Molecular analysis among a group of Egyptian Duchenne muscular dystrophy patients using real-time PCR

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Background
Duchenne muscular dystrophy (DMD) is a neuromuscular disease of children caused by dystrophin protein deficiency encoded by dystrophin gene that is localized to the short arm of the X chromosome, position 21.1q. dystrophin gene mutation results in this disorder. Dystrophin is necessary for keeping the integrity of both skeletal and smooth muscles.

Aim
Identify mutations in the group of Egyptian DMD patients, namely exons 48, 49, and 51, and to create a genotype–phenotype correlation from analyzing these patients clinically as well as genetically.

Patients and methods
The study included 50 children, 25 DMD patients selected from the Neuropediatric Outpatient Clinic, Abo El Reesh Hospital, Cairo University, selected based on clinical, biochemical, and electromyography findings suggestive of a myopathic picture and 25 normal healthy children matched for age and sex constituted the control group, analysis was done using q-real-time PCR.

Results
About 75% (18/24) showed single-exon deletion, while 25% (6/24) showed multiple-exon deletions, of which 12.5% were double deletions and 12.5% were triple deletions, the clinical severity of the condition seems to be independent on the number of deleted exons. The most common single deletion was that of exon 48 followed by exon 51.

Keywords:
deletion, Duchenne, dystrophin, q-real-time PCR

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Introduction

Duchenne muscular dystrophy (DMD) is the most common inherited neuromuscular disorder, affecting 1: 3500 live-born males [1]. It is an X-linked recessive disease caused by mutations in the dystrophin gene (DMD gene) encoding for a protein called dystrophin, which is essential for the integrity of the membrane of the skeletal muscle fiber. The DMD gene is mapped to the short arm of the X chromosome at the position Xp21.1. Mutations in the DMD gene result in a truncated dystrophin protein that causes the severe phenotype of DMD. Patients present with delayed walking, frequent falling, and progressive muscle weakness. Mutations occur predominantly in two high-frequency-mutation regions termed hotspots; proximal hotspot extends from exon 1 to exon 20, while the distal hotspot extends from exon 42 to exon 53 [2]. Identification of mutations in patients confirming a clinical diagnosis, allows carrier testing and prenatal diagnosis. Therapies being tested for DMD are absolutely dependent on precise knowledge of the mutation [3]. In this study, genomic DNA will be analyzed for exons 48, 49, and 51 located in the distal hotspot of the short arm of the X chromosome and creating genotype–phenotype correlation from analyzing these patients clinically as well as genetically.

Patients' selection

The study was conducted on 25 DMD patients. They were recruited from the Neuropediatric Outpatient Clinic, Abo El Reesh Children Hospital, Cairo University, presenting with delayed walking, frequent falling, and difficulty in climbing the stairs. Electromyography was showing evidence of myopathic changes with serum creatine kinase (CK) more than or equal to 10-fold the upper limit of its normal level, the control group consisted of 25 normal healthy children matched for age and sex. Informed written consent was obtained from all participants, the Ethical Committee of Faculty of Medicine Cairo University Hospital approved the study.

Molecular biology

Four-to-five milliliters of whole blood were withdrawn from each participant and divided equally into two

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parts: 2 ml withdrawn on a plain vacutainer and used for analysis of CK and lactate dehydrogenase (LDH) after separation of serum, analysis was done using 'Dimension' autoanalyzer. Two milliliters of EDTA blood for DNA extraction that was done immediately and the extracted DNA stored in an Eppendorf tube at − 80°C till working time using MagNA Pure-DNA LC total isolation kit (Cat. No. 03038505001). The procedure is fully automated (Roche, Germany), samples were first lysed with a buffer containing chaotropic salts and proteinase K. Magnetic glass particles were then added to bind the DNA to their surfaces. Unbound substances were removed by several washing steps and the purified DNA was removed. Genetic analysis was done by real-time PCR using Taqman probes and primers done on Applied Biosystem Step One Real-time PCR, the system primers used were:

Exons	Sequence
Exon 48	F: 5'TGCTGCTAAAATAACACAAATC
	R: 5'CCTTAACGTCAAATGGTCC
Exon 49	F: 5'ACCAGGCAGAAATTGATCTG
	R: 5'CAGTATGGCCAGTATTTCCT
Exon 51	F: 5'CAACCTGTGGTTACTAAGGA
	R: 5'GAGAGAAACAGTTGCCCTAAGA

Allelic-discrimination plate was read and analysis was done after PCR amplification, an endpoint-plate read was performed using Applied Biosystem Real-time PCR system. The Sequence Detection System software used the fluorescence measurement made during plate read to plot the fluorescence value based on the signals from each well.

Statistical analysis

Data were analyzed using SPSS statistics, version 17 (SPSS Inc., Chicago, Illinois, USA). Normality of numerical data distribution was examined using the D'Agostino–Pearson test. Parametric numerical variables were presented as mean ± SD and intergroup differences were compared using one-way analysis of variance. Nonparametric numerical variables were presented as median and interquartile range. Categorical variables were presented as number and percentage and intergroup differences were compared using χ^2 test. *P* value less than 0.05 was considered statistically significant (Table 1 and Fig. 1).

Results

Patients presented with variable complaints, the majority presented with difficulty in climbing the stairs 19/25 (76%), while 4/25 (16%) presented with repeated falling down and the least-frequent presentation was walking on toes 2/25 (8%) (Fig. 2).

Family history of a similar condition in the family was elicited in five families (5/20, 25%), families of case nos. 2, 9, 11, 20, and 22, three of which showed affected siblings (families of case nos. 9, 20, and 22). One family had an affected sibling and an affected maternal uncle who died by the age of 18 years (case no. 11). One family had only an affected maternal uncle who died by the age of 17 years (case no. 2) (Table 2 and Fig. 3).

Only one patient showed no deletions in any of the three tested exons: case no. 19. He was a 7-year-old boy, who presented at the age of 4½ years with difficulty in climbing the stairs. He started walking by the age of 1½ years, had bilateral hypertrophy of calf muscles, and a muscle power of grade 4 in both the upper and lower limbs. His serum CK and LDH were 9520 and 1120 U/l, respectively. Owing to this classic picture of DMD and since our molecular analysis did not include all 79 exons of the dystrophin gene, further genetic analysis for dystrophin gene is recommended for this patient (Table 3).

A statistical significance was found between molecular analysis of exon 48 and the age (3.1 ± 1 years) of onset among the DMD cases, the *P* value being 0.045. This means that DMD cases presenting at this mean age are likely to have a deletion in exon 48. No statistical significance was found for other clinical and biochemical aspects as shown below (Table 4).

Table 1 Frequency distribution of age of onset among Duchenne muscular dystrophy cases (25 cases)

Age at onset (years)	*n* (%)
9	1 (4)
6	5 (20)
5	9 (36)
4	4 (16)
4.5	3 (12)
3	2 (8)
2	1 (4)

Table 2 Biochemical indices of Duchenne muscular dystrophy cases and control group

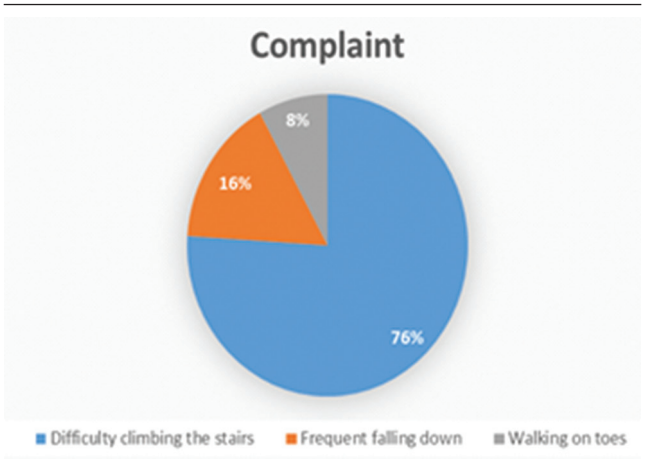
Biochemical indices	Cases (25 DMD cases)	Controls (25 controls)	*P*
Serum CK (IU/l)	9364±5518.5	95±43.9	<0.01
Serum LDH (IU/l)	862.2±250.5	191±53.7	<0.01

Values are given as mean±SD. CK, creatine kinase; DMD, Duchenne muscular dystrophy; LDH, lactate dehydrogenase.

Table 3 Frequency of distribution of the tested deletions within the distal hotspot of the dystrophin gene exons (48, 49, and 51) among the 25 Duchenne muscular dystrophy cases

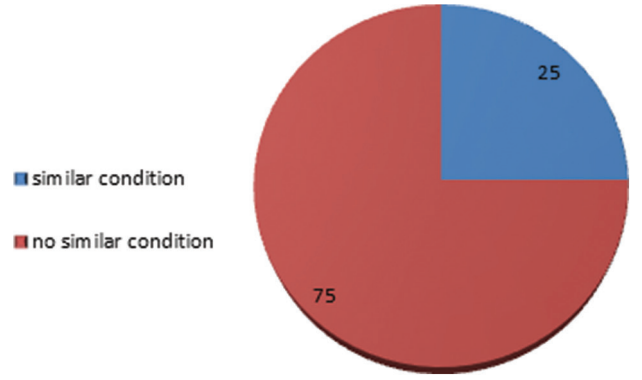
Genotype	Exon 51 [*n* (%)]	Exon 49 [*n* (%)]	Exon 48 [*n* (%)]
Homozygote	3 (12)	1 (4)	7 (28)
Heterozygote	3 (12)	3 (12)	17 (68)
Wild type	19 (76)	21 (84)	1 (4)

Figure 1



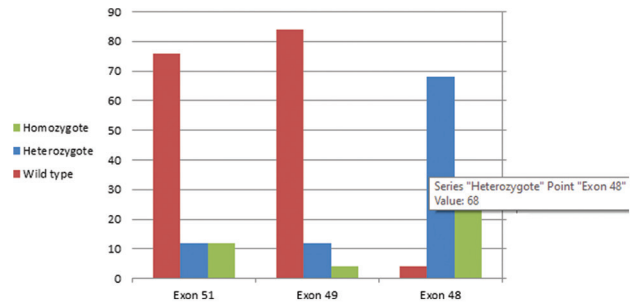
Frequency distribution of the various complaints of the 25 DMD cases. DMD, Duchenne muscular dystrophy.

Figure 2



The percentage of families having a similar condition of DMD in the family. DMD, Duchenne muscular dystrophy.

Figure 3



Frequency distribution of exon mutations among DMD cases (25 cases). DMD, Duchenne muscular dystrophy.

A statistical significance was found between molecular analysis of exon 49 and the age of onset among the DMD cases, the *P* value being 0.002. This means that DMD cases presenting at this mean of age are likely to have a deletion in exon 49. No statistical significance was found for other clinical and biochemical aspects as shown below (Table 5).

There was no statistical significance found between the molecular analysis of exon 51 among the DMD cases and the various clinical and biochemical aspects. This is shown in Table 6.

Discussion

DMD is an X-linked recessive neuromuscular disorder. Its prevalence is 1: 3500 live-born males [4]. It is caused by deficiency of dystrophin protein that is essential for sarcolemma muscle integrity. It is coded by dystrophin gene that consists of 79 exons [2]. The aim of this work was to analyze three common exon deletions among a group of Egyptian DMD patients, namely exons 48, 49, and 51. Analysis was done using q-real-time PCR. The detection of DMD mutations is important for diagnosis of the disease, providing accurate genetic counseling and prenatal identification of DMD patients. Moreover, identification of DMD mutations could be beneficial for mutation-specific gene therapies, such as 'exon-skipping approach' or suppression of nonsense mutations during translation. Biochemical markers of muscle affection were also analyzed, namely serum CK and LDH. A genotype/phenotype correlation was done to throw light upon more understanding of the nature of the disease.

This study included 50 children. Cases were 25 Egyptian-male DMD patients. Their ages ranged from 4 to 13 years with a mean ± SD of 8.4 ± 2.4 years. They were recruited from the Neuropediatric Outpatient Clinic, Abo El Reesh Children Hospital, Cairo University, during the period from 5/2013 to 5/2014. They were selected on the basis of clinical, biochemical, and electromyography findings. The control group consisted of 25 normal healthy children matched for age (mean ± SD 7.2 ± 2.1 years) and sex (males).

The mean age of symptom onset was 4.86 ± 1.33 years and mean age of walking was 1.8 ± 0.46 years. The most common presenting symptom was difficulty in climbing the stairs 19/25, 76%, followed by frequent falling down 4/25, 16%. The remaining patients presented with abnormal gait 2/25 (8%). Ten patients had a history of similar condition in the family 40%. Twelve (48%) patients gave a history of delayed walking. On physical examination, pseudohypertrophy of calf muscles and Gowers' sign were observed in all cases. The serum CK and LDH values were significantly elevated ($P < 0.01$) in all patients. The highest and lowest levels of serum CK were 27 089 and 1113 U/l, respectively, while those of serum LDH were 1637 and 415 U/l, respectively. Comparing the age with these indices, an inverse relation was found between the serum level of CK and the age of the patients. It might be the result of

Table 4 Statistical analysis of clinical and biochemical aspects versus exon 48 deletion

Clinical and biochemical aspects	*P*
Complaint	0.5
Age of walking	0.8
UL muscle power	0.24
LL muscle power	0.09
Serum CK	0.91
Serum LDH	0.55

CK, creatine kinase; LDH, lactate dehydrogenase; LL, lower limb; UL, upper limb.

Table 5 Statistical analysis of clinical and biochemical aspects versus exon 49 deletion

Clinical and biochemical aspects	*P*
Complaint	0.69
Age of walking	0.69
UL muscle power	0.16
LL muscle power	0.07
Serum CK	0.66
Serum LDH	0.69

CK, creatine kinase; LDH, lactate dehydrogenase; LL, lower limb; U, upper limb.

Table 6 Statistical analysis of chemical and biochemical aspects versus exon 51 deletion

Clinical and biochemical aspects	*P*
Age of onset	0.96
Complaint	0.24
Age of walking	0.68
UL muscle power	0.18
LL muscle power	0.17
Serum CK	0.69
Serum LDH	0.6

CK, creatine kinase; LDH, lactate dehydrogenase; LL, lower limb; UL, upper limb.

progressive elimination of dystrophin protein in the muscle fibers, resulting in decreased levels of muscle markers. It starts to decrease, particularly in advanced cases. The biochemical indices thus have a potential role in screening and followup of patients, but cannot be relied on for diagnosis. Our findings are similar to those in a previous Indian study [5].

Deletions in one or more of the tested exons (48, 49, and 51) were detected in 96% (24/25) of the cases. This puts this percentage of deletions within the distal hotspot of the dystrophin gene, which extends from exons 44 to 55. Out of the 24 DMD patients with deletions, 75% (18/24) showed single-exon deletion, while 25% (6/24) showed multiple-exon deletions, of which 12.5% were double deletions and 12.5% were triple deletions. Looking at a genotype/phenotype correlation, it was found that patients with multiple-exon deletion were by no means having a more severe phenotype than those with single-exon deletions. Hence, the clinical severity of the condition seems to be independent on the number of deleted exons. The most common single deletion was that of exon 48, 18/24. A statistical significance was observed between the age of onset and the deletion of exon 48 ($P = 0.045$). This means that a patient with a deletion in this exon shall manifest by the mean age of the onset of symptoms of the studied patients (3.1 ± 1 years). The second most frequently detected exon deletion was exon 51; 24%, followed by exon 49; 16%. A statistical significance was also found between the age of onset and the deletion of exon 49, $P = 0.002$. This means that cases harboring this deletion shall manifest by the age of 6.7 ± 2 years.

Rao *et al.*[5] investigated 81 Indian DMD patients using multiplex PCR targeting the two hotspots of the dystrophin gene. The mean age of onset was 4.09 ± 0.15 years and the mean age of presentation was 10.75 ± 0.39 years. Twenty (24.7%) cases had a family history of the disease. Clinically, hypertrophy of the calf muscles was observed in 71 cases, Gowers' sign in 42 cases, and scoliosis in 20 cases. The state of ambulation was concluded to be supported in 43 cases, while the remaining 38 were wheelchair bound. Serum CK and LDH were significantly elevated ($P < 0.05$) in all patients compared with the control group. The mean serum CK and LDH were 7218.41 ± 6977.77 and 798.68 ± 49.07 IU/l, respectively. An inverse relation was observed between the age of the patients and the serum level of CK, which comes in accordance with our findings. The deletion rate was 76.5% (62/81). The deletion rates in the distal and proximal hotspot regions were 89.2 and 10.8%, respectively. The majority of the patients, 89.2% (58/81), showed deletions in the downstream region of the gene. Most of them (80%) had deletions in the central-rod domain between exons 45 and 52 and out of these, 25.8% (16/62) of the deletions started at exon 45. Exon 50 (58.5%) was the most frequently observed individual exon deletion. This also coincided with previous Western–Indian studies. Thus, this part of the gene is more deletion prone in the Indian-Gujarat population, which is similar to the findings we found in our study population [5].

Lee *et al.*[6] studied 25 Korean DMD patients using multiplex PCR and multiplex ligation-dependent probe amplification (MLPA). The mean age of the patients at the time of genetic testing was 5.1 ± 2.2 years (10 months–7.7 years), and the mean age of symptom onset was 3.5 ± 2.2 years (5 months–7.2 years). The most common presenting symptom was muscle weakness, which was seen in 13 (52%) patients, followed by incidentally detected elevated hepatic enzymes in nine (36%) patients. The remaining patients presented with developmental delay ($n = 2$) and abnormal gait ($n = 1$). Nine (31%) of 25 patients had a family

history of similar condition in the family. On physical examination, pseudohypertrophy of calf muscles was presented in 21 (85%) patients and Gowers' sign was observed in 17 (68%) patients. The mean level of CK was 14 048 ± 8925 IU/l (4605–39 318 IU/l). The overall mutation-detection rate was 76% (19/25), identifying deletions in 68% (17/25). Most of the deletions were confined to the central hotspot region between exons 44 and exon 55 (52.9%), putting this percent in the distal hotspot, which is different from our findings [6].

Verma *et al*.[7] studied 217 Indian DMD patients using combined multiplex PCR and MLPA. They identified a deletion rate of 79.2% (172/217). The deletions detected by MLPA were checked using reading-frame checker ver. 1.9, at the Leiden database (www.dmd.nl) correlated clinically with patient phenotype. In agreement with our study, clinical phenotype did not depend on the size of the deletion, but rather on the exact location of mutation in gene. An out-of-frame deletion, irrespective of size, was correlated with a severe phenotype, whereas in-frame deletion was correlated with a mild phenotype. Large in-frame deletions removing up to 35 exons in the central-rod domain have been described for the mild BMD [7].

Further studies by Chen *et al*(2013), analyzed 160 Chinese DMD families using combined PCR and Sanger sequencing. The frequency of deletions was 85% (136/160). Among them were 115 (71.9%) with more than one exon deletion. No statistical significance was found between disease severity and the extent of deletions (P = 0.429). Attempting to identify the hot-mutation region in the dystrophin gene, the calculations revealed that most of the deletions (43.7%) occurred in exons 45–52 [8]. Away from the frameshift hypothesis, the number of deleted exons did not seem to influence the progression of the disease, which is similar to the findings of Magri *et al*. (2011) and that of our study [9].

Chen *et al*.[10] investigated 119 Chinese DMD cases using combined MLPA, denaturing high-performance liquid chromatography and Sanger sequencing found 53.8% (64/119) deletion frequency. This included 15 (22.6%) single-exon deletions and 49 (41.2%) multiple-exon deletions. Deletion hotspots appeared to be 44–53 in the central region and 3–21 in the 5' end of the DMD gene. To confirm the potential DMD-deletion hotspot, they reanalyzed all 64 cases with DMD deletions using their breakpoint frequency (per kb), that is, by calculating exon-deletion events of certain exon intervals. The identified two deletion hotspots were at 43–55 and 10–23, which has different hotspot boundaries from that of cumulative event analysis. It is believed that the breakpoint frequency (per kb) is more convictive. However, both methods came in accordance with our findings, confirming the prevalence of deletions within the central hotspot region [10].

Hallwirth *et al*.[11] screened 53 South African DMD patients for 18 exons using multiplex PCR. Like our study, a complete representation of the size of the deletion in the dystrophin gene could not be determined. Deletions were clustered in the high-frequency deletion region, located at the 5'proximal end of the gene, with deletions spanning exons 3–7, and at the distal hotspot encompassing deletions of exons 45–51. The age range of DMD patients was 3–26 years. The study group included different races within KawaZulu Natal in South Africa. A deletion was found in 62.3% (33/53) of the patients: 19/29 black, 10/17 Indian, and 4/7 white. The most frequently affected region encompassed exons 47–51, which resemble our results [11].

Basak and colleagues analyzed 90 Indian DMD patients using multiplex PCR. The CK values among the patients were abnormally elevated (5–10×–100 × or even more). But at the late stage of the disease, CK values came down to normal due to loss of muscle mass, which is similar to the findings in our study. The highest and lowest CK values were 35 200 and 195 IU/l, respectively. Age of onset ranged from 1 to 16 years and a median of 5 years. He found 26% of cases giving a family history of the disease. Deletions were detected in 59 (65.5%) out of 90 patients. This included 48 (53.3%) localized in the central hotspot region, that is, between exons 42 and 53, and eight (8.8%) at the proximal hotspot region, that is, between exons 1 and 20. There were three (3.3%) cases who extended from the proximal hotspot region to the central hotspot region [1].

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Conflicts of interest
There are no conflicts of interest.

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