The Egyptian Journal of Medical Human Genetics (2014) **15**, 235–240

Ain Shams University

The Egyptian Journal of Medical Human Genetics

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ORIGINAL ARTICLE

Non-deletion mutations in Egyptian patients with Duchenne muscular dystrophy

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Received 9 March 2014; accepted 29 March 2014
Available online 19 April 2014

KEYWORDS

Duchenne muscular dystrophy;
Deletion;
Duplication;
Egypt;
MLPA;
Sequencing

Abstract Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophies affecting approximately 1:3500 male live births. Deletion of the dystrophin gene accounts for approximately 65% of mutations, duplications occur in 6–10% while the remaining 20–30% are point mutations, small deletion/insertions, or splicing mutations.

Aim: To study non-deletion mutations in a sample of Egyptian patients with DMD as most previous studies focused on deletion mutations.

Patients and methods: The study included 25 patients with DMD from 18 different families from the genetics clinic, Children's Hospital, Ain Shams University. Diagnosis was made based on typical clinical findings, high CPK and EMG result. Molecular analysis included Polymerase Chain Reaction (PCR) followed by multiplex ligation-dependent probe amplification (MLPA) to those patients with no deletion by PCR. Direct sequencing of the whole dystrophin gene was done to those patients who had no deletion or duplication by the previous 2 methods.

Results: Non-deletion mutation included duplications (5 families (27.8%)) which are higher than previously reported and point mutation (c.583C > T) in only one family. Deletion mutations were found in 9 families (50%) and no mutation found in 3 families (16.7%). Interestingly, 60% of the duplications were located in the distal region of the dystrophin gene. A frame shift mutation

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Peer review under responsibility of Ain Shams University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.ejmhg.2014.03.004

was identified in most patients (93%) except one with duplication of exons 50–51 who had an unexpected severe disease with an early age of onset. Also, an intragenic deletion involving the 5′ end of the dystrophin gene (deletion of muscle protomor and exon 1) was found in another patient with severe disease without cardiac involvement.

Conclusion: The relative higher frequency of duplication mutations in Egyptian patients with DMD may indicate that MLPA and not PCR should be preferred for molecular testing of Egyptian patients with DMD.

1. Introduction

Duchenne muscular dystrophy (DMD; MIM# 310200) is the most common form of all muscular dystrophies caused by mutations within the dystrophin gene and is inherited as X-linked recessive. It is a serious condition with progressive muscle wasting and weakness with most affected boys becoming wheelchair-bound by the age of 12 years and dead by their 20s. A similar but milder condition (known as Becker muscular dystrophy (BMD; MIM# 300376) is caused by mutation in the same gene. The incidence of DMD is approximately 1:3500 male live births [1].

The DMD gene is structurally complex, with 79 exons and 7 promoters, comprising 2.4 million base pairs, making it one of the largest genes known to date [2]. Previous reports suggested that large deletions account for approximately 65% of DMD mutations and 85% of BMD mutations. Duplications occur in roughly 6–10% of males with either DMD or BMD. The remaining 20–30% of mutations are point mutations, small deletion/insertions, or splicing mutations [3]. Most of point mutations lead to premature translational termination due to nonsense (34%), frameshift (33%), splice site (29%), and missense (4%) mutations in the dystrophin gene [4]. Unlike the large deletions that cluster in just two regions, point mutations are more randomly distributed throughout the dystrophin gene [3]. To date, 2556 unique point mutations have been documented in the dystrophin gene [5].

The identifications of the causing mutation in the dystrophin gene is considered very important because it may provide new insights into the function of dystrophin and direct information for genetic counseling, prenatal diagnosis and carrier studies [3,6]. Furthermore accurate molecular diagnosis is essential for different recent mutation specific therapeutic modalities [7].

The aim of this work was to study the non-deletion mutation spectrum in Egyptian patients with DMD because all previous studies focused on deletions only [8–11] and to find the most appropriate molecular method for accurately diagnosing the largest number of our patients.

2. Patients and methods

The study included 25 patients with DMD from 18 different families from the genetics clinic, Children's Hospital, Ain Shams University. Diagnosis was made based on typical clinical findings (progressive symmetric muscular weakness starting $\leqslant$5 years (proximal greater than distal) often with calf hypertrophy), high CPK ($>10\times$ normal) and EMG showing myopathic pattern.

Molecular analysis included

1. Detection of deletion mutations by using Polymerase Chain Reaction (PCR) and amplifying the 79 exons and the Dp427m promoter of the dystrophin gene [12,13].
2. Multiplex ligation-dependent probe amplification (MLPA) was done to those patients with no deletion by PCR using commercial MLPA kits (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions (http://www.mrc-holland.com) [14].
3. Sequencing of the whole dystrophin gene was performed in those patients who had no deletion or duplication by the 2 previous methods using ABI 3037 [15].

The study was approved by the ethics committee of the institute and informed consent was obtained from the parents. The study was carried out in accordance to the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

3. Results

3.1. Clinical findings

Patients' ages ranged between 4 and 20 years. The mean age at diagnosis was 4.5 ± 1.8 years (range 2–9 years). The age of onset was before 5 years in 16 (64%) patients and at the age of 5–10 years in 9 (36%). The main presenting symptoms at the time of diagnosis was abnormal gait in 9 cases (36%), frequent falls in 7 cases (28%), difficulty in climbing up stairs in 6 cases (24%), standing up difficulties in 2 cases (8%) and delayed motor milestones in one case (4%). The mean age of walking was 18 months (range 12–30 months). Six patients (24%) were wheelchair-bound at the time of the study; they lost independent ambulation at a mean age of 10 years (range 9–12 years). Mild to moderate mental retardation was detected in 10 DMD cases (40%). Family history revealed a similar affected member in 9 families (3 sibs and 6 maternal cousins).

3.2. Laboratory investigations

a. Serum CPK levels ranged between 2134 IU/L and 24,000 IU/L with a mean of 11,273 ± 5426 IU/L (normal: 38–173 IU/L).
b. Echocardiography revealed cardiomyopathy in four patients (16%), all of them were above the age of 10 years.
c. Mutation analysis, Table 1:

Table 1 Distribution of mutation found in the studied 18 DMD patients multiplex.

Family no.	Mutation type				Method(s) of detection	In frame/out of frame shifting
	Exon(s) deleted	Exon(s) duplicated	Point mutation	No mutation found by PCR/MLPA/sequencing		
1	–	–	–	+	–	–
2	–	E 50–51	–	–	MLPA	In frame
3	–	–		c.583C > T	–	Sequencing
–						
4	–	E 16–17	–	–	MLPA	Out of frame
5	–	E 45–52	–	–	MLPA	Out of frame
6	E 49–50		–	–	PCR	Out of frame
7	–	E 50–52	–	–	MLPA	Out of frame
8	E 45		–	–	PCR	Out of frame
9	E 45		–	–	PCR	Out of frame
10		E 49–62	–	–	MLPA	Out of frame
11	E 1 & prom (DP427 m)	–	–	–	PCR	Out of frame
12	E 45	–	–	–	PCR	Out of frame
13	E 49–54	–	–	–	PCR	Out of frame
14	–	–	–	+	–	–
15	–	–	–	+	–	–
16	E 52	–	–	–	PCR	Out of frame
17	E 46–55	–	–	–	PCR	Out of frame
18	E 45	–	–	–	PCR	Out of frame
Total	9 (50%)	5 (27.8%)	1 (5.5%)	3 (16.7%)	–	–

- PCR analysis of all exons and exon/intron boundaries revealed deletions within the dystrophin gene in 9 families (50%). This included single exon deletion in 5 families (55%) and multiple exon deletions in 4 (45%). Exon 45 was the most common deleted exon accounting for 44.4% of all deletions. 89% of the deletions were located in the distal hot spot region (exons 45–55) of the dystrophin gene; only one patient (11%) had deletion in the 5′ end region.
- MLPA analysis identified duplication mutations in 5 families (27.8%), which were all multi-exon duplications, Table 1.
- Sequencing of the dystrophin gene revealed a single nucleotide substitution (c.583C > T) which predicted to create a stop codon in exon 7 (p.Arg195X) in only one family (Fig. 1). No mutation was found in 3 families (16.7%).
- Reading frame rule and genotype–phenotype correlations.

The reading frame rule using frame shift checker of Leiden Muscular Dystrophy website (www.dmd.nl/) was applied to the 14 patients with deletions or duplications. A frame shift was detected in 13 (93%) patients while an in-frame mutation was found in only one patient presenting with a duplication of exons 50–51 (patient no. 2, Table 1). The latter variant is exceptional in respect of the frame shift hypothesis because he had a relatively severe disease with an early age of onset. An intragenic deletions involving the 5′ end of the dystrophin gene (deletion of muscle protomor and exon 1) in patient no. 11 was observed to be associated with earlier onset of disease (2 years) and more severe symptoms (wheel-chaired at age of 9 years) but without any cardiac involvement at the time of examination. Although four of the patients carried the same mutation (exon 45 deletion), they had variable ages of onset and variable disease course. In the remaining mutations identified, no clear phenotype–genotype correlation could be found.

4. Discussion

This is the first Egyptian study applying sequencing of dystrophin gene searching for non-deletion mutations in DMD patients. Interestingly, it revealed a higher frequency of duplication mutations (27.8%) than previously reported (10.3%) [16]. Although this high frequency can be attributed to the small number of patients, a true high frequency of duplication mutations among Egyptian DMD patients cannot be excluded. It also indicates that the MLPA and not PCR should be the best method to start with for a molecular diagnosis of Egyptian DMD patients.

Similar high frequencies of duplication mutation were previously reported in the Bulgarian population (27%) and Taiwanese population (24.7%) [17,18].

A previous Egyptian study revealed duplication in only 2 out of 41 patients (5%) [11]. This low percentage can be explained by the lower number of exons studied (only 18 exons were analyzed).

Another interesting finding is that 60% of duplications found in this study were located in the distal region of dystrophin gene. This is in contrast to what was reported in other populations where a higher percentage of duplications is located in the proximal region [19,20].

Regarding deletion mutations, the frequency found is almost similar to previous studies (Table 2). Higher frequencies were reported only by Bastaki et al. and El sheriff et al. (75% and 61%, respectively) [11,21]. This high incidence is probably due to the fact that both studies were performed on only immunohistochemistry confirmed DMD patients, a technique that we did not include in this analysis due to its local unavailability.

The frequency of deletion mutations also lies between highest deletion frequencies of Kuwait, Canada and Greece (86%,

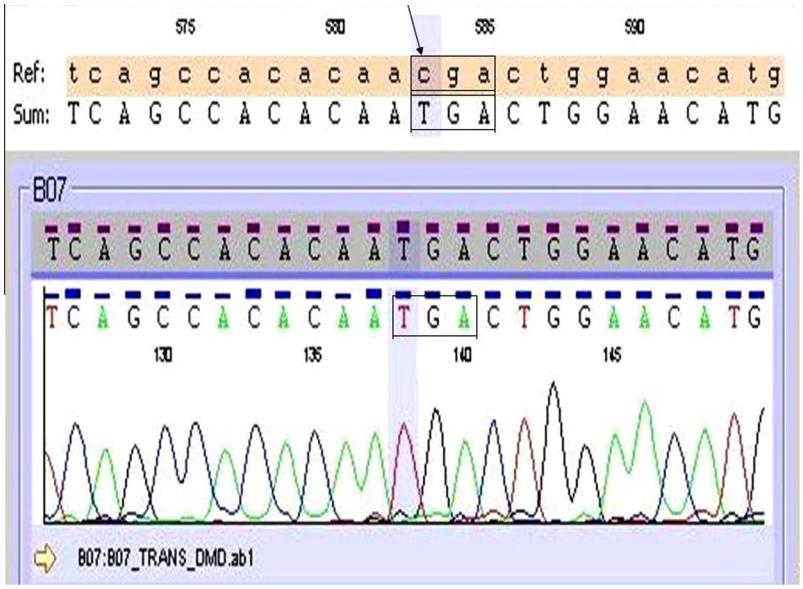

Figure 1 Sequencing of patient No. 3 showing a nonsense point mutation (c.583C > T) in exon 7.

Table 2 Percentages of deletions and duplication found in different Egyptian studies.

Study	No. of Samples	No. of exon tested	Method used	Deletion%	Duplication%
Baskati et al., [21]	26	25	mPCR	75	–
Effat et al., [8]	100	18	mPCR	55	–
Elhawary et al., [9]	152	10	mPCR	51.3	–
Shawky et al., [10]	59	9	mPCR	55.9	–
El Sherif et al., [11]	41	25	qPCR	61	5
Present study	25	All 79	PCR, MLPA	50	27.8

mPCR: multiples PCR, qPCR: quantitative PCR, MLPA: multiplex ligation-dependent probe amplification.

73% and 63%, respectively) and lowest frequencies of Vietnam, Russia and Singapore (32%, 40% and 41%, respectively), Table 3. A variation was noted in deletion rates among studies carried out even in the same country which could be explained by the differences in the techniques used, number of exons studied and different ethnic groups, Tables 2 and 3.

Single exon deletion constituted more than half of the deletion mutations (56%). This is consistent with a previous Egyptian study of Shawky et al., while Effat et al. reported on single exon deletions in only 40% of Egyptian patients [8,10].

Exon 45 was the most frequent single deleted exon in our patients, which has also been reported by others [8,10,33]. On the other hand, exon 50 was the most common single deleted exon in Indian and Pakistani populations [34,35] while exon 44 was found more often deleted in a Turkish population [33].

Noteworthy was that patient No. 2 presented with severe symptoms in spite of maintaining the reading frame which could be explained by exon skipping or deletion at the exon–intron boundaries affecting splicing behavior [36]. Also, intragenic deletions involving the 5′ end of the dystrophin gene (deletion of muscle protomor and exon 1) in patient No. 11 was found to be associated with earlier disease onset (2 years) and more severe symptoms (wheel-chaired at age of 9 years) but without any cardiac involvement at the time of study. The same finding was previously reported [37]. This rare type of deletion has been linked in earlier reports with severe dilated cardiomyopathy with or without skeletal muscle weakness [38–40]. It is known that the effect of deletion of exon 1 is not predictable on the basis of the effect of the deletion on the reading frame [41].

A nonsense point mutation was observed in two brothers from one family (c.583C > T) creating a stop codon in exon 7 (p.Arg195X). To the best of our knowledge, this is the first report of point mutation in Egyptian DMD patients, although it was reported in different ethnic groups [42]. These two brothers were similar regarding muscle weakness but completely different in their cognitive abilities (while the older child is the top in his class, the younger was mentally retarded). Previous studies reported a correlation for IQ values in affected siblings with the same deletion mutation, but a poor correlation between unrelated affected individuals carrying the same mutation [43–45].

Out of the 18 families, 3 (16.6%) did not show any pathogenic mutations by using the methods as outlined. This percentage is higher than that previously reported who used the same testing approach but had only 4% cases with no detectable mutation [17]. This could be explained that these cases represent another type of muscular dystrophy rather than DMD and can be confirmed only by immunohistochemistry of muscle biopsy which is considered as a gold-standard for DMD diagnosis despite its invasiveness. Another explanation is that we missed the variants such as deep intronic changes, which Del Gaudio et al. reported approximately 2% of DMD cases [46].

Table 3 Comparison of frequency of dystrophin gene deletions in different countries.

Country	Authors	Methods	No. of exons tested	% of deletion
Kuwait	Haider et al., [22]	mPCR	25	86
Saudi Arabia	Al-Jumah et al., [23]	mPCR	25	78
Canada	Stockley et al., [24]	qPCR	All 79	73
China	Wang et al., [25]	MLPA	All 79	66
Greece	Florentin et al., [26]	mPCR	18	63
Morocco	El Sbiti et al., [27]	mPCR	18	59
Turkey	Battalogue et al., [28]	mPCR	18	59
China	Lu et al., [29]	MLPA	All 79	49
Russia	Baranov et al., [30]	mPCR	10	41
Saudi Arabia	Tayeb, [31]	mPCR	9	40
Singapore	Lai et al., [32]	mPCR	19	40
Vietnam	Lai et al., [32]	mPCR	19	32
Present study (Egypt)		PCR, MLPA	All 79	50

mPCR: multiples PCR, qPCR: quantitative PCR, MLPA: multiplex ligation-dependent probe amplification.

5. Conclusion

Our study revealed higher frequencies of duplication mutations (27.8%) in Egyptian patients with DMD than previously reported which indicates that MLPA should be included in the genetic screening of affected Egyptian patients. Furthermore, we confirm in this analysis that there is some variability in the phenotype even in carriers of the same genotype. Since newly introduced molecular therapies may revolutionize outcome in patients, genetic diagnostics is essential to achieve to identify the molecular defect in each patient if possible. MLPA serves as an excellent tool to improve the rate of molecular diagnosis in DMD patients also in Egypt.

Declaration of conflicting interests

The authors declare no conflicts of interest. There is no financial and personal relationship with other people or organizations that could inappropriately influence this work.

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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