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DISTROPHIN GENE MUTATION SPECTRUM IN CHILDREN WITH DUCHENNE/BECKER PROGRESSING MUSCULAR DYSTROPHIES IN UZBEKISTAN

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Abstract

Background. Duchenne and Becker progressing muscular dystrophies (D/BPMD) are the one of the most common congenital diseases of the neuromuscular system of juniors in the world. Along with the improvement in medical and genetic service and the quality of prevention and early diagnosis of congenital and hereditary diseases, there are a number of tasks that are pending, including medical and genetic counseling of hereditary diseases of the neuromuscular system in children.

Methods. Direct DNA diagnostics was performed for 99 patients with D/BPMD from 86 families, 92 (92.9%) patients with DPMD, 7 (7.1%) patients with Becker PMD. The analysis was carried out on 20 exons of the dystrophin gene — the promoter region, 3, 4, 6, 8, 13, 17, 19, 32, 42, 43, 44, 45, 47, 48, 50, 51, 52, 53, 60 exons. Indirect diagnosis was performed in 21 families weighed by D/BPMD using intragenic highly polymorphic markers located in the 45th (STR-45), 49th (STR-49), 50th (STR-50) gene introns. Mutations in 18 exons out of 20 studied were identified, which necessitates further study of the deletion spectrum of mutations in the dystrophin gene in our population.

Results. In 35 patients (35.4%) of 32 families (37.2%), no deletions were detected, and in 64 patients (64.6%) of 54 families (62.8%), dystrophin gene deletions were detected with various lengths - from one to nine exons: in 70.4% of families extended deletions were verified (44 patients), deletions of one exon were found in 29.6% of families (20 patients). The heterozygous genotype STR-45 (CA) 28 is highly informative in identifying heterozygous carriers of the damaged dystrophin gene among relatives of the D/BPMD patient in Uzbekistan.

Conclusion. There is huge demand for sequencing of a limited portion of the DMD gene to determine the point mutation of 32 exon in the dystrophin gene. Furthermore, carrying out prenatal diagnosis in high-risk families according to D/BPMD allowed to prevent the birth of sick children and reduce disability among children for this disease.

Key words: progressive Duchenne and Becker myodystrophy, direct DNA diagnostics, indirect DNA diagnostics, children.

BACKGROUND

Duchenne and Becker progressive muscular dystrophies (D/BPMD) are the most common congenital diseases of the neuromuscular system among children (Arupova, 2016; and Basak et al, 2011). According to experts of the World Health Organization (WHO), the incidence of DPMD is 1: 3500 and BPMD is 1: 20,000 live birth boys (Illarioshkin et al, 2002; Gatta et al, 2015; and Zimowski et al, 2017).

Patients with Becker and Duchenne muscular dystrophy have a mutation in the DMD gene that leads to a deficiency or absence of dystrophin protein. This results in skeletal muscle weakness and loss of ambulation, usually in the second decade of life in Duchenne muscular dystrophy and later in life in the milder Becker muscular dystrophy. Both reduced and absent dystrophin also leads to the development of cardiomyopathy resulting in progressive left ventricular dysfunction (cited in Soslow et al, 2019).

Being aware of the clinical importance of Becker and Duchenne muscular dystrophy and cardiomyopathy has increased during the last few decades. This is mostly owing to progress in healthcare, which have increased life expectancy in patients with Duchenne muscular dystrophy. The preponderance of Becker and Duchenne research focuses on skeletal muscle weakness, but cardiovascular disease is now one of the leading causes of death in patients with Duchenne muscular dystrophy (Bach and Martinez, 2011). Identifying risk factors leading to increased length of stay, cost of hospitalization, and readmission rates in this population can increase recognition and potentially improve outcomes in future admissions (Soslow et al, 2019).

Along with the development in the Republic of Uzbekistan of a modern medical and genetic service and improving the quality of services in the field of prevention and early diagnosis of congenital and hereditary diseases, there are a number of tasks that are pending, including medical and genetic counseling of hereditary diseases of the neuromuscular system in children.

PURPOSE OF THE STUDY

To study the dystrophin gene mutation spectrum in families of patients with D/BPMD in Uzbekistan.

MATERIAL AND METHODS

The molecular-genetic part of this research was carried out at the Department of Molecular Medicine and Cellular Technologies of Scientific Research Institute of Hematology and Blood Transfusion of the Ministry of Health of the Republic of Uzbekistan.

STUDY POPULATION

Molecular genetic testing was carried out using direct and indirect DNA diagnostic methods. Direct DNA diagnostics was carried out by detecting 20 frequent deletions of the dystrophin gene in sick and pregnant women from families weighed by D/BPMD bearing a male fetus. Indirect

DNA diagnostics was carried out with the study of intragenic highly polymorphic markers located in the 45th (STR-45), 49th (STR-49), 50th (STR-50) introns of the gene to determine the carriage of the mutant gene among proband relatives. Direct DNA diagnostics were performed for 99 patients with D/BPMD from 86 families, 92 (92.9%) patients with Duchenne PMD, 7 (7.1%) patients with Becker PMD. The analysis was carried out on 20 exons of the dystrophin gene — the promoter region, 3, 4, 6, 8, 13, 17, 19, 32, 42, 43, 44, 45, 47, 48, 50, 51, 52, 53, 60 exons. Indirect diagnosis was performed in 21 families weighed by D/BPMD using intragenic highly polymorphic markers located in the 45th (STR-45), 49th (STR-49), 50th (STR-50) gene introns.

Genomic DNA from peripheral blood samples (Vacutainer Becton Dickinson International with EDTA) was isolated using the DIAtom ™ DNA PreplOO DNA Isolation Kit, Molecular Genetics Center LLC (Moscow) according to the instructions. Amplification was performed using GeneAmp PCR-system 2720 thermal cyclers (Applied Biosystems, USA) using DMD-del and DMD-CA kits (Center for Molecular Genetics LLC, Moscow).

RESULTS

According to the results of molecular diagnostics, in 35 patients (35.4%) of 32 families (37.2%), no deletions were detected, and in 64 patients (64.6%) of 54 families (62.8%), dystrophin gene deletions were detected with various lengths - from one to nine exons: in 70.4% of families extended deletions were verified (44 patients), deletions of one exon were found in 29.6% of families (20 patients).

Mutations of the dystrophin gene found in 4 families with related marriages were characterized by deletions of one exon in the distal region of the 3'-end (deletions of 48, 50 and 53 exons), and in 3 families Duchenne muscular dystrophy was determined for the first time in this generation (Fig. 1). Mutations de novo in our studies were found almost 2 times less than in the detection of family cases of diseases in the “hot spots” of the dystrophin gene (32.6% versus 67.4%).

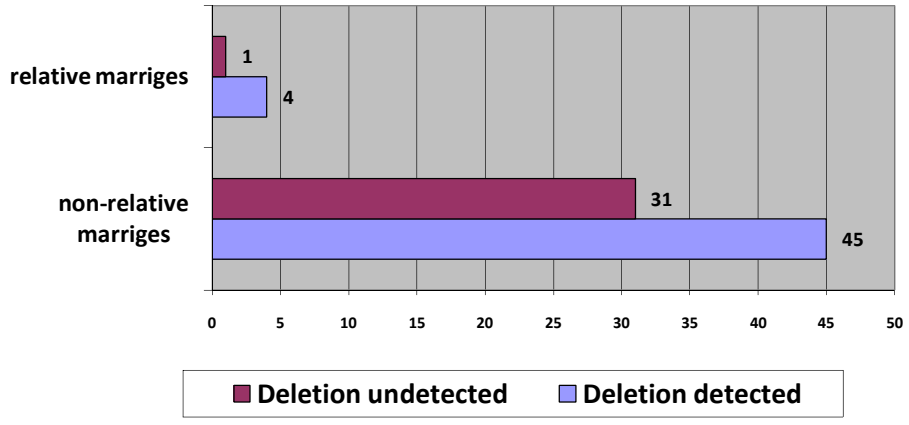

Fig. 1. The frequency of family marriages in families with D/BPMB

When analyzing deletions, deletions in the distal part of the dystrophin gene were most often detected, which accounted for 79.6% of all identified mutations in families: 50 exon in 24 cases, 51 exon in 22 cases, 52 exon in 16 cases, 48 exon in 17 cases, 53 exon - in 11 cases; 32 exon - 1; 42 exon - 1; no deletions of the promoter zone and 60 exon were detected. With proximal mutations, deletions of 19 (in 7 cases), 6 (in 7 cases), 4 (in 6 cases) and 17, 13, 8 in equal amounts (in 5 cases, respectively) of exons were more common (Fig. 2).

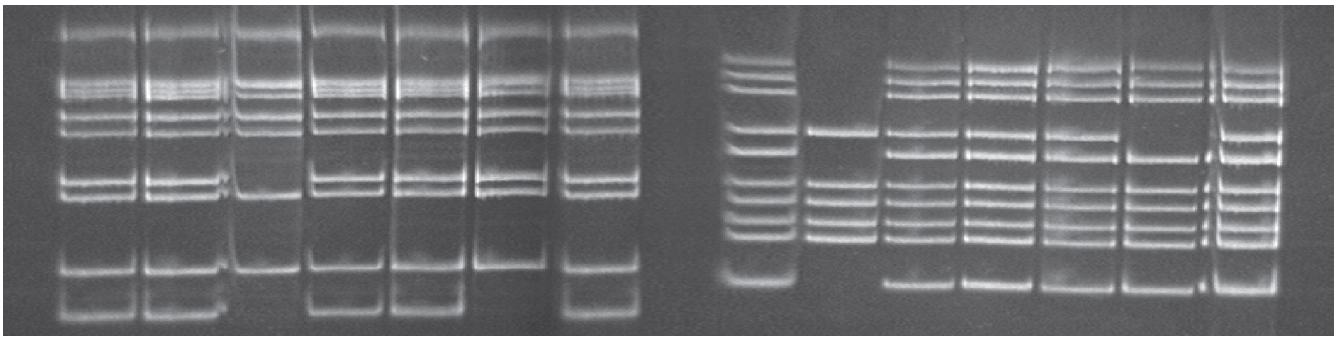

Fig. 2. The results of multiplex PCR diagnostics of the dystrophin gene on DNA samples in 7 patients with D/BPMD.

In sporadic cases and familial forms, in most cases deletions in the distal part of the dystrophin gene — the 3'-end (deletion of 40-60 exons) were more often detected, almost 3.6 times more often than the frequency of occurrence of proximal deletions of the dystrophin gene in 5'- end (deletions of 3-19 exons) (Fig. 3).

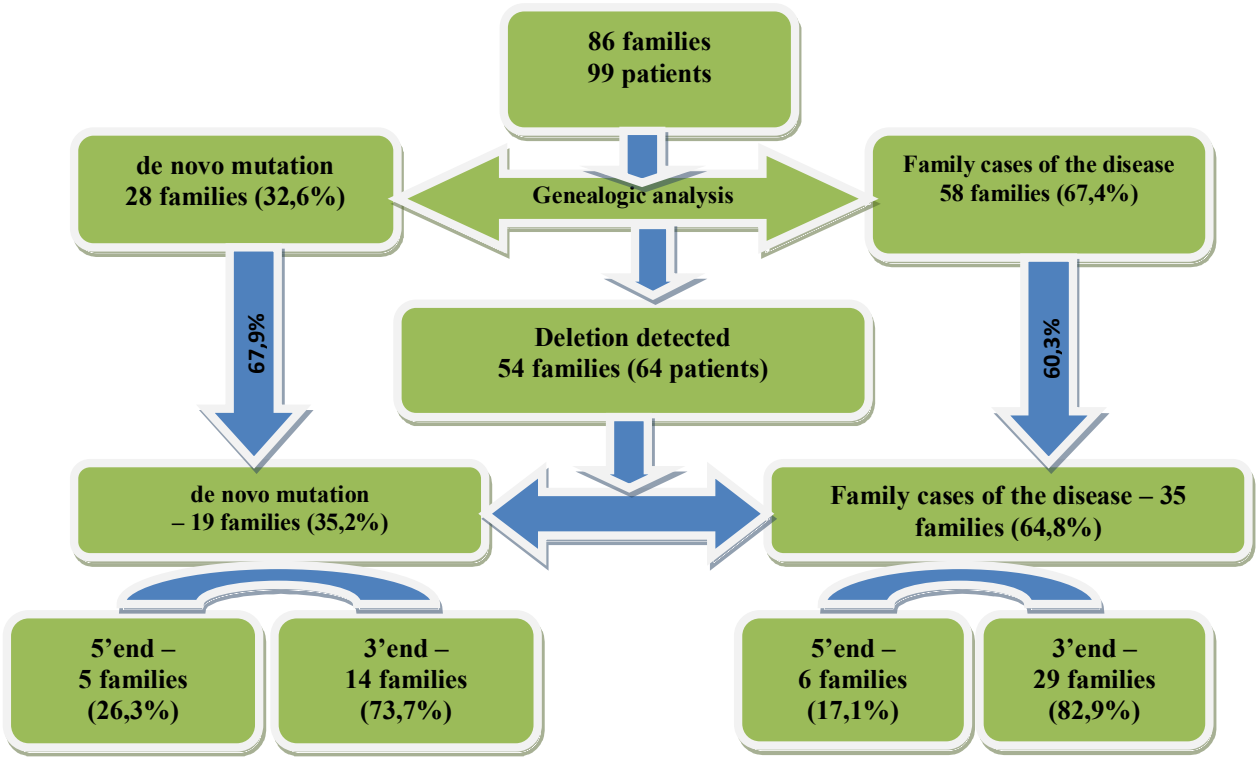

Fig. 3. The ratio of de novo mutations and family cases of diseases according to the "hot spots" of the dystrophin gene.

Indirect DNA diagnostics using intragenic highly polymorphic markers located in the 45th (STR-45), 49th (STR-49), 50th (STR-50) gene introns was performed by 61 relatives from 21 families burdened with D/BPMD. Of 21 families in 33.3% (7 families) when conducting direct DNA diagnostics, deletions in the dystrophin gene were not detected, in 14.3% (3 families) deletions were detected at the 5'-end, in 52.4% (11 families) - deletions were detected at the 3'-end.

The linkage analysis of STR-45, STR-49, and STR-50 loci with exon deletion of the DMD gene was performed: a total of 26 healthy chromosomes without deletions (13 unrelated women) and 43 chromosomes of patients with STR hemisygote deletion were studied. A clear tendency was found to increase the level of heterozygosity of women according to STR-45, with the presence of deletion of X chromosomes in comparison with women with no deletion of X chromosomes. The heterozygosity level of STR-45 in the X chromosomes with no deletion was 23.1%, and in the group with deletion 41.9% ($\chi 2 = 2.5$; P = 0.1; OR = 2.4; 95% CI 0.8028-7.175). The difference in the heterozygosity index for the STR-49 locus in the X chromosomes with and without deletion was significant and amounted to 34.9% and 11.5%, respectively ($\chi 2 = 4.6$; P = 0.03; OR = 4.1 ; 95% CI 1,058-15,9). The level of adhesion of heterozygotes at the STR-49 locus to a deletion of exons of the DMD gene was 4.1 times significantly higher compared to heterozygotes of this locus with no deletion. The frequency of the STR 50 polymorphic locus in the X chromosomes with and without deletion was 23.2% and 15.4%, respectively ($\chi 2 = 0.6$; P = 0.4; OR = 1.7; 95% CI 0.464 -5.987). Moreover, the difference in frequency in the compared groups of X chromosomes was not significant (Table 1).

Table 1

The frequency of distribution of exon deletion of the DMD and STR gene exons in patient groups

Total informative results for STR	STR 45	STR 49	STR 50	Total
X-chromosome with no deletion	6 (23,1%)	3 (11,5%)	4 (15,4%)	26 chrom
X chromosomes with deletion	18 (41,9%)	15 (34,9%)	10 (23,2%)	43 chrom

As can be seen from the table, a tendency towards an increase in the level of the heterozygous state in chromosomes with the presence of distal mutations as compared with proximal ones ($\chi 2 = 1.8$; p=0.2) was revealed. Of the 12 women examined who were carriers of the proximal mutation, heterozygous and STR-45 homozygous genes were identified in 7 (58.3%) and 5 (41.2%). The heterozygosity of the

multiallelic STR-45, STR-49 and STR-50 loci in the subgroups of women with proximal and distal deletions of the dystrophin gene was 22.7% and 77.3%, respectively.

To determine the level of information content, i.e. heterozygosity of the dinucleotide $(CA)_{28}$ repeat of the STR-45 dystrophin gene, we studied 44 unrelated healthy X chromosomes. The observed heterozygosity frequency of this marker was 59.1% (0.59; 26/44) (Table 2).

For the heterozygous genotype of STR-45 polymorphism, it was found that the observed frequency of heterozygotes (H_{obs}) is statistically significantly higher than the expected H_{exp} calculated according to Hardy-Weinberg law (0.59 versus 0.42%, respectively, $\chi 2 = 3.9$; $p = 0.049$). The deviation coefficient F of the actual heterozygosity from the theoretical one was +0.4.

For STR-45 (CA) 28, D was positive and maintained >0. The H_{obs} value was close to 0.6, which indicates a high level of informational content of this microsophyllite polymorphism for detecting heterozygous carriage and prenatal DNA diagnostics in high-risk D/BPMD families.

Table 2

Expected and observed frequencies of the distribution of genotypes by PXB of the genetic marker STR-45 $(CA)_{28}$

Genotype	Genotype frequency		χ^2	P
	Observed H_{obs}	Expected H_{exp}		
-/-	0,41	0,5	0,34	0,049
-/+	0,59	0,42	1,6	
+/+	0,00	0,09	1,92	
Total	1,0	1,0	3,9	

Microsatellite polymorphism repeats $(CA)_{24}$ and $(AC)_{16}$ are located respectively in introns 49 and 50 of the dystrophin gene. These polymorphisms also have a certain number of CA repeats (24 and 16, respectively), inherited according to the laws of Mendel. It was found that the STR-49 $(CA)_{24}$ polymorphism is in strong disequilibrium in adhesion with STR-50 $(AC)_{16}$, and therefore with the STR-45 $(CA)_{28}$ microsatellite polymorphism, which makes these markers useful when then of these markers will not be informative.

In determining the level of heterozygosity of these polymorphisms, 44 unrelated X chromosomes were also studied. In both cases, the calculated actual frequency of heterozygotes was 45.5% (0.45; 10/22). Based on the obtained data, the calculation of the level of theoretical heterozygosity, i.e. the information content of multiallelic systems in our studied samples, according to the Hardy-Weinberg

equation, amounted to - 0.35. As can be seen, for both polymorphisms, the theoretical number of heterozygotes is not statistically significantly reduced compared to the actual one (0.35 and 0.45, respectively, $\chi 2 = 1.9$; $p = 0.2$). The relative deviation of the actual heterozygosity from the theoretical one turned out to be positive and amounted to D = + 0.3.

DISCUSSIONS

The results of molecular genetic studies have shown that family cases of Duchenne / Becker PMD over the de novo mutation, as well as extended deletions at the 3'-end of the dystrophin gene, predominate in the population sample of Uzbekistan. Deletions of the gene were found in 64.6% of the examined chromosomes, of which 21.9% are located in the proximal part of the gene, 78.1% - in the distal part, which corresponds to the data for the Russian and Kazakh populations.

The heterozygosity of the multiallelic loci STR-45 $(CA)_{28}$, STR-49 $(CA)_{24}$ and STR-50 $(CA)_{16}$ is a marker for detecting carriers and prenatal DNA - the diagnosis of Duchenne/Becker myopathies in high-risk families. The calculated index of the total theoretical heterozygosity of these loci was 0.71. This means that 71% of women in the study sample are theoretically informative of one of the three polymorphic markers for indirect DNA diagnostics. However, in a joint study of three multiallelic polymorphic markers, the efficiency of detecting heterozygous gene carriage among women was 83.3%, while the difference between the total expected and observed heterozygous frequencies was statistically insignificant.

A positive correlative relationship was revealed between the carriage of various deletion mutations and the heterozygous state of the polymorphic loci STR-45, STR-49, and STR-50. The heterozygous genotype STR-45 $(CA)_{28}$ is highly informative in identifying heterozygous carriers of the damaged dystrophin gene in relatives of the D/BPMD patient in Uzbekistan.

In the absence of frequent deletions according to the results of direct and indirect DNA diagnostics, the search for mutations can be carried out by dystrophin gene sequencing.

During the study, dystrophin gene sequencing was performed on 2 D/BPMD patients; the indication for sequencing was the presence of clinical symptoms and biochemical abnormalities of D/BPMD, as well as primary muscle lesions according to the results of EMG studies. Dystrophin gene sequencing is a method for confirming the diagnosis of D/BPMD in the presence of clinical and laboratory signs of the disease and negative results obtained using the available methods of direct and indirect DNA diagnostics.

Sequencing of the dystrophin gene in the first case revealed the previously described mutation - duplication of exons 3-7 in the dystrophin gene in a hemizygous state, leading to a shift in the reading frame, which is typical for the clinical form of DPMD disease. In the second case, the previously undescribed mutation C.4518 + 2T>A was revealed in the dystrophin gene

in the hemizygous state, leading to replacements in the sites of donors of splicing sites.

A prenatal invasive diagnosis of D/BPMD was carried out on the basis of the Republican Center "Screening for Mother and Child" for 7 pregnant women who had a male fetus from high-risk families for this pathology. Of these, in 2 cases, fetal biomaterial for further DNA research was obtained by amniocentesis and cordocentesis was performed in 5 cases.

According to the results of DNA diagnostics, deletions in the dystrophin gene with a high risk of clinical realization of the disease after birth were identified in 3 cases (42.8%) in the fetus, families were recommended to terminate the pregnancy for medical and genetic reasons. In the remaining families, no dystrophin gene mutations were detected in the fetuses, the pregnancy was prolonged, and subsequently a postnatal examination of the children was performed to confirm the absence of mutations in the dystrophin gene.

According to results of the research conducted by LeRumeur (2015), therapies in progress will not cure the DPMD disease but slow down its progression. The purpose now is to focus therapy on the BPMD-like dystrophin sequences with the less severe disease gene or exon skipping therapy. We have now to increase our knowledge about BPMD disease genetics and time course and dystrophin structure consequences after BPMD deletions. The diagnosis of BMD is highly relevant to anticipate and to understand data resulting from human clinical trials. It is also likely that certain severe patients should benefit from therapy strategies or from compensatory strategies elaborated for DPMD patients.

Cho et al (2017) analyzed the dystrophin gene in 507 Korean D/BPMD patients by multiple ligation-dependent probe amplification and direct sequencing and identified 117 different deletions, 48 duplications, and 90 pathogenic sequence variations, including 30 novel variations. Deletions and duplications accounted for 65.4% and 13.3% of Korean dystrophinopathy, respectively, suggesting that the incidence of large rearrangements in dystrophin is similar among different ethnic groups. We also detected sequence variations in >100 probands. The small variations were dispersed across the whole gene, and 12.3% were nonsense mutations.

CONCLUSION

Thus, a positive correlative relationship was revealed between the carriage of various deletion mutations and the heterozygous state of the polymorphic loci STR-45, STR-49 and STR-50. The heterozygous genotype STR-45 $(CA)_{28}$ is highly informative in identifying heterozygous carriers of the damaged dystrophin gene among relatives of the D/BPMD patient in Uzbekistan. Sequencing of the dystrophin gene revealed a new mutation C.4518 + 2T>A in the DMD gene of chromosome X in the hemizygous state, which indicates the need for sequencing of a limited portion of the DMD gene to determine the point mutation of 32 exon in

the dystrophin gene. Furthermore, carrying out prenatal diagnosis in high-risk families according to D/BPMD allowed to prevent the birth of sick children and reduce disability among children for this disease.

STUDY LIMITATIONS

We could not perform sample size calculation for this research and the number of participants to prove the null hypothesis might be less than it supposed to be. Thus, further researches may be required with an adequate sample size to show more clear and exact results.

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ETHICAL APPROVAL

The ethical approval for the study was granted by the Committee of Ethical Approval for Researches under the Ministry of Health of the Republic of Uzbekistan.

CONSENT

Written informed consent was obtained from all participants' parents of the research for publication of this paper and any accompanying information related to this study. A copy of the written consent is available for review by the authors.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

FUNDING

No funding sources to declare.

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