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GENETIC HETEROGENEITY OF HEREDITARY METABOLIC DISEASE PHENYLKETONURIA

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Abstract. Hereditary metabolic disorders are called diseases caused by mutations in the genes and representing numerous metabolic diseases. Metabolic diseases are the main cause of hereditary human diseases. Depending on the nature of the mutation occurring in the gene, the activity of the synthesized enzyme is completely or partially disrupted. Depending on the degree of impairment of enzyme activity, the conversion of the substrate to other substances is impaired. In most cases, there is an accumulation of pathogenic, toxic intermediate products in tissues and vital organs or a violation of the synthesis of compounds necessary for the normal development of the body. Inherited metabolic disorders are also called "congenital metabolic errors" or fermentopathies. Currently, this group includes about 2500 metabolic diseases. The term hereditary metabolic disorders was introduced at the beginning of the 20th century by a British physician, Sir Archibald Garod (1857-1936). Traditionally, hereditary metabolic disorders are mainly divided into diseases associated with impaired accumulation of sugars, amino acids or organic acids. Phenylketonuria and galactosemia are also metabolic diseases. With phenylketonuria, there is a violation of the metabolism of amino acids, and with galactosemia - sugars. Classical phenylketonuria is inherited in an autosomal recessive manner and results from a mutation of the phenylalanine hydroxylase (PAH) gene in the q22-q24 zone of the long arm of the 12th chromosome. The protein coding sequence consists of 13 exons, approximately 90 kb in length. In different world populations, the frequency and nature of mutations in the PAH gene have different genetic heterogeneity, which also affects the clinical manifestations, diagnosis and treatment of phenylketonuria.

Keywords: *PKU, PAH gene, protein, primer, exon, screening.*

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Most PAH missense mutations impair enzyme activity by causing increased protein instability and aggregation. Gjetting et al. (2001) described an alternative mechanism by which some PAH mutations may render phenylalanine hydroxylase defective. They used database searches to identify regions in the N-terminal domain of PAH with homology to the regulatory domain of prephenate dehydratase (PDH), the rate-limiting enzyme in the bacterial phenylalanine biosynthesis pathway. Naturally occurring N-terminal PAH mutations are distributed in a nonrandom pattern and cluster within residues 46-48 (amino acids GAL) and 65-69 (amino acids IESRP), 2 motifs highly conserved in PDH. To examine whether N-terminal PAH mutations affect the ability of PAH to bind phenylalanine at the regulatory domain, wildtype and 5 mutant forms (including G46S, A47Vand I65T) of the N-terminal domain (residues 2-120) of 612349 human PAH were expressed as fusion proteins in E. coli. Binding studies showed that the wild type form of this domain specifically binds phenylalanine, whereas all mutations abolished or significantly reduced this phenylalanine-binding capacity.

The data suggested that impairment of phenylalanine-mediated activation of PAH may be an important disease-causing mechanism of some N-terminal PAH mutations.

Most missense mutations found in PKU result in misfolding of the phenylalanine hydroxylase protein, increased protein turnover, and loss of enzymatic function. Pey et al. (2007) studied the prediction of the energetic impact on PAH native-state stability of 318 PKU-associated missense mutations, using the protein-design algorithm FoldX. For the 80 mutations for which expression analyses had been performed in eukaryotes, in most cases they found substantial overall correlation between the mutational energetic impact and both in vitro residual activities and patient metabolic phenotype. This finding confirmed that the decrease in protein stability is the main molecular pathogenic mechanism in PKU and the determinant for phenotypic outcome. Metabolic phenotypes had been shown to be better predicted than in vitro residual activities, probably because of greater stringency in the phenotyping process. All the remaining 238 PKU missense mutations compiled in the PAH locus knowledgebase (PAHvdb) were analyzed, and their phenotypic outcomes were predicted on the basis of the energetic impact provided by FoldX. Residues in exons 7-9 and in interdomain regions within the subunit appeared to play an important structural role and constitute hotspots for destabilization.

Phenylalanine hydroxylase (PAH) catalyzes the hydroxylation of phenylalanine to tyrosine, the rate-limiting step in phenylalanine catabolism. The reaction is dependent on tetrahydrobiopterin (BH4), as a cofactor, molecular oxygen, and iron. Phenylketonuria (PKU) is an autosomal recessive inborn error of metabolism resulting from a deficiency of PAH (Zurfluh *et al*., 2008).

Scriver (2007) stated that the PAH protein contains regulatory, catalytic, and tetramerization domains. They noted that the 452-amino acid monomer assembles to form functional dimeric and tetrameric forms of the enzyme.

Scriver (2007) stated that the PAH genomic sequence and its flanking regions span about 171 kb. The 5-prime UTR covers about 27 kb, and the 3-prime sequence downstream of the poly(A) site in exon 13 covers about 65 kb.

Using PAH enzyme assays, Lichter-Konecki et al. (1999) demonstrated enzymatic hydroxylation of phenylalanine to tyrosine in human liver and kidney lysates, with increasing tyrosine formation over time. The results indicated 40 to 45% as much enzymatic activity in kidney lysates as in liver lysates.

Zschocke et al. (1999) described a child in whom PKU was apparently caused by homozygosity for the E390G mutation in exon 11 of the PAH gene. However, the clinical severity of the disease was not as mild as expected, the mutation was not identified in the father despite confirmed paternity, and the paternal allele showed a highly unusual pattern of polymorphic markers in the PAH gene. The patient was found to have a large deletion involving exons 9, 10, and 11 of the PAH gene, and was thus a compound heterozygote, accounting for the more severe phenotype.

Corsello et al. (1999) determined the plasma amino acids in 48 Sicilian women with 1 or more microcephalic children. As a result, 2 families came to their attention. Unexpectedly, maternal phenylketonuria (PKU) in these 2 families was responsible for the microcephaly and was caused by untreated classic PKU rather than mild hyperphenylalaninemia. The mothers were mentally retarded, with blood phenylalanine levels more than 1,200 micromol/l. DNA studies demonstrated a pro407-to-leu (P407L) mutation due to a C-to-T transition at the second base of codon 407. The second family had a previously known mutation, R111. The parents of this mother (the grandparents of

the microcephalic child) were related as first cousins once removed; both were heterozygous for the R111X mutation.

For discussion of the deletion involving exons 9, 10, and 11 of the PAH gene that was found in compound heterozygous state in a patient with phenylketonuria (PKU) by Zschocke et al. (1999).

Kaufman (1999) described the derivation of a quantitative model of phenylalanine metabolism in humans. The model was based on the kinetic properties of pure recombinant human PAH and on estimates of the in vivo rates of phenylalanine transamination and protein degradation. Calculated values for the steady-state concentration of blood phenylalanine, rate of clearance of phenylalanine from the blood after an oral load of the amino acid, and dietary tolerance of phenylalanine all agreed with data from normal as well as from phenylketonuric patients and obligate heterozygotes. Kaufman (1999) suggested that these calculated values may help in the decision about the degree of restriction of phenylalanine intake that is necessary to achieve a satisfactory clinical outcome in patients with classic PKU and in those with milder forms of the disease.

Hillert et al. (2020) evaluated genotypes and metabolic phenotypes of patients with PKU from several databases, including PAHvdb, ClinVar, HGMD, and LOVD. Of 16,092 patients, 61.7% had classic PKU, 21.9% had mild PKU, and 16.4% had mild hyperphenylalaninemia. Of 16,196 patients, 72.9% were compound heterozygous and 27.1% were homozygous for PAH mutations. Of the mutations, 58.3% were missense, 13.9% were frameshift, and 13.1% were splicing. Most mutations (59.2%) were located in the central catalytic domain. The 3 most prevalent genotypes were homozygosity for R408W, found in 4.8% of patients; homozygosity for c.1066-11G-A, found in 2.6% of patients; and compound heterozygosity for R408W and IVS12+1G-A, found in 1.6% of patients.

Waters et al. (2000) characterized 4 PKU-associated PAH mutations that change an amino acid distant from the enzyme active site. Using 3 complementary in vitro protein expression systems and 3D structural localization, Waters et al. (2000) demonstrated a common mechanism, i.e., PAH protein folding is affected, causing altered oligomerization and accelerated proteolytic degradation, leading to reduced cellular levels of this cytosolic protein. Enzyme-specific activity and kinetic properties are not adversely affected, implying that the only way these mutations reduce enzyme activity within cells in vivo is by producing structural changes which provoke the cell to destroy the aberrant protein. The mutations were chosen because of their associations with a spectrum of in vivo hyperphenylalaninemia among patients. Waters et al. (2000) concluded that their in vitro data suggests that interindividual differences in cellular handling of the mutant but active PAH proteins contributes to the observed variability of phenotypic severity.

Using recombinant proteins expressed in E. coli, Gersting et al. (2008) characterized 10 BH4-responsive PAH mutations, including arg408 to trp (R408W) and tyr414 to cys (Y414C). Residual activity was generally high, but allostery was disturbed in almost all variants, suggesting altered protein conformation. This hypothesis was confirmed by reduced proteolytic stability, impaired tetramer assembly or aggregation, increased hydrophobicity, and accelerated thermal unfolding, which primarily affected the regulatory domain, in most variants. Three-dimensional modeling revealed that the misfolding was communicated throughout the protein. Gersting et al.

(2008) concluded that global conformational changes in PAH hinder the molecular motions essential for enzyme function.

Jung-KC et al. (2019) found that expression of different HPA-associated human PAH mutants in COS-7 cells correlated with expression of endogenous Dnajc12. Analysis of liver samples from HPA mice homozygous for the Pah val106-to-ala (V106A) mutation showed that expression of mutant Pah was not changed at the transcriptional level. Instead, the mutant Pah protein showed increased aggregation and degradation compared with wildtype. Further analysis demonstrated that mutant Pah interacted with Dnajc12, likely leading to its degradation through a ubiquitin-dependent pathway.

Guttler et al. (1999) reported findings from the maternal PKU collaborative study concerning genotype, biochemical phenotype, and cognitive performance in females with phenylalanine hydroxylase deficiency. PAH gene mutations were examined in 222 hyperphenylalaninemic females, with the discovery of a total of 84 different mutations, and complete genotype was obtained in 199 individuals. Based on previous knowledge about mutation-phenotype associations, 78 of the mutations could be assigned to 1 of 4 classes of severity: severe PKU, moderate PKU, mild PKU, and non-PKU mild hyperphenylalaninemia.

Benit et al. (1999) tested the activity of the mutant gene products from 11 PAH-deficient patients in a eukaryotic expression system. Two mutations, ala259 to val and leu333 to phe, markedly reduced PAH activity; 1 mutation, glu390 to gly, mildly altered the enzyme activity, and most of the mutant genotypes reduced the in vitro expression of PAH activity to 15 to 30% of controls. Comparing the predicted residual activity derived from expression studies to the clinical phenotypes of the PAH-deficient patients, Benit et al. (1999) found that homozygosity for the L333F/E390G mutations resulted in severe and mild PAH deficiencies, respectively, both in vivo and in vitro, while compound heterozygosity (L333F/E390G) resulted in an intermediate dietary tolerance. Similarly, in vitro expression studies largely predicted dietary tolerance in compound heterozygotes for other mutations. Taken together, these results supported the view that expression studies are useful in predicting residual enzyme activity and that the mutant genotype at the PAH locus is the major determinant of metabolic phenotype in hyperphenylalaninemias.

At least half of patients with phenylketonuria have a mild clinical phenotype. Muntau et al. (2002) explored the therapeutic efficacy of tetrahydrobiopterin for the treatment of mild phenylketonuria. Tetrahydrobiopterin significantly lowered blood phenylalanine levels in 27 of 31 patients with mild hyperphenylalaninemia (10 patients) or mild phenylketonuria (21 patients). Phenylalanine oxidation was significantly enhanced in 23 of these 31 patients. Conversely, none of the 7 patients with classic phenylketonuria had a response to tetrahydrobiopterin. Long-term treatment with tetrahydrobiopterin in 5 children increased daily phenylalanine tolerance, allowing them to discontinue their restricted diets. Seven mutations were classified as probably associated with responsiveness to tetrahydrobiopterin, including V245A and E390G. Six mutations were classified as potentially associated with responsiveness, including F39L, D415N, R158Q, and I65T. Four mutations were inconsistently associated with responsiveness, including Y414C, L48S, and R261Q. Mutations connected to tetrahydrobiopterin responsiveness were predominantly in the catalytic domain of the protein and were not directly involved in cofactor binding. Muntau et al.

(2002) concluded that responsiveness could not consistently be predicted on the basis of genotype, particularly in compound heterozygotes.

Lassker et al. (2002) reported 2 new patients with tetrahydrobiopterin-responsive PKU and compared their PAH genotypes to those of previous cases from the literature. These patients carried missense mutations in the PAH gene, confirming the suggestion of Erlandsen and Stevens (2001) that tetrahydrobiopterin-responsive patients are frequently carriers of missense mutations within the DNA region coding for the catalytic domain of the enzyme. Both patients showed no effect of tetrahydrobiopterin at 7.5 mg/kg/day on plasma phenylalanine levels in the newborn period, and the authors suggested that a normal neonatal tetrahydrobiopterin test does not necessarily exclude tetrahydrobiopterin responsiveness in all such patients.

Pey et al. (2004) analyzed the kinetics and cofactor binding properties of 7 mild PKU mutations, including I65T, P244L, R261Q, V388M, and Y414C. BH4 prevented degradation of the V388M and Y414C protein variants by acting as a chemical chaperone. In addition, in all the mutants, BH4 increased PAH activity and protected the protein from rapid inactivation. Pey et al. (2004) concluded that the response to BH4 substitution therapy by PKU mutations may have a multifactorial basis, involving chemical chaperone and protective effects.

Zurfluh et al. (2008) analyzed data on 315 patients with BH4-responsive PKU from a large PKU database. The average residual activity for 57 BH4-responsive mutations was 46.8%, and the most common variants included R261Q, Y414C, and V245A. Combined genotype data additional from other genetic databases and published reports yielded population-specific figures for the percentage of PKU patients predicted to be BH4 responders: 58% in Germany, 76% in Northern Ireland, 55% in South Korea, and 57% in northern China. The genotype-predicted prevalence figures were generally higher than data generated from BH4-loading test data.

Smith and Kang (2000) used the ENU-induced mouse model of PKU to study cerebral protein synthesis. They suggested that ultimately a more thorough understanding of the role of protein synthesis in the ability of the brain to grow and develop normally and to undergo plasticity will help in the understanding of the etiology of mental retardation in PKU and the formulation of new treatments.

Gersting et al. (2010) found that loss of function in Pah-enu1 mice was a consequence of misfolding, aggregation, and accelerated degradation of the enzyme. Tetrahydrobiopterin (BH4) attenuated this triad by conformational stabilization augmenting the effective PAH concentration, which led to rescue of the biochemical phenotype and enzyme function in vivo. Combined in vitro and in vivo analyses revealed a selective pharmaceutical action of BH4 confined to the pathologic metabolic state.

Tighe et al. (2003) stated that the R408W mutation in Europe arose by recurrent mutation and is associated with 2 major PAH haplotypes. R408W associated with the 2.3 haplotype exhibits a west-east cline of relative frequency reaching its maximum in the Balto-Slavic region, whereas R408W associated with the 1.8 haplotype exhibits an east-west cline peaking in Connacht, the most westerly province of Ireland. Spatial autocorrelation analysis demonstrated that the 2 clines are consistent with a pattern likely to have been established by human dispersal. Stojiljkovic et al. (2006) identified the R408W mutation in 18% of mutant alleles among 34 unrelated patients with PKU from Serbia and Montenegro. Gersting et al. (2008) stated that the R408W mutation occurs within the catalytic domain of PAH. Unlike wildtype recombinant PAH, which

formed tetramers when expressed in E. coli, PAH with the R408W mutation formed high-molecular-mass aggregates, indicative of severe distortion of the protein's oligomeric state.

Gersting et al. (2008) stated that the Y414C mutation occurs within the dimerization motif of the PAH oligomerization domain, which interacts with the catalytic domain of the same PAH subunit. They found that tetramerization of recombinant PAH with the Y414C mutation resembled that of the wildtype protein. The reduction in activity resulting from the Y414C mutation appeared to be due to a global conformational change in the protein that reduced allostery.

An A-to-T substitution at cDNA nucleotide 1197 of the PAH gene had been regarded as a silent mutation because both the wildtype (GUA) and the mutant (GUU) alleles encode a valine residue at codon 399. The nucleotide is located at the 3-prime end of exon 11 at position -3 of the exon-intron junction. Chao et al. (2001) demonstrated that skipping of exon 11 occurred with the allele containing the 1197A-T substitution. Thus, this mutation is not a neutral polymorphism but a mutation that induces posttranscriptional skipping of exon 11 leading to a PKU phenotype.

In patients with PKU from the Old Order Amish in Lancaster County, Pennsylvania, Wang et al. (2007) identified compound heterozygosity for 2 PAH mutations: R261Q and the 3-bp deletion at codon 94. The incidence of PKU in the Lancaster County Amish was 1 in 10,000, similar to that in other populations.

In PKU patients from the Old Order Amish in Geauga County, Ohio, Wang et al. (2007) found homozygosity for the splice site mutation in intron 10. The incidence of PKU in this group was estimated to be 1 in 1,000, much higher than in other populations.

Esfahani and Vallian (2019) found that this splice site mutation was the most common among 140 Iranian patients with PKU, with a frequency of 26.07%.

Stojiljkovic et al. (2006) found that the L48S mutation was the most common among 34 unrelated patients with PKU from Serbia and Montenegro, occurring in 21% of mutant alleles. This mutation was exclusively associated with the classical severe PKU phenotype, defined as having pretreatment plasma phenylalanine levels above 1200 micromol/liter.

Chen et al. (2002) studied a case of non-phenylketonuria hyperphenylalaninemia detected by a national newborn screening program in Taiwan. The paternally inherited allele harbored a de novo E76G mutation. The basal promoter and the mRNA processing were normal in the PAH allele inherited from the mother. However, a 3.7-kb deletion was identified in the 5-prime flanking region of the maternally inherited PAH allele. Characterization of the deleted sequence led to the identification of a novel liver-specific DNaseI hypersensitive site located 3.3 kb upstream of the RNA initiation site of the PAH gene. They showed that this site comprises a liver-specific enhancer with cAMP responsiveness. They further showed by mutation analysis that the enhancer carries a major hepatocyte nuclear factor-1 (HNF4A)-binding site important for the enhancer function but not for cAMP responsiveness. In transient transfection assays with a reporter gene, they demonstrated that a PAH plasmid construct carrying the deletion, designated as -4173_-407del, was severely impaired in phenylalanine hydroxylase transcriptional activity.

In a case of non-phenylketonuria hyperphenylalaninemia, Chen et al. (2002) found a de novo glu76-to-gly (E76G) substitution in the PAH protein. They

detected an A-to-G transition at position 227 of the patient's PAH cDNA. This mutation was found in compound heterozygosity with a 3.7-kb deletion.

The present study aimed to determine frequencies of mutations in the phenylalanine hydroxylase gene (PAH) in 30 patients diagnosed with phenylketonuria 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). The R261Q mutation in exon 7 was observed in seven patients from 30 patients (Aghayeva *et al*., 2018; Aliyeva *et al*., 2018; Qarayeva *et al*., 2020; Huseynova *et al*., 2019a; 2019b).

Identified mutations in the PAH gene and to evaluate the genetic heterogeneity of PKU disease in 30 Azerbaijanian patients who had been referred different regions of Azerbaijan. From this experiment, 7 of 30 PKU patients had the same mutation. Our analysis of the R261Q mutations was nearly similar to that observed in Southern region of Azerbaijan. 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 another 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. 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 glutamine amino acid. One of the families (G.M family) with identified inherited metabolic disease of phenylketonuria lives in Masally administrative area. Masally area itself is located in South-East of Azerbaijan Republic on the slopes of Talysh mountains in subtropical zone. Members of proband's family possess deficiency of glucose-6-phosphatedehydrogenase. Phenylketonuria gene has an identified R261G (G-A) mutation (Huseynova *et al*., 2020; Huseynova *et al.*, 2021). The study of erythrocyte enzyme preparation for family members has shown low electrophoretic mobility for G6PD which was unknown in the world studies. Homozygous form was identified in 4-year-old girl. Heterozygous form carriers were both parents and one sibling. 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 consanguineous parallel marriage type.Identified 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 (Huseynova *et al*., 2019a; Huseynova *et al*., 2020; Mammadov *et al*., 2019; Huseynova *et al*., 2021).

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.

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