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GENETIC ANALYSIS OF DYSTROPHIN GENE IN AFFECTED MALES AND FEMALES CARRIERS WITH DUCHENNE/BECKER MUSCULAR DYSTROPHY IN REPUBLIC OF MOLDOVA BETWEEN 1992 AND 2012.

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Summary

ANALIZA GENETICĂ A GENEI DISTROFINEI LA BĂRBAȚII BOLNAVI ŞI FEMEILE PURTĂTOARE CU DISTROFIE MUSCULARĂ DUCHENNE/BECKER ÎN REPUBLICA MOLDOVA (1992 – 2012)

Cuvinte ceie: *distrofie musculară Duchenne/Becker, gena distrofinei, multiplex PCR, site-urilor polimorfe, statutul de purtător*

Introducere. *În cadrul Centrului Național de Sănătate a Reproducerii şi Genetică Medicală s-a efectuat analiza mutațiilor în gena distrofinei, folosind multiplex PCR, a probelor colectate în perioda 1992-2012. Acest studiu a inclus 206 familii cu risc pentru distrofia musculară Duchenne/Becker (DMD/BMD), necesitînd consultație medico-genetică. Analiza ADN a fost efectuată la familiile cu cel puțin un copil bolnav de DMD sau la rudele apropiate ale acestuia.*

Material şi metode. *Studiul molecular a fost efectuat la 190 bolnavi DMD/BMD prin multiplex PCR, cu seturi de primeri specifici care favorizează amplificarea a 20 exoni diferiți, şi prin metoda analizei site-urilor polimorfe RFLP: pERT87-8/Tag1, pERT87-15/BamH1, 16intron/Tag1.*

Rezultate. *Efectuarea amplificării „Multiplex" a genei distrofinei a permis identificarea a 81,5% din pacienți cu deleții în genă. Cea mai frecventă regiune supusă ruperii a fost intronul 44. Cu o frecvență înaltă a fost identificată deleția exonului 48 (14%), exonului 45 (17%), exonului 47 (10%) şi a exonului 50 (10%). Din cele 206 familii cu risc inalt de DMD/BMD 198 au fost găsite informative cel puțin după un site polimorfic pentru analiza ADN. Statutul de purtător a fost confirmat la 81 % femei înrudite şi infirmat la 17% din ele (nivelul de încredere de 90-95%). La 2% din femei a fost imposibil de stabilit statutul de purtător, familiile fiind neinformative. Şase pacienți cu DMD/BMD au fost diagnosticați prenatal prin analiza directă a deleților sau prin testul RFLP.*

Concluzii. *Aplicarea ambelor metode de testare molecular genetică au asigurat detectarea a cca 94% de cazuri informative. Diferențele în frecvența deleților exonilor implicați împreuna cu instabilitatea regiunilor genei distrofinei observate, în comparație cu cele observate anterior în alte studii, probabil că sunt specifice populației Republicii Moldova. Variațiile interpopulaționale respective în pattern-urile deleților genei distrofinei sunt discutate.*

Резюме

ГЕНЕТИЧЕСКИЙ АНАЛИЗ ГЕНА ДИСТРОФИНА У БОЛЬНЫХ МИОДИСТРОФИЕЙ ДЮШЕННА/БЕККЕРА МАЛЬЧИКОВ И У ЖЕНЩИН НОСИТЕЛЬНИЦ В РЕСПУБЛИКЕ МОЛДОВА ЗА ПЕРИОД С 1992-2012 ГГ.

Ключевые слова: *Миодистрофия Дюшенна, ген дистрофина,, мультиплексная ПЦР, полиморфные сайты, носительство.*

Введение. *В Национальном Центре Репродуктивного Здоровья и Медицинской Генетики был проведен анализ мутаций гена дистрофина, используя мультиплексную ПЦР. В исследование вошли 206 семей высокого риска Миодистрфии Дюшенна/Беккера, прошедшие медико-генетическое консультирование в Национальном Центре Репродуктивного здоровья и медицинской генетики (Республика Молдова) с 1992 по 2012 годы. ДНК анализ был проведен в семьях с минимцм одним больным мальчиком или у его ближайших родственников*

Материал и методы. *Прямой поиск протяженных делеций гена дистрофина проводился методом мультиплексной ПЦР (МПЦР) у 190 больных мальчиков МДД/Б со специфичными олигопраймерами на 20 экзонов. Косвенная ДНК диагостика проводилась с использованием внутригенных полиморфных сайтов pERT87-8/Tag1, pERT87-15/BamH1, 16intron/Tag1.*

Результаты исследования. *Методом МПЦР в 81,5% случаев в гене DMD у больных были обнаружены протяженные делеции, захватывающие от одного до нескольких соседних экзонов. .Так, чаще всего точка разрыва делеций наблюдается в интроне 44. Высокая частота делеций наблюдается у экзона 48 (14%), экзона*

45 (17%), экзона 47 (10%) и экзона 50 (10%). Из 206 семей высокого рискоа МДД/Б 198 семей были информативны хотябы по одному полиморфному варианту. для проведения косвенной диагностики. Гетерозиготное носительство было установлено у 81 % женщин родственниц и отклонено у 17% (с вероятностью 90-95%). В 2% случае было невозможно установить носительство из-за неинформативности семьи. В шести случаях был установлен диагноз МДД пренатально посредством прямого поиска делеций или методом ПДРФ.

Выводы. *Применение этих двух методов молекулярной генетики позволяет повысить информативность семьи до 94% Установленные различия в частотах делеционных экзонов, анализ нестабильных районов гена дистрофина и сравление полученных результатов с данными исследований проведенных в других странах позволяет говорить о популяционной особенности паттерна делеций в Республике Молдова. Обсуждены межпопуляционные варианты паттерна делеций гена дистрофина.*

Introduction. Duchenne muscular dystrophy (DMD) is a severe X-linked recessive neuromuscular disorder which primarily affects the muscle. DMD is among the most common human genetic disease, occurring approximately once in every 3.500 male births by Emery A.E. in 1987. In contrast to the mild allelic Becker muscular dystrophy (BMD), DMD is progressive and, usually, results in death during the second decade of life. Normally the DMD males are diagnosed at age of about 4 years, based on elevated serum concentration of creatine kinase (CK) and characteristic myopathy upon histological analysis of muscle biopsy. The prognosis for the children is poor with progressive muscle-wasting, resulting in loss of ambulation by 11 years of age. The ensuing muscle-wasting affects all muscle groups and results in respiratory failure by the third decade of life.[3,4]

The first evidence that further defined the location of the DMD gene came in the late 1970's and early 1980's when rare females who were each balanced X-autosome translocation carries described by Boyd et al. in 1986. In each instance, the breakpoint on the human X stick to one tense in the dark Xp21 band of the short arm region of the gene and the entire cDNA was cloned by Monaco in1985; Ray in 1985 and Roema et al. in 1987,1988. Construction of a long-range restriction map of Xp21 implied that the chromosomal mutation of DMD patients was distributed over a locus of 2.3 megabases in size by Iove D.K., Davies K.E., 1989. This exceptionally large gene size (appr. 0.1% of the total genome) could, by itself, account for the high incidence of DMD [5].

Chromosomal clones from the DMD locus identified as conserved exons by interspecies comparison, have been used to screen cDNA libraries [4]. The mRNA transcript of 14 kb encompasses more than 65 exons and contains a single large reading frame of 3685 aminoacids in length corresponding to a protein of 474 kDa, was named dystrophin [3,5]. The majority (65%-70%) of all DMD mutations are partial gene deletions, leading to the severe form of the disease, to the production of a truncated and, presumably, unstable dystrophin molecule by Monaco A. P. in 1989. It is also well established that the distribution of the deletions across the gene region is not random with the two most vulnerable "hot spot" regions found at the 5'-terminus and in the distal half of the central rod domain [6].

The method of choice in Moldova for DMD/BMD diagnosis is multiplex PCR which target about 12 to 17 exons of the DMD gene to look for whole exon deletions [11,12]. Multiiplex PCR is mostly qualitative or quantitative and serves for the exons in the hotsport regions [1,6,7].

We describe here the results of a molecular study in 206 families with DMD/B from Moldova including carrier detection and 20 PD cases since January 1992, following requests of prenatal diagnosis in pregnant women from high risk group for birth child with DMD.

Materials and methods. Since January 1992, 206 families with increased risk of DMD appearance in offspring required medico-genetic counselling at the National Centre of Medical Genetics, Chisinau, Republic of Moldova. Most of these families were Moldavian by origin. Nearly 188 families manifested sporadic cases of DMD/BMD, while genetically proved (multiple affected individuals) DMD/BMD families represented a minor fraction (18 out of 206). All families passed clinical neurological, biochemical (CK-NAC, CK-MB, LDH, AlAt, AsAt) analyses, EMG. The diagnosis and degree of physical disability was put by the criterion suggested by World Association Muscular Dystrophy (WAMDA) [10]. DNA analysis has been suggested to the families with at least one affected child or with an affected close male relatives, especially of the woman at risk of being a DMD/BMD carrier.

Genomic DNA samples were extracted by standard methods from blood cells (Phenol-chloroform extraction) and standard protocol (Flexi Gene DNA Handbook, Quiagen). The chorion villus samples (CVS) were taken by the transabdominal route under ultrasonic guidance at an average gestational age of 10-13 weeks. Transabdominal placentocentesis with concomitant amniocentesis and withdrawal of about 25-30 ml of amniotic fluid was performed between 16-18 weeks

of gestation. Multiplex PCR with DNA extracted from peripheral blood samples, CVS or fresh amniocytes were performed for deletion detection in both post- and prenatal studies. Twenty different exons of DMD gene were tested in patients DNA (3, 6, 4, 8, 9, 13, 17, 19, 42, 43, 44, 45, 47, 48, 49, 50, 51, 52, 53, 60).

Oligo-sequences corresponded to original sets of primers, as introduced by Beggs et al. (1990), Abbs (1991), Ashton EJ(2008) [1,6,17]. The genomic DNA samples were amplified on thermal cycler PHC-1A (London) or/and Eppendorf Mastercycler pro (Germany) with DNA polymerase ("Promega", USA).

Studied polymorphic sites included pERT 87-85/ TagI, 16intron/Tag1 and pERT/87-15 /BamH1. The PCR technique has been applied for pERT 87-85/ TagI, pERT/87-15 /BamH1 with oligoprimer sequences suggested by Roberts et al. (1989) [8].

Then, samples were put to electrophoresis in 5%-7.5% polyacrylamide gel.

Written informed consent for genetic study was obtained from each patient and participant.

Results and discussion. Pedigree details of 190 males, clinically diagnosed as DMD/BMD, tested for DMD gene mutations, were available for all cases, of which 9% had family history of DMD. There was detected a large spectrum of mutations picked up in our study using the methods of mPCR (tab. 1).

Table 1.

Consolidation of all mutations identified in this study

Size of deletion	Indepentent cases (N=155)	Exon deleted	Fragment deleted
1 exon (n=72)	4	DEL EX 3	c94-?_186+?del
	3	DEL EX 6	c.358-?_530+?del
	2	DEL EX 8	c.650-?_831+?del
	2	DEL EX 13	c.1483-?_1602+?del
	2	DEL EX 19	c.2293-?_2380+?del
	9	DEL EX 43	c.6118-?_6290+?del
	4	DEL EX 44	c.6291-?_6438+?del
	12	DEL EX 45	c.6439-?_6614+?del
	5	DEL EX 47	c.6763-?_6912+?del
	18	DEL EX 48	c.6913-?_7098+?del
	4	DEL EX 50	c.7201-?_7309+?del
	2	DEL EX 52	c.7543-?_7660+?del
	5	DEL EX 53	c.7661-?_7872+?del
2 exons (n=17)	1	DEL EX 3-4	c.94-?_264+?del
	2	DEL EX 43-44	c.6188-?_6438+?del
	1	DEL EX 44, 53	c.6291-?_6438+?del& c.7661-?_7872+?del
	7	DEL EX 47-48	c.6763-?_7098+?del
	2	DEL EX 49-50	c.7099-?_7309+?del
	1	DEL EX 50-51	c.7201-?_7542+?del
	3	DEL EX 52-53	c.7543-?_7872+?del
3 exons (n=23)	2	DEL EX 6-8	c.358-?_831+?del
	2	DEL EX 43-45	c.6118-?_6614+?del
	8	DEL EX 45-47	c.6439-?_6912+?del
	5	DEL EX 48-50	c.6913-?_7309+?del
	2	DEL EX 48,50,52	¿
	3	DEL EX 50-52	c.7201-?_7660+?del
	1	DEL EX 51-53	c.7310-?_7542+?del
4 exons (n=12)	1	DEL EX 3-6	c.94-?_530+?del
	3	DEL EX 48-51	c.6913-?_7542+?del
	4	DEL EX 47-50	c.6763-?_7309+?del
	4	DEL EX 45-48	c.6439-?_7098+?del
5 exons (n=6)	1	DEL EX 45-49	c.6439-?_7200+?del
	3	DEL EX 47-51	c.6763-?_7542+?del
	2	DEL EX 48-52	c.6913-?_7660+?del
6 exons (n=10)	1	DEL EX 3-8	c.94-?_831+?del
	2	DEL EX 8-13	c.650-?_1602+?del
	6	DEL EX 45-50	c.6439-?_7309+?del
	1	DEL EX 47-52	c.6763-?_7660+?del

7 exons (n=4)	1 1 2	DEL EX 48-53 DEL EX 13-19 DEL EX 45-51	c.6913-?_7872+?del c.1483-?_2380+?del c.6439-?_7542+?del
9 exons (n=1)	1	DEL EX 44-52	c.6291-?_7660+?del
10 exons (n=1)	1	DEL EX 44-53	c.6291-?_7872+?del
16 exons (n=1)	1	DEL EX 45-60	c.6439-?_9084+?del
17 exons (n=2)	2	DEL EX 3-19	c.94-?_2380+?del
Double deletion (n=6)			
2 exons (n=3)	1 1 1	DEL EX 8&45 DEL EX 19&43 DEL EX 8 &48	c.650-?_831+?del & c.6439-?_6614+?del c.2293-?_2380+?del & c.6118-?_6290+?del c.650-?_831+?del & c.6913-?_7098+?del
3 exons (n=1)	1	DEL EX 43-44 & 51	c.6188-?_6438+?del& c.7310-?_7659+?del
4 exons (n=2)	1 1	DEL EX 3&50-52 DEL EX 43-45 & 50	c94-?_186+?del& c.7201-?_7660+?del c.6118-?_6614+?del& c.7201-?_7309+?del

Deletion detection

mPCR used in this study amplified 2 sets for ten exon-specific sequences in the normal dystrophin gene (Fig 1). Multiplex PCR was able to pick up deletions in 155 cases, which accounted for 81,5 per cent of all cases. The deletions are nonrandomly distributed and occur primarily in the central rod domain (66,5%) and less frequently near the 5' end (15,5%) and 3'end (14,2%) of the gene. In 3,8 % of identified deletions there were complex rearrangements involving two separate regions of the DMD gene (5'& cental, central & 3' and 3'&5')

The commonest breaking point was intron 44. The most frequently deleted were exon 48 (14%), exon 45 - 10,17%, exon 47 - 10% and exon 50 - 10 per cent of all detected deletions (Fig.2)

Southern blot analysis with cDNA probes identifies deletions in about 65% of all DMD patients (Koenig et al., 1988). The multiplex reactions of Chamberlain et al. and Beggs et al. would together detect 94,5% of these deletions (S. Abbs et al., 1991). There were registered exons deletions in only 81,5% of our patients (155 out of 190). Thus, the efficiency of deletion recovery in our study seems to be less than might be expected from other studies (Beggs et al.,1990; Abbs et al., 1991), but correspondent to data from Italian surveys - 78% (Politano L.,1998), French surveys - 68% (Claustres M.,1991), Hungarian surveys 73%, (Herczegfalvi A., 1999), Canadian surveys 73% deletions and 12% duplication (Stockley et al., 2006) [14].

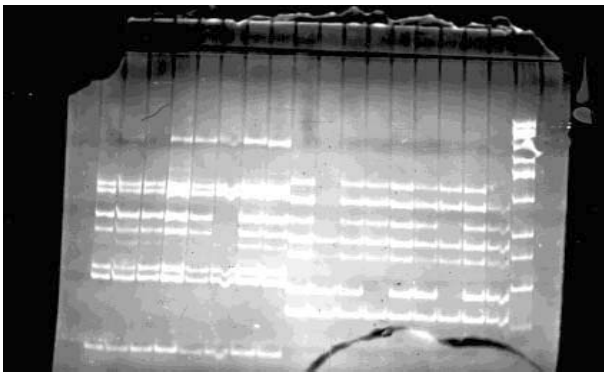

Figure 1. *Analysis of the dystrophin gene by multiplex PCR using DNA from Moldova DMD patients. (The primers sets of Chamberlain et al. and of Beggs et al)*

Moreover, according to present data, the number of deletions in the central region of DMD gene is four times more than in the 5'-end region (103 and 24, respectively). These data differs substantially from the ratio 3.5:1, known from collaborative data (Beggs et al., 1990b; Abbs et al.,1991), are similar to data from Russia (Baranov V.S. et al.,1993) and data from Spanish DMD families research (H. Kruyer et al., 1994).

It should be mentioned that exon 45 deletions account of almost 10,17% of all deletions detected in the present study. This figure higher than the frequency of the same exon deletions reported by Baranov V.S. et al., 1993 and coincide with data from Spanish DMD families research (H. Kruyer et al., 1994). But, in Indian DMD cases the most frequently deleted exons were exon 49 and exon 50 [9].

A significant proportion (3,8%) of deletions extending across both 'hot spot' regions is another unusual finding of this study. All these deletions were encountered in the patients with the most severe forms of DMD. All these types of deletions are described for the first time. We found the same data established double deletion in one patient and double duplications (n=2) in Indian DMD cases [9]. All these samples will be further tested by MLPA (multiplex ligation-dependent probe amplification) for conformation.

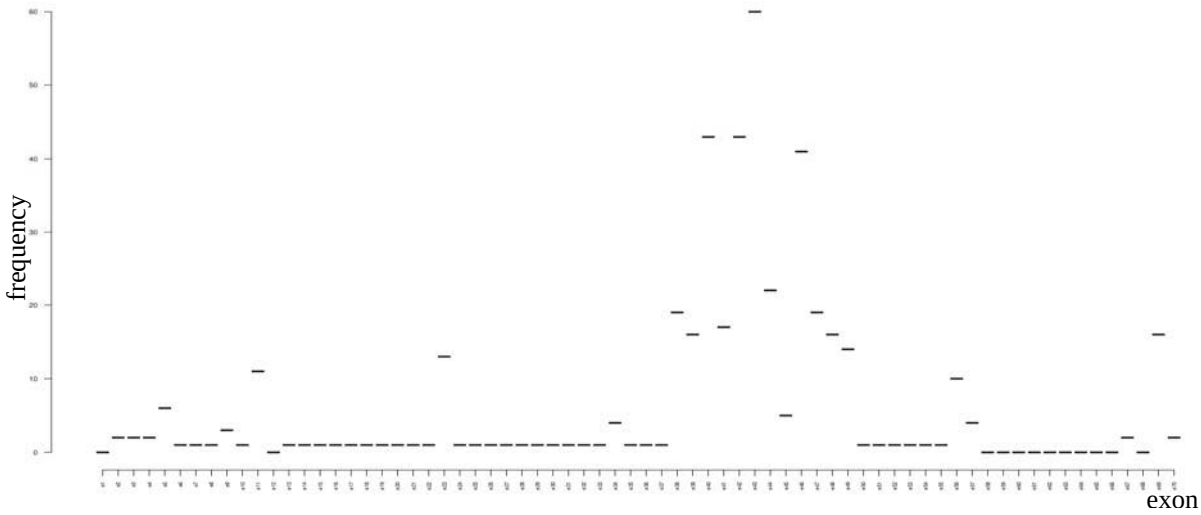

Fig.2 *The frequency of each exon in dystophin gene involved in the deletion process in Moldavian DMD/BMD patients.*

RFLP analysis

No obvious deletions were detected in 35 DMD patients and their families were used for RFLP-analysis (pERT87-8/Tag1, pERT87-15/BamH1 and 16 intron/Tag1). RFLP-analysis was also applied to the deletion group which requested carrier detection. 198 from 206 families referred for carrier detection were found to be informative at least to one polymorphic site for DNA analysis. With a confidence level of about 90-95 per cent, carrier status has been confirmed in 81% female relatives (fig.2a) and rejected in 17% of them. In 2% of females it was impossible to establish carrier status - the families were non-informative.

In two of the families has been performed presymptomatic diagnosis: affected and "normal" younger males relatives had the same haplotype for 16intron/Tag1 site polymorphism.

Thus, using both (MPCR and RFLP) methods in over group affords an opportunity of prenatal diagnose is at 93,8 per cent.

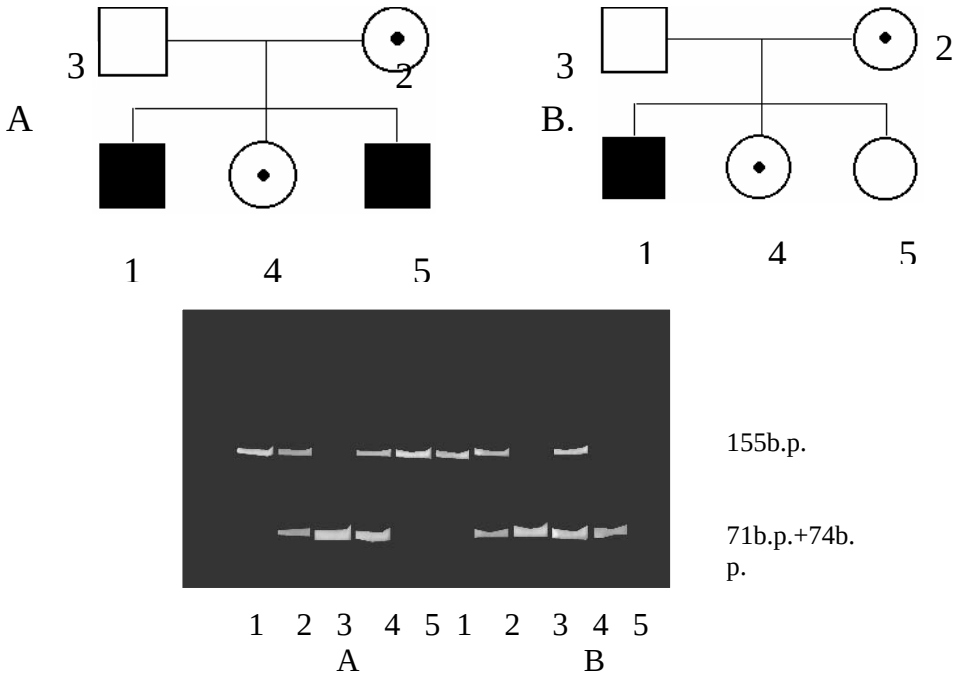

Fig 2. A. Pedigree and RFLP analysis of the polymorphic site pERT87-8/Tag1. Preclinic diagnosis. B. Pedigree and RFLP analysis of the polymorphic site pERT87-8/Tag1.

Prenatal diagnosis

According to the data in Table 2, molecular analysis was efficiently applied to 19 of a total of 20 fetuses of the first or second trimester of gestation, and among them, to 6 of 13 male fetuses at risk of DMD. The absence of a detectable deletion in the affected child and the inability to distinguish two maternal X chromosomes by RFLP analysis made PD impossible in one male fetus at the second trimester of pregnancy, which was soon terminated. Carrier status could be established in four from seven female fetuses (fig 2b). The accuracy of PD in one male fetus informative for a particular deletion should be considered close to 100%. PD in other at-risk male fetus relied exclusively on RFLP analysis of intragenic polymorphic sites and the accuracy of diagnosis was around 95%.

Table 2

Results of prenatal diagnosis of Duchenne muscular Dystrophy

№	Informativeness		Diagnosis		
	MPCR	***RFLP***	***Fetal sex***	***prenatal***	***postnatal***
1.	48-50 ex	pERT87-8/Tag1	46, XY	normal	normal
2.	Not detected	PERT87-8/Tag1, 16intron/ Tag1	46, XX	Non carrier	normal
3.	47-50 ex	PERT87-8/Tag1, 16intron/ Tag1	46,XY	normal	normal
4.	45 ex	PERT87-8/Tag1	46,XY	normal	normal
5.	19,43-48 ex	-	46,XY	affected	TOP
6.	Not detected	PERT87-15/BamH1	46,XX	carrier	normal
7.	48 ex	16 intron/Tag1	46,XY	normal	normal
8.	Not detected	Non-informative	46,XY	Unknown	TOP
9.	Not detected	PERT87-8/Tag1	46,XX	carrier	normal
10.	Not detected	16 intron/Tag1	46,XY	affected	TOP
11	45-50 ex	pERT87-8/Tag1	46, XY	normal	normal
12	Not detected	PERT87-8/Tag1, 16intron/ Tag1	46, XX	Non carrier	normal
13	47-50 ex	PERT87-8/Tag1, 16intron/ Tag1	46,XY	affected	TOP
14	45 ex	PERT87-8/Tag1	46,XY	affected	TOP
15	49-50	PERT87-8/Tag1	46,XY	normal	normal
16	- Not detected	16 intron/Tag1	46,XX	carrier	normal
17	48 ex	16 intron/Tag1	46,XY	affected	TOP
18	Not detected	PERT87-8/Tag1	46,XY	affected	TOP
19	Not detected	16 intron/Tag1	46,XX	Non carrier	normal
20	Not detected	16 intron/Tag1	46,XX	carrier	normal

Note – TOP - *termination of pregnancy*

Deletion detection and carrier testing is a decisive step in elaborating a reliable strategy of prenatal diagnosis in families at high risk of DMD. This straightforward approach is currently applied to all DMD families with living affected probands, referred to our diagnostic centres.

Whether our data should be treated as occasional findings or whether they actually reflects some interpopulational difference regarding the frequency of deletions and their distribution along the dystrophin gene remains obscure and deserves thorough examination. Analysis of the breakpoint distribution pattern in the dystrophin gene showed that, similarly to that observed in several Western European populations, intron 44 was involved most frequently as a starting breakpoint. In Moldavian population intron 50 is the second most frequently observed hot spots at 3'end; these seem to be characteristic for the Moldavian patients.

Tactics, developed by us, for using the methods of molecular genetics (mPCR, RFLP- pERT78-8/Tag1, pERT87-15/BamH1, 16intron/Tag1) allows to determine informative approximately 94% cases and to conduct accordingly clinical, preclinical and prenatal diagnosis.

Thus, in Republic of Moldova, as is apparent in the present study, significant advances in reaching this goal might be achieved through the application of multiplexes for other exons, starting from complete sets of oligoprimers as recently suggested by Beggs et

al. (1990) and Abbs et al. (1991) to the Ashton et al. (2008). Introduction and adaptation of other diagnostic approaches for proper DMD family in Moldova is very necessary for molecular analysis of the dystrophin gene and for higher efficiency of both PD and carrier detection in DMD families.

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