplex PCR for deletion. In 14 cases out of 41 showed intragenic deletions as shown in Table 1. Majority of the deletions were located in distal hotspot region (26/39 ~66.66%) which includes the exons 45-55 and 15.38% of deletions were located at the proximal hotspot region (2- 19 exons). Rest 08 deletions were observed in Promoter region (06 cases) and exon 06 (02 cases).

There were a total of 39 exons (1 in isolated cases, 38 in familial cases) deleted in 41 patients studied. In 27 cases, no deletion of any exon was found whereas single exon was deleted in 3 cases and in 11 cases multiple deletions were observed. The most frequent deletion was of exon 48, 49 and Promoter (15.38% each) as shown in Figure 3. Largest deletions were observed in 02 patients extending from exon 19 to 51.

When familial and isolated cases were compared, deletion was observed in one case only, whereas in familial cases, deletion was detected in 13 patients.

Contribution of Immunohistochemical analysis and mutation analysis on diagnosis of DMD/BMD

Based on the expression of various muscle proteins- Dystrophin-C, Dystrophin-N, α-Sarcoglycan, β-Sarcoglycan,

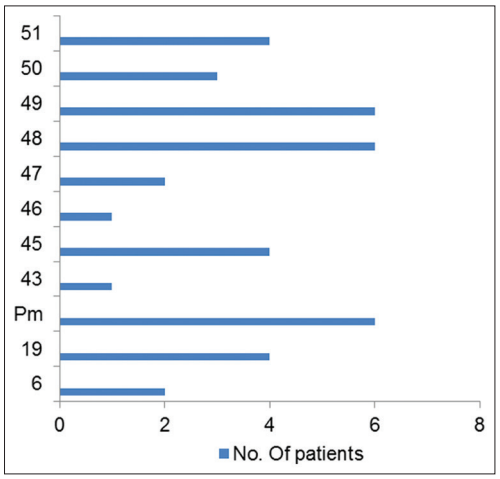

Figure 3: Graphical representation of the frequencies of deletions in 41 patients.

Table 1: Clinical data & genetic testing results for all patients

S. No.	Age (years)	Clinical data	Deleted exons
1.	8	Not given	6
2.	10	Not given	-
3.	8	Calf muscle hypertrophy	Pm, 19, 48, 51
4.	10	? BMD	-
5.	7	Proximal myopathy	-
6.	36	? BMD	Pm, 19, 43, 48
7.	4	Muscle weakness	Pm, 19, 48
8.	12	Not given	-
9.	13	? LGMD	Pm, 19, 45, 48, 51
10.	15	Not given	-
11.	6	Difficulty in sitting	49
12.	4	No family history of DMD	-
13.	5	Difficulty in walking & weakness in lower limbs	-
14.	10	Difficulty in getting up	-
15.	11	Mild mental retardation	49, 50, 51
16.	9	Calf muscle hypertrophy, Gower sign +	-
17.	7	Not given	49
18.	25	? Calpanopathy	Pm, 19
19.	9	Not given	-
20.	3	Delayed developmemtal milestones, Calf muscle hypertrophy	-
21.	8	Not given	-
22.	4	Not given	Pm, 45, 48, 49, 50, 51
23.	9	Gower sign present, scapular winging present	
24.	16	Weakness of all four limbs	48, 49
25.	32	LMN type quadriparesis ? calpanopathy	-
26.	31	Weakness of all four limbs proximal part	-
27.	8	Scapular winging, delayed milestones	-
28.	8	Delayed milestones	-
29.	9	Weakness of lower limbs	-
30.	17	Pain in legs	45, 46, 47
31.	7	Calf muscle hypertrophy, Gower sign present	-
32.	13	Calf muscle hypertrophy, Gower sign present	-
33.	10	Delayed milestones	6, 50
34.	7	Not given	-
35.	20	Not given	-
36.	10	Not given	-
37.	12	Delayed milestones	-
38.	20	Not given	-
39.	10	Not given	-
40.	12	Calf muscle hypertrophy	45, 47, 49
41.	7	Calf muscle hypertrophy	-

Table 2: Genetic testing & immunohistochemical results for 09 patients

S. No.	Age (years)	Immunohistochemistry									Deleted exons
		Dys1	Dys-C	Dys-N	αSG	βSG	γSG	δSG	Dysferlin	Merosin	
1.	10	+++	+++	+++	+++	+++	+++	+++	-	+++	-
2.	36	+++	+++	+++	+++	+++	+++	+++	+++	+++	Pm, 19, 43, 48 (?BMD)
3.	15	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
4.	6	+++	+++	+++	+++	-	-	-	-	+++	49
5.	32	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
6.	8	+++	-	+	+++	-	-	-	+++	+++	-
7.	7	+	+	-	-	-	-	ND	+++	-	-
8.	20	+++	-	+	-	-	-	+++	-	+++	-
9.	10	+	+	-	+++	+++	+++	+++	+++	+++	-

Dys- dystrophin, SG- sarcoglycan

γ-Sarcoglycan, δ-Sarcoglycan, Dysferlin and Merosin, patients could be grouped in four groups (Table 2).

In group I, Case 1, 3 and 5 were included which showed preserved architecture of muscle with normal expression of dystrophins (rod domain, C-terminal & N-terminal), sarcoglycans (α, β, γ, δ), dysferlin and merosin protein. Case 1 was the only exception, which showed complete absence of dysferlin. On multiplex PCR testing, no deletion of any exon of dystrophin gene was observed.

Group II had four patients (case 6-9), showing irregular pattern of dystrophin immunoreactivity with complete preservation of all other muscle proteins and no deletion of any exon of dystrophin gene. The staining pattern of case 6 and 8 showed normal expression of dys-rod domain with patchy dys-N and absence of dys-C, whereas case 7 and 9 showed irregular staining for dys-N along with irregular expression for dys-rod domain and dys-C.

In group III (case 2), normal distribution of all the muscle protein seen on immunohistochemical analysis, but four (Pm, exon 19, 43 and 48) deletions in the dystrophin gene were seen on multiplex PCR.

In last group, group IV (case 4), βSG, γSG, δSG and dysferlin were severely reduced and staining for dystrophin (C, N & rod domain) were clearly detectable. Further, screening of the dystrophin gene revealed the deletion of exon 49.

DISCUSSION

In the present study, multiplex PCR method was used to detect deletions in dystrophin gene in 41 patients. Out of 41 patients, 14 patients (34.1%) showed deletion, which is much lower than the proportion of deletions reported in different population of India using same methodology (multiplex PCR method). There is a wide variation in reported frequency of deletion in various parts of India and it ranges from 62.1% to 74% (8-10). Such wide variation in deletion rate is attributed to population based differences in mutations of dystrophin gene which could be due to accumulation of differences in intronic sequences and their distribution of differences over a period of time as a consequence of genetic drift. Such sequences unique to the population may lead to intragenic deletions due to mismatch at locus.[11] As deletion rate detected was much lower than reported in literature in the present study, it is difficult to draw a conclusion. However, all the patients tested for deletion in the present study did not have characteristics clinical features of DMD/BMD, whereas in literature the deletion rate was reported in cases having characteristics clinical features of DMD/BMD. Also, our study had a small sample size.

In our study 66.6% of the deletions were located at distal hotspot (exon 45-55) and 15.38% of the deletions were located at proximal hot spot region (exon 2-19). Our data conforms to Basumatary et al,[8] which showed 85.7% and 14.3% deletions in distal and proximal hotspot regions respectively.

Correlation of immunohistochemical analysis & mutation analysis on diagnosis of DMD/BMD

In Case 1, 3 & 5(Group I), there was a family history of muscular dystrophy. However, all subjects were more than 10 years and were ambulatory. In clinical notes, there was suspect of DMD in case 3 and sarcoglyconopathy in case 5. On immunohistochemistry, expression of all muscle proteins was normal except case 1 where dysferlin protein was absent. On multiplex PCR no deletion of dystrophin gene was detected. Hence in case 3 and 5, diagnosis was incomplete and

requires further work up to rule out limb girdle muscular dystrophy (LGMD).

In Group II (case 6-9), there is irregular staining for dystrophin protein, whereas other muscle protein i.e., sarcoglycan, dysferlin and Merosin showed normal staining with no deletion of dystrophin gene. As clinical notes were available only for case 6, it is difficult to correlate these lab findings with phenotype of patients, and diagnosis remains incomplete. Therefore, further investigations should be done to look for the duplications or point mutations on the dystrophin gene.[12]

In case 2 (Group III) histopathology findings were suggestive of calpainopathy, whereas on multiplex PCR, deletion of Pm, exon 19, 43 and 48 was detected. In this patient, the deletions maintain the reading frame as these deletions are located at the region of central rod domain of dystrophin, thereby supporting the diagnosis of BMD. As per rule of Monaco,[13] the difference between DMD and BMD depends on the reading frame. If the mutation occurring in dystrophin gene does not disturb the reading frame, a shortened but functional protein may present as milder phenotype (BMD). Bellayou et al also showed similar kind of findings in the case of BMD.[12] However, western blotting analysis needs to be done to detect the truncated proteins, which may provide useful information regarding BMD[14] or calpain protein to rule out/confirm calpainopathy.

In case 4, muscle biopsy showed large areas of inflammatory infilterate and myofibre necrosis. Expression of dytrophin protein is normal on immunohistochemical analysis with deficiency of sarcoglycan and dysferlin protein. But on multiplex PCR, deletion of exon 49 was observed. Though the histological findings are suggestive of inflammatory myopathy, but it is well known that many histopathology overlaps exist between inflammatory myopathy and dystrophy with inflammation. Positive immunostaining can be misleading and it needs to be supplemented by mutation analysis to rule out DMD and western blotting to quantify the dystrophin protein to rule out BMD especially if it is clinically indicated.

CONCLUSION

In the present study, 34% patients showed deletion with 66.6% of the deletions located at distal hotspot and 15.38% of the deletions at proximal hot spot region. However, being the largest gene, whole dystrophin gene cannot be screened for deletion/point mutation/duplication. Hence complete work up of any muscular dystrophy requires immnohistochemical analysis to see the expression of muscle proteins along with multipleplex PCR test to detect any exon deletion, multiplex ligation-dependent probe amplification (MLPA) to detect point mutation and duplication and western blotting to quantify the dystrophin protein.

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Authors Contribution:
RA- Conceptualized study and design of the study, reviewed the literature, manuscript preparation and critical revision of the manuscript; **SC-** Manuscript preparation and critical revision of the manuscript; **NC-** Manuscript preparation and critical revision of the manuscript; **IP-** Collecting data for immunohistochemical profile of DMD patients and review of study; **A-** Collecting data for dystrophin gene deletion studies and literature search.

Orcid ID:
Dr. Rachna Agarwal: http://orcid.org/0000-0003-2604-9809
Dr. Sujata Chaturvedi: http://orcid.org/0000-0002-2680-2947
Dr. Neelam Chhillar: http://orcid.org/0000-0001-7948-3022
Dr. Ishita Pant: http://orcid.org/0000-0003-4222-0207
Ms. Anuradha Sharma: http://orcid.org/0000-0003-4192-6689

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