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Phenylalanine hydroxylase deficiency: Molecular epidemiology and predictable BH_4-responsiveness in South Portugal PKU patients

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ABSTRACT

Hyperphenylalaninemia (HPA, OMIM #261600), which includes phenylketonuria (PKU), is caused by mutations in the gene encoding phenylalanine hydroxylase (PAH), being already described more than 600 different mutations. Genotype–phenotype correlation is a useful tool to predict the metabolic phenotype, to establish the better tailored diet and, more recently, to assess the potential responsiveness to BH_4 therapy, a current theme on PKU field.

The aim of this study was the molecular analysis of the *PAH* gene, evaluation of genotype–phenotype relationships and prediction of BH_4-responsiveness in the HPA population living in South Portugal. We performed the molecular characterization of 83 HPA patients using genomic DNA extracted from peripheral blood samples or Guthrie cards. *PAH* mutations were scanned by PCR amplification of exons and related intronic boundaries, followed by direct sequence analysis. Intragenic polymorphisms were determined by PCR-RFLP analysis.

The results allowed the full characterization of 67 patients. The mutational spectrum encompasses 34 distinct mutations, being the most frequent IVS10nt-11G>A (14.6%), V388M (10.8%), R261Q (8.2%) and R270K (7.6%), which account for 46% of all mutant alleles. Moreover, 12 different haplotypes were identified and most mutations were associated with a single one. Notably, more than half of the 34 mutations belong to the group of more than 70 mutations already identified in BH_4-responsive patients, according to BIOPKU database. Fifty one different genotypic combinations were found, most of them in single patients and involving a BH_4-responsive mutation. In conclusion, a significant number (30–35%) of South Portugal PKU patients may potentially benefit from BH_4 therapy which, combined with a less strict diet, or eventually in special cases as monotherapy, may contribute to reduce nutritional deficiencies and minimize neurological and psychological dysfunctions.

1. Introduction

Hyperphenylalaninemia (HPA, OMIM #261600), which includes phenylketonuria (PKU), is the most common inborn error of amino acid metabolism with an average incidence among Caucasian and Oriental Asian populations of 1 per 10,00 newborns, while the mutant allele frequency in the population is polymorphic (~0.01). This pathology displays autosomal recessive inheritance and its cause is multifactorial: mutations in the gene encoding phenylalanine hydroxylase (PAH; EC 1.14.16.1) and exposition to dietary phenylalanine are both necessary and sufficient conditions to trigger it [1].

PAH is responsible for the conversion, in the presence of the cofactor tetrahydrobiopterin (BH_4) and dioxygen, of phenylalanine into tyrosine, which becomes an essential amino acid when the hydroxylating activity is absent or impaired. Then a simultaneous elevation in phenylalanine levels can be observed altogether with an impairment in tyrosine ones and both events contribute to the features characterizing the metabolic and clinical phenotypes displayed by the patients.

PKU and related HPA represent the paradigm of a genetic disease that can be treated. For the last 50 years, the traditional treatment has been a phenylalanine restricted diet for all lifelong with all the problems associated, namely nutritional deficits and socialization troubles, just to mention two different aspects [1–3]. However, some new approaches are being tried, as the use of large neutral amino acids and macroglycopeptide, as well as enzyme replacement therapy with phenylalanine ammonia lyase, but these therapeutic roads are not yet fully effective [3]. Additionally, more sophisticated approaches, like chaperone and gene

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therapies, are still far in the horizon. Recently, however, a new therapy is becoming widely used, the supplementation with pharmacological doses of the PAH cofactor, which can alleviate, and in special cases to avoid, the diet burden [4–7]. The molecular basis of BH_4 action is not fully understood, but the effect upon *PAH* gene expression was discarded. BH_4 can act as a chemical chaperone helping the stabilization of some mutant forms or, by increasing the cofactor concentration, it can promote the enzymatic activity of low-affinity mutants [8,9]. Accordingly, BH_4 seems to display a multifactorial mechanism of action.

PAH gene located in the long arm of chromosome 12 (12q24.1), covers ~100 kb of genomic DNA and is structured in 13 exons separated by introns. The messenger is 2448 bp long, being translated into 452 amino acid polypeptides, which are assembled onto functional homotetramers [1]. More than 600 different mutations have already been described (*PAH*db, www.pahdb.mcgill.ca), being the majority missense ones, and every population displays a mutational *spectrum* characterized by a reduced number of prevalent and public mutations and a large number of private pathogenic alterations. Moreover, each mutation impairs PAH activity in a specific manner and, as a consequence of most patients being compound heterozygotes, phenotypes range from the most severe form of classic PKU to the non-PKU hyperphenylalaninemia *status*. Moreover, several polymorphic markers (RFLP, VNTR and STR) have been defined within the *PAH* gene. These intragenic polymorphisms have proven to be a useful guide to mutation detection and, besides, have been extensively used in molecular anthropology studies to improve our understanding of the ancestral migratory movements which underlie the present geographic distribution of the most frequent *PAH* gene mutant alleles [10,11].

As PAH enzyme displays a hepatic expression and liver needle biopsies are no longer justified in PKU patients, the primary source of information concerning residual enzyme activity relies on *in vitro* expression and analysis of recombinant mutant proteins [12]. The data thus obtained is then used to estimate the patients' phenotype. Genotype–phenotype correlations are difficult to realize in most inherited metabolic disorders, but in PKU and related HPA they revealed to be a strong and reliable predictive tool [12,13]. Accordingly, the characterization of each patient genotype can greatly help to predict the metabolic phenotype, to establish the better tailored diet and, more recently, to assess the potential responsiveness to BH_4 therapy.

Herein is presented the molecular analysis of the *PAH* gene, the genotype–phenotype relationships evaluated and the rate of BH_4-responsiveness predicted in the HPA population living in South Portugal.

2. Materials and methods

2.1. Patients and phenotypic classification

Eighty-three patients, encompassing four pairs of siblings and all living in the Southern region of Portugal, were investigated. Most patients were detected by the newborn screening program, running in Portugal since 1979, and whose current cut-off value is 180 μM of phenylalanine. Diagnosis was confirmed after exclusion of BH_4 deficiency by evaluating urinary pterin levels and erythrocyte dihydropteridine reductase activity.

Metabolic phenotype was assigned to each patient according to pre-treatment blood phenylalanine concentrations and, when available, dietary tolerance at 5 years of age. Patients were classified as having classic PKU (pre-treatment Phe levels >1200 μM and Phe tolerance <20 mg/kg/day), moderate PKU (pre-treatment Phe levels between 900 and 1200 μM and Phe tolerance between 20 and 25 mg/kg/day), mild PKU (pre-treatment Phe levels between 600 and 900 μM and Phe tolerance above 25 mg/kg/day) and non-PKU HPA if they keep their Phe levels below 600 μM on a free diet.

This study was approved by the local Ethics Committee and informed consents were obtained from the patients or from their parents, who were also enrolled in the study.

2.2. Genotype analysis

Genomic DNA was isolated from peripheral blood samples or from Guthrie cards according to a salting-out procedure (Puregene Cell and Tissue kit, Gentra Systems, Minneapolis, MN, USA). After PCR amplification of individual exons and related intronic boundaries, *PAH* gene (GenBank accession no. AF404777) was scanned for mutations by direct sequence analysis, using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.3. Haplotype analysis

Mini-haplotypes were established after PCR-RFLP analysis of the intragenic bi-allelic polymorphisms *Bgl*II, *Pvu*IIa, *Pvu*IIb, *Msp*I and *Xmn*I and of the multi-allelic VNTR system at *Hind*III site at the 3′ region of *PAH* gene [14]. Haplotype numbering followed the rules of Eisensmith and Woo [15].

2.4. Calculation of homozigosity (*j*)

Homozigosity (*j*) at the *PAH locus* in a given population is determined by $j = \sum x_i^2$, where x_i is the frequency of the *i*th allele. In our population, where ascertainment of mutations was not 100%, each of the uncharacterized alleles was defined as having a frequency of 1/N, where N is the total number of mutant chromosomes investigated.

2.5. Relative residual PAH activity and genotype–phenotype correlation

Relative residual PAH activity was calculated from data compiled from *PAH*db which displays values calculated from *in vitro* expression of recombinant mutant proteins. PAH activity is defined as the average sum of activities of both individual mutant alleles, and expressed as the percentage of the wild-type enzyme.

3. Results

3.1. Mutation analysis

This study involved the molecular characterization of 83 HPA patients living in South Portugal, corresponding to 158 mutant alleles. The results revealed a mutational *spectrum* encompassing 34 distinct mutations which were distributed along the *PAH* gene sequence (Table 1). Most mutations were nucleotide substitutions corresponding to 27 missense (79.6%), 3 nonsense (8.8%) and 2 at splicing sites (5.8%); additionally, two deletions were found. Two mutations displayed a relative frequency >10% (IVS10nt-11G>A and p.V388M); a group of five mutations had a frequency between 3.8 and 8.2% (p.R261Q, p.R270K, p.P281L, p.I65T and p.R158Q); another group of seventeen mutations had a frequency in the range 1.3–2.5% and the remaining eight mutations were present in only one mutant allele (0.6% each). The majority of the mutations were situated in the catalytic domain (85%), while two were located in the regulatory domain (6%) and three in the tetramerization domain (9%).

A detection rate of 89.9% was achieved, with complete genotyping of 67 patients, while in the remaining sixteen individuals only one causative mutation was identified. Among the fully genotyped patients, only 14 (20.9%) were homozygous, the majority displaying compound heterozigosity for two different mutations. Among the 14 homozygous patients, three individuals carried the IVS10nt-11G>A mutation, two individuals carried the p.R261Q mutation and another two the p.R270K allele. The remaining seven patients harbored p.I65T, p.R176X, p.E280K, p.P281L, p.L311P, p.T323del and p.V388M mutations in homozigosity. Moreover, it was interesting to note the wide array of genotypes (Table 2). Among the 67 fully genotyped patients we could observe 51