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Territorial and ethnic distribution of mutant alleles of BCKDHB and DBT genes of Azerbaijani patients

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Abstract

Maple Syrup Urine Disease (MSUD) is an inherited metabolic disorder caused by mutations in the genes BCKDHA, BCKDHB, DLD, and DBT, which are involved in the breakdown of branched-chain amino acids (BCAAs). The research aimed to characterize these mutations among MSUD patients from different regions and ethnic groups within Azerbaijan. Polymerase Chain Reaction (PCR) was used to amplify the genes BCKDHA, BCKDHB, DLD, and DBT. Blood samples were collected from 940 patients diagnosed with MSUD, and exome sequencing along with Sanger sequencing were employed for detailed analysis. The study involved patients from various regions of Azerbaijan, including Sheki-Zagatala, Guba-Khachmaz, Lankaran-Astara, and Baku. These areas have diverse ethnic groups, with the study specifically noting patients from the Azerbaijani Turk and Lezgi ethnic groups in the Guba-Khachmaz zone. DBT gene: The 1199A>G mutation was found in a homozygous form in one patient. BCKDHB gene: The 508C>T mutation was found in a homozygous form in a second patient. BCKDHB gene: In a third patient, the 972C>T (exon 9) and 1221A>G (exon 10) mutations were found in a compound heterozygous form. The 508C>T mutation (BCKDHB gene) caused a Lys-Gly change at position 332 in the corresponding polypeptide. The 972C>T and 1221A>G mutations in the BCKDHB gene caused changes at positions 673 and 947, resulting in Arg673Ser and Phe947Gly substitutions. The 1199A>G mutation in the DBT gene caused a Lys508Gln change at the corresponding position. Using Swiss modeling software, the protein structures were analyzed. The 508(C>T) mutation affected the protein helix structure. The Arg673Ser and Phe947Gly mutations were observed in the protein coils. The Lys907Gly mutation resulted in an extension of the β-sheet structure. The study highlights the importance of genetic testing for MSUD, especially in regions with diverse ethnic populations like Azerbaijan. The novel mutations discovered provide essential information for genetic counseling, diagnosis, and treatment of MSUD. Furthermore, the territorial and ethnic distribution of these mutations offers valuable insights into molecular diagnostics and understanding the genetic diversity of the disease in Azerbaijani populations. The findings underline the critical role of identifying genetic mutations in MSUD, facilitating early diagnosis and personalized treatments. Additionally, the study demonstrates the significance of regional and ethnic variations in genetic diseases, which can inform better-targeted healthcare strategies for diverse populations.

Keywords: Heterogenic disease, MSUD, Mutant alleles, Novel mutation, Protein, Territorial and ethnic distribution.

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1. Introduction

MSUD is a metabolic disorder. In normal human metabolism, the 22 amino acids from which proteins are produced are very important to body functions and develop the structure of the body organs and muscles. MSUD is due to a lack of an enzyme in cells. In cells, a cell enzyme combination breaks down the three amino acids into their individual chemical parts. In people who have the disease, the enzyme does not work. The chemical parts of the amino acids build up and harm the tissues of the body, particularly the brain. This can cause problems with body functions [1-3]. Disease is transmitted by autosomal recessive inheritance [4].

MSUD is caused by mutations in the BCKDHA, BCKDHB, DLD, and DBT genes. The BCKDHA gene is located at the 19q13.1-q13.2 locus, the BCKDHB gene is located at the 6q14 locus, the DBT gene is located at the 1p31 locus, and the DLD gene is located at the 7q31-q33 locus. Mutation in BCKDHA (branched-chain keto acid dehydrogenase E1, alpha polypeptide), BCKDHB (branched-chain keto acid dehydrogenase E1, beta polypeptide), DLD (dihydrolipoamide dehydrogenase), DBT (dihydrolipoamide branched-chain trans acylase) genes disrupts the metabolism of leucine, isoleucine and valine amino acids, causing accumulation of these amino acids in the organism. The BCKDHA gene is located at the 19q13.1-q13.2 locus, the BCKDHB gene is located at the 6q14 locus, the DBT gene is located at the 1p31 locus, and the DLD gene is located at the 7q31-q33 locus. Excessive accumulation of these amino acids in the body has a toxic effect on organs and tissues [5, 6].

Mutations in the BCKDHB and DBT genes can significantly affect protein structure and function, leading to metabolic disorders, particularly Maple Syrup Urine Disease (MSUD), which is caused by defects in the branched-chain amino acid (BCAA) degradation pathway. The BCKDHB gene encodes the E1β subunit of the branched-chain alpha-keto acid dehydrogenase complex (BCKDC), which plays a crucial role in the catabolism of the branched-chain amino acids: leucine, isoleucine, and valine. Mutations caused alterations in the amino acid sequence of the E1β subunit, which can impact the protein's folding, stability, and interactions within the enzyme complex. These structural changes can impair the BCKDC's ability to decarboxylate the branched-chain alpha-keto acids, resulting in a buildup of toxic intermediates (such as leucine, isoleucine, and valine) and an inability to properly metabolize BCAAs. As a result, the activity of the BCKDC complex is reduced, leading to disruptions in amino acid metabolism and contributing to the symptoms of MSUD, such as neurological damage, developmental delay, and in severe cases, death if untreated. The DBT gene encodes the dihydrolipoamide branched-chain transacylase (E2 subunit), another essential component of the BCKDC. The E2 subunit is responsible for transferring the acyl group from the branched-chain alpha-keto acids to the lipoamide group of the enzyme. Mutations in the DBT gene can affect the protein's ability to form proper protein-protein interactions within the BCKDC. This could impair the E2 subunit's role in transferring the acyl group, destabilizing the entire complex. The E2 subunit's role is critical for the overall function of the BCKDC complex. When the E2 subunit is malfunctioning due to mutations, it disrupts the whole enzymatic pathway, leading to the accumulation of branched-chain amino acids and their toxic byproducts. Mutations may cause misfolding or destabilization of the E2 subunit, which can lead to decreased BCKDC activity, exacerbating the metabolic imbalance seen in MSUD. Both BCKDHB and DBT mutations lead to impaired BCKDC function, a crucial enzyme complex for BCAA metabolism [7-9].

The mutations typically cause protein misfolding, reduced stability, or impaired enzyme activity, resulting in the accumulation of branched-chain amino acids and their corresponding keto acids. This dysfunction leads to the clinical manifestations of Maple Syrup Urine Disease, including neurological and developmental symptoms due to the buildup of toxic metabolites in the blood and brain. Treatment for MSUD typically involves a strict diet low in BCAAs to manage the accumulation of toxic compounds, but the underlying issue of enzyme dysfunction remains tied to the genetic mutations in BCKDHB or DBT. Mutations in these genes impair the function of key enzyme subunits in the BCKDC, leading to a buildup of toxic metabolites that disrupt normal brain function and metabolism [10-13].

190 different mutations have been identified in these genes. These mutations should be detected early and treated immediately. Otherwise, the disturbance of amino acid metabolism leads to the aggravation of the patient's condition and, in turn, may result in death [14].

Symptoms of MSUD appear immediately after birth. Physical examination may reveal dystonia, hepatomegaly, and motor-postural delay. Early molecular-genetic diagnosis and medical intervention are important for these patients. These important revelations are significant contributions that have led to an understanding of MSUD, with many families and children now profiting from insight [15-18].

2. Material and Methods

2.1. Materials

Nine hundred forty people involved in the study were from different zones of Azerbaijan (Baku, Sheki-Zagatala, Guba-Khachmaz, Lankaran-Astara). Blood samples from 940 patients diagnosed with MSUD were analyzed using biochemical and molecular genetic methods. Patients from whom these samples were obtained agreed to participate in the study. Data such as age, gender, and family history of patients were collected.

2.2. Biochemical Analysis

Biochemical analysis was performed to determine the amounts of valine, leucine, and isoleucine amino acids in blood and urine. Liquid chromatography was used to conduct the biochemical analysis. A Thermo Scientific LTQ XL (Massachusetts, US) LC-MS with a Dionex Ultimate 3000 liquid chromatograph interfaced with a linear ion trap analyzer was utilized. MS/MS and chromatographic method development were performed using Thermo Scientific XCalibur and Thermo Scientific Chromeleon software.

2.3. DNA Extraction

2 ml of blood was taken from the patients, and leukocytes were obtained through centrifugation. 200 µl of QIAamp genomic DNA and RNA kit (QIAGEN, Germany), buffer solution, 200 µl of venous blood, and 20 µl of protease enzyme (QIAGEN) were used to isolate DNA from leukocytes.

2.4. PCR of BCKDHA, BCKDHB, DLD, DBT genes

The PCR method was used to amplify all four studied genes. PCR was performed in the "T100TM Thermal BIO-RAD" (Germany) amplifier. A reaction mixture of 25 µL consisted of 0.05 U/µL Taq polymerase and PCR reaction buffer (Taq PCR Core Kit, Qiagen), 2 mM MgCl2, 0.25 mM dNTP, and 0.10 µM of each primer. A pair of Forward (F) and Reverse (R) primers was used for each genome fragment. The PCR conditions were set as follows: one cycle of 94°C for 5 min; 35 cycles of 94°C for 45 s, 56°C for 45 s, 72°C for 45 s, and one cycle of 72°C for 7 min [19].

2.5. Electrophoresis

Positive PCR samples were checked by electrophoresis on 1.5% agarose gel. For this purpose, Power PacBasic Gel DocIM EZ (BioRad, USA) electrophoresis and Lambda DNA Mixed Digest marker were used. DNA fragments were stained in an aqueous solution of ethidium bromide (Figure 1).

2.6. DNA Sequencing

The purified product was preceded to the exome sequencing and Sanger analysis. The exome sequencing was carried out using a HiSeq2500 sequencer. Variations were screened with indicators Sift_Pred=Damaging. The sequence of DNA fragments was studied on "Applied Biosystems (Hitachi) 3130xl Genetic Analyzer Sequencing" sequencer. The data obtained in Sanger sequencing were compared with the reference gene using the "SeqScape TM" program

2.7. In Silico Modeling

For simulating mutations in studied genes, the following steps in silico modeling were followed: the sequences for these genes were obtained from the NCBI databases. We used the ClinVar and HGMD databases for the identification of specific mutations. For restoring protein structure, experimental structures from the PDB (Protein Data Bank) were used. To introduce mutations into the 3D structure, the software PyMOL was utilized. To predict the effect of mutations, we used PolyPhen-2. To assess how mutations affect protein stability, we employed FoldX. For simulating interactions of the mutant protein with cofactors or substrates, the AutoDock program was used. To simulate the behavior of wild-type and mutant proteins, we utilized GROMACS molecular dynamics and analyzed stability, flexibility, and conformational changes. Mutations and structural effects were visualized with PyMOL, and we used the mutation modeling Missense3D databases for automated structural impact predictions. We used KBase to study how mutations in the BCKDH complex genes affect protein structure.

2.8. Statistical Analysis

Pearson Chi-square (X^2) Test and Fisher's Exact Probability Method were applied during the statistical analysis of the study results. Both tests provide a p-value to assess the significance of the results. $P \leq 0.05$ generally indicates that there is a significant association between the variables

3. Results

3.1. MSUD Patient Distribution

In a 4-year-old girl from the Khachmaz region of Azerbaijan, a homozygous mutation resulting from the replacement of adenine with guanine at position 1199 of the DBT gene was detected by molecular genetic methods. Among 1199 patients was a three-year-old girl who was the second child in the family and a newborn boy who was the third child in the family

from the Khinalig village of Guba. In newborns, the disease manifested with characteristic symptoms from the day of birth. There is no difference between the number of samples by gender and age.

3.2. Result of Biochemical Research

The urine and blood levels of valine, leucine, and isoleucine amino acids were higher than normal in patients diagnosed with leucinosis. In 1 out of 3 patients, valine-result 498.66 μmol/gKre (norm 99-316 μmol/gKre), isoleucine-result 395.97 μmol/gKre (norm 175.06-1340.0 μmol/gKre) and leucine-result 2032.98 μmol/gKre (norm 7000 57000 μmol/gKre), in 2 patients, valine 498.66-511.16 μmol/gKre, isoleucine 395.97-388.95 μmol/gKre and leucine result 2155.33-2232.90 μmol/gKre were recorded in the urine.

In the blood of 1 patient, the valine content was 808.55 μmol/L (normal 164.00-296.00 μmol/L), the isoleucine content was 636.13 μmol/L (normal 31.00-81.20 μmol/L), and the leucine content was 3782.02 μmol/L (normal 47.00-150.00 μmol/L). In the blood of 2 patients, the valine content was 728.50-917.76 μmol/L, the isoleucine content was 646.00-731.03 μmol/L, and the leucine content was 3782.02 μmol/L. As a result of biochemical research, the amount of valine, isoleucine and leucine amino acids in the blood and urine of the newborn showed the necessity of conducting a molecular-genetic analysis complex.

3.3. İdentification of Novel Mutations

Sequences were amplified for each four genes. A mutation was detected in position 508 of the BCKDHB gene in the sick child. By molecular genetic methods in a newborn was observed replacement of cytosine by thymine at position 972 and a replacement of adenine by guanine at position 1221 of the BCKDHB gene (Figure 1).

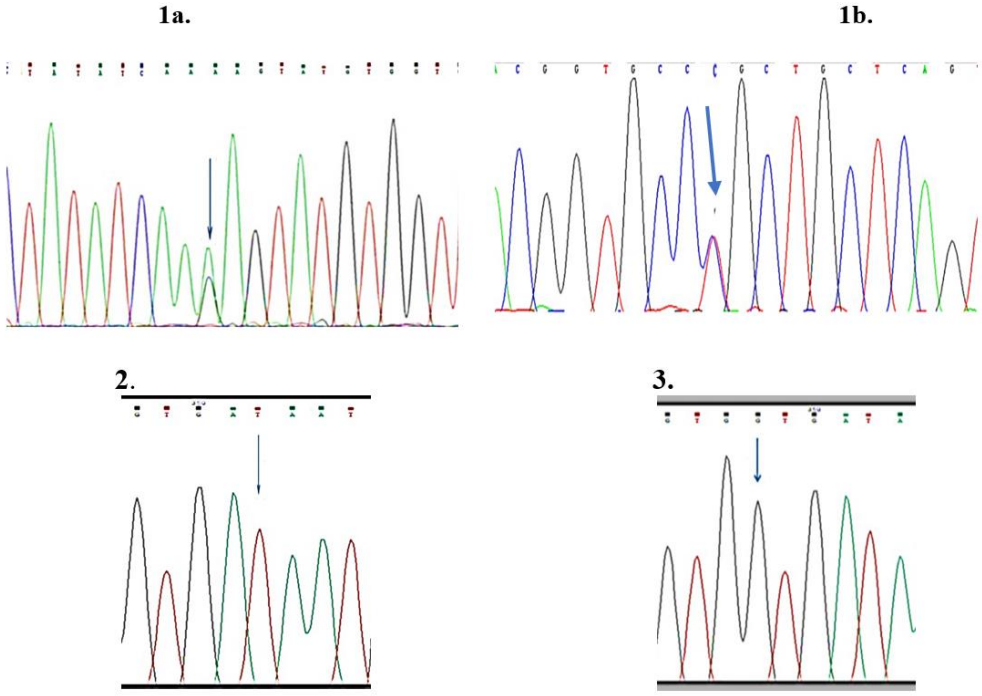

Figure 1.
Electropherogram of changes in the nucleotide sequence of *BCKDHA* and *BCKDHB* genes: 1-Heterozygous compound form of *BCKDHB* gene: a -1221(A>G) mutation in exon 10; b-972 (C>T) mutation in exon 9; 2-Homozygous form of 508(C>T) mutation in exon 2 of *BCKDHB* gene; 3 - Homozygous form of 1199(A>G) mutation in exon 10 of *DBT* gene.

Out of 800 experimental samples included in the study, leucine-causing mutations were found in 3 patients. Each of these mutations are novel mutation. None of the 140 control samples included in the study had any mutations in the studied genes.

3.4. Territorial and Ethnic Distribution of Mutant Alleles of BCKDHB and DBT Genes

As a result of molecular genetic research, information was obtained about the territorial and ethnic distribution of mutant alleles of the BCKDHB and DBT genes in Azerbaijan. The disease was detected in patients living in the northern region of Azerbaijan. Thus, 2 out of 3 patients are from Khinalig village in Guba, and one is from Khachmaz district.

To perform a Chi-Square test to check if there is a significant difference in the distribution of MSUD patients between Khinalig village and others, we broke it down as follows: A total number of MSUD patients is 940, 2 out of 3 MSUD patients

are from Khinalig village, so the proportion of patients from Khinalig is 2/3. The expected number of patients from Khinalig is 626. We used the formula for the Chi-Square test: $\chi^2 = \sum_{i=1}^{k} \frac{\left(m_i - m_i^{'}\right)^2}{m_i^{'}}$

The result for Khinalig was 0,00072, the total Chi-Square Value was 0,00215. Degrees of freedom (df) was 1. We used the Chi-Square distribution table or a statistical software to find the P-value associated with a Chi-Square value of 0,00215 and 1 degree of freedom. The P-value is extremely high, indicating that the difference between observed and expected values is not statistically significant.

Ethnic specificity was determined in the frequency and spectrum of studied mutations in the BCKDHB and DBT genes on the territory of Azerbaijan. One of these patients is an Azerbaijani Turk, and two are from the Lezgi ethnic group. Thus, the frequency of MSUD disease is 0.001 among Azerbaijani Turks and 0,0021 in the Lezgi ethnic group. The frequency of mutations of these genes studied in the population of Azerbaijan was equal to 0.001.

3.5. Analysis of Protein Structure

In the BCKDHB gene 508(C> T) mutation affects the protein helix to some extent, with an extra small helix. A C> T substitution at position 508 in exon 2 of the BCKDHB gene caused a (Lys-Gly) lysine-glutamine change at position 332 of the corresponding polypeptide. Two missense mutations (p. Arg673Ser, p. Phe947Gly) are present in coils. In the second patient, a missense mutation (compound form) was detected: C>T substitution at position 972 of exon 9 of the BCKDHB gene, arginine-serine change at position 673 of the polypeptide, and A>G substitution at position 1221 of exon 10, phenylalanine-glutamine at position 947 of the polypeptide. The next mutation (p. Lys907Gly) is present in β-sheets as a result of extends the β-sheet. In the third patient, an A> G substitution at position 1199 of exon 10 of the DBT gene caused a lysine-glutamine change at position 508 of the polypeptide (Figure 2).

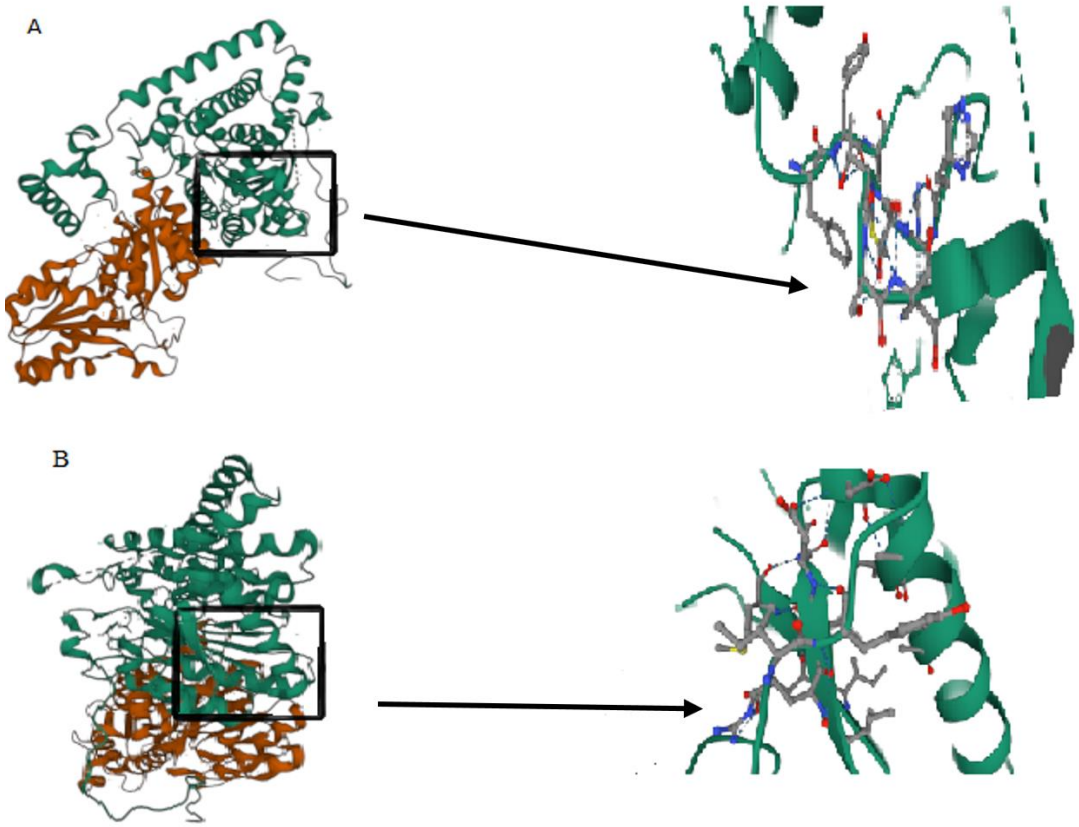

Figure 2.
Predicted 3D protein structure of E1β compotent complex colored in green. A- Normal structure of branched-chain ketoacid dehydrogenase; B- Lys907Gly mutation of the *BCKDHB* gene causing β-sheet expends.

4. Discussion

The objective of this research was to study of the mutations among Azerbaijani patients which diagnosed with MSUD. The identification of these mutations will have great importance for molecular diagnosis of patients from Azerbaijan for prevent (testing, prenatal testing, pre-marital screening and pre-implantation genetic diagnosis) this disease. A study analyzing 52 MSUD patients from Saudi Arabia identified 20 novel mutations in the BCKDHA gene. These mutations were spread across the entire coding region, and all were predicted to be disease-causing. Notably, no mutations were found in the DLD gene among this cohort [19].

Research involving Vietnamese families with MSUD uncovered novel pathogenic variants in the BCKDHA gene. These findings contribute to the understanding of the genetic diversity of MSUD across different populations [20].

In a cohort of Brazilian MSUD patients, novel variants such as p. Gly131Val, p. Glu146Glnfs13, p. Phe149Cysfs9, p. Cys207Phe, and p. Lys211Asn were identified in the BCKDHB gene. These mutations expand the known mutational spectrum associated with MSUD.

The same Brazilian study that identified novel mutations in BCKDHA and BCKDHB also found new variants in the DBT gene, including p. Glu148Ter and p. Glu417Val. These discoveries enhance the understanding of DBT gene mutations in the context of MSUD.

Collectively, these studies highlight the genetic heterogeneity of MSUD and underscore the importance of comprehensive genetic analysis in affected individuals. Identifying novel mutations not only aids in accurate diagnosis but also informs genetic counseling and potential therapeutic strategies [21].

A Chinese case study reported a novel c.391G > A mutation in the BCKDHB gene, leading to a p. Gly131Arg substitution. This mutation was identified alongside a large gene deletion, providing insights into the complex genetic mechanisms underlying MSUD [21].

For amplification of BCKDHA, BCKDHB, DLD and DBT genes was used the polymerase chain reaction (PCR).

Exome sequencing analysis of the three patients identified new mutations. DNA samples of the patients were analyzed for the 2, 9, and 10 exons of the BCKDHB gene and the 10 exon of the DBT gene.

The 1199A>G mutation of the DBT gene was detected in a homozygous form in one patient by the Sanger method. In the second patient, the 508C>T mutation of the BCKDHB gene was found in a homozygous form. In the third patient, 972C>T (exon 9) and 1221A> G (exon 10) mutations of the BCKDHB gene were found in compound heterozygous form. Additionally, we analyzed the protein structure and discussed the clinical implications. In the BCKDHB gene 508(C> T) mutation affects the protein helix to some extent with an extra small helix. A C> T substitution at position 508 in exon 2 of the BCKDHB gene caused a (Lys-Gly) lysine-glutamine change at position 332 of the corresponding polypeptide. Two missense mutations (p. Arg673Ser, p. Phe947Gly) are present in coils. In the second patient, a missense mutation (compound form) was detected: C>T substitution at position 972 of exon 9 of the BCKDHB gene, arginine-serine change at position 673 of the polypeptide, and A>G substitution at position 1221 of exon 10, phenylalanine-glutamine at position 947 of the polypeptide. The next mutation (p. Lys907Gly) is present in β-sheets as result of extends the β-sheet. In the third patient, an A> G substitution at position 1199 of exon 10 of the DBT gene caused a lysine-glutamine change at position 508 of the polypeptide [22].

In this study, we report the identification of novel mutations in 3 patients with MSUD. We identified 4 different novel mutations in the BCKDHB and DBT genes among 3 Azerbaijani MSUD patients. These mutations have not been identified previously. No mutation was found in these genes of the Azerbaijan population. All three patients in which identify mutations were from the Guba-Khachmaz zone. One of the 3 MSUD patients with novel mutations is an Azerbaijani Turk, and two were from the Lezgi ethnic group.

5. Conclusion

In this report, we describe the molecular genetic analysis of patients from Azerbaijan diagnosed with MSUD. For the purpose of research, samples were taken from 940 patients (140 control and 800 experimental), and molecular genetic analyses were performed.

1. Four novel mutations were detected, two of which were compound heterozygous: heterozygous compound form - 1221(A>G) and 972 (C>T) mutations of BCKDHB gene; homozygous form of -508(C>T) mutation of BCKDHB gene; homozygous form of 1199(A>G) mutation of DBT gene. Mutations were identified in homozygous, heterozygous and compound heterozygous forms (there was no significant difference between the revealed $p>0.05$), which is expected due to the high degree of consanguinity in our patients.

2. Considering the symptoms, the results of analyses of the investigated MSUD patients were summarized and compared according to the severity of the disease. MSUD disease was observed only in severe and mild forms. There is no significant difference between the number of severe and mild forms of MSUD patients ($p>0.05$). A moderately severe form was not observed among MSUD patients.

3. A 3D structure protein model was created and mutant versus wild type model was compared. Then, protein structure was analyzed.

4. In a result of molecular genetic research, information was obtained about the territorial and ethnic distribution of mutant alleles of the BCKDHB and DBT genes in Azerbaijan. Samples of patients from different ethnic groups who live in the Sheki-Zagatala, Guba-Khachmaz, Lankaran-Astara and Baku cities were studied. The disease was detected in patients living in the northern region of Azerbaijan. Thus, 2 out of 3 patients are from Khinalig village of Guba, and one from Khachmaz district. All 3 patients (1 male and 2 females) with detected mutations were from the Guba-Khachmaz region.

5. Ethnic specificity was determined in the frequency and spectrum of the studied mutations in the BCKDHB and DBT genes in the territory of Azerbaijan. One of these patients was an Azerbaijani Turk, and two were from the Lezgian ethnic group. Thus, the frequency of MSUD disease is 0.001 among Azerbaijani Turks, and 0.0021 in the Lezgian ethnic group.

None of the 140 control groups included in the MSUD study showed any mutations in the studied genes. To date, genetic studies of MSUD have not been conducted in the population of Azerbaijan. The identification of novel mutations by us was a scientific breakthrough in the molecular study of MSUD.

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