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Detection of the Duplication in Exons 56-63 of Duchenne Muscular Dystrophy Patients with MLPA

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Abstract

Background
Duchenne Muscular Dystrophy (DMD) is a deadly X-linked recessive disorder. This genetic disorder affects 1 among 3,500-5,000 males in the world. The majority of the patients are male, due to the type of inheritance. It affects most of the skeletal, the respiratory, and cardiac muscles, causing these vital organs to contract and eventually mortality.

Materials and Methods
This study was performed on two boys of 7 and 9 years old with DMD, which were belonged to one family who was consanguinity married. These tests were conducted during a year including LDH, cK, AST, ALT, MRI, EMG, NCV, and MLPA. These tests evaluate the production and function of dystrophin proteins in muscles.

Results
The amount of cK and LDH increased during one year of study in patients. According to the positive family history of hydrocephaly and anencephaly, MRI patients were normal. Regarding the fact that the patients have DMD, the result of EMG and NCV showed Myopathy. MLPA test identified duplication in 56-63 exons.

Conclusion
This study investigated the duplication mutation in Duchenne Muscular Dystrophy (DMD) in two members of the same family. Other mutations such as duplication and point mutations consisted of a small percentage of the disease. Therefore, it is recommended that programs of the prevention measures and pre-marital genetic counseling be specifically trained by the Ministry of Health and Medical Education and relevant organizations.

Key Words: Duchenne muscular dystrophy, Dystrophin, X-linked recessive, Duplication MLPA.

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1- INTRODUCTION

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive genetic disorder (1). It is one of the most severe types of muscular dystrophy and is characterized by the progressive degeneration of muscles at an early age (2). The disease is caused by a mutation in the dystrophin-encoded gene, which consists of 79 exons (3). Genetic defects in the DMD gene are divided into deletion (65%), amplification (5-10%), and point mutation (10-15٪) (**Table.1**) (4). Because of the X-linked inheritance, only boys are exposed to this dystrophy (5). It is being estimated that 1 among 3,500 boys in the world suffer from this type of dystrophy. The DMD gene has been identified on the X chromosome, which encodes a large protein called dystrophin (6). Biochemical symptoms of DMD are elevated transaminases (such as ALT, AST), elevated serum creatine kinase, and abnormal muscle function (7).

There are two types of muscle dystrophies including, Duchenne and Becker. The symptoms of Duchenne dystrophy are walking, frequent falls, GOWERS phenomenon (this means a patient he has to use his body and even his hands of squatting status), and muscle disorders (5). DMD and Becker dystrophies are very similar, while among the progressive degeneration of muscles are slower, the muscle weakness begins later, and the patients lose their ability to walk later among Becker's patients (8). Sometimes they can walk up to 3 years and sometimes up to 4 years. Symptoms: Initial symptoms, boys with this condition, are difficult to walk between the ages of 2 and 4 years and are usually unable to run or jump, unlike their peers, often having difficulty climbing stairs, and Fences help. These patients do not have the definitive treatment and only the side effects can be partially healed. The loss of ability to walk usually occurs at the age of 10. During the wheelchair period, muscle contractions of the pelvis, knees, and scoliosis develop (9). Good nutrition and mild physiotherapy are the best treatment options. The major causes of mortality among the patients are the first pulmonary infections (due to reduced capacity of the lungs, scoliosis, pneumonia, respiratory insufficiency in sleep, and sometimes airway obstruction). Drug treatment, according to current guidelines, includes glucocorticoids, which have the effect of delaying muscular dystrophy. However, long-term use of glucocorticoids can cause side effects like severe osteoporosis, obesity, and even myopathy (8, 10, 11). Nowadays, various techniques such as exon skipping and suppression of gene termination in different countries such as Japan, the USA, and France are used to relieve pain among the patients with muscular dystrophy (12). The crucial note concerning these treatments is that they are applied to specific types of gene defects in muscular dystrophy, and these new treatments are quite specific (13). The results of these therapies demonstrate their significant effect on improving muscle weakness in patients with muscular dystrophy (14). The present study aimed to identify the main cause of genetics in patients with DMD. This reduces the risk of relapse and prevention in affected and non-affected families; also manage and treat the disease by diagnosing it.

Table-1: Type and number of mutations in DMD gene (Further options available in HGMD professional 2019.3).

Type of mutation	Number of mutation
Missense/nonsense	848
Splicing	361
Regulatory	1
Small deletions	540
Small insertions	212
Small indels	57
Gross deletions	1385
Gross insertions/duplications	608
Complex	113
Total	4125

2- MATERIALS AND METHODS

A family consisted of seven members was evaluated in Iranshahr, Sistan and Baluchistan, Iran. They had two affected patients, 7 and 9 years old, and one girl with hydrocephaly who died in the first year of life and a son with anencephaly who died in the first days of birth. Two patients were normal at birth. They were walking until they were six years old, but difficult walking after six years. They use a wheelchair for daily works now. They were biochemically evaluated using cK, Ca, Cr, AFP, LDH, Lactate, Ammonia, AST, ALT, evaluation of amino acids, and aldolase tests, initially. After obtaining results and a significant increase in LDH and cK in DMD patients, they were plotted pedigree with software to identify the type of inheritance **(Figure.1)**.

Then a 9-year-old patient was nominated for genetic testing. Moreover, about 5-10 mL of blood samples were collected from parents and affected and unaffected individuals in the families. DNA was extracted from whole blood. Therefore, laboratory tests including genetic testing have been performed. For these patients, genetic testing of MLPA was also performed. MLPA is a technique used to quantify the number of copies of a specific nucleotide sequence in genomic DNA.

Uses of MLPA include detection of genome deletions and duplications, exon deletions, and screening for SNPs. In this method, the probes are first added to the sample. If two oligonucleotide probes are correctly hybridized to the target sequence, these two probes can be linked and form a complete probe and then replicate with the PCR. MLPA can be used as a multiplex concurrently to evaluate the number of spectral copies of different genes using multiple probes. Also, considering the positive family history of two children with hydrocephaly and anencephaly and two cases of recurrent miscarriage, more precise evaluation was performed using the MRI, EMG, and NCV.

3- RESULTS

Two 7 and 9-year-old male patients suspected of Duchenne Muscular Dystrophy (DMD) were examined in terms of clinical symptoms and biochemically. Clinical examination showed proximal limb weakness, peculiar gait, difficulty rising from the floor (Gower's sign), inability to run properly, and unable to hop. The 9-year-old patient was initially nominated at the age of 8 years for biochemical tests. The results of the tests were as follows, AST (181 U/L), ALT (273 U/L), CPK (4555 U/L), and LDH (553 U/L). Then at age 9, the biochemical analysis was performed again **(Table.2)**.

Significant changes in CPK and LDH were observed during the one-year evaluation of the patient from 8 to 9 years. Nerve and muscle evaluation was then performed. Conduction velocity and distal latency were within normal. EMG showed amplitude, polyphagia, and short duration motor units. Easy recruitment was observed in most of the examined muscles. Spontaneous activity was absent in the examined muscle. Thus EMG and NCV are consistent with a diagnosis of the Myopathy process. The patient was then evaluated for brain MRI examination.

According to the results of MRI, no evidence of space-occupying lesion in supra and infratentorial structures was observed. The white and gray matter in both hemispheres had normal signal intensity. Ventricular size and shape were normal. 7/8 nerves complex were normal which confirmed the results of the MRI on the health of the patient's brain regarding the birth of children with hydrocephalus and anencephaly in the intended family. The results of the EMG were summarized in **(Table.3)**. In the one pathogenic mutation, duplication of exons 56-63; Homozygous was detected in the DMD gene by deletion/duplication analysis

(Figure.2). Thus, this individual is predicted to be affected by Duchenne/Becker Muscular Dystrophy. Female offspring will be carriers of the disease and may be variably affected. Male offspring will be neither carrier nor affected with the disease.

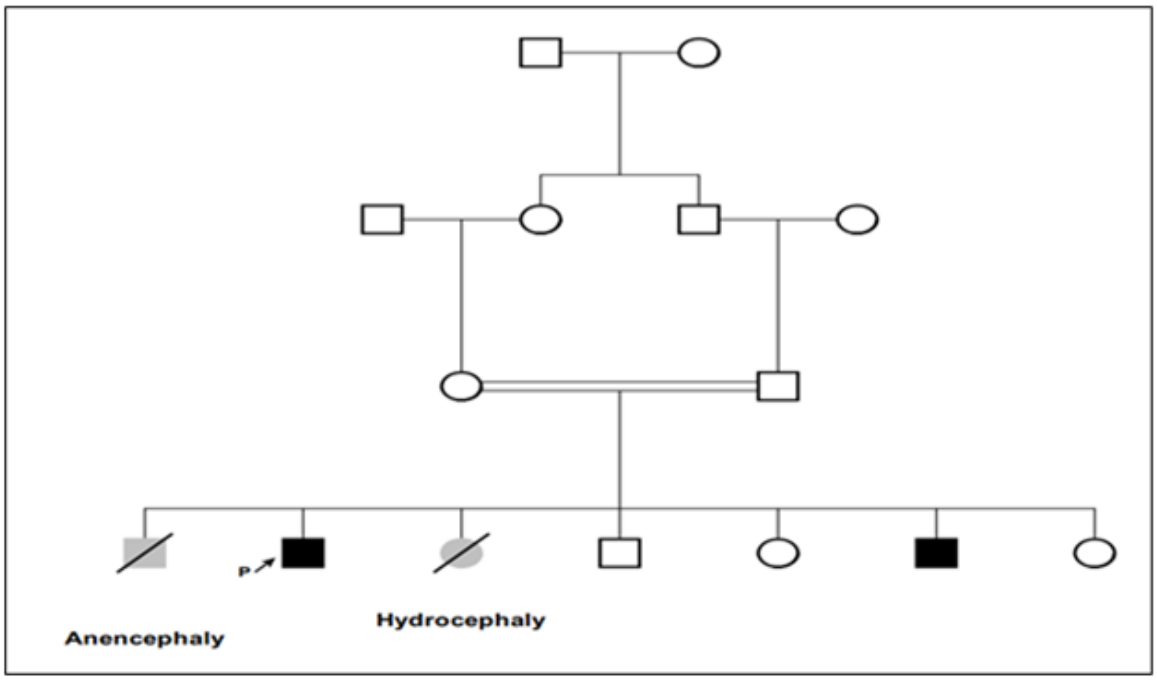

Fig.1: Pedigree of this family, in the drawn pedigree, patients are marked with black. This pedigree indicates that the mother is the carrier for this mutation.

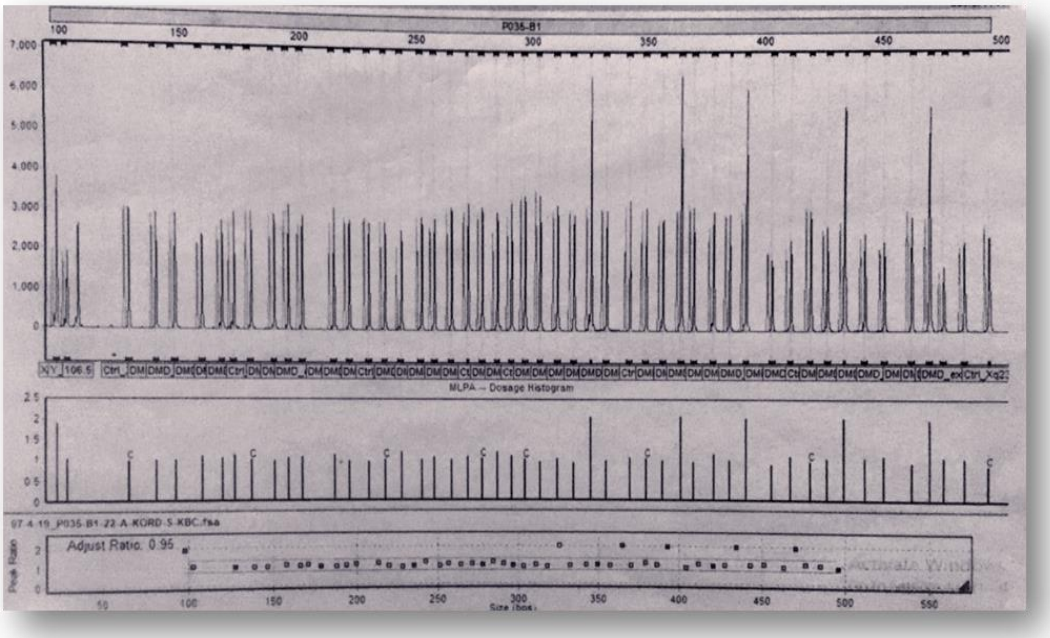

Fig.2: MLPA identified the patient duplication of exons 56-63.

Table-2: Result of laboratory tests of patient.

Test	Result	Flag unit
Blood Biochemistry		
Creatin kinase	4734	U/L
Lactate Dehydrogenase	1483	U/L
Lactate in Blood	17.2	mg/dL
Ammonia in Blood	198	µg/dL
Hematology		
Platelet	501000	mm^3
Immunology and Hormone		
Vitamin B12	26.1	pg/ml
Alfa Feto Protein	0.5	IU/mL
Folic acid	10.9	ng/mL

Table-3: Result of EMG test of patients.

EMG Report								
Side	Muscle	Nerve	Root	Recu	Amp	Dur	Poly	Comment
Rt	Deltoid	Axillar	C5-6	Full	Low	Sho	3	Myopathi
Lt	FCR	EDC	C7-8	Ade	N	N	0	___
Lt	Gastr	Tibial	S1-2	Full	N	N	0	Myopathi
Rt	RectFe	Femo	L2-4	Full	Low	Sho	3	Myopathi
Rt	GluMed	SupGl	L4-5	Full	Low	Sho	2	Myopathi
Lt	TibAnt	DPN	L4-5	Full	N	N	0	Myopathi

4- DISCUSSION

In the present study, a family of 7 and 9-year-old boys with DMD duplication of exons 56-63 on the dystrophin gene on the X chromosome was performed using the MLPA technique. The family also had a hydrocephalus boy who died in the first year of life and a girl with anencephaly who died in the first days of birth. Increased levels of CPK, LDH, AST, and ALT were observed in proband biochemical tests. An Electromyogram also indicated that the patient also had myopathy. But there was no abnormality in the MRI results of the brain. The dystrophin gene (Omim: 310200) has 79 exons. Its genomic DNA contains 3.2 million bp, which is only 14 kb transcribed to mature mRNA. Its protein weighs 427 kDa, which functions as intracellular actin to extracellular laminin around the muscle cell membrane. Dystrophin binds to the F-actin at the amine end and via the carboxyl end to the DAPC (Dystrophin-Associated Protein Complex) **(Figure.3)** (15). There is no definitive treatment for this dystrophy and only corticosteroids and CPAP (continuous positive airways pressure) to delay Respiratory problems and relative prolongation of life are used. The side effect of corticosteroids is osteoporosis.

Several studies were conducted on DMD treatment such as CRISPR / Cas9, exon skipping, utrophin modulation, and dystrophin protein restoration. CRISPR/Cas9- based gene therapy has low cost in the long-term in contrast to antisense permanently corrects patients with DMD. One of the studies on CRISPR/Cas9-based therapies is the modification of duplication mutations in patients. Another therapeutic method is the reproducibility of dystrophin protein using exon skipping, non-sense suppression, and micro-dystrophin gene therapy. Moreover, by increasing the expression of utrophin, a Dystrophin-Related Protein (DRP) partly healed these patients.

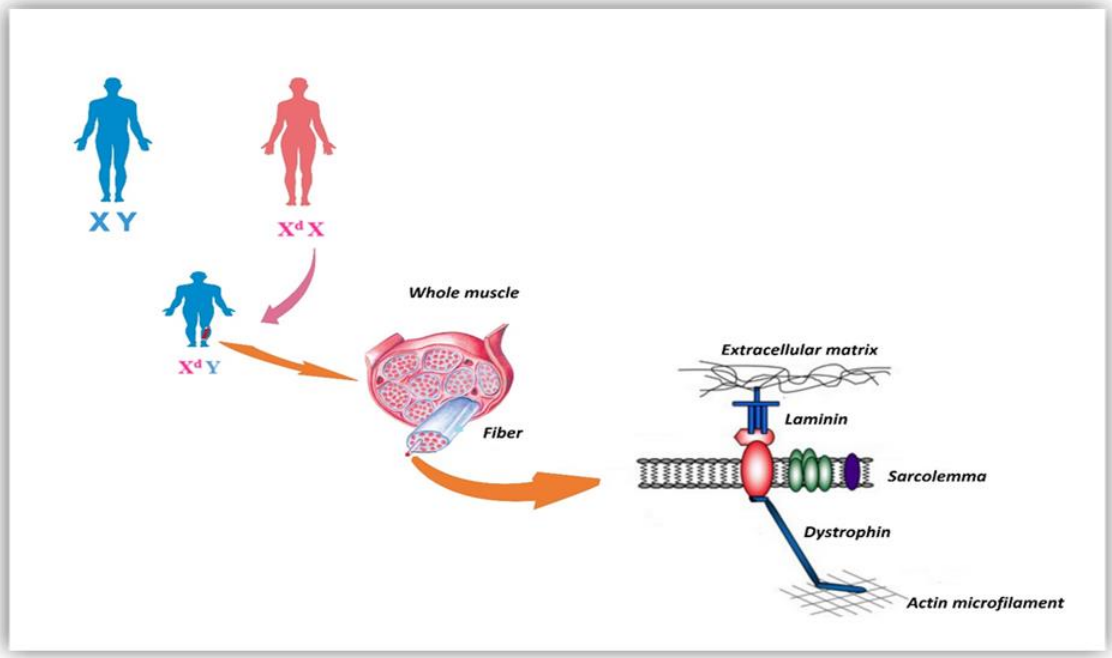

Fig.3: Schematic image of dystrophin protein in muscle cells.

5- CONCLUSION

The present study, which examined two people with Duchenne Muscular Dystrophy in a family, showed a high incidence of this disease in areas with low public awareness, lack of awareness, and consanguinity marriages. Therefore, policy shifts and revisions to preventive measures such as public health education, genetic counseling, and raising public awareness, especially in remote and border regions of the country, where low levels of awareness and health prevail.

6- ABBREVIATIONS

DMD: Duchenne muscular dystrophy, LDH: lactate dehydrogenase, ck: Creatin kinase, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, MRI: Magnetic resonance imaging, EMG: Electromyography, NCV: Nerve conduction velocity, MLPA: Multiplex ligation - dependant probe amplification, DAPC: dystrophin-associated protein complex, CRISPR/Cas9: Clustered regularly interspersed short palindromic repeat/CRISPR associated 9, AFP: alpha-fetoprotein, CPAP: continuous positive airways pressure, DRP: dystrophin-related protein, Cr: creatinine, Ca: calcium, PCR: polymerase chain reaction.

7- CONFLICT OF INTEREST: None.

8- ACKNOWLEDGMENTS

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