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MOLECULAR GENETIC STUDIES OF THE DISEASES DUCHENNE MUSCULAR DYSTROPHY, PHENYLKETONURIA AND FAMILIAL MEDITERRANEAN FEVER IN THE POPULATION OF THE AZERBAIJAN REPUBLIC

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Annotaion. The genealogical analysis of hereditary diseases of Duchenne muscular dystrophy at the level of the N.A. family tree who are residents of the Balakan region of Azerbaijan Republic has been presented. Two, of the 18 family members studied, had Duchenne muscular dystrophy, and 6 of them were heterozygous carriers of the disease as detected. The total activity of the creatinphosphokinase enzyme in the patients' blood was > 2000 U/L, in the heterozygotes - at the range of 877.6-1271.0 U/L. The enzyme average activity for heterozygotes was 1005 U/L. It is recommended to use the total CPK enzyme activity for the diagnostics of Duchenne muscular dystrophy in heterozygous women. The family with identified inherited metabolic disease of phenylketonuria lives in Masally administrative area. Phenylketonuria gene has an identified R261G (G-A) mutation.

Purpose of the work was to study mutations of MEFV gene of the Familial Mediterranean Fever disease in the population of the Republic of Azerbaijan. For this purpose, a complex of modern molecular-genetic methods based on polymerase-chain reaction has been used.

The 7 mutations of the MEFV gene have been identified: R761H M694I, M694V, V726A, R202Q, M680I and E148Q. Two mutations - E148Q and R202Q were located in exon 2, five mutations - R761H M694I, M694V, V726A, and M680I were found in exon 10. 9 polymorphisms have been identified in the exons 2,3 and 5 of the MEFV gene.

To prevent Familial Mediterranean Fever hereditary disease in the population of the Republic of Azerbaijan, it is planned to carry out the medical-genetic counseling for families with genetic risk with the following prenatal diagnosis of the fetus in the next pregnancy.

Key words: Muscular dystrophy, enzyme, gene, exon, mutation, disease**.**

INTRODUCTION

The Duchenne muscular dystrophy was first described by English anatomy-surgeon Charles Bella in 1830. The disease occurs in approximately one of 4,000 newborn boys [9,11].

Dystrophin gene is the largest of the known genes and consists of at least 4 promoters consisting of 2.6 million nucleotides, 79 exons, and 78 introns. The length of the dystrophin protein, which is a cytoskeletal protein, is 147 kDa. All gene mutations account for approximately 60% starting of a few exons' deletions up to dozens of exons, 30% point mutations, and 10% duplications, 98% of all mutations are discovered. In the promoter portion of the gene and 27 of the first exons, and this part is called the "hot spot" of the gene. Mostly deletions of 48, 49, 50 and 51 exons are observed. The translocations of the dystrophin gene are also found. Dystrophin gene is found in X sex chromosomes (locus Xp21.2), the inheritance type is linked with X- chromosome. Dystrophin gene was cloned for the first time in 1987 [6,10].

In 1985 the gene phenylalanine-4-hydroxilase (PAH) responsible for phenylketonuria disease was identified. The gene is located on the long shoulder of chromosome 12 in q22-24.1 site. The lendth is 90 thousand nb and consists of 13 exons. Synthesized protein consists of 451 amino acid residues. Phenylalanine amino acid coming with food in oxidation process turns into different amino acide -thyrosine as a result of phenylalanine hydroxidation process. In the result of mutation in PAH gene this phenylalanine into thyrosine transformation fails. Up to 1 % cases of phenylketonuria are presented with atypical forms. The disease is inherited as to autosome-recessive type [3].

It is worth mentioning that genetic heterogeneity and biochemical polymorphism for Phenylketonuria and Duchenne muscular dystrophy were not researched. The obtained results will provide early diagnostics, proper and effective treatment of the disease and early prenatal diagnostics. Familial Mediterranean Fever Gene (MEVF) is located in chromosome 16, being precise in 16.13.3. locus. The MEFV gene belongs to the RoRet family of genes and contains 10 exons, which is 10,000 nucleotide sequences long. The synthesized pyridine protein consists of 761 amino acids, though the length of the transcript is 3.7 thousand nucleotide sequences,. The word pyrin is a Greek word for "flame", or marenostrin expressed in mieloid cells means "our sea" in Latin. MEFV gene is located between the genes responsible for the kidney polycystosis and Rubinstein Teybi syndrome. It has autosome-recessive type of heredity. Autosome-dominant hereditary species were also recorded [1].

Out of 177 MEFV gene mutations found, 154 are missense mutations. The most common mutation is M694V, which occurs in 30-67% cases. The disease has a severe clinic and results in amyloidosis [4].

The mutation V726A stays in the second place and occurs in 5-35% of patients. M694V and V726A constitute 75% of all mutations found. The molecular-genetic analysis of these mutant-carrying haplotypes revealed that they belonged to the same ancestor haplotype. In the process of evolution, the ancestor haplotype has been diverted [4,5].

Most mutations occur at the exon 10. Approximately 70% of patients living in the Mediterranean Sea have one of five mutations (M694V, V726A, M694I, M6801, E148Q) [7,8].

The Family Disease Fever was first studied by Raymond in 1948 and was termed "periodic disease" based on periodic recurrence of the disease. Disease usually gives up to 30 years of age. It is a rare frequency hereditary disease [7].

The disease occurs mostly on the Mediterranean coasts and in the Asia Minor communities: the majority in Armenians, Turks, Seafard and Ashkahazi Jews, Arabs, and less in Greeks, Spaniards and Italians. The heterogeneity of the disease among the people living on the Mediterranean Sea is 20%, and the rate of births of homozigous children equals 1: 1000-1: 2500. It is sporadicaly met in other ethnic groups [5].

The following mutations of the MEFV gene have been identified in Turkey: E148Q, R202Q, P369S, F479L, M680GA, M680GC, M694V, M694I, K695R, V726A, A744S and R761H [7].

The diagnostics of the disease: what ethnic group they and their ancestors belong to - is of great diagnostic significance. [5,7].

For the first time in the population of the Republic of Azerbaijan, We for the first time in Azerbaijan put up the goal: to study the molecular-genetic characteristics of the MEFV gene for the Familial Mediterranean Fever disease in ethnic Azerbaijanis.

MATERIALS AND METHODS

The material was collected during field studies in 2017-2018 being in Balakan and Masally regions of Azerbaijan Republic.

Venous blood samples with heparine anticoagulant were used as the study subjects. Blood was sampled from G.M. (proband) family members, who are inhabitants of Tekle village of Masally area.

PKU diagnostics was carried out by means of IFA method. In identification of PKU gene mutations, complex of molecular-genetic methods were used [2].

Genomic DNA was isolated from venous blood, using readymade kits by QIAGEN (Germany) company. Intactness and quantity of isolated genomic DNA were identified by means of electrophoresis in 1.7% agarose gel, as well as gene fragments after

polymerase chain reaction (PCR). Electrophoretic apparatus and power source were BioRad (USA) manufactured. Marker for identification of synthesized DNA fragments was DNA Ladder 100 bp .

The content of PCR: 0,1-1,0 µg of genomic DNA, 0,25 µM of each dNTP, 25 µl buffer (67 mM Tris-HCL, pH 8,8: 16,6 M $(NH_4)_2SO_4$, 0,01% Twin-20, 1,5 unit DNA-polymerase. 2 µg of primers for each of exons 3,5,7,11 and 12.

Regime of PCR for PKU gene was as follows: 95°C-2 minutes, (94°C-45^I, 58°C-45^I, 72°C-45^I 30 cycles), 72°C-7 minutes and pause at 4°C for 10 minutes. PCR was conducted in amplifier – Professional Thermocycler, Biometra, (Germany).

Purification of DNA fragments after the first PCR stage a set of magnets was used: «AgencourtAMPure XP PCR purification» and SPRIPlate 96 Super Magnet Plate. After that purified DNA fragments were used for the further researches. The second PCR was conducted in the regime: 95°C-2 minutes, (95°C-30^I, 52°C-58°C - 30^I, 78°C-2 minutes 30 cycles), 72°C-10 minutes and pause on the amplifier at 4°C for 10 minutes. Then the standard procedure on the apparatus GENOMELabGeXP^{TM} Sequencing for the identification of nucleotide sequence of each DNA fragment was carried out [2].

The total creatine phosphokinase (CPK) and creatine phosphokinase MB (CPK MB) enzymes were identified in the "Beckman" biochemical analyzer manufactured in the US, with a total volume of 2 ml venous blood from each of the 18 family members diagnosed with Duchenne muscular dystrophy. N.A. family tree has been designed as generally accepted [1].

The genome DNA was separated from the 200 µl venous blood. For this purpose, venous blood samples were taken from 18 patients. Patients were between 2.5-8 years old (female-5, male-13). The concentration and intactness of the separated genome DNA was tested in 0.7% agarose gel. The genome DNA was PCR-ed separately for protein-encoding exons of the MEFV gene. Positive PCR samples, that have been got by electrophoresis in the agarose gel, were purified by enzymatic method. Positive Cycle Sequencing PCR samples, got by agarose gel electrophoresis, are purified by BIGDye XT dye remover. The purified gene samples were read by the Automatic DNA sequencing AB13130xI Analysis System. The obtained nucleotide sequences were read out with Seqscape V.2.7. programme, compared to normal MEFV nucleotide sequence by Blast Ce NCBI, and then polymorphisms and relative mutations were identified. [4,5,7].

RESULTS AND DISCUSSION

The world scientific literature researches show that European populations have mainly R408W, P281L, R261Q, R158Q, R252W, I65T, IVS10nt546, IVS12ntl. PAH gene mutations prevail over the others. These mutations are located in 3,5,7,11 and 12 exons of the gene (6,10,11). With this purpose we have done amplification of PAH gene exons 3,5,7,11 and 12 genomic DNA fragments, got from lymphocytes of the G.M. family members: two parents and six their children, by means of polymerase chain reaction with 5 primer groups.

Fragments of exon 3 of 112 nb, exon 5 of 162 nb, exon 7 of 218 nb, exon 11 of 222 nb and exon 12 of 177 nb were amplified.

Total nucleotide sequencing was done only for exon 7 as an example, where R261G mutation was identified. Being a point mutation we have found a substitution of guanine with adenine. The result of mutation was on protein level, and arginine amino acid was substituted with gluthamine amino acid.

Homozygous form was identified in 4-year-old girl (proband – III-6). Heterozygous form carriers were both parents (II-1, II-2) and one sibling (III-2). So, family members manifested one homozygous and three heterozygous forms of R261G mutation.

It's worthwhile noting, that proband's parents are children of two sisters. Marriage is identified as a 3rd cosanguineous parallel marriage type. G.M. family tree is presented in the

Figure 1.

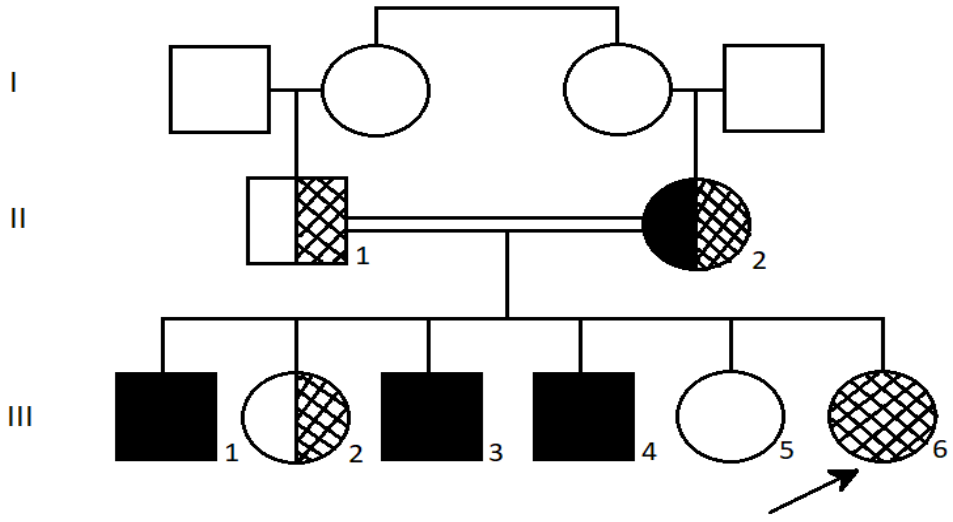

Figure 1. Family tree of G.M. family

Father (II-1) – heterozygous carrier of R261G mutation, mother (II-2) – heterozygous carrier of R261G mutation and G6PD enzyme deficiency, proband (III-6) – homozygote of R261G mutation, siblings (III-1), (III-3) and (III-4) – hemizygotes of G6PD enzyme deficiency, sibling (III-2) – heterozygous carrier of R261G mutation, sibling (III-5) – healthy.

In the Figure 2, the Duchenne muscular dystrophy patient N.A.'s family tree is presented.

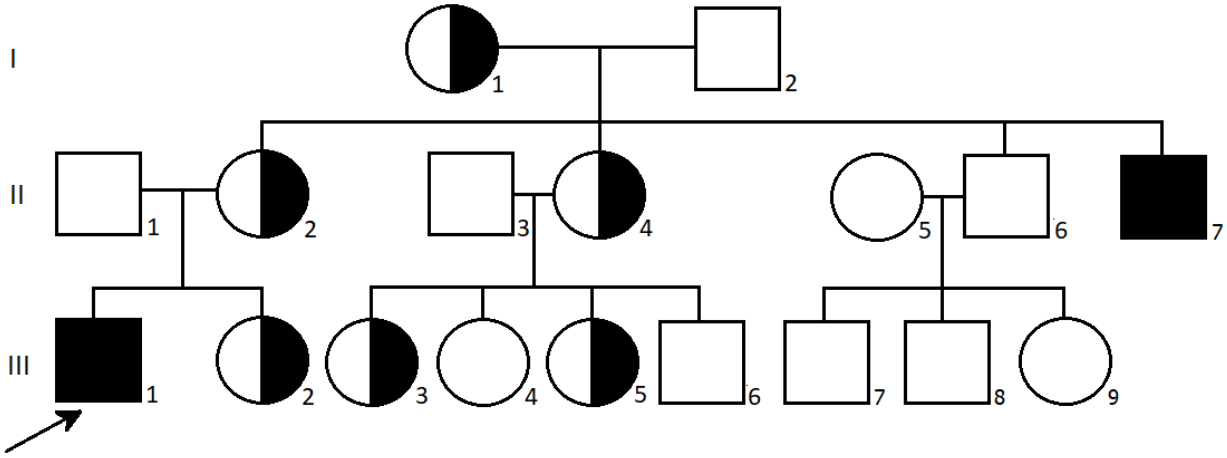

Figure. 2. Duchenne muscular dystrophy patient N.A.'s family tree.

III-1 – proband – Duchenne muscular dystrophy affected person,II-1 – father of the proband, II-2 – proband's mother, III-2 – proband's sister, II-4 proband's aunt, II-6 and II-7 – proband's maternal uncles, III-3, III-4, III-5, III-6, III-7, III-8 and III-9 – proband's second cousins, I-1 – proband's grandfather and I-2 – proband's grandmother.

Since the gene of inherited Duchenne muscular dystrophy has X-linked recessive type, the proband's mother (II-2) is the heterozygous carrier of the disease. The proband's uncle (II-7), manifested Duchenne dystrophy, and thus has been diagnosed. The heterozygous carriage of the disease was found in Proband's sister (III-2), grandmother (I-1), aunt (II-4) and two siblings (III-3, III-5). Results of activity of CPK and CPK MB enzymes in the blood serum of N.A. proband's family members are given in Table 1. In the Proband (III-1) and maternal uncle (II-7), the total CPK and CPK MB enzymes were higher than normal. The total CPK in both cases was higher than 2000 U/L. The amount of CPK MB was also high at 68.3 U/L and 70.8 U/L.Total activity of the CPK in heterozygotes was noted in the range of 877.6-1271.0 U/L. The average activity of the enzyme for heterozygotes was 1005 U/L. The activity of CPK MB enzyme in heterozygotes was in the range of norm in three cases: II-4 (22.8 U/L), III-3 (18.4 U/L), III-5 (23.0 U/L) : I-1 (32.3 U/L), II-3 (33.4 U/L) III-2 (35.2 U/L). It is recommended to use the total CPK enzyme activity in diagnostics of Duchenne muscular dystrophy in heterozygous women. Nearly 50% of boys in women with heterozygocity are ill.

Because of the three heterozygous girls are found in the N.A.'s family carriage of, they are part of the genetic risk group in future when having their own families, and the probability of birth with Duchenne muscular dystrophy kids is 50%. Thus, in families with Duchenne muscular dystrophy for preventive purposes, it is advisable to determine the activity of the total CPK enzyme in girls. If indications are high, prenatal diagnostics of fetus should be performed. First of all, the sex of the fetus should be determined. In the case of a boy, mutation should be sought in the dystrophin gene. As a result of preventive measures, a birth ofan affected child could be prevented.

The molecular-genetic study of the MEFV gene has identified 7 mutations: R761H M694I, M694V, V726A, R202Q, M680I and E148Q. The seven mutations discovered were previously identified in the communities living in the Mediterranean region, mainly in the Turkish population [4]. Three of 18 examined patients were heterozygotes, eight homozygotes, and seven double heterozygotes (compounds).

Table 1 lists the polymorphisms found in the MEFV gene - nucleotide substitutions, frequencies, and the located in exons.

Table 1.

No	Polymorphism	Exon	Number	Frequency (%)
1.	306 T/C (D102D)	2	8	22.22
2.	414 A/G (G138G)	2	4	11.11
3.	442 G/C (E148Q)	2	2	5.56
4.	495 C/A (A165A)	2	4	11.11
5.	605 G/C (R202Q)	2	2	5.56
6.	942 C/T (R314R)	3	6	16.67
7.	1422 G/A (G474G)	5	4	11.11
8.	1428 A/G (G476G)	5	4	11.11
9.	1530 T/C (D510D)	5	2	5.56

As it shown in Table 1, nine polymorphisms were found in three exons of the MEFV gene. Five polymorphisms were observed in exon 2, one polymorphism in exon 3 and three polymorphisms in exon 5.

The polymorphisms of the MEFV gene 414 A/G, 442 G/C, 495 C/A, 1422 G/A and 1428 A/G of the patients with their parents in consanguinous marriages were heterozygous.

High polymorphism rates were for 306 T/C (22.22%) and 942 C/T (16.67%) mutations. The gene frequencies for the polymorphisms of 942 C/T, for 605 G/C and

1530 T/C they were the lowest - 5.56%. The gene frequency of the remaining four polymorphisms was equal 11.11%.

Table 2 lists the mutations causing the disease, gene frequencies, and exons in which the MEFV gene is identified.

Table 2.

MEFV gene mutations, frequencies and exons in Azerbaijan Republic

Mutations	Mutation number	Frequency (%)	Frequency (fraction)	Exon
E148Q	3	9,1	0,0909	2
R202Q	5	15,2	0,1515	2
M680I	4	12,1	0,1212	10
R761H	9	27,3	0,2727	10
M694I	5	15,2	0,1515	10
M694V	1	3,0	0,0303	10
V726A	6	18,2	0,1818	10

The highest frequency of the MEFV gene among the 18 patients was 27.3% for the R761H mutation. mutation V726A (18.2 %) was in the second place, and M694I (15.2%) was in the third place.

Two R202Q and E148Q mutations were found in exon 2 (28.57%) of MEFV gene, and five mutations - M860I, R761H, M694I, M694V and V726A – were reealed in the exon 10 of the gene (71.43%).

R202Q mutation was in two patients, mutation E148Q was heterozygous in one patient, and two patients had compound form (R202Q/E148Q). The homozygous form of the R761H mutation was recorded in two cases, but the same mutation in compound condition (R761H/M694I) was found in four cases with M694I mutation. The M694I mutation was separately met with two mutations - M694V and R202Q (M694I/M694V and M694I/R202Q) as compounds. M680I mutation was found to be homozygous in two patients (M680I / M680I). The mutation of the V726A was identified in three cases to be homozygous. It should be noted that in patients with the homozygous form of mutations parents had consanguinous marriges.

According to world literature, five mutations: M694V, V726A, M694I, R202Q, M6801 and E148Q found up today, constitute 75% of all mutations [5,7]. Five of the seven mutations found in our studies belong to the same group, and constitute 57.6% of total mutations found.

In order to prevent Familial Mediterranean Fever disease, parents of 18 patients have been consulted by geneticist for a healthy child prognosis for the next pregnancy and 25% of the risk of affected child. As the majority of families are in reproductive age, we have got their consents to carry out fetus prenatal diagnosis during their next pregnancies.

CONCLUSION

1. Heterozygous and homozygous genetic types of phenylalanine-4-hydroxylase gene mutation R261G (G-A) were identified.
2. Identification of the CPK enzyme total activity for the diagnostics of Duchenne muscular dystrophy, than the CPK MB enzyme, is more informative
3. In heterozygous women, the activity of the total CPK enzyme was in the range of 877.6-1271.0 U/L. The average activity of the enzyme for heterozygotes is 1005 U/L.
4. For preventive purposes, it is advisable to identify the activity of CPK enzyme in pregnant women from genetic risky families.
5. Results of molecular genetic researches for the MEFV gene in patients with the diagnosis: periodic disease - are presented. Seven mutations of MEFV gene were identified, they are: R761H, M694I, M694V, V726A, R202Q, M680I and E148Q. Two mutations E148Q and R202Q are located in exon 2, and the rest R761H, M694I, M694V, V726A, M680I five mutations in exon 10.

To carry out prophylaxes of periodic disease to families with genetic risk of affected child birth, medical genetic consultation is planned to be conducted with the following prenatal molecular genetic diagnostics of fetus in the first trimester of pregnancy.

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