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Genotype-predicted tetrahydrobiopterin (BH_4)-responsiveness and molecular genetics in Croatian patients with phenylalanine hydroxylase (PAH) deficiency

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ABSTRACT

Specific mutations in the gene encoding phenylalanine hydroxylase (*PAH*), located on chromosome 12q22-24.1, are linked to tetrahydrobiopterin (BH_4; sapropterin)-responsive phenylketonuria (PKU). Diagnosis is usually done through the newborn screening for PKU, followed by a BH_4 loading test. So far, more than 60 mutant alleles, presenting with a substantial residual PAH activity (average ~47%), were identified in more than 500 patients worldwide. We investigated the predictive value of BH_4-responsive *PAH* mutations in Croatian population. From a group of 127 PKU patients, 62 were selected (based on the genotype) as potentially BH_4-responsive and 39 loaded with BH_4 (20 mg/kg). The overall frequency of BH_4-responsiveness (>30% blood phenylalanine reduction within 24 h) was 36% (14 out of 39 patients with 23 different genotypes), significantly less than expected. The best responders were patients with mild hyperphenylalaninemia (4/4; 100%), followed by mild PKU (8/9; 89%), and classical PKU (2/26; 8%). The most common BH_4-responsive genotypes were p.E390G/p.R408W and p.P281L/p.E390G. These genotypes correspond for approximately >30% residual PAH activity. The p.E390G mutation was 100% associated with BH_4-responsiveness, regardless of the second allele (p.R408W, p.P281L, p.F55Lfs, p.L249P). With regard to the predicted relative PAH activity of recombinantly expressed mutant alleles, there was a significant ($p < 0.002$) difference between BH_4-responders and non-responders.

In a general Croatian PKU population, disease-causing mutations were identified on 226 alleles (99%). There were 35 different mutations: 21 missense, 8 splice site, 3 nonsense, 2 single nucleotide deletions, and 1 in-frame deletion. Four mutations are reported for the first time: p.E76D, p.L333P, p.G346E, and IVS8-2A > G. Five mutations accounted for over two-thirds of investigated alleles: p.L48S, p.R261Q, p.P281L, p.E390G, and p.R408W. Thus, the Croatian PKU population seems to be more homogenous than some other Mediterranean or Central European populations.

This study reveals the importance of a full genotype for the prediction of BH_4-responsiveness. In contrast to previous assumption and with exception of the p.E390G mutation, single allele mutations are not reliable for the selection of potential PKU candidates for pharmacological therapy with BH_4.

Introduction

Phenylketonuria (PKU; OMIM #261600) is an autosomal recessive metabolic disease caused by hepatic phenylalanine hydroxylase (PAH; EC 1.14.16.1) deficiency [1]. Over 500 different mutations, identified on *PAH* gene, are responsible for a large spectrum of clinical phenotypes [2], from mild hyperphenylalaninemia (MHP), a variant that does not require treatment, to classical PKU that leads to severe neurological impairment when untreated. Although phenylalanine restriction has been the mainstay of successful dietary treatment since 1953 when first initiated [3], it imposes a substantial burden on individuals with PKU and the family. This synthetic, highly restrictive diet is associated with a risk of nutritional deficiencies and phenylalanine control, despite good compliance, is sometimes difficult to achieve. However, compliance is often poor, particularly as individuals reach adolescence [4]. Moreover, there is information on poor phenylalanine control before and during pregnancy in women with PKU, which can adversely influence fetal health [5]. Hence there is a need for an alternative treatment of PKU.

BH_4, a catalytic cofactor for PAH, has been shown to activate residual PAH activity and partially restore oxidative Phe

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metabolism in a substantial number of PKU patients. Although this finding was suggested many years ago [6,7], not much attention has been paid to this issue until 1999 when Kure et al. [8] reported patients with PAH deficiency who had responded to oral BH_4 intake by lowering their blood Phe levels. Since then, an increasing number of BH_4-responsive PAH-deficient patients has been reported [9–22]. Continued treatment with BH_4 in responding patients has been shown to increase Phe tolerance, reduce or eliminate the need for Phe-free protein supplements or even to completely replace the diet [23–29]. One of the greatest issues still remains how to identify BH_4-responsive individuals in a large and heterogeneous pool of PAH-deficient patients.

BH_4 loading test result depends on many methodological factors such as preload plasma Phe level, the patient's age at test (newborn vs. older) [12,18,30–32], Phe intake during the test, amount of administered BH_4 and dosage scheme, cut-off levels of Phe reduction and duration of BH_4 test. An optimized 48-h BH_4 loading protocol, with two BH_4 administrations (20 mg/kg/d) on two consecutive days and with four blood samplings (T_0, T_8, T_{16}, and T_{24}) after BH_4 administration, has been proposed for this reason [33]. Nevertheless, one study reported a significant number (>50%) of initially positive BH_4 responders (Phe reduction > 30% in short-term loading test lasting up to 24 h) who did not respond to long-term BH_4 treatment [27]. Also, there are several reports on patients with no significant response to single-dose loading test, but with a marked decrease in plasma Phe after several days of BH_4 administration [12,34]. These inconsistencies stress the need for an additional approach to evaluation of BH_4-responsiveness.

Specific mutations in the *PAH* gene, many of them characterized by substantial residual activity when recombinantly expressed in different cell systems, are repeatedly found to be associated with BH_4-responsiveness [10,35,36]. This is in accordance with data from BH_4 loading tests indicating an incidence of BH_4-responsiveness of >80% in mild variants of PKU patients with an overall incidence of >40% in general PKU population [37]. Up to 10% of classical PKU patients respond in BH_4 loading test (with a usual 30% cut-off in blood Phe reduction) and they are a more difficult target to properly evaluate BH_4-responsiveness. This is because some severe PKU patients had responded to BH_4 by lowering Phe levels for 20%, which was defined as a significant response for this phenotype. Thus, Fiege and Blau [30] propose to modify the cut-off level for BH_4-responsiveness accordingly to the patient's clinical phenotype. However, there is no accurate correlation between genotype and BH_4-responsiveness, still with many reported responding inconsistencies within the same genotype [36]. So far, mutational analysis provides useful information on potential non-responders comprising two null mutations but the prediction of BH_4 responders remains incomplete [37].

The aim of our study was to provide more information on predictive value of genotype for BH_4-responsiveness and to summarize the mutation spectrum of the *PAH* gene in Croatian PKU population. We initially suggested that the presence of a mutation with *in vitro* substantial residual activity, compared with the wild type enzyme, on at least one *PAH* gene copy would be sufficient for BH_4-responsiveness.

Patients and methods

Patients

From a group of 127 patients diagnosed with hyperphenylalaninemia (HPA) (the highest blood phenylalanine 300–3630 μmol/L) from Croatia in whom *PAH* gene mutation analysis had been done, 39 patients were included in BH_4 loading test. Although we selected 62 patients, only 39 individuals accepted to perform the BH_4 loading test. In four families two sibs were included. Selection criteria were only based on genotype. Inclusion criteria were: (a) presence of at least one BH_4-responsive mutation; or (b) presence of at least one mutation termed as unclear in correlation to BH_4-responsiveness; or (c) presence of at least one mutation with so far unknown response to BH_4. A mutation was classified as BH_4-responsive if it was present either in homozygous or functional heterozygous form in BH_4 responders from data in different publications (for further explanation on definition of BH_4-responsive or unclear mutations see Zurflüh et al. [37]). Patients with two null mutations (with no residual activity) were excluded from the study. For mutation classification in relation to BH_4-responsiveness and for additional information on *PAH* gene mutations we used data from BIOPKU database (www.bh4.org/BH4DatabasesBiopku.asp) and a locus-specific knowledgebase PAHdb (www.pahdb.mcgill.ca). There was an almost equal distribution between females (19/39) and males (20/39) (age ranged 1–24 years; mean 11 years) entering BH_4 trial. BH_4 deficiency was excluded in all patients by measuring urinary pterins and dried blood dihydropteridine reductase activity. Patients were assigned to one of the three phenotype categories according to the highest plasma Phe concentration before introducing the diet or after protein loading test (180 mg/kg/d of Phe intake over 5 days): 4/39 patients (10%) were classified as MHP (phenylalanine levels $\leqslant$600 μmol/L), 9/39 patients (23%) were assigned to mild PKU (Phe levels 601–1200 μmol/L) and 26/39 patients (67%) to classical PKU (Phe levels >1200 μmol/L).

BH_4 loading test

BH_4 loading was performed at Department of Pediatrics, University Hospital Center Zagreb, after obtaining an informed consent from all participants or their parents including the approval of the institutional ethics committee. Three or four days before BH_4 loading (classical PKU vs. milder forms) and during the entire testing period patients had no dietary restrictions, moreover, they were encouraged to consume Phe-rich food. BH_4 (6R-BH_4 dihydrochloride; Schricks Laboratories, Jona, Switzerland) was administered orally to all patients as a single dose of 20 mg/kg body weight. Blood was collected just before BH_4 administration (T_0), and 8 (T_8), 24 (T_{24}), and 48 h (T_{48}) after the loading. We simplified the criteria suggested by Fiege et al. [17] to define BH_4-responders as follows: "responder", reduction of blood Phe by $\geqslant$30% within 24 h and "slow responder", reduction of blood Phe by <20% at T_8, and $\geqslant$20% but <30% at T_{24}. One patient was classified as "not clear" with the reduction of blood Phe by $\geqslant$30% at T_8, and <20% at T_{24}. No side effects were observed during the BH_4 loading test. Phe and BH_4 were measured from dried blood spots; Phe was analyzed using tandem-mass spectrometry and BH_4 was measured according to the method previously published [38].

Mutational analysis

One hundred and fourteen families with HPA (127 patients), all but four patients detected by Guthrie test within neonatal screening program, were enrolled in a comprehensive analysis of *PAH* gene mutations in Croatia in the last 17 years. According to the previously mentioned criteria, 78% of patients suffer from classical PKU, 14% from mild PKU whilst MHP phenotype is present in only 8%. Analyzed patients comprise 78% of total Croatian PKU population. Patients and/or parents signed informed consent for mutational analysis. Genotyping was performed as follows: DNA was isolated from dried blood spots using the QIAamp DNA Micro Kit (Qiagen). PCR was performed using Hot FirePol DNA Polymerase (Solis Biodyne) and standard thermal cycling, i.e., 15 min denaturation at 95 °C followed by 37 cycles of 30 s at 95 °C, 45 s at 56 °C,