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Molecular genetics and impact of residual *in vitro* phenylalanine hydroxylase activity on tetrahydrobiopterin responsiveness in Turkish PKU population

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ABSTRACT

Background: The prevalence of phenylalanine hydroxylase (PAH)-deficient phenylketonuria (PKU) in Turkey is high (1 in 6500 births), but data concerning the genotype distribution and impact of the genotype on tetrahydrobiopterin (BH_4) therapy are scarce.
Objective: To characterize the phenotypic and genotypic variability in the Turkish PKU population and to correlate it with physiological response to BH_4 challenge.
Methods: We genotyped 588 hyperphenylalaninemic patients and performed a BH_4 loading test (20 mg/kg bw) in 462 patients. Residual PAH activity of mutant proteins was calculated from available *in vitro* expression data. Data were tabulated in the BIOPKU database (www.biopku.org).
Results: Eighty-eight mutations were observed, the most common missense mutations being the splice variant c.1066-11G>A (24.6%). Twenty novel mutations were detected (11 missense, 4 splice-site, and 5 deletion/insertions). Two mutations were observed in 540/588 patients (91.8%) but in 9 patients atypical genotypes with >2 mutations were found (8 with p.R155H *in cis* with another variant) and in 19 patients mutations were found in BH_4-metabolizing genes. The most common genotype was c.1066-11G>A/c.1066-11G>A (15.5%). Approximately 22% of patients responded to BH_4 challenge. A substantial *in vitro* residual activity (average >25% of the wild-type enzyme) was associated with response to BH_4. In homozygous genotypes ($n=206$), both severity of the phenotype ($r=0.83$) and residual PAH activity ($r=0.85$) correlate with BH_4 responsiveness.
Conclusion: Together with the BH_4 challenge, these data enable the genotype-based classification of BH_4 responsiveness and document importance of residual PAH activity. This first report of a large-scale genotype assessment in a population of Turkish PKU patients also documents a high prevalence (47%) of the severe classic phenotype.

1. Introduction

Phenylketonuria (PKU; OMIM# 261600) is an autosomal recessive disorder associated with deficient hepatic phenylalanine hydroxylase (PAH) activity [1]. PAH converts phenylalanine (Phe) to tyrosine in the presence of the essential cofactor tetrahydrobiopterin (BH_4), molecular oxygen, and Fe^{2+}. BH_4 is synthesized from guanosine triphosphate (GTP) in a biosynthetic pathway including the enzymes GTP cyclohydrolase I (GTPCH; gene *GCH1*), 6-pyruvoyl-tetrahydropterin synthase (PTPS; gene *PTS*), and sepiapterin reductase (SR; gene *SPR*). The oxidized cofactor is regenerated in two enzymatic steps involving pterin-4a-carbinolamine dehydratase (PCD; gene *PCBD1*) and dihydropteridine reductase (DHPR; gene *QDPR*) [2]. Mutations in genes coding for PAH and BH_4-metabolizing enzymes result in hyperphenylalaninemia (HPA) [3]. *SPR* deficiency and autosomal dominant *GCH1* deficiency present without HPA [4]. BH_4 deficiencies are more severe than PKU, and in addition to HPA present with catecholamines and serotonin deficiency [5].

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The overall prevalence of PKU in Europe and the United States is about 1 in 10,000 live births. Higher disease incidence is observed in cultures where consanguinity is practiced (e.g., Turkey, Saudi Arabia, or Gaza; ca. 1 in 3500–6500); however, in regions such as Finland the incidence is low (1 in>100,000). Prevalence of BH_4 deficiencies is about 1–2% of all HPAs [6].

Late-diagnosed, untreated PKU leads to severe neurological impairment including mental retardation, microcephaly, autistic behavior, eczema, and seizures [7], particularly in the most severe forms of PAH deficiency, "classic PKU" (blood Phe concentrations >1200 μmol/L). Less severe forms include mild PKU (blood Phe concentrations 600–1200 μmol/L) and mild HPA without any clinical findings (blood Phe concentrations <600 μmol/L). Hyperphenylalaninemic patients are identified through prospective newborn screening and follow-on diagnostic procedures will identify the defective gene, enabling early initiation of appropriate therapy [8]. Not every HPA patient is routinely tested for DNA mutations.

The observation that serum Phe concentration may be controlled in a subset of PKU patients through oral administration of synthetic 6R-BH_4 [9] and reports of a relatively high incidence (20–30%) of BH_4 responsiveness [10,11] provided an alternative to the traditional low Phe diet [14]. A number of studies documented that PAH-deficient patients with mild to moderate phenotypes are more likely to benefit from BH_4 therapy [10–14]. In some patients Phe concentration may be controlled with BH_4 monotherapy; however, others require a combination of BH_4 and dietary restrictions to maintain blood Phe in the therapeutic range while increasing daily Phe tolerance [15–18]. Mechanisms of BH_4 responsiveness are multifactorial [19]. Current data suggest the most common mechanism by which BH_4 rescues PAH function is by acting as a pharmacological chaperone promoting proper enzyme folding, which in turn reduces enzyme degradation and inactivation [20,21].

The *PAH*db (www.pahdb.mcgill.ca/) has cataloged over 500 mutations in the *PAH* gene [22], while the BIOPKU database (http://www.bh4.org/BH4DatabasesBiopku.asp) describes an approximately equal number of *PAH* genotypes and their association with BH_4 response [23]. A systematic review of PKU in Europe identified 29 mutations that may be regarded as prevalent in European populations [24], but there are very few reports on the molecular basis of PKU in Turkey [25,26].

Herein are presented *PAH* genotypes of 588 Turkish PKU patients where 88 mutations were identified; among these are 20 novel mutations. Data from oral BH_4 challenge in 462 patients are reported. Comparisons are made relating BH_4 response with the genotype, residual *in vitro* PAH activity, and disease phenotype. The results extend the knowledge of the genotypic PKU variation in the Turkish PKU population and document a high prevalence of classical PKU (47%), a relatively high proportion (22%) of potential candidates for the BH_4 therapy, and the common occurrence of BH_4 deficiencies (2.4%) within this study.

2. Patients and methods

2.1. Patients and samples

A total of 588 hyperphenylalaninemic patients were investigated. At the time of diagnosis, 165 patients presented with mild HPA (blood Phe <600 μmol/L), 130 with mild PKU (blood Phe 600–1200 μmol/L), and 274 patients presented with classic PKU (blood Phe >1200 μmol/L). Nineteen patients with BH4 deficiencies presented with a variable range of blood Phe (9 mild HPA, 7 mild PKU, 3 classic PKU). Forty-six percent of patients were the offspring of consanguineous mating (Table 1); however, an even higher percentage (48.7%) displayed mutation homozygosity, suggesting inbreeding (Suppl. Table 1). Nine pedigrees, where >2 mutations were identified, are included in this study.

Table 1
Consanguinity in Turkish PKU patients investigated in this study.

Related marriage	Number of families	%
No consanguinity	221	43.1
No consanguinity, but parents from same village	57	11.1
1st grade cousin	149	29.0
1.5 grade cousin	10	1.9
2 grade cousin	36	7.0
2.5 grade cousin	2	0.4
3 grade cousin	27	5.3
3.5 grade cousin	2	0.4
4 grade cousin	9	1.8

The majority of patients (~75%) were identified through prospective newborn screening, while the remainders were identified by selective screening. Blood specimens were collected on filter paper cards by finger or heel prick, and all tests were performed within routine clinical and biochemical investigation and in accordance with local regulations. Blood phenylalanine was measured using a fluorometric method until 2003, and tandem mass spectrometry was used afterwards. The first confirmatory quantitative phenylalanine was performed during clinical assessment when the child was provided a normal diet. Informed consent for genotype assessment was obtained from all subjects. The University of Utah Institutional Review Board approved the plan to receive de-identified specimens for assessment of the PAH gene and genes of the BH_4 synthesis/recycling pathways.

2.2. Loading test with BH_4

A single-dose BH_4 challenge (20 mg/kg body weight) was performed on 462 PKU patients (81%) (Schircks Laboratories, Switzerland). Three different protocols were used: A) Prior to 1999, a partially active formulation of BH_4, containing a mixture of the active R enantiomer and inactive S enantiomer (66.6% 6R-BH_4 and 33.3% 6 S-BH_4), was used to challenge 166 patients. Thus a 20 mg tablet contained 13.3 mg of biologically active BH_4. In this subset, serum Phe was monitored over 8 h. B) A fully active formulation of BH4 (6R-BH_4) was utilized post-1999. Among the 296 patients challenged, Phe was monitored over an 8-h period (0, 4, and 8 h) in 104 patients and C) over a 24-h period (0, 4, 8, and 24 h) in 192 patients. Data from patients whose plasma Phe concentration was monitored over 24 h were used for genotype–phenotype correlation and determination of residual PAH activity. In all BH_4 challenge protocols, response was defined as a sustained reduction of blood Phe concentration by ≥30% from the pre-challenge baseline [27].

2.3. Assessment of the PAH, PTS, and QDPR genes

DNA was prepared from dried blood on filter paper as previously described [28]. The *PAH* gene was assessed utilizing a previously described system involving high-resolution melt profiling and follow-on DNA sequencing of regions displaying aberrant melting profiles [29,30]. DNA sequence data were analyzed using Mutation Surveyor software (Softgenetics, State College, PA, USA). The protocols utilized in assessment of *PTS* and *QDPR* also involved high-resolution melt profiling and follow-on DNA sequencing of regions displaying aberrant melting profiles. The specifics of these assessments will be included in a separate study.

In several instances, PAH-deficient patients were identified with >2 mutations in the *PAH* gene. When family participation could be recruited in such cases, blood samples were obtained from parents and other first-degree relatives for performance of pedigree studies to determine the *cis*/*trans* relationship between the mutations.