Study of Dystrophinopathy in Eastern Uttar Pradesh Population of India

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Introduction: The frequency and distribution of dystrophin gene deletions vary in patients with Duchenne/Becker muscular dystrophy (DMD/BMD). Objective: In this study, we aimed to analyze clinical, biochemical, and dystrophin gene deletion pattern, by using multiplex polymerase chain reaction (PCR) in the population of eastern Uttar Pradesh and the adjoining districts of Bihar and Madhya Pradesh. Material and Method: After clinical assessment, 225 patients of DMD/BMD were analyzed for deletion in dystrophin gene. Clinical features and biochemical parameters were noted. For genetic study, all samples were tested for deletion from 25 exons of DMD gene by using multiplex PCR. Result: Deletions were detected in 169 (75.1%) patients of DMD/BMD. Deletions were observed in both proximal and mid-distal hot spot regions with maximum deletion localized in the mid-distal hot spot region of the gene. The most frequent deletions were observed in exon 50 (14.9%) and exon 49 (10.8%). Conclusion: This study concludes that mid-distal region of dystrophin is highly polymorphic in the population of eastern Uttar Pradesh and responsible for pathogenesis of DMD. The population of eastern Uttar Pradesh shows similar pattern of deletion in dystrophin gene when compared with other ethnic groups of the Indian population.

Keywords: Becker muscular dystrophy, Duchene muscular dystrophy, dystrophin, multiplex polymerase chain reaction

BACKGROUND

Dystrophin gene deletion is the main leading cause of X-linked recessive neuromuscular disorder Duchenne muscular dystrophy (DMD) and its allelic form Becker muscular dystrophy (BMD).1 The incidence ratio of DMD is 1 in 3,500 and BMD is 1 in 18,000 male births.2,3 DMD is characterized by muscle weakness, calf hypertrophy, and Gower's sign.4 Dystrophin is the largest gene that spans more than 2.5 billion base pairs, which consists of 79 exons. Deletion of exons is nonrandomly distributed in specific hot spot areas of the gene. These hot spot areas are divided into two regions: (1) proximal hot spot region, which contains exons 1–19 and leads to about 20% of the deletions in this area, and (2) mid-distal hot spot region, which covers exons 42–55 and leads to approximately 80% of deletions in this region.5,6 Due to the deletion in the gene, it leads to shift in reading frame (out of frame) and results in very low production of functional dystrophin protein, and subsequently results in severe form of DMD phenotype. In BMD, reading frame is preserved (in frame) and that leads to the production of semi-functional protein.7 Due to altered protein, muscle degradation occurs and it leads to destruction of muscle proteins such as creatine kinase. Overall, approximately 65% large-scale deletions, 5% duplications, and 30% point mutations conform to the reading frame. Due to the bigger size of gene and highly polymorphic nature of deletions, direct sequencing of gene is very expensive. Yet several techniques are discovered for detection of mutation in the gene. Multiplex polymerase chain reaction (MPCR) is one of the cost-effective and

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highly specific techniques for the detection of large gene deletions. Recently, multiplex ligation-dependent probe amplification (MLPA) method is also used to detect deletion and duplication as well as distribution and extent of deletion and duplication in the DMD gene.\[8\] This study has been undertaken to evaluate the types and distributions of deletions, clinical, and biochemical parameters in patients of DMD/BMD in the population of eastern Uttar Pradesh by using MPCR assays. After deletion analysis, we compared our study with the other ethnic groups of the Indian population.

**MATERIALS AND METHODS**

**Sample collection**

A total of 225 patients who were clinically confirmed or suspected for DMD/BMD were recruited from the outpatient department of neurology of Sir Sunderlal Hospital Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India, which provides health services to the population of eastern Uttar Pradesh, Bihar, Jharkhand, Chhattisgarh, and the adjoining districts of Uttar Pradesh. Detailed clinical evaluation was noted by clinicians regarding the age of onset, family history, and strength of muscle. We also evaluated some blood parameters such as creatine phosphokinase (CPK) level, and lactate dehydrogenase (LDH) level, and electromyography (EMG) data for strengthening the clinical evaluation. Blood samples from the patients were collected in ethylenediaminetetraacetic acid–coated vials after obtaining informed consent from the patients and/or their parents in case of minors.

**Genomic DNA isolation and multiplex PCR**

Genomic DNA was extracted by using the standard method,\[9\] which includes slight changes according to the standardization in the laboratory. Genomic DNA was quantified to check the quality and quantity of DNA (Nanodrop Spectrophotometer; BioTeK, Winooski, Vermont USA). MPCR method was performed as per the study by Chamberlain et al.,\[10\] modified by Beggs et al.\[11\] and Sinha et al.\[12\] For dystrophin gene deletion analysis, primers of promoter and 24 exons were designed for DMD gene that was highly polymorphic in nature. To facilitate our result, we combined the primers into five primer set or groups (described in Table 1): group 1 included exons 53, 47, 42, and 60; group 2 included exons 45, 48, 49, 43, and 44; group 3 included promoter, exons 19, 3, 8, and 13; group 4 included 51, 50, 6, 21, and 55; and group 5 included 17, 4, 46, and 34. Exon 52 and exon 12 were run separately. The MPCR conditions included initial denaturation at 94°C for 7 min, denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension at 65°C for 4 min, and final extension at 65°C for 7 min followed by 24 cycles. After MPCR, PCR products were analyzed using standard agarose gel electrophoresis (3% agarose gel) and visualized under ultraviolet transilluminator (Bio-Rad Mumbai, India). Deleted exons that were not amplified were reconfirmed by using a single-reaction PCR.

**RESULTS**

Of the total 225 samples of patients, 169 showed deletion in exons of DMD gene, of which 162 samples were diagnosed with DMD and 7 were diagnosed clinically with BMD. Fifty-six samples that did not show any deletions in 24 exons were categorized under unknown diagnosis [Figure 1].

The deletion pattern for the multiplex set is illustrated in agarose gel loaded with PCR products [Figure 2]. Amplified products represent the presence of exon in DMD gene and missing bands represent deletion of that exon in DMD gene. As shown in Figure 2, deletion of exon 44 was reported because exon 44 was not amplified in that patient. Deletion was confirmed by running (twice) single reaction of PCR for deleted exon. Similar results were obtained in other patients also.

Clinical and biochemical indices of the patients with DMD and BMD are shown in Table 2.

To determine physical disability, we used a Gardner–Medwin and Walton Scale that has been previously assigned to each patients: grade 0, all normal activities and hyperCKemia; grade 1, normal gait, unable to run freely; grade 2, abnormal gait; grade 3, muscle weakness, climbing stairs with support; grade 4, positive Gower’s sign; grade 5, unable to rise from floor; grade 6, unable to climb stairs; grade 7, unable to get up from chairs; grade 8, unable to walk independently; and grade 9, unable to eat, drink, or sit without support.\[13\] Gower’s sign and calf hypertrophy were reported in majority of cases. Maximum cases presented with complaint of lower limb muscle weakness. In Figures 3 and 4, clinical and biochemical data of few samples explained

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**Table 1: Distribution of DMD gene exons in different groups**

| Group | Exons | Product size (bp) |
|-------|-------|------------------|
| Group 1 | 53, 47, 42, 60 | 212, 181, 155, 139 |
| Group 2 | 45, 48, 49, 43, 44 | 547, 506, 439, 357, 268 |
| Group 3 | Pm, 19, 3, 8, 13 | 535, 459, 410, 360, 238 |
| Group 4 | 51, 50, 6, 21, 55 | 388, 271, 202, 178, 119 |
| Group 5 | 17, 4, 46, 34 | 416, 196, 139, 102 |
| Exon 52 | 52 | 113 |
| Exon 12 | 12 | 311 |
the basis on which these samples had been selected for DMD gene deletion analysis.

Patients A-1 and B-1 represent lower limb weakness because of which the patient was unable to stand without support. A-2 represents the back side of the patient showing muscle weakness. Serum CPK of Patient A was 46,556 U/L and value of LDH was 7,634 U/L. In Patient A, exons 46–55 of dystrophin gene were deleted. In case of Patient B, value of CPK was 11,632 U/L and exons 49 and 50 were deleted.

Patients C and D represent winging of scapula and calf hypertrophy. Serum CPK level of Patient C was 25,678 U/L and that of Patient D was 17,289 U/L. Exons 12, 13, and 17 were deleted in Patient C, and exons 49, 50, and 51 were deleted in Patient D. Patient D was a familial case, his maternal uncle was also diagnosed with DMD.

In Patient E, serum CPK level was 16,563 U/L and LDH level was 513 U/L, and exon 51 was deleted in gene deletion analysis. Patient F represents scoliosis; his serum CPK level was 17,240 U/L and exons 48–51 were deleted. Patient G represents BMD sample; his CPK level was 6654.6 U/L and LDH level was 595 U/L. In this patient, exons 6, 13, and 43 were deleted.

After MPCR, deletions were detected in 169 patients (75.1%); among these, 23 patients (13.6%) had single exon deletions and 146 patients had more than one deletion (range, 2–10). Twenty-one patients (12.4%) had two exon deletions, 24 (14.2%) had three deletions, 45 (26.6%) had four deletions, 36 (21.3%) had five deletions, and 16 had (9.42%) six deletions, 3 (1.77%) had nine deletions, and 1 (0.59%) had ten exon deletions [Figure 5].

The most frequently deleted exons were 50 and 49. No deletion was detected on exon of promoter. Approximately 75.5% of all deletions were located on exons 42–52 in mid-distal hot spot region [Figure 6]. In case of BMD, 6 patients reported deletion in exons 42–52 in the mid-distal hot spot region, whereas 1 showed deletion in the proximal hot spot region.
DISCUSSION

DMD and BMD are the most common X-linked inherited neurological disorders. Mapping data and molecular genetics study indicate that both forms are the result of mutation in gene that encodes for the protein dystrophin. Various methods have been used in various laboratories to assess deletion in the dystrophin gene. Before the genetic study was developed, DMD was diagnosed by clinical features and various biochemical methods such as serum CPK levels, LDH levels, and EMG data. Skeletal muscular biopsy and Western blotting were applied in several laboratories. Few laboratories used southern blotting, but this method is difficult and time-consuming, and further, it did not provide data of deletion and duplication of gene and in determining effect of mutation on reading frame. Beggs et al.[11] first developed method MPCR that was cheaper and faster than southern blotting, but this method was applied only to identify deletions in gene. QPCR (quantitative PCR) is another method to determine gene dosage in carrier testing. The most suitable method to detect both deletion and duplication is MLPA technique, which now replaces MPCR by detecting the extent and distribution of deletion and duplication.

| Features          | DMD              | BMD              |
|-------------------|------------------|------------------|
| Total case        | 169 cases        | 7 cases          |
| Age of onset      | 4.2–5 years      | 10–16 years      |
| Consanguinity     | 1                | 0                |
| Electromyography  | Myopathic pattern| Myopathic pattern|
| CPK value         | 2,901–42,260 U/L | 990–8910 U/L     |
| LDH value         | 854–2486 U/L     | 606–1717 U/L     |
| Gower’s sign      | 157              | 1                |
| Calf pseudohypertrophy | 135        | 3                |
| Contracture       | 15               | 0                |
| Scoliosis         | 140              | 0                |

Table 2: Representation of clinical features and biochemical indices of DMD and BMD samples

Figure 3: Clinical representation of patients with DMD showing lower limb muscle weakness, winging of scapula, and calf hypertrophy

Figure 4: Clinical representation of patients with DMD (E, F) is showing contracture in both legs (Patient E) and scoliosis (Patient F). Patient G represents clinical features including winging of scapula and prominent calf hypertrophy in BMD sample
duplication and by facilitating genotype and phenotype correlation.

In India, several previous studies have been carried out to analyze dystrophin gene deletion [Table 3]. In 1992, Sinha et al. [12] performed DMD gene deletion analysis on two of the three families through PCR. In 1997, Khalap et al. [20] worked on 25 patients from western India by using PCR screening method to detect the deletions in 18 samples. Singh et al. [13] in 1997, analyzed 121 unrelated patients with DMD/BMD from North India with MPCR and southern hybridization and reported intragenic deletions in 88 patients. Mallikarjuna Rao et al. [14] in 2003, worked on 66 patients of south Indian population; of these, 41 patients showed intragenic deletion in dystrophin gene. Kumari et al. [15] in 2003, performed deletion analysis of 8 DMD and 10 BMD samples by MPCR and southern blotting using cDNA probes, and detected deletions in 5 DMD and 7 BMD samples, and through quantitative multiplex PCR, they performed carrier detection in 7 families. In 2006, Basak et al. [17] worked on 70 samples from eastern Indian population, of which 46 patients showed large intragenic deletions in dystrophin gene. Dastur et al. [18] in 2008, worked on 347 samples from Western India. Of this, 46 were patients of BMD, and all showed deletions in dystrophin gene. Swaminathan et al. [19] in 2009, tested 112 patients from southern India. A total of 101 patients were confirmed for DMD by histopathology and genetics test analysis. Kohli et al. [16] in 2010, compared gene changes in MPCR and MLPA techniques. They analyzed 180 samples from all over India with both techniques. MPCR technique detected deletions in samples of 90 patients and MLPA detected 3 additional deletions, 16 duplications, and 2 point mutations. In 2010, Murugan et al. [21] used both techniques MPCR and MLPA on 150 male patients, and through MPCR they identified 103 patients of the 150 samples. In 2012, Verma et al. [22] also used MPCR and MLPA for deletion detection, and reported that MLPA detected additional 5.6% cases of DMD and correctly confirmed the carrier status. Mandava et al., in 2014, through MPCR investigated the deletion pattern in 88

![Figure 5: Bar diagram represents distribution of number of exons deleted in the number of patients](image)

![Figure 6: Distribution of various exons deletions among the studied population](image)
patients of DMD/BMD from Gujarat. Of 88 samples, 65 showed deletions in dystrophin gene.[23,24]

In our study, by using multiple PCR, we detected intragenic deletions in 169 individuals (75.11%) (DMD/BMD). No deletion of the promoter region was found. Fifty-six samples (24.88%) that did not show deletion in dystrophin gene were diagnosed for muscular dystrophy only on the basis of clinical findings such as Gower’s sign, lumber sign, calf hypertrophy, biochemical data, and EMG reports. On the basis of our mentioned data, these samples were selected for gene deletion analysis but deletion was not reported for 24 exons in these 56 samples. These 56 cohorts may belong to gamma-sarcoglycanopathy because autosomal-recessive limb girdle muscular dystrophy 2C clinically resembles dystrophinopathy.[25] Further, immunohistochemistry and MLPA tests were suggested for confirming the diagnosis of these samples. In our study, of 169 samples, deletion was reported in both proximal and mid-distal hot spot regions. This study analyzed that maximum deletions were located on exons 42–52 of mid-distal hot spot regions of dystrophin, which code for the rod domain consisting of spectrin-like sequences and play important role in the stability of protein. Very few deletions are reported in proximal hot spot region, which lead, subsequently, to mild disease expression. To evaluate all the existing mutations in the dystrophin gene, they may be subjugated to MLPA; though maximum exons were covered through MPCR method, it does not rule out every mutation in the gene, thus few mutations may have escaped, although clinically, the patients were suspected for DMD/BMD. In such cases, MLPA or direct sequencing can be used. This study helps clinicians in the diagnosis of DMD/BMD among patients, because diagnosis through muscle biopsy is painful and patients are not comfortable with this procedure. There is no genotype-phenotype correlation because severity of disease varies in patients reported for same deletion. So this study supports clinicians to spread awareness regarding genetic disease. In present study maximum cases are familial in nature, in maximum cases maternal uncle of patients are affected with same disorder. Carrier testing is required in these families.

**CONCLUSION**

Deletion of exons is the most common mutation in dystrophin gene, and MPCR identifies majority of mutations and it is relatively economical for the Indian population. In our study, 25 exons were selected for mutational analysis that is highly polymorphic in nature. In eastern Uttar Pradesh population, maximum deletion was reported in mid-distal hot spot regions of dystrophin and exons 50 and 49 were mostly deleted. For the other negative cases, where deletion is not identified by MPCR, multiplex ligation probe alignment or direct sequencing can be used. After the comparison of our result with the other ethnic groups of Indian population, we can conclude that mid-distal hot spot region is highly responsible for the pathogenesis of DMD and BMD in the Indian population, as our data show that the maximum deletion is reported in mid-distal region of dystrophin. By this study, we suggest researchers to develop drugs specific to exons that code for mid-distal region (exons 42–52).

**Declaration of patient consent**

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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**Conflicts of interest**

There are no conflicts of interest.

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**Table 3: Mutational analysis of dystrophin gene deletion pattern in different ethnic groups**

| Indian population studied | Mutation analysis |
|---------------------------|------------------|
| Western (Mumbai, India)   | Most deletions reported in exon 44 and exon 51 in central hot spot region[14] |
| Northern                  | 73% of deletion frequency reported[13] |
| Southern                  | Majority of the deletions (78%) reported in central hot spots and mostly deletion reported in exon 50[15] |
| Eastern (parts of West Bengal, a few eastern states, and Bangladesh) | Maximum deletions reported in (79%) central hot spot and 17.91% at the proximal hot spot region[16] |
| Western (Mumbai, India)   | Most deletions reported in exon 45; most deletions (84.8%) reported in central hot spot region[17] |
| Southern                  | Most deletions reported in exon 50 and 49, and majority of deletions (75.1%) detected in mid-distal hot spot region |
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