Archives of Razi Institute, Vol. 78, No. 3 (2023) 949-954

DOI: 10.22092/ARI.2022.360050.2534

Original Article

Preliminary Results of a Genetic Study of Children with Duchenne Myodystrophy in Aktobe, Kazakhstan

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Received 18 September 2022; Accepted 18 October 2022
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Abstract

Duchenne muscular dystrophy (DMD) is an X-linked recessive neuromuscular illness with a progressive course caused by mutations in the gene encoding the protein dystrophin (DMD; locus Xp21. 2). This study aimed to investigate the clinical aspects of DMD progression according to the mutation type. Included in the study were 38 boys aged 3 to 11 years. Laboratory (biochemical evaluation of the level of creatinine phosphokinase, multiplex ligation-dependent probe amplification [MLPA], and next-generation sequencing [NGS] analysis of the DMD gene), genealogical, and clinical approaches were utilized (including adapted Hammersmith Functional Motor Scale, the study of auditory-speech memory by the "Memorizing 10 words" method, and a general neurological assessment). The MLPA revealed deletion in 22 cases (57.8%), duplication in 6 (15.7%), and negative results in 11 (26.5%). To discover point mutations, 11 infants with negative MLPA results were sequenced. According to the results of the NGS, point mutations were identified in six boys (four single-nucleotide deletions and two single-nucleotide duplications), and five boys lacked mutations. Due to the high proportion of neuro-hereditary diseases in the general structure of neurological pathology, the profound disability of patients with progressive mental and physical disadaptation, as well as the generally fatal course of these incurable afflictions, molecular genetics research is of particular importance.

Keywords: Duchenne myodystrophy, Genetic research, Mutations, Neuromuscular disorders, Sequencing

1. Introduction

Duchenne muscular dystrophy (DMD) is the most severe condition within the spectrum of inherited primary muscle damage (1). DMD is an X-linked recessive neuromuscular disorder caused by mutations in the DMD gene, resulting in the lack or insufficiency of dystrophin, a cytoskeletal protein that supplies myofibrils with strength, stability, and function (2). The absence of complete dystrophin causes DMD. The gene responsible for dystrophin production is located on the X chromosome and contains 79 exons. When this gene mutation exists, muscle degeneration occurs as adipose and connective tissue gradually replace the muscle tissue. Its progression culminates in the loss of independent mobility at age 10. In a steady development, cardiovascular and respiratory failure typically culminate in mortality between the ages of 20 and 25 (3, 4). The protein dystrophin is found not only in skeletal muscles but also in the diaphragm, heart muscle, some isoforms, and the brain, which explains the presence of cognitive impairment in 30-35% of cases. The clinical picture depends on the type of mutation, the location of the mutation in the gene, the size of the mutation, and the therapy. Historically, there has been no treatment for DMD, except for a syndromic approach in which the death of muscle

fibers is slowed down; for this, glucocorticoid therapy is often used in addition to rehabilitation measures (5).

In more than half of the cases, one or several exons experience deletion. Only 10% of cases are duplicated gene regions, and a point mutation occurs in other cases. Today, when modern pathogenetic and/or gene therapy methods are being developed and implemented, the most interesting is the early and accurate diagnosis of changes in the DMD gene. Concerning the diagnosis of DMD, the multiplex ligation-dependent probe amplification (MLPA) method allows the testing of all 79 exons of the dystrophin gene for deletions and duplications; however, polymerase chain reaction (PCR) can detect only a limited number of exons, which is a disadvantage of this method (6, 7). In Kazakhstan, genetic methods are not routine, so to optimize the diagnostic search, only MLPA is indicated in the Kazakhstan DMD protocol. The disadvantage of this method is that it cannot detect point intra-zone and/or intron mutations. Considering the prevalence of point mutations in the range of 10-15%, the importance of conducting next-generation sequencing (NGS) becomes obvious (8). Before genetic testing, a clinical evaluation of the illness is undertaken based on its symptoms.

The mutated gene is typically passed from the mother to the carrier when boys get ill. In one-third of instances, the mutation is de novo. In 10% of cases, the mutation occurs in the parental gametes. DMD typically manifests between the ages of four and eight when children learn to walk. In this instance, we see stumbling, falling, a "duck" stride, difficulty mounting stairs, and pseudohypertrophy of the calf muscles. This symptomatology is caused by muscle weakness and muscle tissue degradation, as evidenced by laboratory tests (increased creatine kinase levels), multiple clinical scales, imaging of the muscle tissue, and electromyography (9, 10). Creatine kinase elevation is a preclinical sign in this family's medical history. The range of clinical symptoms necessitates a multidisciplinary team monitoring this group of children. Medical genetic counseling is the foundation of secondary prevention, which requires knowledge of the types of mutations, as well as the carrier status of the mother. In the traditional form of DMD, the chance of having boys with DMD is 50%, and the risk of having daughters with DMD is 50% (11). The present study aimed to figure out how the symptoms of DMD change depending on the type of mutation.

2. Materials and Methods

The study involved 38 boys aged 3 to 11 years (mean age=5.7 years). All boys complained about muscle weakness, rapid fatigability, and various gait disturbances (movement disorders). The following methods were used: laboratory (determination of the level of creatinine phosphokinase by biochemical means, MLPA, and NGS analysis of the DMD gene), genealogical, and clinical (including the adapted Hammersmith Functional Motor Scale [HFMS], the study of auditory-speech memory by the method of "Memorizing 10 words", and a general neurological assessment). The diagnosis was verified based on the current clinical protocol for diagnosing and treating “Progressive Muscular Dystrophy Duchenne/Becker".

2.1. Methods of Clinical and Neurological Examination of Patients

All patients underwent a comprehensive clinical and neurological examination. Informed consent was obtained from each patient (parent). When collecting anamnesis, clinical and genealogical information was taken into account, and attention was paid to the onset of the disease, the course of the disease, clinical manifestations for the entire period of the disease, the progression rate of the disease, and the diagnoses with which the neurologist observed patients.

2.2. Genealogical Method

As it is known, the type of inheritance in DMD is recessive and linked to the X chromosome, and the transfer of pathological genes occurs from the mother to the son. According to our sample, 73.5% of patients with DMD were familial cases, and 26.5% of those studied had this diagnosis for the first time. As a rule, it was possible to trace the pedigree in two or a maximum

of three generations of relatives they remembered and knew. This can be explained by the gradual reduction of families in which there are patients with DMD. One recommended scale for assessing the degree of activity limitation in patients with DMD or Becker muscular dystrophy (BMD) is the adapted HFMS. The scale consists of 20 points, each rated according to the degree of completion of 2, 1, or 0 points.

The neuropsychological examination methodology included conversation, as well as the study of memory, attention, and thinking indicators. For a high-quality test, it was necessary to comply with specific conditions, such as silence in the room, preventing external stimuli from distracting the child's attention, and excluding parental help during the tests. Working with children began with getting to know each other and establishing contact. The researcher specified the child's age, date of birth, and family composition. The study of auditory-speech memory was conducted according to the "Memorizing 10 words" method (A. R. Luria) to analyze the indicators of memory, attention (voluntary), and exhaustion due to the progression of the disease, as well as the dynamics of the course of the disease. The child was asked to remember the number of words he memorized out of the 10 types suggested in the first task.

Interpretation:

-4 points (norm): a high rate, remembered 9-10 words after the third presentation with an 8-9-word delayed recall

-3 points (mild cognitive impairment): medium, remembered 6-8 words after the third presentation with a 5-7-word delayed recall

-2 points (moderate cognitive impairment): below medium, remembered 3-5 words after the third presentation with a 3-4-word delayed recall

-1 point (pronounced cognitive impairment): a low rate, remembered 0-2 words after the third presentation with a 0-2-word delayed recall

2.3. Molecular Genetic Methods

The DNA samples were obtained from peripheral blood leukocytes according to standard procedures. Mutations in the DMD gene were detected using the MLPA method according to the manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands). To analyze all 79 exons of the dystrophin gene, two sets of reagents were used: a mixture of SALSA 034 probes (exons 1-10, 21-30, 41-50, and 61-70) and a mixture of SALSA 035 probes (exons 11-20, 31-40, 51-60, and 71-79). The material was processed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA).

2.4. Next-Generation Sequencing

The DMD gene is typically analyzed by the amplicon-based NGS methodology. Amplicons cover the entire coding region and highly conserved Exon-Intron compounds. The minimum coverage is >20× for each amplicon, and the technical sensitivity (SNV/InDels) is 99.9%. The Copy Number Variation (CNV) allows the detection of deletions and duplications using the NGS methodology.

3. Results

3.1. Results of Clinical and Laboratory Studies

The mean age of the children was 5.7 years. The general condition and neurological status depended on the degree of disease development. The onset of the disease varies from 2.8 to 5 years. The most common complaint in patients was muscle weakness in the lower extremities (thigh) and pelvic girdle with a transition to the muscles of the upper shoulder girdle, back, and proximal arm parts. The severity of muscle weakness varied from 4 points to 1 and depended on the degree of progression of the patient's age. Walking "on tiptoe" causes more anxiety among mothers than among the subjects themselves. During a detailed questioning of the patient to find out the cause of the gait disturbance, the patient replied that it was so convenient for him and that he did not even notice how it turned out. Over time, there was a complaint about a violation of support on foot. The HFMS results showed that 8 cases (21%) scored 3 points (the mild-degree impairment of motor functions), and a moderate-degree impairment of motor

functions was observed in 16 boys (42.1%), which corresponded to 2 points. Moreover, 1 point was observed in 14 cases (36.8%), which is characteristic of a pronounced-degree impairment of motor functions.

Creatine phosphokinase indicated a significant increase up to 7-8 thousand U/L, with a norm of 15-190 U/L mainly at the beginning and during the progression of the disease.

When interviewing subjects with DMD, about 40% of patients complained about poor memory, inattention, and distraction. In the "Memorizing 10 words" study, 10 cases (26.3%) showed decreasing attention and memorization of words. They memorized 6 out of 10 words, corresponding to mild cognitive impairment. A moderate cognitive impairment was detected in 10 patients (26.3%), and the investigation showed decreased attention, impaired recall of word order, and memorization of words reproduced by the researcher more than three times. Inattention, decreased memory and receptivity of instructions, and the repetition of 2 out of 10 words were observed in 18 patients (47.3%), which corresponds to a pronounced cognitive impairment.

3.2. Molecular Genetic Results.

According to the MLPA results, deletion was observed in 22 cases (57.8%), duplication in 6 (15.7%), and negative results in 11 (26.5%). This ranking is shown in figure 1.

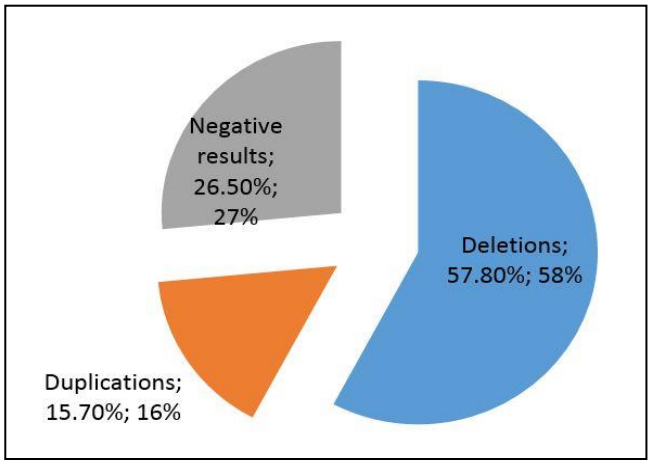

Figure 1. Ranking of the types of mutations in the DMD gene

Among deletions, we distinguished two categories, including the deletion of one exon and the deletion of two or more exons. Among the 22 cases, large elongated deletions of more than eight exons accounted for 6 cases (27.3%), from two to eight exons accounted for 9 (40.9%), and one exon deletion for 7 (31.8%). These characteristics are shown in figure 2.

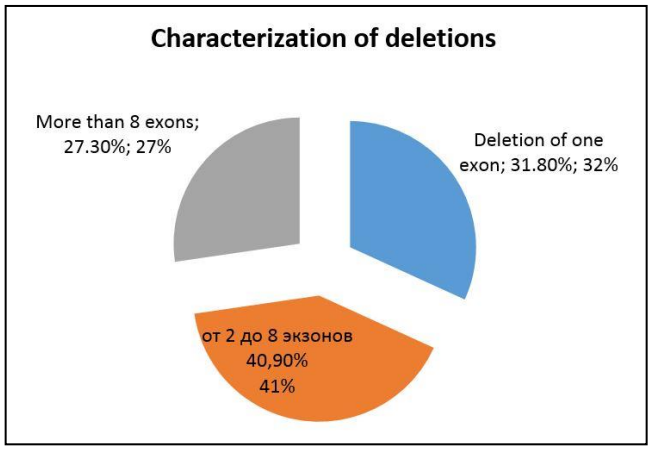

Figure 2. Ranking of deletions by characteristic

Figure 2 shows the characteristics of the deletions since the severity of the condition depended on both the length of deletions and the state of the reading frame of the nucleotide sequence.

We performed sequencing in 11 children with negative MLPA results to identify point mutations. The NGS results revealed point mutations in six boys (four single nucleotide deletions and two single nucleotide duplications), and no mutations were detected in five boys.

It is known that when a protein reading frame is disturbed, extremely unstable dystrophin is synthesized, which can be subsequently destroyed by proteolytic enzymes or is absent. A more in-depth analysis of genetically proven variants was carried out in 33 children, where we evaluated the results of genetic tests with a reading frame analysis. For children with negative genetic results (MLPA and NGS), muscle biopsy and complete exome sequencing are recommended. These results are shown in figure 3.

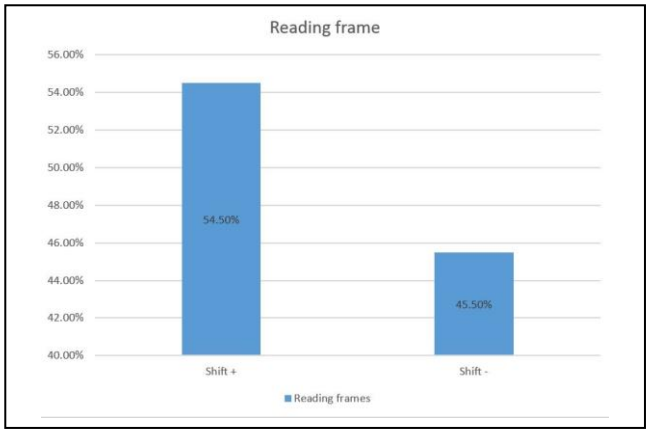

Figure 3. Analysis of protein reading frame

4. Discussion

The most common molecular defect in the DMD gene is the deletion of one or more exons, occurring in 65% of DMD cases, while duplication occurs in 6-10% of cases. The remaining cases (approximately 25%) are associated with minor mutations. However, a lower incidence (approximately less than 2%) is caused by complex rearrangements and profound intronic changes (12).

The minimum level of diagnostic testing is for quantitative analysis of DMD genes to detect the majority of gene changes that are exon deletions or duplications, followed by a qualitative approach represented by complete gene sequencing.

Among the quantitative methods available, MLPA is currently the most widely used. This method, which simultaneously tests all 79 exons of the DMD gene, determines the CNV in a reaction based on a multiplex PCR (13). The introduction of NGS technology can lead to the development of a unique diagnostic method; moreover, a specific computational structure can simultaneously manage the identification of CNV and single nucleotide variations. The MLPA diagnostic workflow may not identify 2% of complex rearrangements or deep intronic changes; therefore, sequencing may become necessary for genetic diagnosis in this subtype of rare mutations (14). To summarize, we can say that the optimal procedures for molecular diagnostics of DMD consist of quantitative analysis for the detection of CNV followed by genomic sequencing or the NGS strategy (15).

Research in molecular genetics is of particular relevance due to the high proportion of neuro-hereditary diseases in the general structure of neurological pathology, profound disability of patients with progressive mental and physical disadaptation, and the fatal course of these incurable sufferings in most cases. For the first time in the Aktobe region, children's molecular genetic diagnostics of DMD was carried out. To date, this method is the only preventive measure, making it possible for prenatal diagnosis in high-risk families. Considering that diseases from this group are mainly characterized by a steadily progressive course and the absence of effective treatment methods, neuromuscular diseases should be recognized as one of the most pressing problems of clinical neurology. Prevention of the recurrence of neuromuscular diseases in high-risk families is currently the only effective means of combating these severe and often fatal ailments. Furthermore, DNA diagnostics is central to the system of preventive measures.

Authors' Contribution

Study concept and design: A. U.
Acquisition of data: D. A.
Analysis and interpretation of data: A. M.
Drafting of the manuscript: B. D.
Critical revision of the manuscript for important intellectual content: A. D.
Statistical analysis: A. U.
Administrative, technical, and material support: A. U.

Ethics

The human study was approved by the ethics committee of the West Kazakhstan Marat Ospanov Medical University, Aktobe, Kazakhstan.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

The authors express their gratitude to West Kazakhstan Medical University for the financial support provided in this scientific work.

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