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Molecular Genetics and Gene Therapy Aspects of Phenylalanine Hydroxylase (*PAH*) Related Hyperphenylalaninemias

Maria Grigoriadou[1], Kleopatra H Schulpis[2] and Haris Kokotas[1]*

[1]*Department of Genetics, Institute of Child Health, Athens, Greece*
[2]*Department of Inborn Errors of Metabolism, Institute of Child Health, Athens, Greece*

Hyperphenylalaninemias (HPAs; OMIM 261600) are related to phenylalanine hydroxylase (PAH; OMIM *612349) deficiency; a hepatic enzyme, and are characterized by moderate and/or high levels of the amino acid phenylalanine and reduction of tyrosine. High levels of phenylalanine and low of tyrosine characterize phenylketonuria (PKU; OMIM 261600) disease whereas moderately increased levels of phenylalanine and/or reduced or normal levels of tyrosine are usually measured in hyperphenylalaninemia patients [1].

Phenylketonuria is an autosomal recessive inborn error of metabolism resulting from a deficiency of PAH, an enzyme that catalyzes the hydroxylation of phenylalanine to tyrosine, the rate-limiting step in phenylalanine catabolism. Phenylketonuria has a mean frequency of 1:10,000 in Caucasian populations [1-3]. If undiagnosed and untreated, phenylketonuria can result in impaired postnatal cognitive development resulting from a neurotoxic effect of hyperphenylalaninemia [4]. Features other than mental retardation in untreated patients include a 'mousy' odor, light pigmentation, peculiarities of gait, stance, sitting posture, eczema and epilepsy [5].

Hyperphenylalaninemia refers to all those clinical conditions leading to abnormally high phenylalanine (Phe) levels. Usually, HPAs are induced by mutations in the *PAH* gene coding for phenylalanine hydroxylase. *PAH* requires tetrahydrobiopterin (BH_4) as co-factor. Mutations in those genes responsible for BH_4 biosynthesis or regeneration lead to about 2% of the HPAs. On the other hand, PKU refers specifically to those HPAs caused by mutations in the *PAH* gene, which are severe enough to require therapeutic intervention. Currently, more than 800 mutations have been described as resulting in PKU or its milder form - a mild hyperphenylalaninemia (www.biopku.org).

The *PAH* gene spans 90 kb and contains 13 exons [6,7]. 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. Phenylalanine hydroxylase catalyzes the hydroxylation of phenylalanine to tyrosine, and apart from BH_4 as a co-factor this reaction is dependent on molecular oxygen, and iron. Two isozymes of phenylalanine hydroxylase were reported to exist in human fetal liver [8]. Isozymes have also been reported in rat liver Pah [9]. Most of this variation is explainable by (i) purified enzyme contains different polymeric structures of a single subunit, i.e., trimers or tetramers; (ii) animals heterozygous for polymorphic variants in the *PAH* gene produce protein subunits with slightly different charge and electrophoretic migration; and (iii) post-translational modification. There is no evidence to support the involvement of more than one locus encoding the apoenzyme for PAH. A full-length cDNA encoding PAH from a human liver cDNA library has been isolated [10]. The predicted protein contains 452 amino acids and shares 96% homology with rat Pah. The PAH protein contains regulatory, catalytic, and tetramerization domains [11]. The 452-amino acid monomer assembles to form functional dimeric and tetrameric forms of the enzyme. The highest expression of a 2.5-kb PAH transcript has been detected by Northern blot analysis in human liver, followed by kidney, pancreas, and brain [12]. A 4.6-kb transcript was also detected in liver, kidney, and pancreas. RNase protection assays confirmed PAH expression in liver and kidney. RNA *in situ* hybridization revealed PAH expression in proximal convoluted tubules of adult and fetal kidney cortex and in the cerebral cortex of fetal brain. Immunohistochemical analysis confirmed expression of PAH protein in proximal convoluted kidney tubules.

Using a cDNA probe for human *PAH* to analyze human-mouse hybrid cells by Southern hybridization, it has been shown that the *PAH* gene is on chromosome 12 and presumably on the distal part of 12q [13]. By *in situ* hybridization, the assignment of the *PAH* gene was narrowed to chromosome 12q22-q24.1 [14].

Most *PAH* missense mutations impair enzyme activity by causing increased protein instability and aggregation. An alternative mechanism by which some *PAH* mutations may render phenylalanine hydroxylase defective has been described [15]. Binding studies showed that the wild-type form of the N-terminal domain of PAH 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. The prediction of the energetic impact on PAH native-state stability of 318 PKU-associated missense mutations, using the protein-design algorithm FoldX has been studied [16]. 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 (PAHdb) were analyzed, and their phenotypic outcomes were predicted on the basis of the

***Corresponding author:** Haris Kokotas, Department of Genetics, Institute of Child Health, Athens, 11527 Greece, Tel: +302132037333; Fax: +302107700111; E-mail: hkokotas@yahoo.gr

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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.

The ideal treatment of genetic diseases would consist of taking a normal copy of the defective gene and transferring it into the patient's cells, which should express it [17]. Since the *PAH* gene is expressed mainly in the liver, vectors derived from a recombinant retrovirus can efficiently transduce the *PAH* cDNA into PAH-deficient hepatocytes *in vitro*, but the transduction efficiency is low *in vivo* [18]. Beyond this barrier, studies have been initiated in which vectors derived from a recombinant adenovirus expressing *PAH* cDNA have been placed in the portal circulation of PAH-deficient mice. This approach allowed the restoration of 10 to 80% of hepatic PAH activity, normalizing the plasma phenylalanine levels. Antibodies against recombinant adenoviral vector are a great obstacle to this strategy [18,19]. However, it has been shown that the concurrent administration of an immunosuppressant blocking host immune response prolongs PAH gene expression, and promotes the reversal of hypopigmentation [20].

Recombinant adenovirus-associated vectors seem to be safer and more effective. They lead to minimal immune response and produce longer lasting therapeutic effects. This treatment was very satisfactory in male PKU mice, inducing a reduction of plasma Phe level from 1800 to 360 μM in two weeks. Unexpectedly, the treatment was less effective in females and further studies are needed to explain this difference [21,22].

In addition to conventional gene therapy, some studies with heterologous therapy (PAH expression in tissues other than liver) have been developed for PKU. According to this strategy, epidermal keratinocytes and dermal fibroblasts were engineered by transducing retroviral vectors expressing genes coding for PAH and GTP-cyclohydrolase (one of the enzymes involved in BH_4 synthesis), and high Phe clearance was obtained [23]. Moreover, it has been shown that concomitant overexpression of enzymes responsible for additional steps in Phe uptake and metabolism, such as LAT1 and 4F2hc subunits of the large neutral amino acid transporter and tyrosinase, also increases Phe transport into human keratinocytes improving its clearance [24]. PAH expression in erythrogenic bone marrow, T-lymphocytes and skeletal muscle has also been explored [25,26]. Skeletal muscle therapy seems promising, but co-expression of BH_4 biosynthesis genes is necessary [26,27].

Apart from the technical difficulties typically associated with gene therapy, some other problems emerge from specific aspects of the illness. Although most PKU mutations lead to loss of function, the protein is frequently present. Allele interactions in PKU are still poorly understood. It is predictable, that in some cases it would also be necessary to inactivate the abnormal alleles [17].

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