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RESEARCH REPORT

Molecular Genetics and Genotype-Based Estimation of BH4-Responsiveness in Serbian PKU Patients: Spotlight on Phenotypic Implications of p.L48S

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Abstract Phenylketonuria (PKU) is caused by mutations in the gene encoding phenylalanine hydroxylase (PAH) enzyme. Here, we report the updated spectrum of *PAH* mutations in 61 Serbian PKU patients. By using both DGGE/DNA sequencing and PCR-RFLP, we identified 26 disease-causing mutations (detection rate 99%). The most frequent ones were p.L48S (31%), p.R408W (16.4%), p.P281L (6%), p.E390G (5.2%), and p.I306V (5.2%). Homozygosity value indicated high heterogeneity of Serbian population.

To overcome possible pitfalls of patients' phenotypic classification, we used two parameters: pretreatment/maximal phenylalanine blood concentration and Phe tolerance. The two phenotypes did not match only for patients with p.L48S. Therefore, we used Mann-Whitney statistical test to compare pretreatment/maximal blood Phe concentration and Phe tolerance detected in patients with p.[L48S];[null] and p.[missense];[null] genotypes. For patients with p.L48S, our results implied that Phe tolerance is a better parameter for phenotypic classification. Also, Fisher's exact test was used to compare p.L48S effect on phenotype of homozygous and functionally hemizygous patients. Our findings showed that effect of p.L48S was altered in functional hemizygotes. Moreover, phenotypic inconsistency found in homozygotes suggested that interallelic complementation and/or additional factors play a role in genotype-phenotype correlation.

Since BH4-supplementation therapy is not available in Serbia, we made the first estimation of its potential benefit based on patients' genotypes. In the analyzed cohort, the total frequency of BH4-responsive mutations was 52.6%. Furthermore, we found a significant number of genotypes (26.2% BH4-responsive and 51% probably BH4-responsive) that may respond to BH4 therapy. This led us to a conclusion that BH4-supplementation therapy could bring benefit to Serbian PKU patients.

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Introduction

Phenylketonuria (PKU, MIM#261600) is the most severe form of hyperphenylalaninemia (HPA). It is a metabolic pathology that represents the paradigm of hereditary disease that can be treated. With the average incidence of 1 per 10,000 newborns, PKU is considered to be the most common inborn error of amino acid metabolism in Caucasians and one of the most studied rare diseases (Scriver et al. 2008).

Hyperphenylalaninemias are a result of deficient hepatic enzyme, phenylalanine hydroxylase (PAH, EC 1.14.16.1). PAH is responsible for the conversion of phenylalanine (Phe) into tyrosine, in the presence of the molecular oxygen and cofactor tetrahydrobiopterin (BH4). If this metabolic pathway is impaired, tyrosine becomes an essential amino acid

while the concentration of phenylalanine rises above normal. Deficiency of the PAH enzyme is caused by mutations in the phenylalanine hydroxylase gene (*PAH*, GenBank accession no. AF404777). *PAH* gene is mapped to human chromosome 12q24.1, it spans approximately 90 kb and contains 13 exons and 12 large introns (Scriver 2007).

PKU is a monogenic disease which is transmitted in an autosomal recessive pattern and the two mutations found in the patient's genotype stand as the main determinant of PKU phenotype (Kayaalp et al. 1997; Guldberg et al. 1998). The knowledge about mutations in the *PAH* gene and their effect on the phenotype is the best example of nutrigenetics – a new scientific discipline with a goal to develop an optimal diet based on genotype analysis (Guttler and Guldberg 2000; Fenech et al. 2011). Nevertheless, the PKU phenotype is not simple and there are numerous factors (genetic and nongenetic) that contribute to its complexity (Scriver and Waters 1999; Dipple and McCabe 2000).

More than 600 different mutations have been identified in *PAH* gene and recorded in the literature and the *PAH* locus knowledgebase (Scriver et al. 2003, http://www.pahdb.mcgill.ca). Depending on mutation type (missense, nonsense, splice site, small or large insertions and deletions) and position (regulatory, catalytic, or tetramerization domain), the effect of a mutation on the structure and activity of the PAH varies greatly. As a consequence, the activity of mutant protein ranges from 0% to almost 100% compared to normal PAH enzyme (Waters et al. 1998). Correspondingly, hyperphenylalaninemic phenotypes range from mild hyperphenylalaninemia (MHP) that does not require treatment to a classic PKU characterized by severe mental retardation and epilepsy in the absence of treatment. Furthermore, the effect of some mutations on PAH activity and PKU phenotype is steady and consistent through numerous in vitro and in vivo studies performed in different populations, while for others its effect is inconsistent and unpredictable. While mutations with a consistent effect enable accurate prediction of PKU phenotype based on genotype, PKU phenotype becomes more complex in case of mutations with an inconsistent effect.

For more than two decades, efforts had been made to fully understand the effect of mutations on PKU phenotype. However, there is ongoing need to further analyze mutations in genotype-phenotype correlation studies, particularly in the group of patients of identical genotype and genetic background.

Recently, a new therapeutic approach is becoming a valid option for the dietary treatment of PKU patients. It relies on the observation that pharmacological doses of the tetrahydrobiopterin (BH4), a natural cofactor of PAH enzyme, can lower blood phenylalanine concentration (Kure et al. 1999). Molecular mechanism of BH4 action is multifactorial, but mainly based on its chaperon-like activity which results in the stabilization of altered conformations of PAH enzyme. By binding to an unstable enzyme, BH4 protects it from degradation, prolongs its half-life and enables it to perform conversion of phenylalanine to tyrosine. (Erlandsen et al. 2004; Pey et al. 2004; Pérez et al. 2005). The BH4-supplementation therapy (Kuvan) can be used to loosen or even replace burdensome dietary treatment of PKU patients (Levy et al. 2007a; Trefz et al. 2009b). However, not all PKU patients are BH4 responsive, meaning that at the molecular level, not all mutated proteins benefit from the increased concentration of BH4. In order to understand which mutations are good candidates, many studies analyzed the enzyme activity of mutated enzymes in vitro in the presence of BH4 precursors (Kim et al. 2006; Aguado et al. 2007). Also, it was shown that PAH function and response to BH4 administration result from interplay between genotype, metabolic state, and cofactor concentration (Staudigl et al. 2011). Several studies analyzed the correlation of genotype and patient's responsiveness to BH4 and made an attempt to predict the BH4-responsiveness on the basis of genotype (Zurflüh et al. 2008; Karacic et al. 2009; Rivera et al. 2011; Sterl et al. 2012). In the Kuvan era, genotypization of patients became important, not only because of the definitive diagnosis and prediction of the optimal diet, but also to point out those patients that could benefit from new therapeutic approach.

BH4 loading test is a definitive diagnostic test for cofactor sensibility. However, estimation based on genotyping answers an important question in countries where BH4 is not an approved drug: Is the frequency of BH4 responsive mutations in a population high enough to consider giving opportunity to patients for a new therapeutic method?

The first study on Serbian PKU population was performed 6 years ago (Stojiljkovic et al. 2006). Here, we presented molecular and phenotypic characteristics of enlarged cohort of Serbian PKU patients with focus on phenotypic effect of the most frequent mutation, p.L48S (c.143T>C). We discussed possible pitfalls of phenotypic classification of PKU patients and genotype clustering that could influence genotype-phenotype correlation studies. Also, we assessed the potential benefit from BH4-supplementation therapy in Serbia.

Subjects and Methods

Patients and Phenotypic Classification

In this study, 61 patients with hyperphenylalaninemia from Serbia (comprising three pairs of siblings) were included. Also, 10 parent samples were analyzed in order to facilitate

and clarify mutation detection of a patient or to explain phenotypic status of a mother. Neonatal screening was established in Central Serbia in 1982, and in 2003 it was expanded to Vojvodina (Serbian Northern province). According to regional agreement, it was recommended to start with dietary regiment if the pretreatment phenylalanine level exceeded 360 μmol/l. Although most patients included in this study were detected by the newborn screening program, 16 children were diagnosed later in life during evaluation of psychomotor retardation or by routine measurement of blood Phe level at the Mother and Child Healthcare Institute "Dr Vukan Cupic" in Belgrade.

Clinical data collected include: pretreatment and maximal phenylalanine blood concentration, phenylalanine tolerance, IQ, age at the diagnosis, the application of low Phe diet, and other relevant data. The phenylalnine tolerance is the highest amount of Phe from food that a patient can tolerate to keep blood Phe concentrations in the safe range (120–360 μmol/l) (Guldberg et al. 1998). It is possible to reliably assess Phe tolerance already at the age of 2 years (van Spronsen et al. 2009).

In this study, patients were classified into three categories according to pretreatment/maximal phenylalanine blood level and according to phenylalanine tolerance: classical PKU (pretreatment Phe >1200 μmol/l; Phe tolerance <20 mg/kg/day – equivalent 250–350 mg/day), mild PKU (pretreatment Phe 600–1200 μmol/l; Phe tolerance 20–25 mg/kg/day – equivalent 350–600 mg/day), and MHP (pretreatment Phe <600 μmol/l; Phe tolerance >25 mg/kg/day – equivalent >600 mg/day) (Trefz et al. 1985; Guldberg et al. 1998). The pretreatment level is a quantitative blood/serum Phe measurement under standardized condition. Phe tolerance was determined in cooperation with the patient/parents or during hospitalization. Maximal Phe level is the highest pretreatment blood Phe concentration (μmol/L) or the highest Phe in the loading Phe test (180 mg/kg/day) if performed.

This study was approved by the Ethics Committee at the Mother and Child Healthcare Institute "Dr Vukan Cupic" and all parents and/or patients gave informed consent. They were further referred to the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, for molecular genetic analysis.

Molecular Genetic Analysis

Genomic DNA was extracted from peripheral blood by QIAamp DNA Blood Mini Kit. Mutations were identified by multiplex "broad range" denaturing-gradient gel electrophoresis (DGGE) (Guldberg and Guttler 1994) followed by DNA sequencing (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). When necessary, PCR-RFLP or PCR-ACRS analyses for several common point mutations (p.L48S (c.143T>C), p.R408W (c.1222C>T), p.R261Q (c.782G>A), p.R261X (c.781C>T), IVS10-11G>A (c.1066-11G>A), and p.R158Q (c.473G>A)) were also performed (Eiken et al. 1993).

We used referent sequence (GenBank accession no. AF404777) to identify changes in *PAH* gene and HGVS guidelines for nomenclature of genetic variants. Also, we used the *PAH* locus knowledgebase (PAHdb, http://www.pahdb.mcgill.ca) and literature found on PubMed to determine if a mutation has already been reported.

Results

Genotyping

This study included molecular characterization of 61 Serbian patients with hyperphenylalaninemia. Given that the cohort included three pairs of siblings, we analyzed the total number of 116 unrelated mutant alleles. On 113 mutant alleles we identified one disease-causing mutation, and, interestingly, on two unrelated mutant alleles we detected two disease-causing mutations. Since 115 out of 116 *PAH* alleles were characterized, the mutation detection rate in this study was 99%. High diagnostic efficiency was achieved by combining PCR-RFLP and DGGE/DNA sequencing analysis of *PAH* gene. Additionally, our genetic analysis established PKU diagnosis for two patients' mothers that have not been diagnosed before.

In this cohort of Serbian patients, we identified 26 different disease-causing mutations. Seven of them were not reported in the previous study on Serbian population. The frequency of the most typical Serbian mutation, p.L48S (c.143T>C), is 31%. Other significant mutations had the following frequencies: 16.4% of p.R408W (c.1222C>T), 6% of p.P281L (c.842C>T), 5.2% of p.E390G (c.1169A>G), and 5.2% of p.I306V (c.916A>G). These five mutations accounted for almost two thirds of all mutant alleles in the Serbian population. The remaining mutations are listed in Table 1. Calculated homozygosity value was expectedly low (0.14), confirming the mutational heterogeneity of *PAH* locus for Serbian population.

Genotypic homozygosity was observed in 12 unrelated patients for the following mutations: p.L48S, p.R111X, p.R243X, p.P281L, and p.R408W. Among them, there were 8 [p.L48S];[p.L48S] genotypes. Interestingly, homozygosity for p.I306V mutation was detected in two additional cases. However, presence of the third disease-causing mutation was also confirmed in these patients' genotypes: p.[R261Q; I306V]; [I306V] and p.[R408W; I306V]; [I306V].

Table 1 Spectrum and frequency of mutations causing PKU in Serbia

Mutations					
Trivial name	Systematic name	Exon	Number of chromosomes	Relative frequency (%)	Type of mutation
p.S16fs	c.47_48delCT	1	1	0.9	Frameshift
p.Q20X	c.58C > T	1	2	1.7	Nonsense
p.L48S	c.143T > C	2	36	31.0	Missense
p.R111X	c.331C > T	3	3	2.6	Nonsense
p.R158Q	c.473G > A	5	4	3.4	Missense
p.V177L	c.529G > C	6	3	2.6	Missense
p.V177M	c.529G > A	6	1	0.9	Missense
p.L213P	c.638T > C	6	1	0.9	Missense
p.P225T	c.673C > A	6	3	2.6	Missense
p.S231F	c.692C > T	6	1	0.9	Missense
p.R243X	c.727C > T	7	2	1.7	Nonsense
p.R252Q	c.755G > A	7	1	0.9	Missense
p.R261Q	c.782G > A	7	4	3.4	Missense
p.R261X	c.781C > T	7	2	1.7	Nonsense
p.P281L	c.842C > T	7	7	6.0	Missense
p.R297H	c.890G > A	8	1	0.9	Missense
p.I306V	c.916A > G	9	6	5.2	Missense
IVS10 + 3A > G	c.1065 + 3A > G	*	1	0.9	Splice site
IVS10-11G > A	c.1066-11G > A	*	2	1.7	Splice site
p.E390G	c.1169A > G	11	6	5.2	Missense
p.A403V	c.1208C > T	12	2	1.7	Missense
p.R408W	c.1222C > T	12	19	16.4	Missense
p.R413P	c.1238G > C	12	1	0.9	Missense
p.Y414C	c.1241A > G	12	2	1.7	Missense
p.P416Q	c.1247C > A	12	1	0.9	Missense
IVS12 + 1G > A	c.1315 + 1G > A	*	3	2.6	Splice site
Unspecified			1	0.9	
Total			116		

* Mutations affecting splice junction, located in flanking intron regions

PKU mutations were variously combined into 39 different genotypes, among which 33 genotypes were not present in more than one family. The relative frequency of unrelated genotypes that included p.L48S mutation was 48% (28/58).

Phenotypic Characterization

All PKU patients were assigned to phenotypic category according to pretreatment/maximal blood Phe concentration level. The analyzed cohort included 34 classical PKU (58.6%), 14 mild PKU (24.2%), and 10 MHP (17.2%) patients.

In the majority of cases, phenylalanine tolerance was used as an additional parameter for classification of PKU patients. We compared phenotype categories based on two different parameters and found that patients without p.L48S in the genotype had matching categories according to both parameters. However, for almost 20% of patients carrying p.L48S, phenotype category based on pretreatment/maximal blood Phe concentration was more severe than the phenotype obtained by Phe tolerance. In order to further analyze observed discrepancies, we listed detailed phenotype characteristics and other relevant data (e.g., start and compliance to the low Phe diet) for homozygous and heterozygous p.L48S patients (Table 2).

Furthermore, we used Mann-Whitney test to compare pretreatment/maximal blood Phe concentration and Phe tolerance for p.[L48S];[null] and p.[missense];[null] genotypes. We have found that patients with p.[L48S];[null] genotype had significantly higher pretreatment Phe concentrations ($p = 0.001$).

Table 2 Phenotypic characteristics of homozygous and heterozygous p.L48S patients

Patient number	Genotype	Max Phe levels (μmol/l)	Phe tolerance (mg/day)	Phenotype		IQ	Other data
				Max Phe levels	Phe tolerance		
17[1]	p.[L48S];[S231F]	>1200	230	cPKU	cPKU	20	LD
18[1]	p.[L48S];[S231F]	>1200	240	cPKU	cPKU	24	LD
10	p.[L48S];[P225T]	>1200	280	cPKU	cPKU	100	DBNS
5	p.[L48S];[I306V]	>1200	280	cPKU	cPKU	110	DBNS
9	p.[L48S];[R158Q]	>1200	280	cPKU	cPKU	85	DBNS
6	p.[L48S];[R158Q]	>1200	300	cPKU	cPKU	87,5	DBNS, BDC
21	p.[L48S];[R158Q]	>1200	300	cPKU	cPKU	90	DBNS
22	p.[L48S];[Q20X]	>1200	300	cPKU	cPKU	45	LD, LPD at the age of 3 years
2	p.[L48S];[R261Q]	>1200	300	cPKU	cPKU	50	LD, LPD at the age of 4 months, seizures
15	p.[L48S];[R408W]	>1200	300	cPKU	cPKU	84	DBNS, PCD
24	p.[L48S];[R408W]	>1200	300	cPKU	cPKU	97,5	DBNS
25	p.[L48S];[R408W]	>1200	340	cPKU	cPKU	100	DBNS
4	p.[L48S];[R408W]	>1200	350	cPKU	cPKU	102,5	DBNS
11	p.[L48S];[R408W]	>1200	350	cPKU	cPKU	105	DBNS
12	p.[L48S];[R408W]	>1200	500	**cPKU**	**mPKU**	110	LD, LPD, from 22 months to 5 years
7	p.[L48S];[P281L]	>1200	350	cPKU	cPKU	60	LD, LPD at the age of 20 months
1	p.[L48S];[P281L]	>1200	400	**cPKU**	**mPKU**	59	LD, LPD at the age of 6 years
23	p.[L48S];[L48S]	>1200	310	cPKU	cPKU	77,5	LD, LPD at the age of 1 year
8	p.[L48S];[L48S]	>1200	330	cPKU	cPKU	80	DBNS, BDC
19	p.[L48S];[L48S]	>1200	>600	**cPKU**	**MHP**	84	LD, never on LPD
20	p.[L48S];[L48S]	>1200	>600	**cPKU**	**MHP**	82,5	LD, never on LPD
13[2]	p.[L48S];[L48S]	1200	450	mPKU	mPKU	115	DBNS, LPD at the age of 11 months
14[2]	p.[L48S];[L48S]	1200	450	mPKU	mPKU	110	DBNS, LPD at the age of 11 months
3	p.[L48S];[L48S]	1200	450	mPKU	mPKU	85	DBNS, refused LPD
26	p.[L48S];[L48S]	900	550	mPKU	mPKU	100	DBNS never on LPD
16	p.[L48S];[V177L]	600	500	**MHP**	**mPKU**	95	DBNS
27	p.[L48S];[V177M]	300	/	MHP	/	100	DBNS

Maximal Phe level is the highest Phe pretreatment blood concentration (μmol/L) or the highest Phe in the loading Phe test (180 mg/kg/day) if performed; cPKU – classical PKU; mPKU – moderate PKU; MHP – mild hyperphenylalaninemia; DBNS – diagnosis by neonatal screening; LD – late diagnosis; LPD – low Phe diet; PCD – poorly control diet; where it was not stated differently, LPD was introduced at the age of 1 month; patients 24–27 are under 2 years of age and Phe tolerance value could be revised later; two pairs of siblings are indicated by superscripts 1 and 2; differences between two phenotype categories based on different parameters are in bold

Genotype-Phenotype Correlation Study

Genotype-phenotype correlation study was performed for patients homozygous for the mutation or functionally hemizygous patients (the mutant allele is combined with null mutation and therefore acts on its own). Nonsense, frameshift, and splice site mutations, as well as missense mutations which resulted in zero enzyme activity in vitro, were considered as null mutations (Kayaalp et al. 1997; Guldberg et al. 1998). Patients with three mutations in the genotype were not taken into account.

The most frequent mutation, p.L48S, is known to have an inconsistent phenotypic outcome (Guldberg et al. 1998). To avoid the effect attributable to interallelic interaction, we separately analyzed genotype-phenotype correlation for 8 p.[L48S];[L48S] and 11 p.[L48S];[null] patients. Also, we analyzed genotype-phenotype correlation when phenotypes were classified according to pretreatment blood Phe concentration and according to Phe tolerance. We used Fisher's exact test to compare the effect of p.L48S on phenotype in homozygous versus functionally hemizygous patients. We found that distribution of phenotypes significantly varied between two genotype groups ($p = 0.018$ and $p = 0.039$, respectively). Interestingly, when functionally hemizygous p.L48S genotypes were correlated with phenotypes determined according to pretreatment blood Phe concentration, these genotypes were associated with classical PKU in 100% cases. On the other hand, p.L48S homozygotes were associated with all three phenotype categories determined according to Phe tolerance.

We also analyzed the effect of several other missense mutations found in functional hemizygotes. We found that p.P225T, p.R261Q, and p.R413P mutations were associated with classical PKU, p.V177L and p.R297H with mild PKU, while p.I306V and p.A403V were associated with MHP. The inconsistency was noticed for p.E390G mutation which was found in patients with one classical PKU, three mild PKU, and one MHP phenotype.

BH4 Responsiveness

The p.L48S mutation as well as seven others (p.R158Q, p.R261Q, p.I306V, p.E390G, p.A403V, p.R413P, and p.Y414C) were characterized as BH4-responsive ones in previous European studies (Zurflüh et al. 2008; Trefz et al. 2009a; Karacic et al. 2009). Accordingly, the sum of relative frequencies of BH4-responsive mutations for Serbian population is 52.6%.

However, some mutations have the status of consistently BH4-responsive while others are considered as inconsistently responsive. According to the classification given in Sterl et al. (2012), we classified all 39 Serbian *PAH* genotypes into BH4 responsive (26.2%), probably BH4 responsive (51%), and non-BH4 responsive (22.8%) (Table 3). Furthermore, we made distinction between genotypes with one and two BH4-responsive alleles.

Discussion

This is an update study on molecular genetics of Serbian hyperphenylalaninemic patients and the first study that estimates potential of BH4-supplementation therapy based on patients' genotypes. Furthermore, we conducted genotype-phenotype correlations to analyze phenotypic effect of mutations.

Unlike recent studies conducted in European PKU patients, all mutations identified in this cohort of patients have already been reported in the literature (Sterl et al. 2012; Groselj et al. 2012). However, the mutational spectrum was enlarged with seven mutations that were not reported in the previous study on Serbian population. Furthermore, five mutations accounted for almost two thirds of all mutant alleles and the homozygosity value was low. All these findings indicated rather high heterogeneity of Serbian population. As it was stated previously, genetics of *PAH* locus is concordant with historically documented migrations across the Western Balkans (Stojiljkovic et al. 2006).

Mutation p.L48S reassured its status as the most frequent in Serbia. Interestingly, its relative frequency (31%) is the highest ever reported in any population. This mutation was initially detected in a Turkish patient with phenylketonuria (Konecki et al. 1991). Since then it has been identified in many European populations usually with a frequency of 2–5% (Zschocke 2003). Higher frequencies have been found in Croatian (10%), Italian (9.7%), and Turkish (7%) populations which are geographically and historically interconnected with the Serbian population (Giannattasio et al. 2001; Karacic et al. 2009; Dobrowolski et al. 2011). In the preliminary haplotype study of patients with p.L48S, it was shown that the mutation was imported to Serbia from populations with different genetic backgrounds (Stojiljkovic et al. 2007).

Mutation p.R408W remains the second frequent Serbian mutation. It is the most prevalent mutation worldwide, which is associated with two different haplotypes and in Slavic populations it follows the cline distribution from Baltic to Mediterranean countries (Zschocke 2003). Since its frequency is 84% in Estonia, 46% in Slovakia, and 36% in Croatia, detected frequency in Serbia (16.4%) fits into the established gradient distribution. Mutations p.P281L and p.E390G are relatively common in Balkans, but rare in other European populations. Thus, p.P281L was found on 10% alleles in Greece, 8.4% in Turkey, 8% in Croatia, and 6% in Serbia, while p.E390G accounted for 7% of alleles in Croatia, 5.2% in Serbia, and 4.1% in Turkey (Zschocke

Table 3 Genotype-based estimation of BH4-responsiveness in Serbian PKU patients

BH4 responsive		Probably BH4 responsive		Non-BH4 responsive
R + IR	R + N	IR + IR	IR + N	N + N
26.2%		51%		22.8%
7%	19.2%	21%	30%	22.8%
p.[I306V];[L48S] (2)	p.[I306V];[R261X] (1)	p.[L48S];[L48S] (8)	p.[L48S];[Q20X] (1)	p.[R408W];[V177L] (1)
p.[I306V];[R261Q] (1)	p.[I306V];[R408W] (1)	p.[L48S];[R158Q] (3)	p.[L48S];[V177L] (1)	p.[R408W];[P225T] (1)
p.[E390G];[R158Q] (1)	p.[I306V];[P416Q] (1)	p.[L48S];[R261Q] (1)	p.[L48S];[V177M] (1)	p.[R408W];[R252Q] (1)
	p.[E390G];[R261X] (1)		p.[L48S];[P225T] (1)	p.[R408W]; [IVS10 + 3A > G] (1)
	p.[E390G];[P281L] (1)		p.[L48S];[S231F] (1)	p.[R408W];[R408W] (1)
	p.[E390G];[R408W] (3)		p.[L48S];[P281L] (2)	p.[P281L];[P225T] (1)
	p.[A403V];[R408W] (1)		p.[L48S];[R408W] (7)	p.[P281L];[P281L] (1)
	p.[Y414C];[IVS10- 11G > A] (1)		p.[R261Q];[L15fs] (1)	p.[P281L]; [IVS12 + 1G > A] (1)
	p.[Y414C];[L213P] (1)		p.[R261Q]; [IVS12 + 1G > A] (1)	p.[R111X];[R111X] (1)
			p.[R413P];[R408W] (1)	p.[R111X];[IVS10- 11G > A] (1)
				p.[V177L];[Q20X] (1)
				p.[R243X];[R243X] (1)
				p.[R297H]; [IVS12 + 1G > A] (1)

PAH mutations are designated as: R – responsive, IR – inconsistently responsive, and N – nonresponsive/unknown to be responsive according to BH4 responsiveness reported in previous studies (Muntau et al. 2002; Zurflüh et al. 2008; Trefz et al. 2009a; Karacic et al. 2009; Sterl et al. 2012) and according to BIOPKU db. Between parentheses is the number of the same genotypes detected in Serbian PKU patients. Genotype with one unidentified mutation was excluded from the table

2003; Karacic et al. 2009; Dobrowolski et al. 2011). Although mentioned frequencies are quite similar, we could observe gradient from southeast to northwest for p.P281L and the inverse for p.E390G.

Interestingly, we found two unrelated patients with three PKU mutations. Although it is rarely found that a PKU patient carries three mutations, our finding highlights the need to screen the complete *PAH* gene in order to accurately define PKU genotype. It is possible to imagine that incomplete genotyping could also lead to inaccurate conclusions regarding genotype-phenotype correlation.

Genotype-Phenotype Inconsistencies

Analysis of mutation's effect, based on genotype-phenotype correlation, could encounter a problem of inadequate phenotype classification. Up to date, two distinct parameters have been widely used for phenotype classification. The first parameter, pretreatment blood Phe level was quite helpful in the past and was convenient because it was known for every diagnosed patient. However, this parameter corresponded mostly to the screening Phe level. The second one, phenylalanine tolerance, depicts more realistically the ability of a patient to metabolize Phe from the food. Unfortunately, there are no universal guidelines which parameter should be used for classification of HPA patients. In order to overcome possible pitfalls of patients' phenotypic classification, we used both parameters.

Among the most frequent mutations in this study, we found two null mutations (p.R408W and p.P281L) and two missense mutations (p.L48S and p.E390G) known to have inconsistent phenotypic effect. Therefore, Serbian cohort was suitable and interesting for investigation of genotype-phenotype correlation in homozygous and functionally hemizygous patients. Given that the inconsistency noticed for p.E390G mutation was found in a small number of patients, we further focused on p.L48S mutation. It is worth noting that other missense mutations, analyzed in genotype-phenotype correlation, showed consistent effects which were already known in the literature.

Our results from Mann-Whitney statistical test for patients carrying p.L48S implied that categorization based on Phe tolerance instead of pretreatment blood Phe concentration is particularly important for patients carrying

p.L48S mutation. Furthermore, Fishers's exact test suggested that the effect of p.L48S mutation is more reliably determined in the correlation of homozygous genotypes and phenotypes based on Phe tolerance. In this study, the majority of p.L48S functionally hemizygous patients had classical PKU phenotype. However, when p.L48S mutation was found in the homozygous form, its inconsistent nature susceptible to different influences became obvious. Therefore, we could conclude that the real effect of p.L48S was masked if the mutation was combined with a null mutation.

For some mutations, such as p.I65T, inconsistency could be explained by interallelic complementation between different subunits of heterotetrameric PAH (Leandro et al. 2006; Leandro et al. 2011). This mutation is frequent in Western European populations (Spanish, Portuguese, French, British, and Norwegian) while it is not frequent in Slavic populations (Zschocke 2003). It is worth mentioning that both mutations (p.L48S and p.I65T) in a homozygous state tend to produce a milder degree of PKU than when associated with a null allele, demonstrating that each of the two mutations encode residual PAH activity (39% and 26.5%, respectively). Although p.L48S has not been studied for interallelic complementation effect, comparison between homozygous vs. functionally hemizygous Serbian patients showed that p.L48S residual activity (39%) was decreased in interaction with null residual activity PAH subunit. Thus, our finding implies existence of negative interallelic complementation in functionally hemizygous p.L48S patients.

Interallelic complementation is only one of the possible explanations for mutation's inconsistency. Phenylalanine concentration in the peripheral circulation is controlled by various genetic loci and modifying factors at least at two different levels (Scriver and Waters 1999; Dipple and McCabe 2000). At the cellular level, a variant PAH enzyme with disturbed stability is susceptible to enhanced degradation. However, if there is a flaw in proteolytic degradation system or a defect in chaperones, both of which could be genetically determined, it would to some extent have influence on the blood Phe concentration. At the level of the whole organism, blood Phe concentration could be influenced by alterations in Phe absorption in the gut or variations in the transport of Phe through the hematoencephalitic barrier. The identification and characterization of modifier genes involved in the determination of phenylketonuria phenotype is the field that needs to be explored.

Furthermore, it has been shown that the genetic variant in the intron of *PAH* gene has the ability to regulate gene expression (Stojiljkovic et al. 2010). Numerous variants are embedded in the large noncoding regions of *PAH* gene, and their effect remains unknown. Therefore, variants found in introns as well as those found upstream and downstream of the gene coding region should be further investigated.

Since we noticed inconsistency in identical genotypes of patients with the same population background, it could be expected that variations in modifier genes would provide an answer to interindividual rather than interpopulation inconsistencies.

BH4 Responsiveness in Serbia

The first study which suggested that BH4-responsiveness could be deduced from *PAH* genotype stated that at least one BH4-responsive mutation would be enough to lead to the physiological BH4 responsiveness (Zurflüh et al. 2008). In this study on Serbian PKU patients, total frequency of BH4-responsive mutations was 52.6%.

Later on, it became clear that BH4-responsive genotype has a greater BH4-responsive predictive value (Trefz et al. 2009a; Karacic et al. 2009). In order to make the most accurate estimation of the potential of BH4-supplementation therapy, we classified Serbian *PAH* genotypes into BH4 responsive, probably BH4 responsive, and non-BH4 responsive (Table 3) (Sterl et al. 2012). Furthermore, we made distinction between genotypes with one and two BH4-responsive alleles. Previously, Zurfluh and colleagues postulated that BH4 responsiveness ought to be in the 17–79% range in European populations with a north to south gradient (Zurflüh et al. 2008). Accordingly, we found that only 22.8% of patients could not benefit from BH4 therapy and that as much as 77.2% of PKU population should be subjected to a standardized BH4 loading test.

Genotype p.[L48S];[L48S] is among the most frequent genotypes reported in the BIOPKU database (www.biopku.org). It was reported that when patients carrying this genotype were subjected to BH4 loading test, they were found to be responders, slow responders, or partial responders. Patients with p.[L48S];[R408W] genotype, which could be regarded as probably responsive according to the genotype, were frequently reported as nonresponders in reality. Interestingly, similar to our findings on phenotypic effect of p.L48S, Karacic et al. observed regularity in p.L48S BH4 responsiveness. They found that p.L48S tends to be a nonresponder in functional hemyzigotes, while it usually acts as a responder in homozygotes (Karacic et al. 2009).

Without a doubt, BH4 loading test is the only way to determine BH4 responsiveness (Blau et al. 2009; Levy et al. 2007b). However, the genotype-based estimation is valuable for countries where BH4 is not yet included in the drug lists. This study, which represents the first estimation on BH4 responsiveness for Serbian population, led us to a conclusion that BH4 supplementation therapy could bring benefit to Serbian PKU patients.

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Synopsis

Analysis of Serbian PKU patients showed that Phe tolerance was a better parameter for phenotypic classification of patients carrying p.L48S mutation and the genotype study identified significant number of individuals that may respond to BH4 supplementation therapy.

References

Aguado C, Perez B, Garcia MJ, Belanger-Quintana A, Martinez-Pardo M, Ugarte M, Desviat LR (2007) BH4 responsiveness associated to a PKU mutation with decreased binding affinity for the cofactor. Clin Chim Acta 380(1–2): 8–12

Blau N, Bélanger-Quintana A, Demirkol M, Feillet F, Giovannini M, MacDonald A, Trefz FK, van Spronsen FJ (2009) Optimizing the use of sapropterin (BH(4)) in the management of phenylketonuria. Mol Genet Metab 96(4):158–163

Dipple KM, McCabe ER (2000) Phenotypes of patients with "simple" Mendelian disorders are complex traits: thresholds, modifiers and system dynamics. Am J Hum Genet 66:1729–1735

Dobrowolski SF, Heintz C, Miller T et al (2011) Molecular genetics and impact of residual in vitro phenylalanine hydroxylase activity on tetrahydrobiopterin responsiveness in Turkish PKU population. Mol Genet Metab 102(2):116–121

Eiken HG, Knappskog PM, Apold J (1993) Restriction enzyme-based assays for complete genotyping of phenylketonuria patients. Dev Brain Dysfunct 6:53–59

Erlandsen H, Pey AL, Gamez A et al (2004) Correction of kinetic and stability defects by tetrahydrobiopterin in phenylketonuria patients with certain phenylalanine hydroxylase mutations. Proc Natl Acad Sci USA 101:16903–16908

Fenech M, El-Sohemy A, Cahill L et al (2011) Nutrigenetics and nutrigenomics: viewpoints on the current status and applications in nutrition research and practice. J Nutrigenet Nutrigenomics 4:69–89

Giannattasio S, Dianzani I, Lattanzio P et al (2001) Genetic heterogeneity in five Italian regions: analysis of PAH mutations and minihaplotypes. Hum Hered 52(3):154–159

Groselj U, Tansek MZ, Kovac J, Hovnik T, Podkrajsek KT, Battelino T (2012) Five novel mutations and two large deletions in a population analysis of the phenylalanine hydroxylase gene. Mol Genet Metab 106(2):142–148

Guttler F, Guldberg P (2000) Mutation analysis anticipates dietary requirements in phenylketonuria. Eur J Pediatr 159:S150–S153

Guldberg P, Guttler F (1994) "Broad-range" DGGE for single-step mutation scanning of entire genes: the human phenylalanine hydroxylase gene. Nucleic Acids Res 22(5):880–881

Guldberg P, Rey F, Zschocke J et al (1998) A European multicenter study of phenylalanine hydroxylase deficiency: classification of 105 mutations and a general system for genotype-based prediction of metabolic phenotype. Am J Hum Genet 63:71–79

Karacic I, Meili D, Sarnavka V et al (2009) Genotype-predicted tetrahydrobiopterin (BH4)-responsiveness and molecular genetics in Croatian patients with phenylalanine hydroxylase (PAH) deficiency. Mol Genet Metab 97(3):165–171

Kayaalp E, Treacy E, Waters PJ, Byck S, Nowacki P, Scriver CR (1997) Human phenylalanine hydroxylase mutations and hyperphenylalaninemia phenotypes: A metanalysis of genotype-phenotype correlations. Am J Hum Genet 61:1309–1317

Kim SW, Jung J, Oh HJ (2006) Structural and functional analyses of mutations of the human phenylalanine hydroxylase gene. Clin Chim Acta 365:279–297

Konecki DS, Schlotter M, Trefz FK, Lichter-Konecki U (1991) The identification of two mis-sense mutations at the PAH gene locus in a Turkish patient with phenylketonuria. Hum Genet 87 (4):389–393

Kure S, Hou DC, Ohura T et al (1999) Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. J Pediatr 135(3):375–378

Leandro J, Leandro P, Flatmark T (2011) Heterotetrameric forms of human phenylalanine hydroxylase: co-expression of wild-type and mutant forms in a bicistronic system. Biochim Biophys Acta 1812(5):602–612

Leandro J, Nascimento C, de Almeida IT, Leandro P (2006) Co-expression of different subunits of human phenylalanine hydroxylase: evidence of negative interallelic complementation. Biochim Biophys Acta 1762(5):544–550

Levy HL, Milanowski A, Chakrapani A et al (2007a) Efficacy of sapropterin dihydrochloride (tetrahydrobiopterin, 6R-BH4) for reduction of phenylalanine concentration in patients with phenylketonuria: a phase III randomised placebo-controlled study. Lancet 370:504–510

Levy H, Burton B, Cederbaum S, Scriver C (2007b) Recommendations for evaluation of responsiveness to tetrahydrobiopterin (BH (4)) in phenylketonuria and its use in treatment. Mol Genet Metab 92:287–291

Muntau AC, Röschinger W, Habich M et al (2002) Tetrahydrobiopterin as an alternative treatment for mild phenylketonuria. N Engl J Med 347(26):2122–2132

Pérez B, Desviat LR, Gómez-Puertas P, Martínez A, Stevens RC, Ugarte M (2005) Kinetic and stability analysis of PKU mutations identified in BH4-responsive patients. Mol Genet Metab 86: S11–S16

Pey AL, Pérez B, Desviat LR et al (2004) Mechanisms underlying responsiveness to tetrahydrobiopterin in mild phenylketonuria mutations. Hum Mutat 24(5):388–399

Rivera I, Mendes D, Afonso Â et al (2011) Phenylalanine hydroxylase deficiency: molecular epidemiology and predictable BH4-responsiveness in South Portugal PKU patients. Mol Genet Metab 104:S86–S92

Scriver CR, Waters PJ (1999) Monogenic traits are not simple. Trends Genet 15:267–272

Scriver CR et al (2003) PAHdb 2003: What a locus-specific knowledgebase can do. Hum Mutat 21:333–344 (http://www.pahdb.mcgill.ca)

Scriver CR (2007) The PAH gene, phenylketonuria, and a paradigm shift. Hum Mutat 28:831–845

Scriver CR, Levy H, Donlon J (2008) Hyperphenylalaninemia: phenylalanine hydroxylase deficiency. In: Valle D, Beaudet AL, Vogelstein B, Kinzler KW, Antonarakis S, Ballabio A (eds) Scriver CR, Childs B, Sly WS (eds emeritus) The online metabolic and molecular basis of inherited disease. McGraw-Hill, New York. Online Chapter 77 (www.ommbid.com)

Staudigl M, Gersting SW, Danecka MK et al (2011) The interplay between genotype, metabolic state and cofactor treatment governs phenylalanine hydroxylase function and drug response. Hum Mol Genet 20(13):2628–2641

Sterl E, Paul K, Paschke E, et al (2012) Prevalence of tetrahydrobiopterine (BH4)-responsive alleles among Austrian patients with PAH deficiency: comprehensive results from molecular analysis in 147 patients. J Inherit Metab Dis. doi: 10.1007/s10545-012-9485-y

Stojiljkovic M, Jovanovic J, Djordjevic M et al (2006) Molecular and phenotypic characteristics of phenylketonuria patients in Serbia and Montenegro. Clin Genet 70:151–155

Stojiljkovic M, Stevanovic A, Djordjevic M et al (2007) Mutations in the PAH gene: a tool for population genetic study. Arch Biol Sci 59(3):161–167

Stojiljkovic M, Pérez B, Desviat LR, Aguado C, Ugarte M, Pavlovic S (2009) The Missense p.S231F phenylalanine hydroxylase gene mutation causes complete loss of enzymatic activity in vitro. Protein J 28(6):294–299

Stojiljkovic M, Zukic B, Tosic N et al (2010) Novel transcriptional regulatory element in the phenylalanine hydroxylase gene intron 8. Mol Genet Metab 101(1):81–83

Trefz FK, Schmidt H, Bartholome K, Mahle M, Mathis P, Pecht G (1985) Differential diagnosis and significance of various hyperphenylalaninemias. In: Bickel H, Wachtel U (eds) Inherited diseases of amino acid metabolism. Thieme, Stuttgart, pp 86–100

Trefz FK, Scheible D, Götz H, Frauendienst-Egger G (2009a) Significance of genotype in tetrahydrobiopterin-responsive phenylketonuria. J Inherit Metab Dis 32(1):22–26

Trefz FK, Burton BK, Longo N et al (2009b) Efficacy of sapropterin dihydrochloride in increasing phenylalanine tolerance in children with phenylketonuria: a phase III, randomized, double-blind, placebo-controlled study. J Pediatr 154:700–707

van Sponsen FJ, van Rijn M, Dorgelo B et al (2009) Phenylalanine tolerance can already reliably be assessed at the age of 2 years in patients with PKU. J Inherit Metab Dis 32(1):27–31

Waters PJ, Parniak MA, Nowacki P, Scriver CR (1998) In vitro expression analysis of mutations in phenylalanine hydroxylase: linking genotype to phenotype and structure to function. Hum Mutat 11(1):4–17

Zschocke J (2003) Phenylketonuria mutations in Europe. Hum Mutat 21:345–356

Zurflüh MR, Zschocke J, Lindner M et al (2008) Molecular genetics of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. Hum Mutat 29:167–175

http://www.biopku.org