Section A -Research paper
ISSN 2063-5346

EARLY MOLECULAR-GENETIC DIAGNOSIS OF PHENYLKETONURIA PATIENTS IN AZERBAIJAN POPULATION

L.S.Huseynova[1*], Ansarova A.H[2], Mahmudova P.A[3]

Article History: **Received: 03.04.23** **Revised: 05.05.23** **Accepted: 20.06.23**

Abstract

Genetic drift and founder effect may have also played a role in the mutation spectrum we observed. The data obtained in our study can also be used in the development of genetic tests for phenylketonuria.
This study was conducted in the patients diagnosed with phenylketonuria who presented to Scientific Research Pediatric Institute of the Ministry of Health of Azerbaijan and hospitals of different regions of Azerbaijan The present study aimed to determine frequencies of mutations in the phenylalanine hydroxylase gene (PAH) in 30 patients diagnosed with phenylketonuria, from 633 person who presented to Scientific Research Pediatric Institute of the Ministry of Health of Azerbaijan and hospitals of different regions of Azerbaijan over the period from 2016 to 2022. Molecular genetic methods for detecting mutations are based on differences in the DNA sequence. More than 600 mutations in the PAH gene in exons 6, 7, 8, 11 and 12 have been detected in various countries around the world. Among them, 18 different mutations were found in Azerbaijani patients (V245V, R261Q, Q232Q, V245V, P281L, R241C, L385L, V399V, E280K, R261X, A434D, R176X, Ex6-96A> G, R241C, R243Q, R256Q). Nutritional therapy and adaptation to this treatment are essential in PKU, a congenital metabolic disease. This study was undertaken to determine the contribution of families in coping with problems that make nutritional adaptation difficult. Difficulties faced by families in achieving low-protein, low-phenylalanine-containing products that are inadequate and expensive to achieve proper nutrition in PKU disease, the ways to cope with these difficulties, the problems that can be or are experienced in harmony with the environment of the patients, and the ways to cope with them consists the subject of the study. Phenylketonuria (PKU) is an autosomal recessive genetic disease, caused by the phenylalanine hydroxylase (PAH) deficiency in the metabolic pathway, which prevents phenylalanine from being converted into tyrosine, leading to a large amount of phenylalanine discharged from the urine. Therefore, it is necessary to establish a simple, fast, accurate and reliable PKU molecular diagnostic method for clinical diagnosis. As a result of the study it is understood that it is necessary and important to educate the families, to prevent the marriage of the relatives and to raise the awareness of the community in order not to increase the prevalence of recessive diseases in general frame. In the private area, the support of the family, the care to be shown about the nutrition, the minimization of the negative effects of the environment, the self-confidence to be given to the patient can create cognitive health and a life without any problems.

Keywords: Phenylketonuria, gene mutation, rare disorder, family, nutrition.

[1, 2, 3] Azerbaijan Medical University, Department of Medical Biology and Genetics. Baku, Azerbaijan.
[*]Corresponding author: L.S Huseynova, Email: royahuseynova2006@gmail.com

Introduction

Phenylketonuria (PKU) is an autosomal recessive inherited metabolic disease in which phenylalanine hydroxylase deficiency results in phenylalanine metabolic disorders. Phenylalanine hydroxylase (PAH) is an enzyme which is necessary to metabolize the amino acid phenylalanine to the amino acid tyrosine. Phenylalanine hydroxylase is a key enzyme in the phenylalanine metabolic pathway. If PKU is not treated, phenylalanine can build up to harmful levels in the body, causing intellectual disability and other serious health problems. It will cause severe retardation of intellectual development due to the hyper-toxic effects of hyperphenylalaninemia and intermediate metabolites in the central nervous system [1].
The *PAH* gene is located on chromosome 12q22-q24.1 and contains 13 exons and 12 introns encoding a protein consisting of 452 amino acids. Phenylketonuria is caused by a highvariety of mutations in the gene for phenylalanine

hydroxylase (PAH) enzyme. At present, more than 600 mutations of *PAH* have been reported worldwide [2].

The prevalence of PKU shows considerable geographic variation. It is estimated to be 1/10,000 live births in Europe with a higher rate in some countries, for example: Ireland, Italy, Turkey and ect. Incidence is as high as 1:2600 live births in Turkey and as low as 1:200,000 in Finland. Common mutations between ethnic groups and geographical areas make PAH deficiency a genetic disease with great allelic heterogeneity [3-6].

Phenylalanine, a protein building block, cannot be metabolized, accumulates in the blood and creates irreversible brain damage. If it is not identified and treated early, it inevitably results in severe mental retardation [7].

PKU, known as a hereditary metabolic disease, where the essential amino acid phenylalanine cannot be metabolized to tyrosine due to lack or inadequacy of phenylalanine hydroxylase enzyme secreted from the liver, where the phenylalanine accumulated in the blood causes brain damage and urinary excretion of residual products such as phenyl pyruvic acid, phenyl lactic acid [8].

In the world, it is stated that on average, one out of every 50 people is a carrier, but the probability that two carriers will come together is 1: 2500 [9].

In the parents of individuals with PKU, there are two genes that are responsible for the production of phenylalanine hydroxylase enzyme, one that is solid and one that is defective and it has been explained that the child who got the bad genes from both parents born as PKU patient, the ones who got only one of the bad genes as the carrier like the mother and father born as carriers, and the child who only got the healthy genes born completely healthy. The likelihood of the carrier mother and father's giving birth to a sick child is as high as 25% [10].

Materials and methods

Patients

We characterized phenylalanine hydroxylase (PAH) genotypes of Azerbaijanian patients with phenylketonuria (PKU). A total of 633 person were included in this study and PKU mutations in 30 Azerbaijanian patients from different regions were identified by molecular-genetic methods. All molecular genetic methods for detecting mutations are based on differences in the DNA sequence.

Obtaining DNA extraction

Material used was venous blood with anticoagulant of 633 patients. Genome DNA was obtained by automatic isolation from 200 ml of venous blood. The DNA concentration was measured by the Digital spectrometer. The integrity of the isolated genomic DNA was detected in a 2% agarose gel. The venous blood for research was drawn into a tube containing EDTA or heparin. Genomic DNA and RNA kits made by Qiagen GmbH (Hilden, Germany) were used for analysis.

Gel electrophoresis

Integrity and quantity of genomic DNA and polymerase chain reaction (PCR) products were identified by electrophoresis on 2% agarose gel (PowerPacBasicGelDoc™ EZ; Bio-Rad Laboratories, Hercules, CA, USA). The genome DNA underwent the PCR procedure for every protein-encoding exon of the *PAH* gene. Positive PCR samples that were checked by electrophoresis in agarose gel were purified by an enzymatic method.

Polymerase chain reaction

Polymerase chain reaction was carried out in a following conditions: denaturation at 96 ^{0}C for 30 seconds; annealing at 55 ^{0}C for 30 seconds; extension at 75 ^{0}C for 1 min. This cycle was repeated 25 times, 72 ^{0}C for 10 min. and 4 ^{0}C pause. The PCR was carried out on a Professional Thermocycler Biometra system (Biometra Biomedizinische Analytik GmbH, Göttingen, Germany). A pair of for-ward and reverse primers was used for each genomic fragment. For the purification of DNA fragments after the first stage of PCR, a set of magnets was used: Agencourt AMPure XP PCR purification and SPRIPlate 96 Super Magnet Plate (Beckman Coulter Inc., Beverly, CA, USA). The second amplification of the purified DNA fragments was carried out in the following condition: denaturation at 95 ^{0}C for 30 seconds; annealing at 55 ^{0}C for 30 seconds; extension at 77 for 2 min. This cycle was repeated 25 times, and 72 ^{0}C for 10 min. and 4 ^{0}C pause.

ISSN 2063-5346

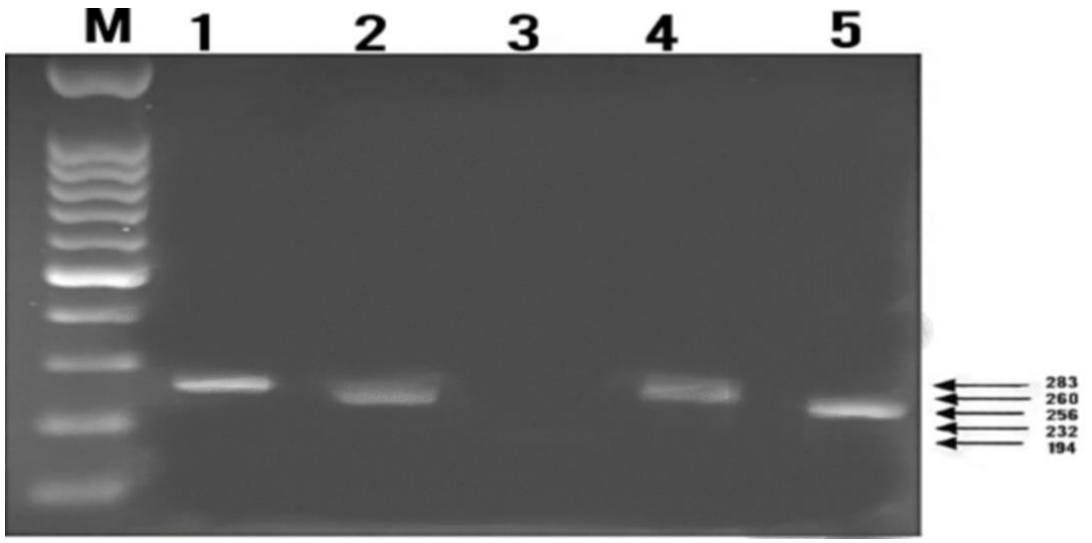

Figure 1. 0.7% agarose gel electrophoresis image of PAH gene exons 6, 7, 8, 11 and 12: M-Marker; 1- exon 6 (283 b.p); 2- exon 7 (256 b.p); 3- exon 8 (normal); 4- exon 11 (260 b.p); 5- exon 12 (232 b.p).

Sequencing

The nucleotide sequence of purified fragments was studied in GENOME Lab GeXP™ Sequencing (SCIEX, Brea, CA, USA). Purified product was dyed with fluorescent dye by BiqDye Terminator V.3.1. (Applied Biosystems, Foster City, CA, USA) and processed by Cycle Sequencing PCR. Positive Cycle Sequencing PCR samples, controlled by electrophoresis in agarose gel, were extracted from the BiqDye XT (Applied Biosystems with dye-purifying agent.

The obtained nucleotide chains were identified through SeqScape® version 2.7 software program (Applied Biosystems, Foster City, CA, USA; http:/tools.thermofisher. com/content/sfs/manuals/4401740.pdf), then compared by means of the National Center for Biotechnology Information (NCBI) Blast Ce, to normal PAH nucleotide chains, and only then were the substitutions and mutations identified. All PCR fragments were sequenced using the same primers as used for PCR amplification. All identified mutations were confirmed using a new PCR product of the abnormal fragment (forward and reverse). For exon 6 used Forward: TGCCCTGCTTGAGACACCTA and Revers: TCCTCCCCCACACTTTCTGC; for exon 7 used Forward: CTCTGACTCAGTGGA ; Revers CTGAGTCTGGCTTGGCTTAA for exon 8 used Forward: CTGAGTCTGGCTTGGCTTAA and Revers: CTCATTTGAGAAATTCAGGT; for exon 11 used Forward: TGCAGCAGGGAATACTGA and Revers: AGATGAGTGGCACCAGT and for exon 12 used Forward: TCCAAATGGTGCCCT and Revers: GGCGATGGTAGGGAA praymers.

The indepth interviews started with the intention to learn the relationship of nutrition and PKU. The study which will search the disease's relationship with the nourishment, as the participants are the mothers of PKU patients, has turned into an emphasis on the contribution of parents to the lives of PKU patients.

Results

Exons 6, 7, 8, 11 and 12 of the PAH gene from 30 patients (between 8 months to 25 years old) with PKU were identificated with DNA sequence. They were from different parts of Azerbaijan. At present, more than 600 mutations of *PAH* have been reported worldwide, mostly on exon 6th, 7th, 8th, 11th, and 12th exon. Among them, the 18 mutations are the most common types of PAH mutations in Azerbaijan population. The rate of consanguine marriage was 50%, and most of the parents were cousins. A total of 18 different mutations (V245V, R261Q, Q232Q, V245V, P281L, R241C, L385L, V399V, E280K, R261X, A434D, R176X, Ex6–96A > G, R241C, R243Q, R252Q, Y356X, R413P) were characterized in Azerbaijanian patients.

These mutations are located in 6, 7, 8, 11 and 12 exons of the gene. With this purpose we have done amplification of PAH gene exons 6, 7, 8, 11 and 12 genomic DNA fragments, got from lymphocytes of the G.M. family members: two parents and their six children, by means of polymerase chain reaction with 5 primer groups.

As can be seen from the gel electrophoresis image given in Figure 1, mutant regions were observed in the studied exons 6, 7, 11 and 12 of the PAH gene. Thus, the mutant region in exon 6 is 283 b.p, in exon 7 is 256 b.p., in exon 11 is 260 b.p., in exon 12 is 232 b.p. matched the part. No mutant region was observed in exon 8.

18 different mutations were found in Azerbaijani patients (V245V, R261Q, Q232Q, V245V, P281L, R241C, L385L, V399V, E280K, R261X, A434D, R176X, Ex6-96A> G, R241C, R243Q, R256Q).

The R261Q mutation in exon 7 was observed in seven patients from 30 patient. Studies have shown that this mutation is most common in the Azerbaijani population. Being R261G (G-A) 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.

It is understood that there is not enough level of consciousness in society yet about the disease of PKU. In order to cope with this disease, which is closely related to consanguineous marriages, the level of education needs to be increased first. It should be ensured that families are more caring about the special situations that the disease requires, teachers and instructors should be more informed and aware about the exclusion behavior. There is also a need to create awareness of special products in food service systems outside home.

In addition to increasing education and training in this disease where treatment problems are frequently encountered due to financial problems, the assistance of the government will also contribute considerably. In this case, what is thought to be a better aid than money is to encourage the main food firms to produce special foods for PKU patients. So, it is thought that the access to special food will increase and the financial problems to reach the products will decrease. It is understood that, in the private area, the support of the family in terms of nutrition is the most important element. It can be said that a problem-free life and cognitive health is possible if the adverse effects of the environment are minimized and the family is committed to feed with the right products.

Disscusion

PKU disease was discovered by Asbjorn Folling in 1934 as the result of research into two mentally retarded children. Folling accepted that phenyl pyruvic acid, a metabolite of the essential amino acid of phenylalanine, is a phenylalanine metabolism disorder in these two children after it has been identified as the metabolite. Later, it was demonstrated that phenylalanine hydroxylase activity, the enzyme that translates phenylalanine to tyrosine in the liver, with the accumulation of phenylalanine in the blood and spinal fluid of individuals with urinary phenyl pyruvic acid and mental retardation, has not been shown [11].

After all these determinations, Lionel Penrose, a mental retardation specialist and a geneticist in August 1935, noticed that PKU was the first form of mental retardation, continued to study, about 10 years later Dr. Horst Bickel has shown that a diet that can prevent mental retardation, a complication of PKU disease [12].

According to Blau et al., until the 1960s, most children born with PKU were totally mentally handicapped and spent most of their lives in treatment centers. In the 1960s, Robert Guthrie developed a diagnostic test for the PKU. This is a screening test, known as the Guthrie Test, made with newborn heel blood [13].

The blood sample for newborn should ideally be taken on the next 3-5 days after birth. Breastfeeding the baby before blood is taken is important for the diagnosis of PKU. Thus, the metabolic development that will take place after phenylalanine, which the baby takes, will be observed.

The original, semi-quantitative test known as the Guthrie test is based on a bacterial inhibition assay, making mass screening simple, cheap and very cost-effective. Gradually (but not entirely) it was replaced by newer methods, such as chromatography, fluorimeter and more recently tandem mass spectrometry. Guthrie's landmark discovery and implementation of the first newborn screening programs for PKU could be – in a 50 year retrospective – easily considered as one of the greatest advances in medicine [14].

There are different sources of information on the prevalence of PKU in the world. In a study conducted in 2008, the incidence of PKU was 1: 200,000 in Finland, 1: 125,000 in Japan, 1: 7.000 in Czechoslovakia, and 1: 2.600 in Turkey. The high incidence of PKU in Turkey is due to the widespread marriage of relatives and the low incidence in Finland and Japan is explained by the ‘negative founder effect’ in Finland and in Japan by genetic drift in the formation of the island population. Negative founder effect is an effect created by the fact that the current population of Finland is the result of the expansion of a very small population established up to 2000 years ago, and that there was little migration in this process. In another source there are prevalence data for PKU. In Europe, the prevalence is about

1/10.000 live births but 1/4000 births in Turkey because of high consanguinity within the population. Finland has the lowest prevalence in Europe with 1/100 000. In the USA the prevalence is 1/ 15 000 but it varies from about 1/ 50 000 to 1/25 000 births for Latin America [15].

In this study, molecular characterization of exons 6, 7, 8, 11, and 12 in 30 Azerbaijanian patients. Ten percent of patients were homozygote for mutations. The R261Q mutation in exon 7 was observed in seven patients in heterozygote form. Studies have shown that this mutation is most common in the Azerbaijani population. This one is the second most common mutation in Turkey and Iran, which is also common in European nations like France (17%), and Italy (16%). The presence of Mediterranean mutations in our patients may prove the historical connection between Azerbaijanian and Mediterranean populations.

This mutation is a common mutation in the Mediterranean and southern Europe but has a very low incidence in Spain. Furthermore, the frequency of R243Q mutation has been reported to be 18.2 % in Chinese and 12 % in Koreans, whiles in the present study the frequency of this mutation was found to be 2.5 %. Most mutant alleles of PAH probably by influencing transcription and translation of the respective gene can decrease the intracellular stability of protein and finally reduce enzyme function completely [16].

Several studies reported that the IVS10-11G>A mutation, a splice mutation in the end of intron 10, observed with a high incidence among in the Mediterranean region, Brazil and some parts of Iran including: East Azarbaijan, Semnan, Khorasan Razavi, however this mutation was not found in the present study. The virtual absence of this mutation in our study may reflect the regional variability of populations. The second most frequent mutation identified in present study, R261Q (7.69 %) occurs on a CpG mutation hotspot on exon 7, leads to the conversion of Arg to Gln at codon 261 of PAH. The majority of mutant alleles (73 %) were located on exon 7 and 6 that is in agreement with previous studies in Iranian. In addition, the novel gene variant c.1069T>C (p.C357R) has been seen in Iranian population for the first time. Thereby to plan detection strategy; the samples will be screened first for mutations in these regions. If mutations were not identified, the other exons and their adjacent regions will be tested populations [17-19].

Conclusion

The R261Q mutation in exon 7 with a relative frequency of 23, 3 % is G → A substitution leads to conversation of arginine amino acid to gluthamine amino acid at codon 782 of PAH. The major mutation in our study was R241C (10%), which is similar to the data obtained in population of Northern region of Azerbaijan, R243Q (6, 6%) and R252Q (6, 6%) which is similar to the data obtained in population Northern region of Azerbaijan. The frequency of other mutations found in this study was 3,3%. (V245V, Q232Q, V245V, P281L, L385L, V399V, E280K, R261X, A434D, R176X, Ex6-96A> G, R241C, R252Q).

The R261Q mutation in exon 7 with a relative frequency of 23, 3 % is G → A substitution lead to conversation of arginine amino acid to glutamine amino acid at codon 261 of PAH. The R241C mutation in exon 7 with a relative frequency of 10 % is C → T substitution lead to conversation of arginine amino acid to cytidine amino acid at codon 241 of PAH.

The results of our study proved the existence of heterogeneous mutations in different ethnic groups in Azerbaijan. The highest rate of mutations was observed in exon 7, and no mutation was observed in exon 8. So by monitoring diverse ethnic groups in Azerbaijan. This data can be useful for genetic counseling and carrier detection tests and as a base to start further research from.

Phenylketonuria and G6PD enzyme deficiency were identified in one family in Tekle village of Masally area of Azerbaijan Republic. Heterozygous and homozygous genetic types of phenylalanine-4-hydroxilase gene mutation R261G (G-A) were identified in one family.

Our results of Azerbaijanian individuals with PKU confirm a heterogeneous spectrum of mutations, displaying different ethnic and geographical origins. Moreover, our findings were slightly different from other ethnic groups. These findings can be useful to genotype/phenotype relationship in patients and provide confirmatory diagnostic testing in future, prognosis and predict severity of the disease in PKU patients.

In PKU, a disease in which the enzyme phenylalanine hydroxylase does not work or underworks, and therefore requires a limited phenylalanine lifetime, patients have to take a lifelong control level of phenylalanine and have a diet to live well and not to lose their intelligence.

PKU dietary therapy is based on keeping blood phenylalanine levels at safe intervals, limiting

this amino acid and relieving the patient's need for tyrosine. Nutritional therapy in each disease is specific to the patient, but PKU is vitally important for each gram of food, so the variety and quantity should be tailored to and specific to the patient's wishes at the limit that will not exceed the allowable limits. Just making a diet is not enough, the appropriateness of the diet needs to be checked at certain intervals. For this purpose, according to the application rules in Azerbaijan, it is necessary to perform phenylalanine control for the newborn 2 times a week for the first 6 months, one time a week for 6 months to 1 year old babies, and once in 10 days after the first year of the baby. These control intervals can vary according to the condition of the patient under the doctor's control.

Conflicts of interest

There is no conflict of interest.

Author Contributions

L.H. study director, conducting experiments, collecting and analyzing data. A.A and L.H. are collecting and analyzing data

Acknowledgments

We would like to acknowledge the Azerbaijan Medical University, Department of Neurology, Laboratory of Human Genetics, Genetic Resources Institute, Azerbaijan National Academy of Sciences for their support in performing some of gene analysis studies and our management staff.

References

1. Bercovich D, Elimelech A, Zlotogora J, Korem S, Yardeni T, Gal N, et al. Genotype–phenotype correlations analysis of mutations in the phenylalanine hydroxylase (PAH) gene. J Hum Genet. 2008 May 1; 53(5):407-18.
2. Song F, Qu YJ, Zhang T, Jin YW, Wang H, Zheng XY (2005) Phenylketonuria mutations in Northern China. Mol Genet Metab 86(Suppl 1):S107–S118
3. Acosta A, Silva W Jr, Carvalho T, Gomes M, Zago M (2001) Mutations of the phenylalanine hydroxylase (PAH) gene in Brazilian patients with phenylketonuria. Hum Mutat 17:122–130
4. Rivera I, Mendes D, Afonso Â, Barroso M, Ramos R, Janeiro P, et al. Phenylalanine hydroxylase deficiency: molecular epidemiology and predictable BH 4-responsiveness in South Portugal PKU patients. Mol Genet Metab. 2011 Dec 31; 104:S86-92.
5. Zare-Karizi S, Hosseini-Mazinani SM, Khazaei-Koohpar Z, Seifati SM, ShahsavanBehboodi B, Akbari MT, Koochmeshgi J (2011) Mutation spectrum of phenylketonuria in Iranian population. Mol Genet Metab 102:29–32
6. Cabalska B, Cyrytowski L, Nowacki P, Walczak M, Koziarska D. Different presentations of late-detected phenylketonuria in two brothers with the same R408W/R111X genotype in the PAH gene. J Intellect Disabil Res. 2003 Feb 1; 47(2):146-52.
7. Erlandsen H, Patch MG, Gamez A, Straub M, Stevens RC. Structural studies on phenylalanine hydroxylase and implications toward understanding and treating phenylketonuria. Pediatrics. 2003 Dec; 112(6 Pt 2):1557-65. Review. Citation on PubMed
8. Güttler F, Azen C, Guldberg P, Romstad A, Hanley WB, Levy HL, Matalon R, Rouse BM, Trefz F, de la Cruz F, Koch R. Impact of the phenylalanine hydroxylase gene on maternal phenylketonuria outcome. Pediatrics. 2003 Dec; 112(6 Pt 2):1530-3. Citation on PubMed
9. MacDonald A, Rocha JC, Van Rijn M, Feillet F. Nutrition in phenylketonuria. Mol Genet Metab, 2011. 104:S10-S18.
10. Scriver CR. The PAH gene, phenylketonuria, and a paradigm shift. Hum Mutat. 2007. 28(9): p. 831-845.
11. Blau N, van Spronsen FJ, Levy HL. Phenylketonuria. The Lancet. 2010 Oct 29; 376(9750):1417-27.
12. Ajami N, Kazeminezhad SR, Foroughmand AM, Hasanpour M, Aminzadeh M. A preliminary mutation analysis of phenylketonuria in southwest Iran. Genet Mol Res. 2013 Jan 1; 12(4):4958-66.
13. Blau N, Hennermann JB, Langenbeck U, Lichter-Konecki U. Diagnosis, classification, and genetics of phenylketonuria and

tetrahydrobiopterin (BH4) deficiencies. Mol Genet Metab. 2011 Dec 31; 104:S2-9.

14. Couce ML, Boveda MD, Fernandez-Marmiesse A, Miras A, Perez B, Desviat LR, Fraga JM (2013) Molecular epidemiology and BH4-responsiveness in patients with phenylalanine hydroxylase deficiency from Galicia region of Spain. Gene 521:100–104
15. Hamzehloei T, Hosseini SA, Vakili R, Mojarad M (2012) Mutation spectrum of the PAH gene in the PKU patients from Khorasan Razavi province of Iran. Gene 506:230–232
16. Bueno MA, Gonzalez-Lamuno D, Delgado-Pecellin C, Aldamiz-Echevarria L, Perez B, Desviat LR, Couce ML (2013) Molecular epidemiology and genotype-phenotype correlation in phenylketonuria patients from South Spain. J Hum Genet 58:279–284
17. Biglari A, Saffari F, Rashvand Z, Alizadeh S, Najafipour R, Sahmani M. Mutations of the phenylalanine hydroxylase gene in Iranian patients with phenylketonuria. Springer Plus. 2015 Dec 1; 4(1):542.
18. Huseynova L.S., Aghayeva S.A., Mamadova S.N., Mahmudova P.A. Molecular genetic studies of the diseases Duchenne muscular dystrophy, phenylketonuria and Familial Mediterranean Fever in the population of the Azerbaijan Republic. Poland. Sylwan. 163 (5)., 2019. p.2-11.
19. Huseynova L.S. Mutation Analysis of The Phenylalanine Hydroxylase Gene in Phenylketonuria Patients from Different Regions of Azerbaijan. Jurnal Biologi Indonesia. 2022. 18(1): 51-58