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Research Article

Molecular and Bio-informatics Analysis of BCKDHA, BCKDHB, DLD and DBT Gene Mutations in Azerbaijani Patients with Maple Syrup Urine Disease

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Abstract
Maple syrup urine disease (MSUD) is a rare autosomal recessive metabolic disorder caused by mutations in the BCKDHA, BCKDHB, DBT, and DLD genes, which encode subunits of the branched-chain α-keto acid dehydrogenase complex. Understanding the genetic basis of MSUD is essential for accurate diagnosis, genetic counseling, and therapeutic interventions. This study aimed to characterize mutations in the BCKDHA, BCKDHB, DBT, and DLD genes among Azerbaijani patients clinically diagnosed with MSUD. Between 2015 and 2020, blood samples were collected from 940 individuals representing various regions of Azerbaijan. Exome and Sanger sequencing were employed to detect variants in the four target genes, with Sanger sequencing used to validate exome-identified variants in BCKDHB and DBT. Structural modeling of mutant proteins was conducted using the SWISS-MODEL platform to predict the functional impact of the mutations. Four novel missense mutations were identified in three unrelated patients from the Guba–Khachmaz region: c.1221A>G, c.972C>T, and c.508C>T in the BCKDHB gene, and c.1199A>G in the DBT gene. All variants were predicted to be pathogenic and associated with amino acid substitutions that potentially disrupt protein conformation. Notably, one patient belonged to the Azerbaijani Turk ethnic group, and two were from the Lezgi community. This study reports, for the first time, novel pathogenic mutations in Azerbaijani MSUD patients, expanding the mutational spectrum of BCKDHB and DBT genes. The findings highlight the importance of ethnically tailored genetic screening and provide valuable data for molecular diagnosis, carrier detection, and the development of personalized management strategies for MSUD in Azerbaijan.

Keywords: Azerbaijani patients, Swiss-Model, exome sequencing, novel mutations, protein modeling

1. INTRODUCTION

Maple syrup urine disease (MSUD) is a metabolic disorder characterized by impaired catabolism of branched-chain amino acids due to genetic mutations. Proper metabolism of the 22 amino acids that constitute proteins is essential for maintaining vital bodily functions and the structural integrity of tissues and organs. Dietary proteins are broken down into amino acids, which are subsequently metabolized within cells by specific enzymes to produce energy and support biochemical synthesis [1]-[3]. Disease is transmitted by autosomal recessive inheritance [4].

In MSUD, however, mutations in genes encoding the branched-chain keto acid dehydrogenase complex result in enzymatic dysfunction, leading to an accumulation of the branched-chain amino acids, i.e., leucine, isoleucine, and valine, and their toxic metabolites. This accumulation causes significant tissue damage, particularly in the brain, resulting in severe neurological and metabolic disturbances [5]-[7]. The MSUD is inherited in an autosomal recessive manner and is primarily caused by mutations in the BCKDHA, BCKDHB, DLD, and DBT genes. These genes encode subunits of the branched-chain keto acid dehydrogenase enzyme complex responsible for the catabolism of branched -chain amino acids. To date, over 190 distinct mutations have been identified in these genes [8] [9].

Early diagnosis is crucial for effective management, as untreated metabolic imbalance can lead to progressive clinical deterioration and potentially fatal outcomes. Clinically, MSUD manifests shortly after birth with symptoms such as motor delay, dystonia, and hepatomegaly. Therefore, timely molecular-genetic diagnosis and

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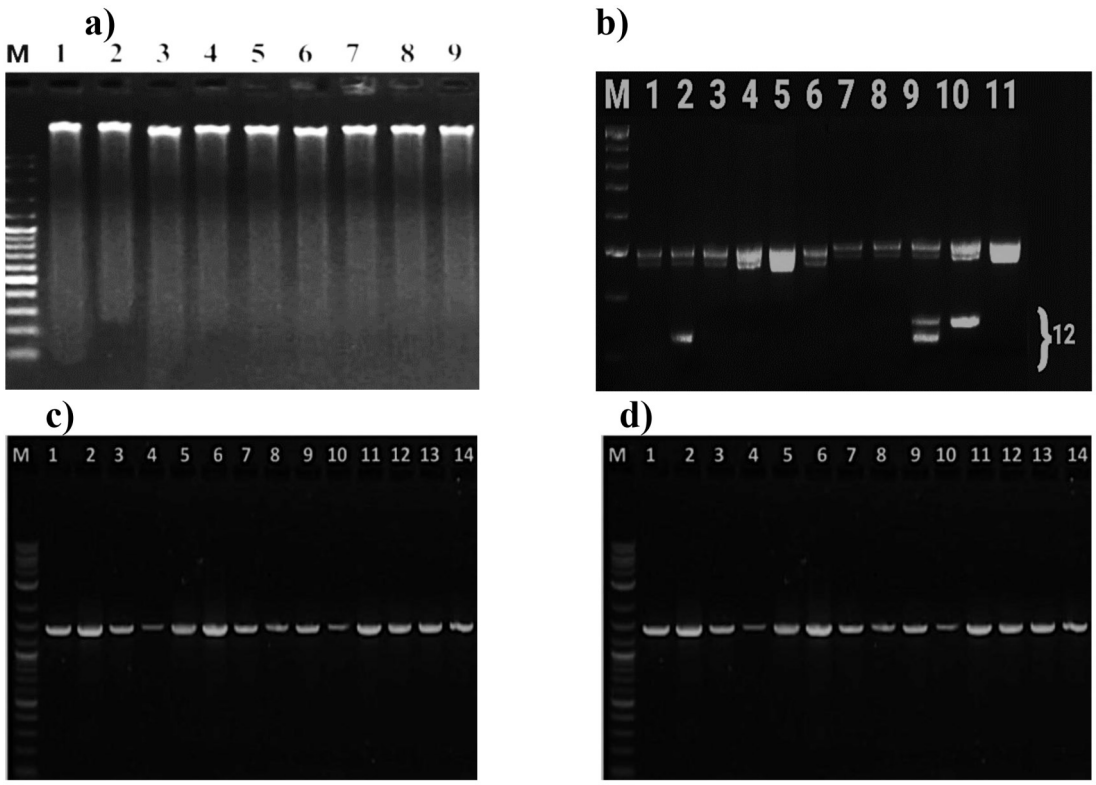

Figure 1. Image of electrophoresis of *BCKDHA, BCKDHB, DBT,* DLD genes in 1.7% agarose gel. (a) Electrophoresis image of *BCKDHA* gene: M-DNA marker, no mutation detected in 1-9-1-9 exons; (b) electrophoresis image of *BCKDHA* gene: M-DNA marker, no mutation detected in 1, 3-8 and 11 exons, mutation in 2,9,10 exons in *BCKDHB* gene , 12-mutant region; (c) *DBT* gene electrophoresis image: M-DNA marker, no mutation detected in 1-9, 11 exons, mutation in exon 10 of 10-*DBT* gene, 12-mutant region; and (d) electrophoresis image of the *DLD* gene: M-DNA marker, no mutation detected in 1-14-1-14 exons.

appropriate medical intervention are essential to improve patient prognosis and quality of life [10] [11]. Recent advances in molecular genetics have significantly enhanced our understanding of MSUD, providing invaluable insights and therapeutic opportunities for affected families and patients worldwide.

2. MATERIALS AND METHODS

2.1. Materials

As much as 940 people that involved in the study were from different zones of Azerbaijan. Blood samples of 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 obtained.

2.2. Methods

2.2.1. Biochemical Analysis

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

2.2.2. Obtaining DNA Extraction

As much as 2 mL of blood was taken from the patients and leukocytes were obtained through centrifugation. A 200 mkl of QIA ampgenomic DNA and RNA kit (QIAGEN. Germany), buffer

solution, 200 mkl of venous blood, and 20 mkl of protease enzyme (QIAGEN) were used to isolate DNA from leukocytes.

2.2.3. Polymerase Chain Reaction 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 $MgCl_2$, 0.25 mM dNTP, and 0.10 µM of each primer. A pair of forward (F) and reverse (R) primers were used for each genome fragment. The PCR conditions were set as follows: one cycle of 94 °C for 5 minute; 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 minute.

2.2.4. 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 (Figures 1 and 2).

2.2.5. DNA Sequencing

The purified product was preceded to the exome sequencing and Sanger analysis. For the exome sequencing was carried out using HiSeq2500 sequencer. Variations were screened with indicators Sift_Pred=Damaging. 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 (Figure 3).

2.2.6. In Silico Modeling

To simulate mutations in studied genes we did *in silico* modeling. The sequences for these genes were obtained from databases NCBI [12]. We used ClinVar and HGMD databases for the identification of specific mutations. For the restoring protein structure were used experimental structures from Protein Data Bank (PDB). For introduce mutations into the 3D structure were used software PyMOL. For predict the effect of mutations were used PolyPhen-2. For access how mutations affect protein stability were used FoldX. For simulating

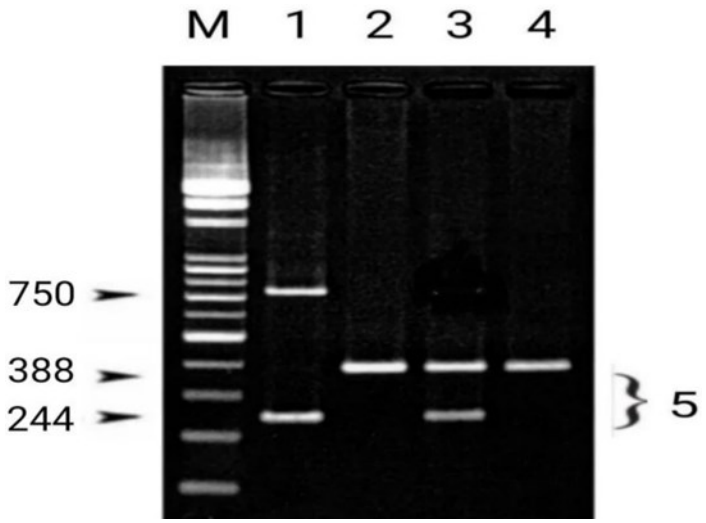

Figure 2. Electrophoresis image of genomics DNA and PCR DNA fragments in intact 1.7% agarose gel: M - DNA marker. (1) Gel electrophoresis image of B*CKDHB* and *BCKDHA* genes in the first healthy daughter (A.Z.) of the first family (750 b.p. and 244 b.p. fragments); (2) the second child of the first family (A.M.) - a homozygote form of 508 (C-T) mutation in *BCKDHB* gene (388c.n.) in a 3-year-old girl; (3) compound heterozygote form of mutations 972(C-T) and 1221(A-G) in the *BCKDHB* gene (388 b.p. and 244 b.p.) in the third child of the first family (A.T.); (4) 1199(A-G) in the *DBT* gene (homozygote form) in the child of the second family (388 b.p.); and (5) mutant region (covers the area where fragments 244 b.p.-388 b.p. are located).

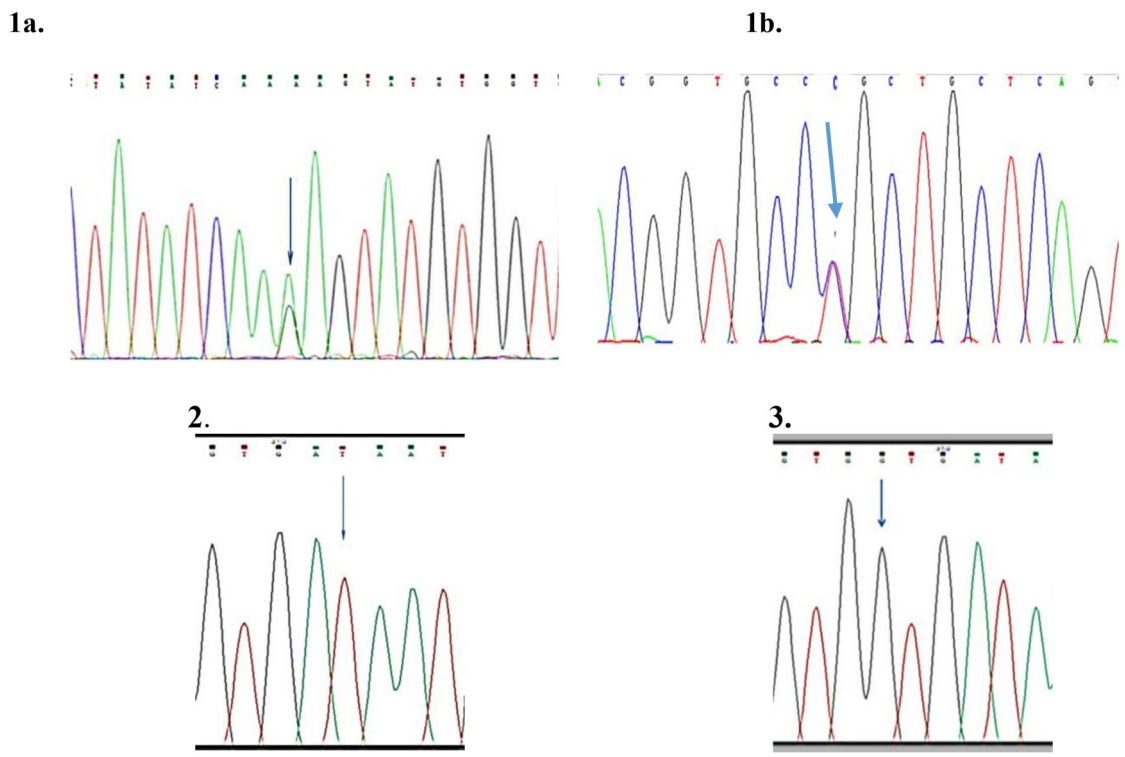

Figure 3. Electropherogram of changes in the nucleotide sequence of *BCKDHA* and *BCKDHB* genes. (1) Heterozygous compound form of *BCKDHB* gene: (a)1221(A>G) mutation in exon 10 and (b) 972 (C>T) mutation in exon 9; (2) homozygous form of 508(C>T) mutation in exon 2 of *BCKDHB* gene; and (3) Homozygote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

interactions of the mutant protein with cofactors or substrates were used AutoDock program. For simulating behavior of wild-type and mutant proteins were used GROMACS molecular dynamics and analyzed stability, flexibility, and conformational changes. Mutations and structural effects were visualized with PyMOL and used mutation modeling Missense3D databases for automated structural impact predictions. We used KBase for study how mutations in the *BCKDH* complex genes affect protein structure [13].

3. RESULTS AND DISCUSSIONS

3.1. MSUD Patient Distribution

In a 4-year-old girl from Khachmaz region of Azerbaijan, a homozygous mutation resulting from replacement of adenine by guanine at position 1199 of the DBT gene was detected by molecular genetic methods. Among 1199 patients were 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.

3.1.1. Results 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 a 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 two 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.

3.1.2. İdentification of Novel Mutations

Sequences were amplified for each four genes. Mutation was detected in position 508 of the *BCKDHB* gene in the sick child. By molecular genetic methods in the newborn was observed replacement of cytosine by thymine at position 972 and replacement of adenine by guanine at position 1221 of *BCKDHB* gene (Figures 1 – 3). Out of 800 experimental samples included in the study, leucine -causing mutations were found in 3 patients. Each of these mutations are novel mutations. None of the 140 control samples included in the study had any mutations in the studied genes.

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

In a result of molecular genetic research was obtained information about the territorial and ethnic distribution of mutant alleles of *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 of Guba, and one from Khachmaz district. Ethnic specificity was determined in the frequency and spectrum of studied mutations in *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.

3.1.4. Analyze of Protein Structure

In *BCKDHB* gene 508(C> T) mutation is affecting 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 poly peptide. 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 poly peptide. 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 (see Figure 4).

3.2. Discussion

The primary objective of this study was to investigate the spectrum of mutations in the BCKDHA, BCKDHB, DLD, and DBT genes among Azerbaijani patients diagnosed with MSUD. This study is particularly significant as it represents the first comprehensive molecular genetic analysis of MSUD patients within the Azerbaijani population. Given the autosomal recessive inheritance of MSUD and the presence of consanguineous marriages in the region, identifying pathogenic mutations is essential for improving early diagnosis, genetic counseling, and targeted intervention strategies. Through exome and Sanger sequencing, we identified four novel missense mutations across three unrelated patients, all originating from the Guba-Khachmaz region of Azerbaijan. These mutations include: BCKDHB gene: 508C>T (homozygous), 972C>T and 1221A>G (compound heterozygous); DBT gene: 1199A>G (homozygous). These variants have not been previously reported in international mutation databases such as ClinVar or HGMD, indicating their novelty and potential relevance as population-specific mutations [14].

In addition to identifying these mutations, we performed *in silico* protein structure modeling to assess their structural and functional impact. Our analysis demonstrated that these mutations lead to significant alterations in protein conformation. For instance: The 508C>T mutation in the BCKDHB gene introduces a small helix into the E1β subunit structure; the 972C>T and 1221A>G compound heterozygous mutations lead to Arg673Ser and Phe947Gly substitutions, respectively, affecting protein coils and potentially impairing enzymatic stability; the 1199A>G mutation in the DBT gene results in a Lys508Gln substitution, extending a β-

sheet structure within the E2 subunit, which could affect substrate binding or complex assembly. These structural changes are consistent with the pathogenic phenotype observed in MSUD, which manifests in the neonatal period and is characterized by elevated levels of branched-chain amino acids (BCAAs), such as leucine, isoleucine, and valine. Biochemical analyses supported the genetic findings, revealing markedly elevated BCAA levels in blood and urine samples of all three patients, confirming the clinical diagnosis of MSUD.

An important dimension of this study is the ethnic and geographic distribution of the mutations. Two of the three patients were from the Lezgi ethnic group, and one was an Azerbaijani Turk. All cases were from the northern region of Azerbaijan, suggesting a potential regional clustering and possible ethnic-specific mutation prevalence. Based on our findings, the estimated carrier frequency of MSUD-related mutations is approximately 0.001 among Azerbaijani Turks and 0.0021 among Lezgi individuals, highlighting the need for further population-wide studies to validate these estimates [15]-[17]. Notably, none of the 140 individuals in the control group showed any mutations in the studied genes, which supports the association between these mutations and disease manifestation. This also underscores the pathogenic nature of the variants and their potential clinical utility in the design of diagnostic panels tailored to the Azerbaijani population.

From a broader perspective, this study emphasizes the critical importance of integrating genetic screening into public health strategies, particularly in regions with elevated rates of consanguineous marriages. The identification of these novel mutations provides a foundation for the development of prenatal testing, carrier screening, pre-marital genetic counseling, and pre-implantation genetic diagnosis (PGD) programs aimed at preventing MSUD. In summary, this research presents the first identification of novel MSUD-related mutations in Azerbaijan, highlights potential ethnic and regional mutational hotspots, and demonstrates the value of combining molecular genetics with structural bioinformatics to elucidate disease mechanisms. The findings pave the way for establishing targeted genetic screening programs and improving clinical management for at-risk populations in Azerbaijan and potentially other Turkic and Caucasian ethnic groups.

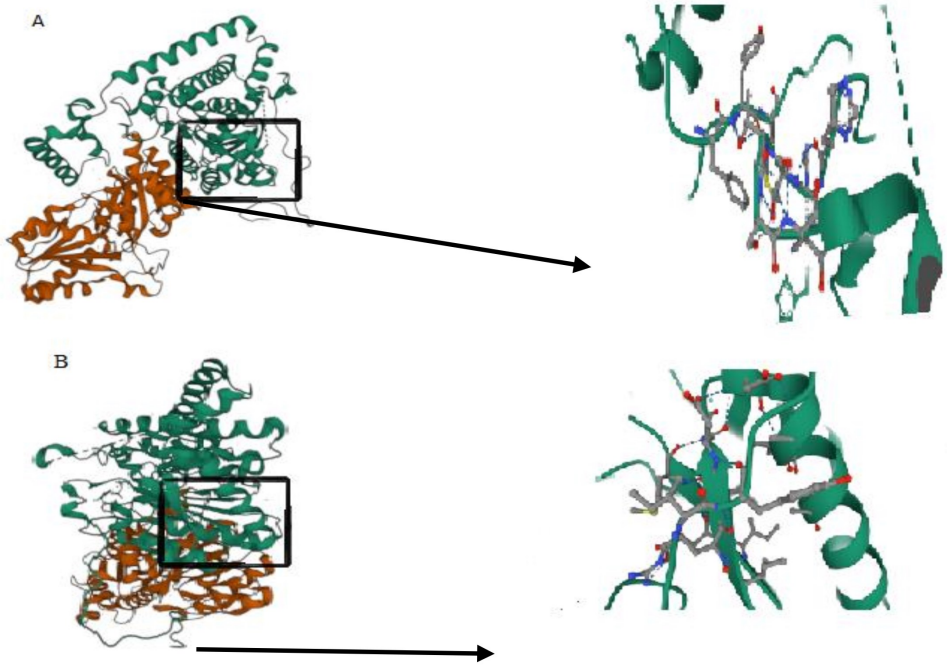

Figure 4. Predicted 3D protein structure of E1β component complex colored in green. (A) Normal structure of branched-chain ketoacid dehydrogenase and (B) Lys907Gly mutation of the *BCKDHB* gene causing β-sheet expends.

The current study represents the first comprehensive molecular genetic investigation of MSUD among Azerbaijani patients, revealing four novel missense mutations in three individuals from the Guba-Khachmaz region. These mutations include 508C>T, 972C>T, 1221A>G in BCKDHB, and 1199A>G in DBT, and all are predicted to be pathogenic based on protein structural modeling. This pattern of discovering novel and population-specific mutations aligns well with global trends in MSUD research. In a Malaysian cohort of 31 patients, researchers identified 21 mutations, 14 of which were novel. Notably, one DBT gene mutation (p.S399C) appeared *recurrent* within the Malay population, suggesting possible founder effects or population-specific mutational hotspots [18]. A study from Iran, involving 7 MSUD patients, revealed four novel mutations across BCKDHA, BCKDHB, and DBT genes. These were accompanied by protein modeling, which underscored likely functional consequences, echoing the methods used in this Azerbaijani study [19]. In the Kingdom of Saudi Arabia, analysis of 52 MSUD patients uncovered 25 mutations, including 20 novel ones spanning BCKDHA, BCKDHB, and DBT. All variants were homozygous—likely reflecting high consanguinity rates—and functional assessments confirmed most as likely damaging [20]. Among Chinese patients, 11 cases identified eight new mutations within BCKDHA, BCKDHB, and DBT genes. Structural modeling was also employed, and importantly, prenatal diagnosis was successfully carried out in some pregnancies, underscoring the clinical application of these findings [21]. A Vietnamese cohort employing whole-exome sequencing identified five novel mutations in BCKDHB and DBT genes in three newborns. As in the current study, these variants were predicted to destabilize protein complexes, again confirming the pathogenic nature of novel mutations across populations [22].

Additionally, a systematic review spanning the Middle East, North Africa, Türkiye (MENAT) region highlighted significant genetic heterogeneity. Some BCKDHB, BCKDHA, and DBT variants were found across diverse ethnicities, but many remained population-specific. Genotype-phenotype correlations were often inconsistent, indicating complex interplay between genetic and environmental factors [23]. Global Consistency of New Mutations states that each study underscores the discovery of new, previously unreported mutations, emphasizing the genetic diversity of MSUD and the importance of building localized mutation databases.

In silico modeling as a standard reveals that protein structure modeling (through tools like PolyPhen-2, SIFT, MutationTaster, SWISS-MODEL, PyMOL, FoldX, etc.) consistently confirms the deleterious impact of novel mutations across studies [24]. This strengthens both diagnostic confidence and biological understanding. The clustering of mutations within specific communities reflects population-level mutational spectra. For example, the recurrent DBT mutation in Malays, the homozygosity in Saudi Arabia, and the community-specific mutations in Azerbaijan underscore the need for tailored genetic screening protocols. Notably, studies from China and Saudi Arabia have translated genetic findings into prenatal diagnostic applications, highlighting a path toward preventive genetic medicine. In Azerbaijan, similar frameworks could be developed based on the novel mutations identified. The MENAT systematic review underscores knowledge gaps in genotype–phenotype correlations. Integrating Azerbaijani data into such regional databases will improve diagnosis, genetic counseling, and epidemiological understanding of MSUD [25].

4. CONCLUSIONS

This study provides the first comprehensive molecular genetic characterization of maple syrup urine disease (MSUD) in Azerbaijani patients, identifying four novel pathogenic mutations—three in the BCKDHB gene (c.1221A>G, c.972C>T, and c.508C>T) and one in the DBT gene (c.1199A>G). These variants, detected in homozygous, heterozygous, and compound heterozygous forms, are consistent with the high level of consanguinity observed within the population. Protein structure modeling confirmed that the mutations likely disrupt normal enzymatic function. Clinically, only severe and mild forms of MSUD were observed,

with no moderate cases detected. All patients carrying the novel variants originated from the Guba–Khachmaz region, specifically within the Azerbaijani Turk and Lezgi ethnic groups, suggesting potential ethnic specificity in mutation distribution. The absence of these mutations in the control group further supports their pathogenic significance. Overall, this study expands the mutational spectrum of BCKDHB and DBT genes and provides essential baseline data for molecular diagnosis, genetic counseling, and population-specific screening strategies for MSUD in Azerbaijan.

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Author Contributions

L. S. H. study director, conducting experiments, collecting and analyzing data, performed the statistical analysis and wrote the manuscript. A. R. H. performed the statistical analysis. S. R. E. conducting experiments. R. R. H. collecting data, S. T. H. collecting data.

Conflicts of Interest

The authors state that there is no conflict of interest regarding the publication of this article.

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DECLARATION OF GENERATIVE AI

Not applicable.

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