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TWO NEW MUTATIONS IDENTIFIED IN AZERBAIJAN: THE GENE BCKDHB 508 (C-T) AND THE GENE DBT 1199 (A-G) IN A HOMOZYGOUS STATE IN TWO FAMILIES WITH MSUD DIAGNOSIS

Abstract. *A family case of inherited disease – maple syrup urine disease – was identified and was accompanied with three amino acids: valine, leucine and isoleucine metabolism disorder. A new previously unknown in Azerbaijan homozygous mutation of 508 (C-T) for BCKDHB gene was identified in two kids of both genders. Presence of three neutral genetic polymorphisms was identified in BCKDHA gene: 972 (C-T), 59 (C-T) and 1221 (A-G), all heterozygous.In the second family, homozygous mutation of the DBT gene in position 1199 with the adenine nucleotide substitution with guanine nucleotide was identified. Taking into account presence of the said disease in the population, the ways of prophylaxis are being discussed as medical-genetic consultancy with the following prenatal diagnostics and disease mass screening in newborns in Azerbaijan Republic.*

Keywords: *inherited metabolic disorder, maple syrup metabolic disorder, polymerase-chain reaction, gene, mutation, amino acid, neutral genetic polymorphisms.*

Introduction. Maple syrup urine disease (MSUD) is a complicated disease that is inherited one. The maple syrup urine disease is accompanied with full or partial disorder of enzyme activity, participating in the metabolism of three amino acids as valine, leucine and isoleucine. If the process of valine, leucine and isoleucine metabolism is interrupted, then stockpiling and decay happens in the body. Decay products of those amino acids are evacuated from the body and are toxic. These toxins relate to biogenic amines – ptomaine.

Maple syrup urine disease is a genetic heterogenic disease which relates to deficiency of keto acids dehydrogenase enzyme complex (BCKAD). Four subunits are in the (E1a, E1b, E2 and E3) are in the content of BCKAD. Mutations in three genes coding those proteins lead to accumulation of organic keto acids in biological liquids and tissues. Gene, which codes E1a subunit BCKDHA, is mapped on the long shoulder of 19 chromosome in 19q13.1-q13.2 position; E1b subunit BCKDHA is mapped on the chromosome 6 short shoulder in position of 6q14; E2 DBT is mapped on the chromosome 1short shoulder in the position of 1p31; E3 DLD is mapped on the chromosome 7 short shoulder in the position of 7q31-q33. Mutation in the E3 DLD gene leads to clinic form which is similar to Lee syndrome [4, 3, 7].

It is known more than 50 mutations of those genes. Frequency for homozygotes in world populations is 1:120000-1:290000, for heterozygotes is 1 for 100-400 newborns. In some isolates frequency of homozygotes is high and comes up to 1:176 newborns. Disease has autosome-recessive type of heritance. An affected child is born in practically sound parents [3,6,8,10].

Objective: Thus, the goal of our researches is molecular genetic research of three affected kids with the disease of maple syrup urine disease in two Baku families.

Materials and methods. Material for the research was venous blood of two kids from the same family A.A. and one patient from T.E.

familytaken in amount of 2ml with anticogulant – heparin. Mutation identification was carried out by means of molecular genetic methods complex.

Genomic DNA was isolated from venous blood, using ready kits made by QIAGEN (Germany). Intactness and quantity of isolated genomic DNA as well as gene fragments after polymerase chain reaction (PCR) were identified by means of electrophoresis in 1.7% agarose gel. Electrophoretic apparatus and power source were from BioRad (USA). Marker for identification of synthesized DNA fragments was DNA Ladder 100 bp[2].

Regime of PCR for BCKDHA, BCKDHBand DBT genes was as follows: 95°C-2 minutes, (95°C-30^{I}, 58°C-30^{I}, 78°C-2 minutes 25 cycles), 72°C-10 minutes and pause at 4°C for 10 minutes, and PCR regime for GAL1 - 95°C-2 minutes, (95°C-30^{I}, 60°C-30^{I}, 76°C-2 minutes 30 cycles), 72°C-10 minutes and pause at 4°C for 10 minutes. PCR was conducted in amplifier – Professional Thermocycler, Biometra, (Germany). Two primers (Forward и Reverse) were used to amplify each BCKDHA gene site (exon 9) and BCKDHB (exon 10).

Table 1

Nucleotide sequences of primers used in stuctural analysis of BCKHDA and BCKHDB genes

Names of primers	Nucleotide primer sequences
1. Sequence- BCKHDA R1	5^{I}-TGA TTC CAT AAACCTTCC ATA-3^{I}
1. Sequence- BCKHDA F1	5^{I}-TAA CAT CCG ACT GAG ATG GTTACA-3^{I}
2.Sequence- BCKHDA F2	5^{I}-GGA ATA GAT CGT AATTGG TAT-3^{I}
2. Sequence- BCKHDA R2	5^{I}-CTA CAG TTA ACA TAG AGG AAT-3^{I}
3. Sequence- BCKHDA F3	5^{I}-CAT AAT CCA TTC AAC TGT TAA-3^{I}
3. Sequence- BCKHDA R3	5^{I}-ACA TAG TCG TGT CGA GTC CAGTAA-3^{I}
4. Sequence- BCKHDA F4	5^{I}-TTC TGGTAA GTA CTT AGAGGA-3^{I}
4. Sequence- BCKHDA R4	5^{I}-GGA TAG ACA AGA GAT GCTGGA-3^{I}
5. Sequence- BCKHDB F1	5^{I}-GGG TCA AAT GTA TAG GGC CAC-3^{I}
5. Sequence- BCKHDB R1	5^{I}-TCG TTT GCG AGT ATAGCA TAT-3^{I}
6. Sequence- BCKHDB F2	5^{I}-ACTGCA CTT CTC TTC ATC CAC CTG-3^{I}
6. Sequence- BCKHDB- R2	5^{I}-TCA AGG TTG GCGATG ATC TAA TGT-3^{I}
7. Sequence- BCKHDB- F3	5^{I}-AGA TAG TCA TGA GAA GCTGGT-3^{I}
7. Sequence- BCKHDB- R3	5^{I}-TTA ACA GAT CTT GATTGG TAG-3^{I}
8. Sequence- BCKHDB- F4	5^{I}-CCAATTTCG AGT ATCGCGTAA-3^{I}
9. Sequence- BCKHDB- R4	5^{I}-CCTGCG CTA CTT GTC GTC CAC CTA-3^{I}

Nucleotide sequences of primers used in stuctural analysis of BCKHDA and BCKHDBgenes are presented in the Table 1.

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 onthe 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.

Results. Family A.A. has three kids: the second child A.M. is a three-year-old girl affected since her birth, another kid A.T. is a newborn boy also affected since his birth. Both kids were born on time with normal weight and height. However, after the first 24 hours of birth the newborn started to have problems related with gastro-intestinal tract. Nonspecific scent in the urine in the newborn, reminding us of maple syrup scent, jogged our memory to the presence of a disease accompanied with metabolic disorder of amino acids: valine, leucine and isoleucine, i.e. maple syrup urine disease. The same case was with the elder child. The urine of the patient had positive reaction to 2.4-dinitrophenylhydrosine that witnessed of presence of maple syrup urine disease.

Maple syrup urine disease gene identification results in position 508 in BCKDHB gene have shown the substitution of cytosine nucleotide by

thymine nucleotide in homozygous state. The given mutation previously was known as one of the pathologic alleles' mutation leading to maple syrup urine disease [7-8].

BCKDHA gene research has revealed three mutations: 1. substitution of cytosine nucleotide by thymine nucleotide in position 59 (59 C-T); 2. substitution of cytosine nucleotide by thymine nucleotide in position 972 (972 C-T); 3. substitution of adenine nucleotide by guanine nucleotide in position 1221 (1221 A-G). All abovementioned mutations were heterozygous. According to literature all three mutations do not cause pathology, in other words they relate to neutral mutations [5, 11].

Hence, BCKDHB gene homozygous mutation 508 (C-T) in patient A.A. has led to the disease named as maple syrup urine disease.

Thus, inherited metabolic amino acids disease – maple syrup urine disease - was found in two members of one family. Identified mutation of BCKDHB gene was homozygous one. For the first time presence of three neutral genetic polymorphisms in heterozygous state was identified in BCKDHA gene: 972 (C-T), 59 (C-T) и 1221 (A-G).

Results of amino acids quantitative identification in A.A.иT.E. patient's urine and blood are presented in Tables 2 and 3.

As seen in Table 2, patient A.A. with homozygous mutation BCKDHB 508 (C-T) demonstrated increase of amino acids as valine, isoleucine and leucine in patient's urine is observed whereas valine is as high as 511.16 mkmol/gKre with norm as 99.00-316.00 mkmol/gKre, isoleucine is 388.95 mkmol/gKre with norm being between 38.00 and 312.00 mkmol/gKre, and leucine – 2155.33 mkmol/gKre with norm between 70.00 and 570.00 mkmol/gKre.

Table 2

Results of amino acid quantitative identification in A.A.and T.E.patients' urine

Amino acids	Quantity		
	Results		Norm
	A.A. family	T.E. family	
Ornithine	76.45 mkmol/gKre	26,15 mkmol/gKre	55.00-164.00 mkmol/gKre
Cysteine	71.13 mkmol/gKre	70,10 mkmol/gKre	68.00-710.00 mkmol/gKre
Lysine	200.10 mkmol/gKre	24,90 mkmol/gKre	189.00-850.00 mkmol/gKre
Tyrosine	388.75 mkmol/gKre	70,76 mkmol/gKre	333.00- 1550.00 mkmol/gKre
Methionine	205.86 mkmol/gKre	20,66 mkmol/gKre	174.00-1690.00 mkmol/gKre
Valine	511.16 mkmol/gKre	498,66 mkmol/gKre	99.00-316.00 mkmol/gKre
Isoleucine	388.95 mkmol/gKre	395,97 mkmol/gKre	175,06-1340,0 mkmol/gKre
Allo-isoleucine	105.08 mkmol/gKre	153,04 mkmol/gKre	0.00-29.00 mkmol/gKre
Leucine	2155.33 mkmol/gKre	2032,98 mkmol/gKre	70.00-570.00 mkmol/gKre
Phenylalanin	241.35 mkmol/gKre	264,35 mkmol/gKre	175.60-1340.00 mkmol/gKre
Tryptophan	60.14 mkmol/gKre	40,51 mkmol/gKre	0.00-93.00 mkmol/gKre

The patient with DBT 1199(A-G) mutation from T.E. family all amino acids' quantities in urine differed from the norm except cysteine, phenylalanine and tryptophan. Valine, leucineand isoleucine amino acids in his urine were below the norm: valine - 498,66 mkmol/gKre (normally 99,00-316,00 mkmol/gKre), isoleucine - 395,97 mkmol/gKre (normally 3800 -31200 mkmol/gKre) and leucine - 2032,98 mkmol/gKre (normally 70,00 - 570,00 mkmol/gKre).

In patients' urine samples with two different mutations, valine, leucine and isoleucine quantities were almost similar. Valin amino acid for the patient A.A. was 511,16 mkmol/gKre, for patient T.E was 498,66 mkmol/gKre; leucine amino acid for A.A. patient manifested as 2155,33 mkmol/gKre, and for T.E. patient – 2032,98 mkmol/gKre; and isoleucine amino acid for A.A. patientwas identified as much as 388,95 mkmol/gKre,for T.E. patient – 395.97 mkmol/gKre.

In patient's blood we have found an increase of amino acids quantity as valine, leucine, isoleucine and allo-isoleucine: valine is as high as 917.76 mkmol/L with norm as 64.00 – 296.00 mkmol/L, isoleucine is 731.03 mkmol/L with norm being

between 31.00 and 81.20 mkmol/L, and leucine – 3782.02 mkmol/L (norm between 47.00 and 150.00 mkmol/L, and allo-isoleucine - 30.22mkmol/L where norm is between 23.00 and71 mkmol/L.

Quantitites of amino acids as valine, leucine and isoleucine in patient A.A. were higher and differed as follows:917,76 mkmol/gKre (in T.E - 808,55 mkmol/gKre), 731,03 mkmol/gKre (in T.E. - 282,05 mkmol/gKre) and 3782,02 mkmol/gKre (in T.E – 276.10 mkmol/gKre).

Table 3

Results of amino acids quantitative identification in blood for patients A.Aand T.E.

Amino acids	Quantity		
	Results		Norm
	family A.A.	family T.Э.	
Cysteine	22.45 mkmol/	26,33 mkmol/L	16.00-87.00 mkmol/L
Lysine	64.18 mkmol/L	58,09 mkmol/L	52.00-90.00 mkmol/L
Tyrosine	46.84 mkmol/L	49,54 mkmol/L	22.00-105.00 mkmol/L
Methionine	16.57 kmol/L	10,67 mkmol/L	9.00-40.00 mkmol/L
Valine	917.76 kmol/l	808,55 mkmol/l	64.00-296.00 mkmol/l
Isoleucine	731.03 mkmol/L	276,10 mkmol/L	31.00-81.20 mkmol/L
Allo-isoleucine	370.12 mkmol/L	378,02 mkmol/L	0.00-290.00 mkmol/L
Leucine	3782.02 mkmol/L	282,05 mkmol/L	47.00-150.00 mkmol/L
Phenylalanine	51.96 mkmol/L	56,26 mkmol/L	31.00-75.00 mkmol/L
Tryptophan	30.22 mkmol/L	16,29 mkmol/L	23.00-71.00 mkmol/L

Discussion. Thus, having target to diagnose maple syrup urine disease we have made a quantitative study of amino acids in urine and blood of the patient. The levels of valine, leucine and isoleucine amino acids specific for the maple syrup urine disease were significantly increased in urine as well as in blood.

Taking into account presence of the given disease in the population, the ways of their prophylaxis as medical genetic consultancy of families with genetic risk of affected newborn birth with following prenatal diagnostics and disease mass screening among newborns in Azerbaijan Republic are being discussed.

Conclusions. 1. By means of molecular genetic diagnostic methods, three members of two families have revealed a disease which is related with amino acids' metabolic breach - maple syrup urine disease.

2. Two new mutations were identified:BCKDHB gene homozygous mutation in position 508 (C-T)and mutation 1199 (A-G) of DBT gene in homozygous statewas identified, and that was the cause of maple syrup urine disease.

3. Forthe first time three neutral polymorphisms: 972 (C-T), 59 (C-T) and 1221 (A-G) of BCKDHA gene in heterozygous state were revealed.

4.Different quantities of valine, leisine, isoleisine amino acids were identified in patients' urine and blood serum.

5. It was shown the importance of newborns prophylactic screening program development and its implementation by means of biochemical and molecular genetic methods to discover and confirm the disease called Maple Syrup Urine Disease in Azerbaijan Republic population.

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