Original Article

Molecular Characterization of Dystrophin Gene in Iraqi Patients with Muscular Dystrophy

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Summary:

Background: Dystrophinopathies are the commonest forms of muscular dystrophy and comprise clinically recognized forms, Duchenne Muscular Dystrophy (DMD), and Becker Muscular Dystrophy (BMD). Mutations in the dystrophin gene which consist of large gene deletions (65%), duplications (5%) and point mutations (30%) are responsible for reducing the amount of functional dystrophin protein in skeletal muscle fibers. This study concentrate mainly at the spectrum of deletions in the 'distal hot spot' region of the DMD/BMD gene in Iraqi DMD/BMD patients using multiplex PCR technique

Objectives: The aim of this study was to investigate the rate, and distribution of deletions in 10 exons of Dystrophin gene in a group of Iraqi dystrophinopathy patients using the multiplex polymerase chain reaction (MPCR).

Patients materials and methods: This is a case prospective study which include 27 clinically diagnosed DMD/BMD patients and six suspected carriers in Medical city /Baghdad . A written consent was obtained from each family for going the research as well as ethical committee approval. Forty six apparently healthy individuals were included as a control group. Blood samples were collected in 5-6 ml EDTA tubes by venepuncture. The DNA was extracted by using the Wizard Genomic purification kit (Promega/USA), and the quantity was estimated by UV-spectrophotometer (Cecil CE7800) . Ten exons of the dystrophin gene were examined (19 ,45 ,46 ,47 ,48 ,49 ,50 ,51 ,52,53) using synthesized primers with complementary sequences and set in five different multiplex PCR groups. The products of PCR amplifications were subjected to electrophoresis and visualized by The UVCI140 & 200 series (advanced CCD gel imaging system from Major Science. It consists of a CCD camera, UV transilluminator UV- light system). All done in college of medicine /Baghdad university.

Results: The rate (relative frequency) of subjects with any positive exonal deletion (among the10 selected and tested exons was significantly higher (85.2%) among patients compared to that among the suspected carriers (33.3%).The distribution of exonal deletions among patients compared to suspected carriers were statistically significant. The frequency of deletions detected in male patients (~82%) was higher than frequencies mentioned in the other studies of comparison. The control group show no deletion in all tested exons.

Conclusions: Multiplex PCR technology was utilized to demonstrate the frequency of 10 exons deletions in a limited group of Iraqi DMD/BMD patients. The overall distribution of deletion mutations in the distal 'hot spot' region was higher than that of DMD/BMD cases investigated elsewhere. The study also serves as a good starting point for further investigations into the genetic aspects of the Iraqi DMD/BMD population.

Keywords: Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), Dystrophin gene, Multiplex PCR.

Introduction:

Muscular dystrophy abbreviated (MD) refers to a group of hereditary and non-hereditary, muscle diseases that weaken the musculoskeletal system and. hamper locomotion, characterized by progressive muscle weakness and degeneration of skeletal muscle The best known muscular dystrophies are the X-linked Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) (1). Muscular dystrophies are characterized by muscle degeneration and regeneration. Clinically, muscular dystrophies are typically progressive, because the muscles' ability to regenerate is eventually lost, leading to progressive skeletal muscle weakness, defects in muscle proteins, and the death of muscle cells and tissue, often leading to use of a wheelchair, and eventually death, which is usually related to cardiac or respiratory weakness(2). The commonest types of hereditary muscular dystrophy include the clinically more severe form, Duchenne muscular dystrophy (DMD), and the

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less severe form, Becker muscular dystrophy (BMD). They are the most common muscle disease in children. Mutations in the large dystrophin gene that consists of 79 exons results in reduced or absent functional dystrophin protein in skeletal muscles, leading to muscle weakness of varying clinical severity(3). DMD has an incidence of 1:3500 male newborns. Physical signs, which usually begin between the ages of 3-5 years, include trouble in walking, running, jumping, calf hypertrophy and lumbar lordosis. Boys with DMD are usually confined to a wheelchair by the age of 12 and die in their third decade of life from cardiac or respiratory failure due to the gradual development of cardiac hypertrophy, as a result of severe myocardial fibrosis typically resulting in premature death (4). Mutations in the dystrophin gene that lead to the production of abnormal , but still partially functional dystrophin protein, results in a display of a much milder dystrophic phenotype in affected patients, resulting in the disease known as Becker's muscular dystrophy (BMD). In BMD, the variant of DMD, with an incidence of approximately 3 per 100,000 male newborns, the clinical course is milder. Symptoms begin at about 11 years of age and progression is slower than DMD (5). Deletions of one or more exons account for approximately of 60%-70% of mutations in individuals with DMD and BMD (6). Duplications account for the disease-causing mutations in approximately 5%-10% of males with DMD and BMD that appear to be evenly distributed throughout the gen(2), while point mutations (small deletions, insertions, single-base changes, and splicing mutations) account for approximately 25%-35% of mutations in males with DMD and about 10%-20% of males with BMD. There are no particularly common point mutations or point mutation hotspots, and each affected family may carry a unique mutation in this enormous gene (termed "private mutations" because they are exclusive to individual families) (2). Sequencing the dystrophin gene is time consuming because of the complex organization of the introns and exons that spans 2.4 million base pairs (Mbp). However, it is fortunate that many of the large gene deletions within the dystrophin gene can be detected in specific "hotspot areas" of the gene. These "hotspots" are clustered in two main regions - at the 5' proximal portion of the gene (exons 1, 3, 4, 5, 8, 13, 19) and within the mid-distal region (exons 42 - 45, 47, 48, 50 - 53, 60).2,5,6 DNA amplification by the multiplex polymerase chain reaction (MPCR) can detect 98% of the large scale deletions in the dystrophin gene . The MPCR differs from the standard PCR in that more than two primer sets are used to amplify different regions of target DNA in a single reaction. The advantage of MPCR is its rapidity as multiple target sequences are amplified simultaneously. MPCR requires only a very small amount of DNA that can be extracted from blood and muscle samples(2).

Best to the presence knowledge, There is no similar research done in Iraq, while similar researches were done in some Arab countries like Saudi Arabia, Morocco.

Patients and Methods:

Blood samples were collected from 27 clinically-confirmed cases of dystrophinopathy by venepuncture, their ages ranged from 4 months to 31 years. Three to four ml of whole blood were aspirated from each individual using a sterile technique and a sterile tube with EDTA as an anticoagulant. Blood samples were stored in a deep freeze at (-20^{o}c) until processing for DNA extraction. Another 2 ml of whole blood were also aspirated in a sterile plane tube and sent directly for CPK values estimation.

Methods :DNA from these cases was extracted from the leukocytes that were obtained from whole blood samples using the Wizard Genomic DNA Purification kit by Promega Corporation/USA, stabilized in DNA rehydration solution, and stored in (-20^{o}c). Ethical consents were obtained from the patients or parents for molecular characterization of the dystrophin gene. Primers (Table 1) sets for amplification of the 10 exons (exon 19, 45,46,47, 48,49, 50,51 ,52and 53) were purchased from (BioCorp /Canada) and synthesized according to published sequences in the Leiden Muscular Dystrophy data pages (7).

The primers were divided into 5 groups (M1, M2, M3, M4 and M5) for 5 separate MPCR assays depending on the optimal annealing temperature obtained for each primer set to allow optimum visualization of the amplified products. Group M1 amplified exons 45 and 51; M2 exons 94 and 52; M3 exons 19 and 46; M4 exons 47 and 48; and M5 exons 50 and 53 respectively.(Table 2)

Table (1): Primers Sequences With Their Lengthy, Arranged According To Their Locations In The Dystrophin Gene.

Exon	Length (in bp)	Forward primer (5'- 3')	Reverse primer (3'– 5')
19	495	GATGGCAAAAGTGTTGAGAAAAAGTC	TTCTACCACATCCCATTTTCTTCCA
45	357	TTCTTTGCCAGTACAACTGC	TCTGCTAAAATGTTTTCATTCC
46	409	CCAGTTTGCATTAACAAATAGTTTGAG	AGGGTTAAGAAGAAATAAAGTTGTGAG
47	399	TGATAGACTAATCAATAGAAGCAAAGAC	AACAAAACAAAACAACAATCCACATACC
48	543	TTGAATACATTGGTTAAATCCCAACATG	CCTGAATAAAGTCTTCCTTACCACAC
49	475	GTGCCCTTATGTACCAGGCAGAAATTG	GCAATGACTCGTTAATAGCCTTAAGATC
50	307	CACCAAATGGATTAAGATGTTCATGAAT	TCTCTCTCACCCAGTCATCACTTCATAG
51	424	GAAATTGGCTCTTTAGCTTGTGTTTC	GGAGAGTAAAGTGATTGGTGGAAAATC

Table (2) Primers Were Grouped According To Their Annealing Temperatures And The Best Observed Yield Of Their Products.

Group	Amplified Exons	Annealing Temp.
M1	45 , 51	52 °C
M2	49 , 25	55 °C
M3	19 , 46	52 °C
M4	47 , 48	52 °C
M5	50 , 53	55 °C
Loading control	Beta-Globin	60 °C

Amplification of the genomic DNA was performed by using the fast ramping Veriti™ 96-Well (conventional PCR Thermal Cycler From Applied Biosystems/Italy) and was carried out using the ready to use KAPA2G™ Fast Multiplex PCR Kit provided by KAPABIOSYSTEMS/USA (Table 3). MPCR was carried out with initial denaturation at (95°c) for 3 min followed by 1 cycle of denaturation at (95°c) for 15 sec, one cycle of annealing at (94°c) for 30 sec, annealing at (60°c) for 30 sec, 30 cycles of extension at (72°c) for 15-30 sec and final extension of 1- 10 min was carried out at (72°c) (cited in the ready to use KAPA2G™ Fast Multiplex PCR Kit leaflet provided by (KAPABIOSYSTEMS/USA).Amplified MPCR products were subjected to electrophoresis in 1% agarose gels at 80 volts for 60 minutes. Appropriate positive DNA controls were included in every MPCR reaction and also DNA blank where no DNA was added to the MPCR reaction to monitor for contamination. Six µl of KAPA Universal DNA ladder, supplied by KAPABIOSYSTEMS/USA was used.

Table (3) Multiplex PCR with the KAPA2G Fast Multiplex PCR Kit reaction

PCR master mix reaction		Volume
KAPA2G Fast Multiplex Mix X1		12.5 µl
PCR grade water		Up to 25.0 µl
Primers	Forward primer	5 pmol
	Reverse primer	5 pmol
Template DNA		10-250 ng
Total Volume		25.0µl

The working solution of primers was prepared by diluting 50 µl of the stock solution in 950 µl of deionized distilled water in new Eppendorf tube, using the equation:
C1 V1 = C2 V2 to obtain a final concentration of (5 pmol/ µl).
Since all of our primers were greater than 14 bases in length, the annealing temperature was calculated for each primer according to the equations provided by Promega Corporation:
Annealing temperature (Ta) = 3(total guanines plus cytosines) + 2(total adenines plus thymines).
Or Ta = [64.9 degrees C + 41 degrees C(total G+C in the primer - 16.4)/N] – 5 where N is the length of the primer

Results:
CPK results in children patients ranged from 29 to >4267 U/L (the highest device reading) with a mean of 1606 ± SE of 414, while it ranged from 71 to >4267 U/L with a mean of 1259 ± SE of 648 in adults patients. The CPK level was normal (29-200 u/l) in 13 patients (48.1%), while high (above 200u/l) in 14 (51.9%) of them . Gel electrophoresis results showed distinct amplified MPCR products for the non-deleted gene sequences in controls and patients. As shown in Figure 1; lanes 1,2,4,5,6,8-12 . Lane M – 1000 bp molecular weight DNA ladder marker. Lane C – DNA from a normal control showing no deletions (presence of exon 47 (399 bp) and exon 48 (543 bp)). Lane LC- Loading control (Beta- Globin) from a random patient DNA (429bp) Lane B - DNA blank where no DNA was added to the MPCR reaction. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 were for 12 patients' samples. Deletions were detected in lane 3 (exon 47), 7 (both exons 47 and 48) and 12(exon 48); in the other lanes no deletions were detected.
In patients with gene deletions, no MPCR products for the respective deleted exon were observed (lanes 3,7and 12). The DNA blanks did not show any MPCR products.
The deletion of..

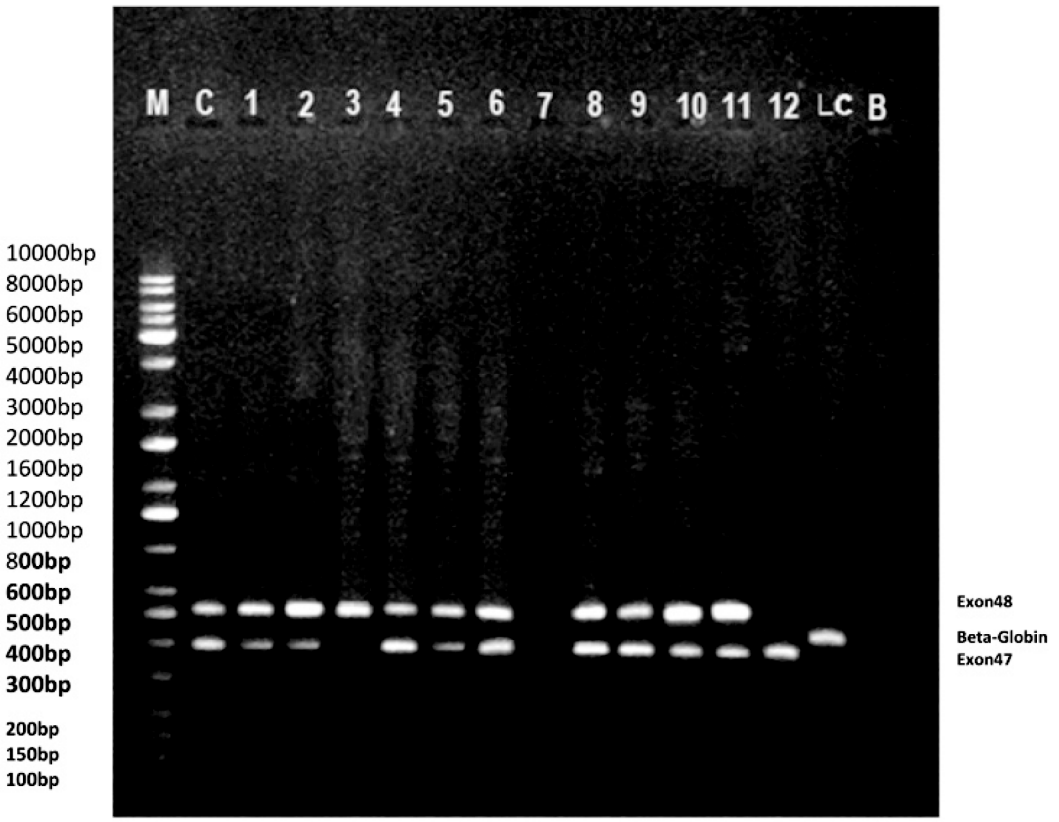

Figure 1: Gel electrophoresis (1% agarose gel at 80 volts for 60 minutes) results after MPCR for the amplification of only exons 47 and 48 in the Dystrophin gene according to the determined optimized primer groups .

Discussion:

Serum creatine phosphokinase (CPK), is a sensitive indicator of skeletal and cardiac muscle injury and is commonly used as marker to test for muscular dystrophy. For this reason it was used as an important inclusion criteria in the present study. The deletion of one or more exons of the dystrophin gene was found in 23 out of 27 Iraqi patients with DMD/BMD (85.2%), varying from (1-5) per patient which was significantly higher than that among the suspected carriers (33.3%).The remaining 4 patients (14.8%) showed no deletions in any exon. Individuals of this group possibly represent the low frequency of DMD/BMD patients with undetectable deletions .[8] One possible explanation could be that deletion may exist in these patients, but occurs in another region of the gene (9). However, it is also possible that other types of mutations such as point mutations, insertions or nucleotide changes and duplications may be responsible for the disease in these patient (10).The frequency of deletions in the present study was higher than that detected in Saudi Arabian studies by Tayeb MT (1), who detected DMD gene deletions in 6 of 15 patients (40%), and who detected DMD/BMD gene deletions in 26 out of 41 patients (63%) (11). This percentage of deletions increased to (78%) when the molecular analysis was restricted to only 27 patients; In a Moroccan study by Sbiti A. (12) , detected exon deletions in the dystrophin gene in 37 out of 72 patients (51.3%). In Chuadhary GA research (10) ,12 out of 15 patients (80%) showed positive exonal deletions which is in agreement with the present study.

The most common deletion among all 10 tested exons was that of exon 51 (10 deletions) with frequency of 37%, followed by exon 48 (6 deletions) 14.8%, exon 50 (5 deletions) 18.5%, and exon 45(4 deletions) 14.8%; while other exons had lower frequencies. These findings are in agreement with Tayeb MT [(1)] who stated that the deletion frequency of exon 51 was the most common deletion (20%), followed by exons 19, 45and 48 that showed a frequency of 6.7% each. In Chuadhary GA research (10), the frequency of exon 51 deletions was also the highest among all tested exons (47%), followed by exon 50 and 49 (13.3%), while exon 48 and 45 showed frequencies of 10.6% and 6.6% respectively. From the total 23 positive patients, only 3 patients (11.1%) showed deletions in exon 19 (which was the only exon tested from the proximal hot region) in combination to other mutations in exons of the distal hot spot. While in the other 20 patients the mutations were confined to the distal hot spot only. Exon 19 was deleted in 6.7% of patients in Tayeb MT [(1)]. In a population of 27 patients, it is not a surprise that

most of the deletions were found in the distal hot spot assuming that the deletion frequencies are comparable to those observed in Chuadhary GA research (10) . Surprisingly, there were no deletions detected in exon 52 in the present study (0.0%) in patients group. This finding was against the others whereby 1 showed that exon 52 deletions were at frequency of 12%. Chuadhary GA (10), and Sbiti A. (12), stated in their results that they detected deleteions in exon 52,; however, the rates of these deletions were not mentioned.

Conclusion:

In Iraq, as in many other countries, this neurodegenerative disorder has extensive implications and places a heavy burden on both the affected ones and their families, where most patients are wheelchair bound after the first decade. The progress in genetic technology and the application of new approaches for DNA analysis, such as multiplex PCR and MLPA (multiple ligation probe amplification), allow rapid molecular-based diagnosis of this disease. However, in the absence of effective therapeutic intervention, such methods may currently be helpful in guiding the patients and their families about the pattern of inheritance, determining female carriers and opening possibilities of prenatal diagnosis in pregnant women with previous DMD/BMD children.

Authors Contributions :

Areen T.Ramadhan Al-Hadithi* (Auther) Study design/ acquisition of data analysis/interpretation of data/ drafting of manuscript, Ban Abbass Abdulmajeed(Supervisor) Study conception/ critical revision and Hula R.Abdulrasool Shareef Data collections / study conception.

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