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Hyperphenylalaninemia in the Czech Republic: Genotype–phenotype correlations and *in silico* analysis of novel missense mutations

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ABSTRACT

Background: Hyperphenylalaninemia (HPA) is one of the most common inherited metabolic disorders caused by deficiency of the enzyme phenylalanine hydroxylase (PAH). HPA is associated with mutations in the *PAH* gene, which leads to reduced protein stability and/or impaired catalytic function. Currently, almost 700 different disease-causing mutations have been described. The impact of mutations on enzyme activity varies ranging from classical PKU, mild PKU, to non-PKU HPA phenotype.
Methods: We provide results of molecular genetic diagnostics of 665 Czech unrelated HPA patients, structural analysis of missense mutations associated with classical PKU and non-PKU HPA phenotype, and prediction of effects of 6 newly discovered HPA missense mutations using bioinformatic approaches and Molecular Dynamics simulations.
Results: Ninety-eight different types of mutations were indentified. Thirteen of these were novel (6 missense, 2 nonsense, 1 splicing, and 4 small gene rearrangements). Structural analysis revealed that classical PKU mutations are more non-conservative compared to non-PKU HPA mutations and that specific sequence and structural characteristics of a mutation might be critical when distinguishing between non-PKU HPA and classical PKU mutations. The greatest impact was predicted for the p.(Phe263Ser) mutation while other novel mutations p.(Asn167Tyr), p.(Thr200Asn), p.(Asp229Gly), p.(Leu358Phe), and p.(Ile406Met) were found to be less deleterious.

1. Introduction

Hyperphenylalaninemia (HPA, MIM# 261600) is the most common inborn error of amino acid (AA) metabolism in Europeans. HPA is transmitted by the autosomal recessive mode of inheritance caused by mutations of the phenylalanine hydroxylase (PAH) gene. In the liver, PAH metabolizes L-phenylalanine (Phe) to L-tyrosine (Tyr) using (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH4) as cofactor. Failure in this conversion leads to an increase of Phe in the body fluids and severe mental retardation unless Phe intake is restricted. Almost 700 different disease-causing mutations in the *PAH* gene have been identified and can be found in the mutation databases PAH and HGMD (http://www.pahdb.mcgill.ca/, http://www.hgmd.cf.ac.uk/ac/index.php), most of which are point mutations scattered throughout the whole *PAH* gene. Mutations vary in their impact on enzyme activity, causing a range of clinical phenotypes. HPA patients can be classified based on their off-diet diagnostic plasma Phe levels as classical PKU (Phe above 1200 μmol/L), mild PKU (Phe between 600 and 1200 μmol/L) or non-PKU HPA (Phe between 120 and 600 μmol/L) [1].

In a subset of HPA patients, Phe concentration is manageable with pharmacological doses of BH4 with either limited or no dietary restriction [2–5]. For patients whose disease responds to BH4, cofactor therapy is an attractive means to increase patient compliance. A number of studies have documented that HPA patients with milder phenotypes are more likely to benefit from BH4 therapy [4–8]. Mechanism of BH4 responsiveness is multifactorial [3]. Current data suggest the most common mechanism by which BH4 rescues PAH function is by acting as a pharmacological chaperone promoting proper enzyme folding, which consequently reduces enzyme degradation [9–11]. About 75% of PAH mutations characterized by high residual activity have been found to be associated with BH4 responsiveness both *in vitro* [12–14] and *in vivo* [4,15]. For this reason, *PAH* genotyping has utility not only in disease categorization (classical PKU, mild PKU, non-PKU HPA) but also

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in predicting the potential for response to cofactor therapy [16,17]. However, there are also reports showing inconsistencies in correlation between BH4 responsiveness and genotype [16,17].

The *PAH* gene, mapped on 12q23.2, consists of 13 exons encompassing 171 kb. The full-length *PAH* cDNA encodes a protein of about 52 kDa (452 AAs) that is assembled as a homotetramer in the mature form. Each monomer consists of three functional domains: an N-terminal regulatory domain (residues 1–142); a catalytic domain (residues 143–410) that includes binding sites for Fe^{3+} ion, which is reduced to the active Fe^{2+} form upon binding of cofactor; and a C-terminal oligomerization domain (residues 411–452) with dimerization (residues 411–426) and tetramerization motifs (residues 427–452) [18]. It is now established that in most HPA cases, the loss of PAH function is due to structural distortion leading to decreased stability [10,19,20], folding deficiency [21], and/or increased susceptibility toward aggregation and degradation [10,22] of PAH mutant proteins. The availability of the crystal structures of PAH [18,23] makes it possible to model missense mutations and their effects on the protein structure [24]. Missense mutations affecting protein structure and stability are referred to as "structural" and are frequently associated with change of AA size, charge, polarity, and more importantly, with a loss of structure-maintaining contacts such as stacking interaction and H-bonding.

In this study, we present results of molecular genetic diagnostics of 665 unrelated Czech HPA patients, genotype-phenotype correlations, and results of the BH4-loading test in selected patients. Further, using bioinformatic approaches and molecular modelling, we carried out structural analysis of selected HPA missense mutations associated predominantly with the PKU or non-PKU HPA phenotype and explored the pathogenicity of six novel HPA missense mutations discovered in our patients. In addition, we utilized widely used mutation prediction tools, such as Polyphen-2 [25], SIFT [26], SNPs3D [27] and FOLDX [28] in order to predict the impact of novel missense mutations.

2. Materials and methods

2.1. DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes by the standard salting-out method and amplified. Primers for amplification of all exons and adjacent intron sequences and the conditions of particular PCRs are available by request. PCR products were sequenced directly using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and analyzed on an ABI 3130Xl Genetic Analyzer (Applied Biosystems). The resulting sequences were compared with the *PAH* NCBI reference sequence (NG_008690.1). All novel missense mutations were analyzed on a control panel consisting of DNA from 200 healthy Czech individuals. Multiple Ligation dependent Probe Amplification (MLPA) was performed using the SALSA P055 PAH MLPA kit (MCR Holland). This kit contained 25 sets of probes; 13 were PAH specific, and the others were control standards from other human genes. In detail, the assay is described in our previous study [29] as well as long-range PCRs for detailed characterization of deletions detected by MLPA. The nomenclature of mutations was implemented according to the current HGVS recommendations (http://www.hgvs.org/mutnomen/).

2.2. BH4-loading test

The BH4 test was performed at two workplaces: 1) the Department of Pediatrics of the Faculty of Medicine, Masaryk University and the University Hospital Brno and 2) the Department of Children and Adolescents of the Third Faculty of Medicine, Charles University and the Faculty Hospital Kralovske Vinohrady, after obtaining informed consent from all participants or their parents. Four days before BH4 loading and during the entire testing period patients were recommended to relax the diet and consume Phe-rich food. A single dose of 20 mg/kg BH4 (Kuvan™, sapropterindihydrochloride) was administered orally after night fasting [7]. In the workplace 1, blood samples for Phe analysis were collected 0 (Phe 0), 4 (Phe 4), 8 (Phe 8), and 24 (Phe 24) hours after administration of BH4. In the workplace 2, blood samples for Phe analysis were collected 0 (Phe 0), 8 (Phe 8), 16 (Phe 16), and 24 (Phe 24) hours after administration of BH4, in some patients the second dose of BH4 was applied and blood samples was collected 32 (Phe 32) and 48 (Phe 48) hours after administration of the first BH4 dose. BH4 responsiveness was defined as a decrease in blood Phe by 30% or more during 24 h. Patients were evaluated as responders (R, reduction of blood Phe by 30% or more during 24 h), late-responders (LR, reduction of blood Phe below 30% during 24 h but this was changed after the second BH4 dose), and non-responders (NR, reduction of blood Phe below 30%).

2.3. Building of 3D protein structures of wild type and mutant PAH

The 3D model of PAH containing the complete catalytic (C) and tetramerization (T) domains was built based on the X-ray structures of truncated forms of human PAH (pdb codes: 1PAH [30] and 2PAH [30]). Based on superposition of these structures in the VMD program [31], we obtained a model comprising residues 117–452. The missing regulatory (R) domain was built using homology modelling with the Modeler program [32] taking the X-ray structure of the truncated form of rat PAH (pdb code: 1PHZ [23]) with R and C domains as a template. A model comprising residues 19–452 was then obtained by superimposing the homology model of human R and C domains and the structure with complete C and T domains over the C domain in VMD. Subsequently, we cut from this model a large portion of the helix Tα1 (α1-helix in the T domain), which participates in oligomerization. We expected this helix to be dynamic in Molecular Dynamic (MD) simulations without the other monomer subunits which could result in formation of incorrect contacts and structural perturbations. Thus, the final model subjected to the mutational analysis and MD simulations consisted only of residues 19–432. The model does not contain Fe^{3+} ion because modelling of divalent/trivalent cations suffers from insufficient parameterization [33]. Since the model was built based on X-ray structures lacking hydrogen atoms, we added these using the Xleap module of Amber 10 [34] which is a package of programs for MD simulations of nucleic acids and proteins. The protonation states of all histidines in the protein were set to allow formation of proper H-bonds. Hence, histidines 146 and 290 were δ protonated. The structure of mutants N167Y, T200N, D229G, F263S, L358F, and I406M carrying mutations p.(Asn167Tyr), p.(Thr200Asn), p.(Asp229Gly), p.(Phe263Ser), p.(Leu358Phe), and p.(Ile406Met), respectively, were generated by truncating the side chains in the wild type structure and building new side chains using the Xleap module.

2.4. Structural analysis of missense mutations

We evaluated 9 sequence and structure features for selected HPA missense mutations associated predominantly with PKU or non-PKU HPA phenotype, and for 6 novel HPA missense mutations discovered in our patients. These parameters characterize the mutations, i.e. they reveal possible structural or functional defects, which indicates the potential causality of a mutation [35,36].

Evaluated sequence and structure features: *1) Formation of specific side chain contacts* (H-bonds, salt bridges, stacking interactions) of AAs in the wild type (wt) structure (based on visual inspection of the 3D model of PAH using the VMD program). Loss of these contacts upon a mutation often results in destabilization of protein folding. *2) Occurrence of AAs on the inner surface of the active site* located in the cavity of the catalytic domain (based on visual inspection of the 3D model of PAH using the VMD program). Replacement of AAs at this site often results in impairment of the catalytic function of a protein. *3) Buriedness of*