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Evaluation of Dystrophin Gene Deletion Patterns in a Large Duchene/Becker Msuscular Dystrophy Patient Sample; 17 Years Experience from one Turkish Diagnostic Center

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ABSTRACT

Background: Duchenne/Becker muscular dystrophy (DMD/BMD) is an X-linked recessive disease results from mutations in the dystrophin gene. We established the deletion pattern profile in unrelated DMD/BMD patients using multiplex PCR (M-PCR). **Methods:** During 1998-2015, 1,385 unrelated DMD/BMD patients were admitted for genetic confirmation and/or exclusion of the disease. Deletion analysis in the dystrophin *(DMD*) gene was performed. **Results:** Of all patients admitted, 42.6% deletion carriers (n=589) were detected, of which 180 (80.3 %)were carrying single exon deletions and 409 (14.8 %) multiple exon deletions. Deletions covering the major hotspot region were 80.3 %, the minor region 14.8% and 2.4% covered both regions. The mean age of diagnosis of patients with out-of-frame deletions (7.27 year) was notably lower than the cases with in frame deletions (17.54 year). No single exon 4 deletion was detected. **Conclusions:** When the known deletion hotspots are considered, the study population showed a similar deletion pattern with other populations. The mean age of patients with out-of-frame deletions were lower than mean age of those with in-frame deletions, in concordance with the reading frame hypothesis. Strikingly, no single exon 4 deletion was found, supporting the hypothesis that absence of it might have no functional consequences.

Introduction:

Duchenne/Becker muscular dystrophy (DMD/BMD) is an X-linked recessive neuromuscular disease with an incidence of 1/3,500-1/18,000 [1-4]. DMD is characterized by rapid progressive degeneration and necrosis of the proximal muscles and calf pseudo-hypertrophy. Most of the DMD patients show muscle weakness in early childhood and become wheelchair-bound at the age of 12 and die of respiratory or cardiac failure in the late teens or early 20's. BMD has a slower rate of progression; affected individuals remain ambulatory beyond the age of 16 years and may lead rather normal lives. During the infantile period, DMD/BMD can be identified in boys by elevated serum creatine kinase activity, even before the manifestation of muscle weakness. The causal mutations reside on the dystrophin gene. Dystrophin gene being the largest known gene with 79 exons, is located at Xp 21.2 and is mainly expressed in skeletal muscle, myocard and brain [5]. Dystrophin protein is involved in the contractile apparatus of the muscle cells and has a molecular weight of 427 kDa. [6]. Dystrophin has of four functional domains: N-terminal region (exons 1 to 8); central rod domain (exons 9 to 63); cysteine-rich region (exons 64 to 68); and C-terminal domain (exons 69 to 79). Mutations inthe dystrophin gene may lead to either DMD or BMD, where the difference can be explained by the reading frame rule [7]. Mutations disrupting thedystrophinmRNA reading frame lead to DMD, while mutations with conserved reading frame produce proteins with some residual function and result in mild BMD. The reading frame rule is applicable in 92% of DMD and BMD patients [6, 7]. In most DMD/BMD cases, mutations are large intragenic deletions (65%) and duplications (5-8%) that encompass one or more exons of the gene [8-10]. Most deletions are clustered on two hot spot regions, the major hot-spot site encompassing exons 44–52 (70%) and a minor hot-spot site including exons 3–19 (30%). Duplications are concentrated in proximal part of the dystrophin gene. Point mutations are rather dispersed to the whole gene therefore they are more difficult to detect [11-14]. Molecular diagnosis of DMD/BMD has been traditionally established by multiplex PCR (M-PCR) analysis of hot spot exons, where exons that fail to amplify are considered deleted in the patient [15, 16]. Using these traditional methods, deletion detection may be achieved in 90-98 % of male patients**[16].**

The aim of this present work was to determine the deletion pattern profile 1385 unrelated of the DMD/BMD patients using M-PCR. Since this informationis necessary in clinical counselling, particularly for differential diagnosis diagnostics; we conducted this study to assess efficiency of M-PCR as an inexpensive rapid method for initial screening. After this initial screening, the patient might be referred to addional advanced tests such as MLPA if necessary.

Material and Methods :

Patient samples

During the period of 1998-2015 June, 1,385 unrelated DMD and BMD Turkish patients were sent to our laboratory for genetic confirmation and exclude of the disease.We performed mutation analysis in the *DMD* gene using M-PCR[15,16].M-PCR primers were used which cover 17 exons and the muscle specific promoter in the deletion hot spots. PCR and agarose gel electrophoresis was performed by standard protocols [15,16]. Genomic DNA was extracted from leukocytes by a salting out procedure [17].

Multiplex PCR deletion screening:

For deletion studies, 17 exons (3, 4, 6, 8, 12, 13, 17, 19, 43, 44, 45, 47, 48, 50, 51, 52 and 60) and the muscle specific promoter (Dp427m) of the Dystrophin gene were tested by using M-PCR technique according to the of Chamberlain et al. [15] and Beggs et al. [16]. The PCR products were separated on 4% agarose gel. Deletions were diagnosed when one of the bands present in the amplified control DNA was absent from the patient DNA.The frame shift hypothesis was checked in the database Leiden muscular dystrophy Pages(www.dmd.nl) according to the extension of the deletions[18].

Statistical methods:

Data are presented as mean ± standard error (SE).The frequencies and distributions data were analyzed using SPSS 10.0 statistical software. Significance was assumed at a probability value of $P < 0.05$. We investigated the relationship between the locations of the deletions and the age diagnosis using the simple chi square test.

Results:

In this study, the dystrophin gene was screened for deletion mutations in 1385 subjects for initial differential diagnostics of DMD/BMD disease based on clinical and electrophysiological findings. The dystrophin gene deletion mutations were detected in 589 (42.6%) patients. The mean age of the admitted patients was determined as

13.92among those, the mean age ofpatients with
and without deletions was 9.41 years (ranged from 0 month to 57 years) and 17.3years(ranged from 2 months to 79 years) respectively. Although we could attribute the age of diagnosis to 589 probands, the age of onset could be determined in only 293 patients, the analysis was done using age of diagnosis. In 3.3 % (n=20) of the patiens another sibling was affected.

The results in are summarized in Table 1, showing the73 deletion patterns in the dystrophin gene and observed frequencies.Single exon deletions were observed in 180 cases and multiple exon deletions were observed in 409 cases.

When multiple exon deletions were taken into account, the most commonly deleted exon was 45. Where fifty one of the patients carried exon 45-47 (8.7%) deletion and fifty of the patients carried exon 45-52 (8.5 %) deletion. The most of the deletions were confined to the central hot spot spanning the region between exons 43 and 52 (80.3 %). The 5'-proximal hotspotlies between the exons 3 and 19(14.8 %), whereas2.4% covered both hot spotsat the same time (Table2).The largest deletion observed in one proband comprised the 3-51 exons. Other large scale deletions spanning over 40 exons covered were found in thirteencases. These large scale deletions ranging exon 3 to 43, exon 3 to 44, exon 3 to 45, exon 3 to 51, exon 8 to 44, exon 8 to 50, exon 12 to 43, exon 12 to 44, and exon 17 to 44 were spreadoverthe both hot spot regions.Six patients had muscle promoter (Dp427m) deletion only and three patients had larger deletions comprising Dp427m.

Single exon deletions were 30.5 % of all deletions, where of exons 50 and 45 accounted the most frequent (19.4 and 18.9 %,respectively). The schematic view of exon deletion frequencies, are displayed in Figure 1. Considering the central region of the gene (exons 43-52), the most frequently deleted exons were 47, 48 and 50 with frequencies of14.9, 14.5 and 13.8 %respectively. Whereas, within the proximal hotspot region (exons 3-19) the most deleted exons were 13(7.2%),12 (7.0 %), 6 and 7 (6.7 %).

The distribution of the deletions among age groups are shown in Table 1.The patients with deletions were grouped as ≤5 years (*I; n*= 194), 6-13 years (*II; n*= 302) and ≥14 years (*III; n*=93) according to the age of diagnosis (Table1). There was no statistically significant relationship between the age of diagnosis and
the deletion hot spots (*p=0.832*). The largest deletions were observed in the ≤5 age group.

Out-of-frame deletions were found in 92cases,whereas in-frame deletions were found to be 63 (Table 3).The mean age of diagnosis was notably lower in cases with out-of frame deletions (7.27) as compared with in frame deletions (17.54).The most common in-frame deletion was found to be 45-47, of which the age range was1 and 57 years.Reading frames were not clear for 434 cases, since determination of the exact breakpoints was not possible with M-PCRmethod.

Discussion:

Identification of deletions indystrophin gene provides information for more accurate genetic counseling and prenatal diagnosis for DMD/BMD patients.We identified the deletionfrequencies, patterns and distributions in the dystrophin gene in 1,385 male patientsfor differential diagnostics.The deletion frequency wasdetermined as 42.6 % in our study group. Different deletion rates were declaredfor several populations, such as European (39% to 63%), Mexican (52%) and Argentine (32%) [19-30]. Furthermore, in Asian populations, namely Singaporean, Japanese and Vietnamese, the deletion rate was 40, 51 and 32 %, respectively [31]. Our results are in concordance with these studies. When patients are initially diagnosed using muscle biopsy and then referred to molecular testing the frequency for deletions are found as 52-64 % in Turkey [32-35].

This current study includespatients that were admitted mostly for non-invasive initial screening for differential diagnosis without muscle biopsyconfirmation.Therefore this slightly lower deletion frequency might be attributed to phenotypical and genotypical heterogeneity.

A very striking outcome was the fact, that no single exon 4 deletion was observed in 1,385 patients. This finding supports Torelli and Muntoni [36], hypothesizing that exon 4 can be spliced out in skeletal and cardiac muscle and that the absence of exon 4 alone is apparently without functional consequences.

It is known that deletions are non-randomly distributed, occurring mainly (~80%) in the central- (exon 44 to exon 60) and less frequently (~20%) at the proximal (5') region (exons 1 to 19), which are referred as the 'major' and 'minor'

deletion hotspots, respectively [8,11]. Our study group displayedapproximately 82.6% deletions within the major hotspot (exons 43-52) and15 % in the proximal 5' hot spot (3-19) region, which wascompatible with previously reported studiesin Turkish as well as other populations [31, 33-35]. The only exception to this distribution pattern was reported for the Filipino population, whereminorhotspot deletions were more common than major hotspot deletions. However, this anomaly could be attributed to the small sample size of that particular study[37].

Five patients having single deletions were younger than 5 years of age and one patient was in 6-13 age group.Also longer deletions spanning the promoter region were detected,being one case in each age group (Table1). The largest deletion was between the promoter and exon 44 and age of diagnosis was two years. The oldest case was 24 years and he was carrying Dp427m-exon3 deletion. The rest of the patients carrying promotor deletions were under age of 14. Another Turkish study[35]reported four promoter deletions in DMD patients, similarly we detected nine casescomprising the promoter region. These findings support that promotor deletions lead to a more severe clinical phenotype as reported by Frisco *et al*[38].

According to the proposed "reading-frame hypothesis", deletions that alter the reading frame of dystrophin mRNA produce no functional dystrophin and therefore cause severe DMD, while in-frame deletions may produce partly-functionaldystrophin leading to the milder Becker disease[7]. After classifying the deletions, our findings also support this hypothesis. The mean age at diagnosis was determined as 7.27 for 92 out-of frame cases, whereas 17.54 years for63 in framecases. Reading frame status might therefore be used as a tool for prediction of prognosis even in the absence of the clinical information.

Multiplex PCR is still the most common and relatively simple screening method for the detection of dystrophin gene hot spot deletions. However, it is not possible to characterize all the deletion breakpoints, to detect duplications and to testcarriershipof females.

To determine the reading frames exact mapping of the brakepointsis necessary. For example, single deletion in exon 45 only creates out of frame mRNA, whereas, a 45-46 exon deletion creates an in frame distorted mRNA molecule.

Another strategy for identification of deletion borders might be usage ofMLPA (multiplex ligation-dependent probe technique, which is methoddetecting the 79 exons. By this method, detection of deletions, duplications and carrierstatus in female individuals is possible.However alterations involving point mutations and short deletion/ duplications requires full gene-sequencing.

In conclusion, the present study suggests that the deletion frequencies and patterns in the dystrophin gene are similar to other populations tested so far. Multiplex PCR,as a fast screening method, identifies most of the mutations in an inexpensive and effective manner. Therefore it is still suitable as initial screening for differential diagnosis prior to more expensive and tedious MLPA method.

Competing interests

The authors declare no competing interests.

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	Allpatients		Age groups					
	(n=589)		≤ 5				≥14	
Exon (s) deletion			(n= 194)		6-13 (n=302)		(n=93)	
	n	Freq. (%)	n	Freq. (%)	n	Freq%	n	Freq(%)
45_47	51	8.7%	6	3.1%	14	4.6%	31	33.3 %
45_52	50	8.5%	16	8.2%	23	7.6%	11	11.8 %
50	35	5.9%	18	9.3%	16	5.3%	1	1.1%
45	34	5.8%	14	7.2%	19	6.3%	1	1.1%
45_48	32	5.4%	6	3.1%	9	3.0%	17	18.3 %
48_50	30	5.1%	9	4.6%	20	6.6%	1	1.1%
45_50	24	4.1%	6	3.1%	15	5.0%	3	3.2%
51	21	3.6%	8	4.1%	13	4.3%	0	0.0%
52	17	2.9%	9	4.6%	7	2.3%	1	1.1%
48_52	17	2.9%	10	5.2%	6	2.0%	1	1.1%
47_48	16	2.7%	7	3.6%	8	2.6%	1	1.1%
51_52	16	2.7%	6	3.1%	10	3.3%	0	0.0%
43	15	2.5%	3	1.5%	10	3.3%	2	2.2%
3_6	15	2.5%	3	1.5%	11	3.6%	1	1.1%
47_51	15	2.5%	5	2.6%	9	3.0%	1	1.1%
44	14	2.4%	3	1.5%	10	3.3%	1	1.1%
47_52	14	2.4%	5	2.6%	9	3.0%	0	0.0%
47	13	2.2%	4	2.1%	9	3.0%	0	0.0%
47_50	13	2.2%	5	2.6%	8	2.6%	0	0.0%

50_52	12	2.0%	5	2.6%	7	2.3%	0	0.0%
12_19	11	1.9%	3	1.5%	7	2.3%	1	1.1%
48	10	1.7%	3	1.5%	3	1.0%	4	4.3%
45_51	10	1.7%	3	1.5%	5	1.7%	2	2.2%
3_19	7	1.2%	2	1.0%	4	1.3%	1	1.1%
Dp427m	6	1.0%	5	2.6%	1	0.3%	0	0.0%
6	4	0.7%	1	0.5%	3	1.0%	0	0.0%
8	4	0.7%	2	1.0%	2	0.7%	0	0.0%
19	4	0.7%	2	1.0%	2	0.7%	0	0.0%
60	4	0.7%	0	0.0%	4	1.3%	0	0.0%
12_13	4	0.7%	1	0.5%	2	0.7%	1	1.1%
3_4	4	0.7%	1	0.5%	1	0.3%	2	2.2%
3_44	4	0.7%	2	1.0%	2	0.7%	0	0.0%
8_19	4	0.7%	1	0.5%	3	1.0%	0	0.0%
13_19	3	0.5%	1	0.5%	0	0.0%	2	2.2%
3_17	3	0.5%	2	1.0%	1	0.3%	0	0.0%
3_43	3	0.5%	0	0.0%	2	0.7%	1	1.1%
3_8	3	0.5%	0	0.0%	3	1.0%	0	0.0%
4_8	3	0.5%	2	1.0%	1	0.3%	0	0.0%
43_44	3	0.5%	0	0.0%	2	0.7%	1	1.1%
6_19	3	0.5%	2	1.0%	1	0.3%	0	0.0%
8_17	3	0.5%	1	0.5%	1	0.3%	1	1.1%
13	2	0.3%	0	0.0%	1	0.3%	1	1.1%
44_51	2	0.3%	0	0.0%	2	0.7%	0	0.0%
44_52	2	0.3%	2	1.0%	0	0.0%	0	0.0%
3	1	0.2%	0	0.0%	1	0.3%	0	0.0%
12	1	0.2%	0	0.0%	0	0.0%	1	1.1%

17	1	0.2%	0	0.0%	1	0.3%	0	0.0%
12_17	1	0.2%	0	0.0%	0	0.0%	1	1.1%
12_43	1	0.2%	0	0.0%	1	0.3%	0	0.0%
12_44	1	0.2%	1	0.5%	0	0.0%	0	0.0%
17_19	1	0.2%	0	0.0%	1	0.3%	0	0.0%
17_44	1	0.2%	0	0.0%	1	0.3%	0	0.0%
3_13	1	0.2%	1	0.5%	0	0.0%	0	0.0%
3_45	1	0.2%	0	0.0%	1	0.3%	0	0.0%
3_51	1	0.2%	1	0.5%	0	0.0%	0	0.0%
4_12	1	0.2%	0	0.0%	1	0.3%	0	0.0%
43_45	1	0.2%	0	0.0%	1	0.3%	0	0.0%
43_50	1	0.2%	1	0.5%	0	0.0%	0	0.0%
43_51	1	0.2%	1	0.5%	0	0.0%	0	0.0%
43_52	1	0.2%	0	0.0%	1	0.3%	0	0.0%
44_45	1	0.2%	1	0.5%	0	0.0%	0	0.0%
44_47	1	0.2%	1	0.5%	0	0.0%	0	0.0%
47_60	1	0.2%	1	0.5%	0	0.0%	0	0.0%
48_51	1	0.2%	0	0.0%	1	0.3%	0	0.0%
51_60	1	0.2%	0	0.0%	1	0.3%	0	0.0%
6_12	1	0.2%	0	0.0%	1	0.3%	0	0.0%
6_8	1	0.2%	0	0.0%	1	0.3%	0	0.0%
8_12	1	0.2%	1	0.5%	0	0.0%	0	0.0%
8_44	1	0.2%	0	0.0%	1	0.3%	0	0.0%
8_50	1	0.2%	0	0.0%	1	0.3%	0	0.0%
Dp427m_19	1	0.2%	0	0.0%	1	0.3%	0	0.0%
Dp427m_3	1	0.2%	0	0.0%	0	0.0%	1	1.1%
Dp427m_44	1	0.2%	1	0.5%	0	0.0%	0	0.0%

Table1. Deletion pattern sand frequencies in the dystrophin gene DMD/BMD patients of our study group.

DeletedRegions	Deletedexons	n	(%)
5' proximalhotspot	43-52	473	80.3
Central hotspot	3-19	87	14.8
Coveringboth hot spots	3-52	14	2.4
	covering Dp427m,		
	exons 51-60, 47-60 and		
Other	60	15	2.5

Table 2. Hotspots of observed deletions.

Table 3. Reading frames assessment and the meanages of diagnosis.

Exon (s) deleted	n	Meanage of diagnosis	Meanage of diagnosis
Out of frameexondeletions			
48-50	30	4	
45-50	24	8	
51	21	6.4	7.27
44	14	8.21	
44-51	2	6.5	
44-47	1	4	
In-frameexondeletions			
45-47	51	19	
45-51	10	11.5	17.54
48-51	1	12	
4-12	1	4	