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Deletion Mutations in DMD Gene and Disease Phenotype Among Saudi Patients with Duchenne Muscular Dystrophy

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Abstract: The importance of deletion mutations in the Duchenne muscular dystrophy (DMD) gene causing DMD have been reported worldwide. This study specifically looks at the distribution spectrum of these mutations in the proximal and distal 'hot spot' regions of the DMD gene in view of disease phenotype among Saudi patients. It further addresses the limitations of multiplex PCR as a widely used screening method to detect these mutations. Fifteen Saudi children aged 2-19 yrs with either clinically confirmed or suspected DMD/BMD, were analyzed for deletions in 26 exons of dystrophin gene using four different sets of multiplex PCR. The study also included normal controls for validation. Disease progression in these patients was clinically monitored since 2007. Twelve out of 15 patients showed deletions in the DMD gene. The remaining three had no deletions in the gene fragments analyzed. Set 1 detected no deletions in any of the patients, whereas each of sets 2, 3 and 4 detected two, four and three deletions respectively. All of these mutations were located in the distal 'hot spot' region and none were detected in the proximal 'hot spot' region. The normal control samples showed no deletions in any of the 26 exons tested. Therefore distribution and frequency of the most common deletions in a group of Saudi DMD/BMD patients using a multiplex PCR method was demonstrated. The overall distribution of deletion mutations in the distal 'hot spot' region was in accordance with cases investigated elsewhere. However, the study suggested that disease progression was directly related to higher incidence of deletions in distal 'hot spot' of the DMD gene which was indicative of severe form of DMD. Failure to detect any mutation in three out of fifteen samples reflected the limitation of the multiplex PCR technique since clinically these patients were DMD/BMD positive. In conclusion, this study emphasizes the need for further investigation into the genotype/phenotype aspects of the Saudi DMD/BMD population through implying alternative methodologies in order to provide better diagnostic, prognostic and prenatal services to the suffering patients and their families.

Key words: DMD gene, multiplex PCR, X-linked recessive, Saudi Duchenne Muscular Dystrophy, genotype/phenotype correlation, deletion mutation

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophies (MDs) as well as the most frequent muscle disease among children [1,2] affecting 1:3500 live male births. DMD is a fatal neuromuscular genetic disease [2] caused by mutations in the DMD gene. The DMD gene is structurally complex, with 79 exons and 8 promoters, comprising of 2.4 million base pairs, making it one of the largest genes known to date [3]. Mutations in this large gene generally result in a disturbance of the open reading frame during dystrophin protein production that either leads to the synthesis of a truncated, degraded protein molecule or complete absence of dystrophin protein [4, 5]. Approximately 65% of the dystrophin mutations in the DMD gene are the most commonly found intragenic deletions. These deletions are mostly clustered in the two known 'hot-spot' regions; one is towards the 5' end, known as proximal 'hot spot' [6] and the other is near the central part of the gene known as the distal 'hot spot'. Point mutations, insertion and nucleotide changes together account 25-30% [7] and duplications account for 5-10% that appears to be evenly distributed throughout the gene [8]. As has been documented by

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Beggs *et al.* [9], the clusters of these two hotspots represent the basis for the use of the multiplex PCR technique where by screening 19-26 exons, allowing 98% of all deletions to be identified.

Becker Muscular Dystrophy (BMD) results from mutations in the same gene and mode of inheritance is also the same but has a much milder presentation than DMD. In BMD, the course of the disease is slower usually presenting at an average age of 12 years and death generally occurs in the third or fourth decade where patients with milder symptoms may even live longer. The clinical discrimination between DMD and BMD is usually linked to age at which the patient becomes wheelchair dependant, although gross muscle wasting at early age and difficulty in walking may be more indicative of DMD. BMD patients remain ambulant until the age of 16 while DMD patients are wheelchair dependant before the age of 13 years. Serum creatinine kinase (CK) level is the commonly used marker to test for muscular dystrophy, although not specific for DMD, nevertheless CK levels tend to decrease substantially in patients with gross muscle wasting. This, together with relevant clinical profile such as disease history and muscle biopsy results determine the diagnosis and possible course of action. By exploring genetic markers in Saudi DMD/BMD patients, the frequency of mutations can be documented in patients followed by risk assessment in immediate relatives and identification of carriers.

Multiplex PCR technology was used in this study to identify and report the spectrum of deletion mutations in clinically diagnosed Saudi DMD/BMD cases by screening for 26 exons in the 'hot spot' regions of the 79 exons DMD gene. The patients were followed up clinically and disease progression was correlated with the distribution and frequency of deletion mutations.

MATERIALS AND METHODS

Patient Samples and Normal Controls: Fifteen samples were collected from either clinically confirmed or suspected DMD/BMD male patients aged 2-19 years and three of them were brothers from same parents. The patients were referred to us from general hospital in Jeddah, Saudi Arabia. The diagnosis was established by clinical assessment, muscle biopsy, family history and serum CK level, as illustrated in Table 1. Serum CK levels were also observed in blood samples collected from immediate relatives of the affected ones. In addition, 15 healthy subjects were used as normal controls.

Table 1: Patients' profile showing serum CK levels and the age of analysis. Other family members including affected brothers* from same parents are also shown

Patient	Age at the time of analysis(years)	CK concentration IU/l	No. of brothers	No. of sisters	Other affected siblings in respective family
1*	2	3000	4	3	2
2	4	32000	-	-	-
3*	5	2000	4	3	2
4	5	22000	2	1	-
5	6	20000	1	-	-
6	6	6000	-	-	-
7	7	12000	1	1	-
8	7	2000	-	-	-
9	8	3000	4	1	-
10	8	8000	2	3	-
11	9	3000	5	2	-
12*	10	900	4	3	2
13	11	3700	-	-	-
14	15	18000	1	1	-
15	19	900	1	1	-

Table 2: Four separate multiplex sets optimized for exon amplifications with corresponding fragment size (bp) for differentiation on PAGE

Multiplex Set-1								No of exons
exon	60	5	4	7	8	3		6
(bp)	139	167	196	315	360	410		
Multiplex Set-2								
exon	42	62	52	17	43	12	49	7
(bp)	155	191	265	326	357	405	439	
Multiplex Set-3								
exon	6	53	50	9	51	1	48	7
(bp)	175	212	271	321	388	419	506	
Multiplex Set-4								
exon	46	47	13	45	44	19		6
(bp)	148	181	238	307	426	459		

Genomic DNA Isolation and Multiplex PCR: Three to four ml of whole blood was collected in sterile purple top tube with EDTA. as anticoagulant factor. Qiagen™ mini column based DNA extraction kit was used to extract high quality DNA from whole blood samples. DMD gene deletions were detected using four multiplex PCR reactions.Using our optimized protocols, as described in Chaudhary *et al.* [12]; a total of 26 exons were analyzed, six with multiplex set-1, seven with multiplex set-2, seven with multiplex set-3 and six with multiplex set-4 as shown in Table 2 with corresponding fragment size in bp.

The reaction mixture was made in a volume of 50 µl containing 1x homemade PCR buffer, 10 mM dNTPs, 1M $MgCl_2$, 200 ng/ml of each primer, 5000 U/ml Taq DNA polymerase (Amersham biosciences, USA) and 200 ng/ml (2µl) of genomic DNA of each sample. The amplification was carried out as a 'hot start' followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 70°C for 2 minutes. Cycling was concluded with a final extension at 70°C for 5 minutes. The PCR products were analyzed by 5 % Polyacrylamide gel. Then the gel was visualized under the UV light and photographed after Ethidium bromide staining.

RESULTS AND DISCUSSION

The deletion pattern for one of the multiplex sets is illustrated in Figure 1. Similar results were obtained for other multiplex sets where all patient samples were analyzed. Exon deletions were observed in twelve out of fifteen patients' samples, where the remaining three samples did not show any deletions for all of the four multiplex sets tested.

The distribution of deletions in the twelve samples is summarized in Table 3. These deletions were confined to the distal 'hot spot' region of the DMD gene that included exons 45 to 53. The number of exons deleted in a given patient varied from three in patient #2, to at least nine exon deletions observed in patients # 1, 3 and 12.

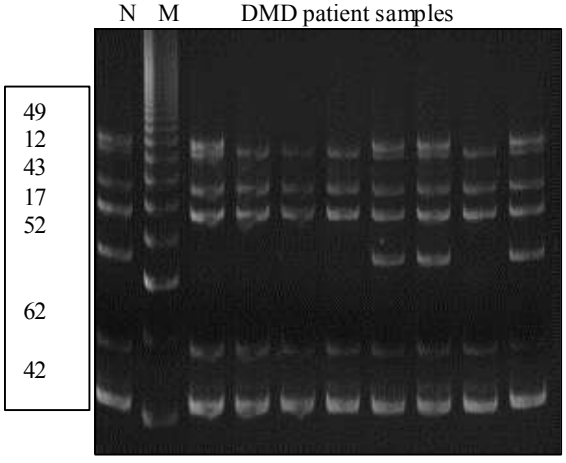

Fig. 1: PAGE shows multiplex PCR for one of the optimized multiplex sets detecting deletion mutations in the DMD gene. Also, shown is normal sample (N) with seven exonic amplifications followed by ladder marker (M) and DMD patient samples, some with deletions

These patients were also brothers from same parents. Clinical symptoms in all the patients tested varied from mild to severe to wheel chair bound. Patients # 12 & 15 had died by the time current clinical manifestations were recorded and each of them had 8 to 9 exon deletions. For all of the fifteen patient samples, no deletions were detected in the proximal 'hot spot' region of the DMD gene, which included exons 1-19.

Although DMD is the most common form of MDs frequently occurring in children across the world [10], yet in Saudi Arabia it is considered a rare disease. This is either attributed to exceptionally low incidence rate or that the positive cases are not reported due to the lack of functional national registry for this disease. The latter emphasizes the need for establishing national registry to allow proper estimation of DMD/BMD status in Saudi Arabia.

Molecular studies addressing mutation analysis of DMD gene in Saudi Arabia are very few [11] [12]. These studies focus mainly on establishing multiplex PCR method for the detection of deletion mutations and to a lesser extent, their distribution in the 'hotspot' regions of the DMD gene. In this study, we report a genotype / phenotype correlation by looking into the frequency of deletion mutations in view of disease progression, over a period of three years, in affected siblings and their first degree male relatives. The method implied in this study was based on multiplex PCR targeting 26 exons. However, it failed to diagnose causative deletions outside the 'hot spot' regions in three out of fifteen cases clinically confirmed as DMD [13], subsequently highlighting the limitation of this approach and suggesting that it be used as screening rather than a confirmatory tool. Other more robust, fluorescent based multiple ligation probe assay (MLPA) should be considered as the method of choice both for confirming deletion mutations by scanning the 79 exons of the DMD gene [14], as well as testing for carrier status in unaffected females without X-autosome translocations [7].

In the remaining twelve patients' samples, deletions were coincidently, confined to distal 'hot spot' region [8, 11, 15], more so in patients 3, 8, 10, 11, 12, 13 and 15, it was demonstrated that large fragment deletions of six or more exons gave rise to severe disease phenotype causing rapid immobilization, cardiomyopathy and subsequent death (Table 3) [16]. The study also suggested that these deletions can be used as predictive markers for disease outcome in first degree male relatives particularly brother(s) of the affected sibling, as in the case of asymptomatic two years old child #1, brother of

Table 3: Deletions in 12 out of 15 Saudi DMD patients are seen in this table. Summarized here is the distribution of these deletions followed by recent clinical status

Patient Sample #	Exons deleted	No. of deletions	Clinical status
1*	45, 46, 47, 48, 49, 50, 51, 52, 53	9	asymptomatic
2	51, 52, 53	3	mild
3*	45, 46, 47, 48, 49, 50, 51, 52, 53	9	severe
4	49, 50, 51	3	mild
5	No deletions found in both distal and proximal regions of DMD gene	0	mild
6	No deletions found in both distal and proximal regions of DMD gene	0	mild
7	48, 49, 50	3	moderate
8	46, 47, 48, 49, 50, 51, 52, 53	8	severe
9	No deletions found in both distal and proximal regions of DMD gene	0	mild
10	45, 46, 50, 51, 52, 53	6	severe
11	47, 48, 49, 50, 51, 52	6	WCB
12*	45, 46, 47, 48, 49, 50, 51, 52, 53	9	dead
13	45, 46, 47, 48, 49, 50, 51, 52	8	WCB[a]
14	46, 49, 51, 53	4	moderate
15	46, 47, 48, 49, 50, 51, 52, 53	8	dead

Mild = fatigue and/or any detectable weakness including clumsiness, falling, abnormal gait, toe walking and slow running in the absence of positive Gower's signs.

Moderate = positive Gower's sign, difficulty with stairs and/or waddling gait.

Severe = inability to rise without assistance and/or walking only with effort and/or severe wasting of muscles.

WCB = wheelchair bound.

[a]Dilated cardiomyopathy.

*With an affected brother

Table 4: Frequency of deletions in the twelve patients suffering from DMD/BMD with most common being deletion 51 occurring a total of 11x followed by 49 & 50 (10x each). The least common deletion was that of exon 45 (5x only)

Exon	Frequency of each deletion	Frequency as Percentage (%)
45	5x	6.67
46	7x	9.34
47	7x	9.34
48	8x	10.67
49	10x	13.34
50	10x	13.34
51	11x	14.67
52	9x	12.00
53	8x	10.67

patients #3 and #12. This predictive assessment in such high risk individuals can help introduce interventional therapies at much earlier stage allowing better quality of life with lesser overall economic burden. Furthermore, these deletions can be used as pro-bands in prenatal diagnosis in subsequent pregnancies as shown in studies carried elsewhere [17]. Patients with fewer deletion mutations suffered from mild to moderate symptoms more suggestive of BMD.

The most common deletion was that of exon 51 (47%) (Table 4), which also coincided with Egyptian DMD patient group [18]. Recent advances in gene therapy emphasize on deletion and frame shift mutations in the DMD gene, because they provide prospect for exploiting siRNA based technology [19] for much needed gene regulation and exon skipping therapy in addressing such debilitating disease [20].

Although CK levels coincided with muscle wasting in our candidates nevertheless studies have shown that *de novo* mutations in the DMD gene can cause DMD/BMD to occur in families without prior disease history [2, 21, 22], where all the family members of the affected ones, present normal serum CK levels. This means that the other brothers of the patient are not affected and even the mothers and sisters may not carry DMD/BMD mutations. It has been reported that 70% of the carrier females show slight elevations in serum CK levels.

CONCLUSION

The study was carried out on a limited number of Saudi DMD/BMD patients and their family, however, distribution and frequency of deletion mutations in the DMD gene with relation to disease progression clearly indicated severity of DMD to be directly proportional to size and region of deleted gene fragment. The study strongly emphasizes the need for further investigation into the genotype / phenotype aspects of the Saudi DMD/BMD population through implying alternative methodologies in order to provide better diagnostic, prognostic and prenatal services to the suffering patients and their families.

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