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PAH AND QDPR DEFICIENCY ASSOCIATED MUTATIONS IN THE NOVOSIBIRSK REGION OF THE RUSSIAN FEDERATION: CORRELATION OF MUTATION TYPE WITH DISEASE MANIFESTATION AND SEVERITY

MUTACIJE POVEZANE SA DEFICITOM PAH I QDPR U REGIJI NOVOSIBIRSK RUSKE FEDERACIJE: KORELACIJA IZMEĐU TIPA MUTACIJE I MANIFESTACIJE I STADIJUMA OBOLJENJA

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Summary

Background: Efficient treatment of inherited hyperphenylalaninemia requires exact identification of mutations defining the trait. Such knowledge is important both for effective individual therapy and understanding of the genetic history and evolution of regional populations.
Methods: DNA sequencing of amplified genome regions was used to identify mutations.
Results: Hyperphenylalaninemia-associated mutations in the phenylalanine hydroxylase locus were identified for 76 unrelated patients from the Novosibirsk region, Russia and for their family members. Twenty-one mutation types were identified, most of them rare and one (IVS2+1delG) not previously described. Common for European populations, the mutation p.R408W appeared to be the most frequent, with allele frequency 63.33%. We also looked for mutations in the quinoid dihydropteridine reductase locus in some patients. For 36 unrelated children PKU patients with known blood phenylalanine levels, we tried to find correlations between this level and the genotype.
Conclusions: Comparative analysis revealed correlations between blood phenylalanine levels and genotypes. The spectrum of phenylalanine hydroxylase mutations in the Novosibirsk region population appeared to be rather com-

Kratak sadržaj

Uvod: Za efikasno lečenje nasleđene hiperfenilalaninemije neophodno je precizno identifikovanje mutacija koje definišu ovu naslednu bolest. Saznanja stečena na taj način važna su kako zbog efikasne individualne terapije, tako i zbog razumevanja genetske istorije i evolucije regionalnog stanovništva.
Metode: Za identifikovanje mutacija upotrebljeno je DNK sekvenciranje amplifikovanih regija genoma.
Rezultati: Mutacije povezane sa hiperfenilalaninemijom na mestu fenilalanin-hidroksilaze identifikovane su za 76 pacijenata, koji nisu u srodstvu, iz regije Novosibirsk u Rusiji i za članove njihovih porodica. Identifikovan je 21 tip mutacija, većinom retkih, kao i jedna (IVS2+1delG) koja dosad nije opisana. Najčešća je bila mutacija p.R408W, uobičajena za evropske populacije, sa učestalošću alela od 63,33%. Takođe smo tragali za mutacijama na mestu kinoid-dihidropteridin-reduktaze kod nekih pacijenata. Za 36 dece obolele od fenilketonurije, koja nisu u srodstvu, sa poznatim nivoima fenilalanina u krvi, pokušali smo da pronađemo korelacije između ovog nivoa i genotipa.
Zaključak: Uporednom analizom otkrivene su korelacije između nivoa fenilalanina u krvi i genotipova. Spektar mutacija fenilalanin-hidroksilaze u populaciji regije Novosibirsk

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List of abbreviations: PKU, Phenylketonuria; *PAH*, phenylalanine hydroxylase gene; PAH, phenylalanine hydroxylase; HPA, hyperphenylalaninemia; MHP, mild hyperphenylalaninemia; DHPR, dihydropteridine reductase; QDPR, quinoid dihydropteridine reductase; BH_4, (6R)-L-erythro-5, 6, 7, 8-tetrahydrobiopterin.

plex, probably as a result of mixed ethnic composition, formed by several multidirectional migration flows.

Keywords: hyperphenylalaninemia, phenylketonuria, phenylalanine hydroxylase, genotype, phenotype

pokazao se kao prilično složen, verovatno usled mešovitog etničkog sastava, nastalog zahvaljujući brojnim migracionim tokovima iz više pravaca.

Ključne reči: hiperfenilalaninemija, fenilketonurija, fenilalanin-hidroksilaza, genotip, fenotip

Introduction

Hyperphenylalaninemia is a result of a defect in the hydroxylation of phenylalanine (Phe) to tyrosine (Tyr), a reaction catalyzed by phenylalanine hydroxylase (PAH, phenylalanine 4-monooxygenase, EC.1.14.16.1) with the cofactor (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) (1). Insufficient phenylalanine hydroxylase activity results in an increase of phenylalanine concentration in organism fluids (up to 900–1200 μmol/L from normal 60–120 μmol/L) and a decrease in tyrosine concentration. Subsequent activation of alternative phenylalanine deamination produces toxic phenylpyruvic, phenylacetic and phenyllactic acids which, being accumulated, lead to chronic intoxication, impairing the central nervous system and subsequently intellectual abilities (2). The disorder can present with different phenotypes classified according to Phe tolerance: classical phenylketonuria (PKU), mild PKU and mild hyperphenylalaninemia (MHP).

In the majority of cases, hyperphenylalaninemia is caused by mutations in the *PAH* gene (OMIM 261600), localized in the q22-q24 region of chromosome 12 long arm and spanning over 90 kbp. The gene includes 13 exons and encodes a 451-amino acid protein (3). PKU caused by *PAH* gene mutations is inherited as an autosomal recessive trait and impaired individuals are in most cases hemizygotes carrying two PKU-associated mutations of different types. The exact type and severity of symptoms manifestation as well as effectiveness of different therapeutic approaches are determined to a significant extent by the exact molecular mechanism of phenylalanine hydroxylase inactivation and its residual activity, which, in turn, depends on the types of PKU-associated mutations that a particular patient carries.

Some other relatively rare forms of HPA are not directly associated with the *PAH* gene, being described as atypical PKU or non-PKU hyperphenylalaninemia (http: //www.bh4.org). For instance, atypical PKU could be caused by mutations in the *QDPR* gene impairing the activity of corresponding enzyme quinoid dihydropteridine reductase (DHPR, NAD(P)H: 6,7-dihydropteridine oxidoreductase, EC 1.6.99.7)). The *QDPR* gene is localized in the 4p15.3 region of chromosome 4 short arm and includes 7 exons encoding a 244-amino acid protein. DHPR deficiency impairs tetrahydrobiopterin (BH_4) reduction, finally resulting in deficiency of the cofactor required for phenylalanine, tyrosine and tryptophan hydroxylation. The main symptoms of atypical PKU caused by DHPR deficiency are quite similar to those of classical PKU, but the therapy used for classical PKU is almost ineffective in most cases of atypical PKU.

The spectrum of the PKU-associated *PAH* gene mutations is rather broad with more than 800 mutation types known up to date (http: //www.biopku.org/pah/), and the list keeps growing. Previously unknown mutation types were discovered recently in populations and ethnic groups residing in Korea, Armenia, Slovenia, India, Morocco, China (4–9). The rate of PKU among the newborn varies broadly in different populations, from high levels up to 1: 4000 – 1: 4500 in Turkey and Ireland (10, 11) to relatively low as 1: 10000 – 1: 11000 in Finland and China (12, 13) and even lower down to 1: 41000 – 1: 80500 in Korea and Japan (14, 15). Overall, PKU is considered to be one of the most widespread monogenic metabolic disorders.

Phenylketonuria symptoms are classified by the severity of hyperphenylalaninemia. The normal range of blood phenylalanine concentrations is considered to be 60–120 μmol/L. Individuals with blood phenylalanine concentrations of 360–1200 μmol/L are classified as mild phenylketonuria (sometimes a moderate classification is included for concentrations of 900–1200 μmol/L) and concentrations above 1200 μmol/L denote classic phenylketonuria (2). Usually, elevated levels of blood Phe are first reported during neonatal PKU tests. Unfortunately, these tests give no data to estimate the molecular mechanisms of hyperphenylalaninemia, and there is no way to distinguish atypical PKU cases from PKU caused by the *PAH* gene mutations.

The present investigation aimed to reveal correlations between the types of *PAH* gene mutations and severity of PKU manifestations in children PKU patients from the Novosibirsk region, revealed during newborn PKU neonatal screening.

Materials and Methods

The studied cohort consisted of 36 unrelated PKU patients aged up to 5 years residing in the Novosibirsk region and registered at the Municipal Center for Reproduction and Family Planning, Novosibirsk. PKU was diagnosed in the course of a neonatal screening program during the period from 2007 to 2012. Phenylalanine concentration in a newborn's blood was measured on the third or fourth day of life. If elevated blood Phe was detected, the test was repeated and PKU was diagnosed after elevated

Table I Primers for *QDPR* gene fragments amplification.

Exon	Direction	Sequence 5'->3'	Amplicon length, bp
1	forward	ACAGTCCCTCCGGGTGGC	412
	reverse	GCAAGCAACACGAGTCAGG	
2	forward	CCTCAAGAGATCCTCCCACATT	330
	reverse	ACATACAGCCAAAGGAAGAACAT	
3	forward	ATTCTCAAAGCATTAATTGCCA	360
	reverse	CGAGCAACTGTAAAGATTCCG	
4	forward	GCCCTGTGCTGTTTGTGTTAGA	435
	reverse	CAGGCCCAAAAGAAGACAAAAT	
5	forward	CCAGAGGCCATAATTCCCAA	448
	reverse	AGAGGTGAGAGCACACCCAG	
6	forward	CAGCGCACCTTATTGAATTATG	413
	reverse	CAGCGCACCTTATTGAATTATG	
7	forward	TTAAACAGTCGCTGCTGTGC	438
	reverse	CTATTTGCAGGCCACCAGTC	

blood Phe confirmation. To confirm trait inheritance, all the identified PKU-associated *PAH* gene mutations were tracked down in all the available family members – in total, 103 persons, not including 36 probands. All patients and/or their parents gave informed consent.

Phenylalanine concentration was measured via the fluorescence assay using a Delfia-Victor (Perkin Elmer, Finland) multifunctional analyzer according to manufacturer's instruction. Capillary blood samples were collected using test paper strips.

Total genomic DNA from blood was isolated as described elsewhere (16). Initially, nucleotide sequences of all the 13 *PAH* gene exons along with adjacent intron regions were determined after PCR-amplification of the corresponding DNA fragments using a set of previously described (19) oligonucleotide primers. Unless both PKU-associated mutations were found in the *PAH* gene, the *QDPR* gene was also examined, as it is known to bear relatively often mutations associated with the non-PKU type (http: //www.bh4.org). To amplify and sequence *QDPR* gene exons with adjacent introns areas, we used oligonucleotide primers summarized in *Table I*.

The PCR temperatures were as follows: 3 min at 95 °C followed by 32 cycles of denaturation during 1 min at 94 °C, annealing during 1 min at 58 °C and elongation during 2 min at 72 °C. PCR products were separated by electrophoresis in regular 1.5–2% agarose gel, target bands were cut and DNA was isolated from the gel slice using a Gene Jet Gel Extraction Kit (Fermentas, EU). Sanger sequencing with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) was done for both DNA strands using the same primers as for PCR. Sanger reaction mix of a total volume of 20 μL contained 150–250 fmole of dsDNA template, 2 pmole of primer, 1 μL of BigDye v.3.1 reagent and 4 μL of 5× sequencing buffer from the kit. Centri-Sep (Princeton Separations, USA) spin columns were used to purify Sanger products from unincorporated fluorescent dyes prior to analysis on an ABI3130×l Genetic Analyser (Applied Biosystems, USA).

Results and Discussion

The Novosibirsk region is a vast area of 178.2 thousand square km situated at the southeast of the West Siberian Plain between the Urals and Lake Baikal. On September 1, 2012 it was inhabited by about 2.7 million residents according to official records. The rate of PKU among the newborn in the Novosibirsk region was estimated at 1: 7000 (16). There are currently 76 PKU patients from the Novosibirsk region listed and receiving care at the Novosibirsk Municipal Center for Reproduction and Family Planning, diagnosed from 1989 till 2012. Sixty-nine of them are treated with strict or moderate low-Phe diet as a protein substitute. Fourteen are residents of distant areas or recent migrants from neighbouring FSU countries in which no neonatal PKU screening is conducted. Those 14 have profound signs of PKU symptoms including mental detention and hyperdynamic syndrome, as a result of late PKU

Table II PKU-associated alleles frequency in the Novosibirsk region PKU patients.

Mutation		Gene, localization	Allele occasions (frequency %)
Protein	cDNA		
p.S16>XfsX1	c.47_48delCT	*PAH*, exon 1	1 (0.67%)
p.L48S	c.143T>C	*PAH*, exon 2	2 (1.33%)
IVS2+1delG	c.169delG	*PAH*, intron 2	1 (0.67%)
IVS2+5G>A	c.168+5G>A	*PAH*, intron 2	1 (0.67%)
IVS2-13T>G	c.169-13T>G	*PAH*, intron 2	2 (1.33%)
IVS4+5G>T	c.441+5G>T	*PAH*, intron 2	3 (2.00%)
p.R158Q	c.473G>A	*PAH*, exon 5	7 (4.67%)
p.R243Q	c.728G>A	*PAH*, exon 7	2 (1.33%)
p.R243X	c.727C>T	*PAH*, exon 7	1 (0.67%)
p.R261Q	c.782G>A	*PAH*, exon 7	12 (8.00%)
p.R261X	c.781G>T	*PAH*, exon 7	1 (0.67%)
p.E280K	c.838G>A	*PAH*, exon 7	1 (0.67%)
p.P281L	c.842C>T	*PAH*, exon 7	4 (2.67%)
p.E390G	c.1169A>G	*PAH*, exon 10	1 (0.67%)
IVS10-11G>A	c.1066-11G>A	*PAH*, intron 10	4 (2.67%)
p.A403V	c.1208C>T	*PAH*, exon 12	1 (0.67%)
p.P407L	c.1220C>T	*PAH*, exon 12	1 (0.67%)
p.R408Q	c.1223G>A	*PAH*, exon 12	1 (0.67%)
p.R408W	c.1222 >	*PAH*, exon 12	95 (63.33%)
p.Y414C	c.1241A>G	*PAH*, exon 12	2 (1.33%)
IVS12+1G>A	c.1315+1G>A	*PAH*, intron 12	3 (2.00%)
X	–	–	4 (2.67%)

X – unidentified (no protein affecting mutation found)

diagnosis. These PKU patients were partly genetically characterized by us previously (16). Sequencing of *PAH* gene exons provided exact knowledge of the PKU-associated mutations spectrum for the Novosibirsk region PKU patients. The mutation p.R408W appeared to be the most common, accounting for 63.33% of the PKU-associated alleles. The second in frequency was mutation p.R261Q with 8.00%. This mutation is quite common across Europe, being the most frequent in Switzerland (32.0%) and France (17.0%) (18). Mutation p.R158Q, also widespread in Europe with 13.0% in the Netherlands (18), was identified in 7 patients from our cohort (4.67% alleles). Mutations IVS10-11G>A and p.P281L, which are also known to be common across Europe, were found in the Novosibirsk region with the frequency of 2.67% each. The following mutations were identified on single occasions: p.S16>XfsX1, p.L48S, IVS2+1delG, IVS2+5G>A, IVS4+5G>T, p.R243X, p.E390G, p.A403V, p.P407L and p.R408Q (*Table II*). The above data supports the hypothesis of presumably European origin of most PKU-associated alleles in the Novosibirsk region, brought here through migration from the European part of Russia or from East Europe (mutations p.R252W, p.R158Q, p.R261Q, p.P281L, IVS10-11G>A, p.R408W, IVS12+1G>A). Still, along with the majority of mutations characteristic for Europe, we also found mutations typical for Southeast Asia (p.R261Q) or Turkey (p.R243Q). A good portion of the identified mutations are known to be quite rare or even unique: IVS2+5G>A, IVS4+5G>T, p.S16> XfsX1, p.A403V, p.P407L, p.R408Q. Therefore, the mixed ethnic composition of the Novosibirsk region population, formed by several multidirectional migration flows, most probably accounts for the diversity of the PKU-associated mutations spectrum.

Precise identification of PKU-associated mutations by DNA sequencing provides the grounds for the analysis of correlation between mutation types and the degree of biochemical and general disease symptoms manifestation. Different mutations affect the structure of the PAH enzyme and its activity differently: some, for instance, distort the structure of the enzyme active center, some alter the coenzyme binding site, others impair the tetramerization domain thus inhibiting the formation of the active tetrameric enzyme form. Such parameters as relative enzymatic activity, the amount of specific immunoreactive protein, mRNA level in certain tissues could provide a link between a mutation type and its impact on overall activity of the gene product, thus allowing more elaborate disease development prognosis and therapy effectiveness prediction. The residual activity of PAH in PKU patients could differ in a wide range, depending on the combination of two PKU-associated

Table III Patient genotypes and blood PA levels.

PAH gene mutations:						
No	Diagnosis	Genotype		Blood PA, μmol/L		TS, mnt
				1*	2**	
1	classic PKU	p.R408W	p.R408W	714	6840	2
2	classic PKU	p.R408W	p.R408W	648	5700	2
3	classic PKU	p.R408W	p.R408W	1014	4800	2
4	classic PKU	p.R408W	p.R408W	834	3600	2
5	classic PKU	p.R408W	p.R408W	756	3540	2
6	classic PKU	p.R408W	p.R408W	342	2226	2
7	classic PKU	p.R408W	p.R408W	1104	1986	2
8	classic PKU	p.R408W	p.R408W	522	1956	2
9	classic PKU	p.R408W	p.R408W	966	1650	2
10	classic PKU	p.R408W	p.R408W	510	1518	2
11	classic PKU	p.R408W	p.R408W	420	1620	2
12	classic PKU	p.R408W	p.R408W	564	1488	2
13	classic PKU	p.R408W	p.R408W	960	>1200	2
14	classic PKU	p.R408W	p.R408W	N/A	>1200	24
15	classic PKU	p.R408W	p.R408W	786	>1200	2
16	classic PKU	p.R408W	p.R408W	678	>1110	2
17	classic PKU	p.R408W	p.R408W	N/A	N/A	18
18	classic PKU	p.R261Q	p.P281L	456	6468	2
19	classic PKU	p.R408W	IVS12+1G>A	516	3210	2
20	classic PKU	p.R408W	p.L48S	810	1998	2
21	classic PKU	p.R408W	p.R261Q	N/A	1944	48
22	classic PKU	p.R408W	IVS4+5G>T	564	1734	2
23	classic PKU	p.R408W	IVS10-11G>A	912	1608	2
24	classic PKU	p.P281L	IVS2+1delG	N/A	1536	2
25	classic PKU	p.R408W	p.R158Q	636	1464	2
26	classic PKU	p.R408W	IVS2+5G>A	816	1428	2
27	classic PKU	p.R408W	IVS12+1G>A	450	1416	2
28	moderate PKU	p.R408W	p.R261Q	636	1152	2
29	moderate PKU	p.R408W	p.P281L	384	1032	2
30	moderate PKU	p.R408W	IVS10-11G>A	576	924	2
31	moderate PKU	p.R408W	p.R158Q	342	756	2
32	moderate PKU	p.R408W	p.R158Q	684	750	2
33	mild PKU	p.R408W	p.R408Q	726	546	2
34	mild PKU	p.R243X	X	342	372	2
35	mild PKU	p.R261Q		150	120	2
QDPR gene mutation:						
36	atypical PKU	p.Y150C	p.Y150C	204	378	2

* – first blood PA measurement was done at day 3 or 4 after birth;
** – second blood PA measurement was done at day 10 to 15 after birth;
TS – treatment start time, months after birth;
X – unidentified (no protein affecting mutation found).

mutations they bear. We tried to find correlations between mutation types and disease manifestation in 36 PKU patients diagnosed during the period from 2007 to 1012 years.

Seventeen of the 36 PKU patients appeared to have the homozygous genotype p.R408W/p.R408W, which is known to cause severe PKU manifestation. Arginine replacement by tryptophan in position 408 leads to a drastic decrease in the distance between the catalytic center and tetramerization domain, virtually preventing normal enzyme tetramerization (3). The residual activity is known to be practically absent – below 0.3% (19). Consistently, we observed a rather high level of blood Phe in p.R408W/p.R408W patients (*Table III*).

Six patients (No. 19, 22, 23, 26, 27 and 30) had a p.R408W mutation in compound with splicing mutations: IVS12+1G>A, IVS4+5G>T, IVS2+5G>A and IVS10-11G>A. Most of the splicing mutations are known to significantly alter the enzyme structure leading to low residual activity and classic PKU manifestation. For instance, mutation IVS12+1G>A, which ruins the donor splicing site, results in synthesis of the enzyme molecule shortened by 52 amino acids leaving less than 1% of residual enzyme activity due to conformation instability (20). Mutation IVS10-11G>A creates a false acceptor splice site causing the insertion of additional 9 nucleotides from intron 10 into mRNA which in turn inserts 3 extra amino acids Gly-Leu-Gln between the 355th and 356th PAH amino acid. The insertion causes a complete loss of enzymatic activity, although the PAH mRNA level in the liver cells is not affected (21). Splice mutation IVS4+5G>T at the boundary between exon 4 and intron 4 is known to cause a frame shift starting from exon 5. Literature provides the examples of patients having this mutation in compound with p.R261Q, p.R158Q, p.R243Q, IVS10-11G>A, and all of them are described as developing classic PKU symptoms (22), although there are no explicit data on residual enzymatic activity. Mutation IVS2+5G>A at the boundary of intron 2 and exon 3 alters the splice site thus altering introns removal from the primary transcript. As a result, a part of exon 3 or the whole exon 3 is cut out of mRNA, and at the same time a part of or whole intron 2 can be inserted into mature mRNA. Most of these mutations in compound with p.R408W have lead to classic PKU in patients from our cohort, save one patient (No. 30) with genotype p.R408W/IVS10-11G>A and moderate PKU manifestation.

Patients with a combination of missense mutations known to be associated sometimes with moderate and sometimes with classic PKU had blood Phe levels from 1020 to 1200 μmol/L in our cohort.

The p.R158Q is an example of such relatively moderate mutations, causing replacement of arginine in position 158 by glutamine. Arginine 158 is involved in salt bridge formation with Glu 280, as well as hydrogen bond formation with Tyr 268. Both bonds are important for maintaining the correct configuration of enzyme catalytic center. According to *in vitro* expression data in a COS eukaryotic system at 37 °C p.R158Q leaves residual PAH activity at the level of 28.5% (21), which is quite high. Mutation p.R158Q is quite common generally, and in our cohort in particular – 3 patients have it in compound with p.R408W. Despite the identical genotype, these patients (No. 25, 31 and 32) had different degrees of PKU manifestation: one (No. 25) had blood Phe at 1440 μmol/L which corresponds to classic PKU, while two others (No. 31, 32) had blood Phe at 720 μmol/L which, according to conventional classification, corresponds to mild PKU. The literature also mentions both mild and classic PKU cases associated with the p.R408W/p.R158Q genotype (6).

Missense mutation p.L48S located at exon 2 of the *PAH* gene is known to significantly (by about 60%) decrease the residual enzymatic activity in human kidney cell culture A293 decreasing intact PAH protein content by even up to 12% of the normal (23). Exact mechanisms of activity decrease are not clear up to date and changes in BH_4 Michaelis constant or downregulation of *PAH* gene expression are speculated. Mutation p.L48S in compound with p.P281L, p.R408W or p.R261Q is known to cause classic PKU, but in compound with p.I306V or p.V177L leads to mild PKU (24). When p.L48S mutation is present in the homozygous form, its inconsistent nature susceptible to different influences could become more profound (25, 26). In our cohort, we had one patient (No. 20) with the p.L48S/p.R408W genotype and a high level of blood Phe (3180 μmol/L) corresponding to classic PKU.

Mutation p.R261Q prevents the formation of hydrogen bonds with Gln 304 and Thr 238, which are essential for the correct active center secondary structure stabilization. The mutation also influences dimerization and tetramerization, leaving residual enzymatic activity at 47.4% of the normal according to *in vitro* expression in COS eukaryotic system data (21), which is still considerably high. Literature gives examples of both classic and mild PKU for genotype p.R261Q/p.R408W (1). Two patients from the cohort presented in this study also had the p.R261Q/p.R408W genotype, one of them (No. 28) demonstrating mild PKU while the other (No. 21) was developing classic symptoms. One should, however, take into account that the patient No. 21 with classic PKU was born in Kazakhstan and was properly diagnosed and treated only at the age of 4 after migration of his family to the Novosibirsk region. The most probable reason for classic symptoms development in this case could be late diagnosis and treatment.

Missense mutation p.P281L was located in exon 7. The replacement of proline by less rigid leucine loosens the enzyme structure that keeps the active center close to the iron atom (27). Conformation changes lead to a drastic drop in residual activity to 1–12% of the normal according to the data obtained in a reticulocytes lysate expression system (http://www.pahdb.mcgill.ca). In the studied cohort, three patients were bearers of p.P281L. The patient No. 18 with genotype p.P281L/p.R261Q had classic PKU with rather high blood Phe (about 6900 μmol/L). Genotype p.P281L/p.R408W (patient No. 29) was connected with moderate symptoms and blood Phe about 1020 μmol/L.

The third bearer of p.P281L (patient 24) had it in compound with a previously unknown mutation IVS2+1delG. We tried to predict the possible consequences of IVS2+1delG mutation using the Automated Splice Site Analysis tool (28). Results indicated a possible shift of the donor splicing site by one

nucleotide in 3′ direction towards exon 2 causing a probable frame shift starting from amino acid 57 and most probably bringing residual activity to zero. In agreement with the above assumptions, the patient (No. 24) showed classic PKU symptoms with blood Phe above 1500 μmol/L.

Mutation p.R408Q in exon 12 is shown to leave rather high residual enzymatic activity – about 55% in a COS *in vitro* eukaryotic expression system (29). The only p.R408Q bearer in the studied cohort – patient No. 33 with the genotype p.R408W/p.R408Q showed very mild PKU with a blood Phe level of about 540 μmol/L which is consistent with high residual enzymatic activity.

We were able to identify PKU-associated mutations in one *PAH* gene allele only for patients No. 34 and 35: a nonsense mutation p.R243X and a missense mutation p.R261Q respectively, both located in exon 7. No PKU-associated mutations were found in the second *PAH* gene allele, although all the 13 *PAH* gene exons with adjacent intron regions were thoroughly sequenced. Both patients showed very mild PKU symptoms with blood Phe levels about 360 μmol/L and 120 μmol/L respectively. Still, there remains the possibility of large deletions in the second *PAH* gene allele which are not detectable via exon sequencing (6).

For the patient No. 36, who was also diagnosed as having rather mild PKU with blood Phe at 240 μmol/L on the 4th day after birth, we did not find any PKU-associated mutations in the *PAH* gene coding regions. While sequencing the coding regions of *QDPR*, we identified a homozygous mutation p.Y150C in exon 5. This mutation is known to cause DHPR deficiency leading to tetrahydrobiopterin (BH_4) regeneration failure (30). The patient could also be an example of simple screening methods limitations in revealing the exact disease nature, clearly illustrating the importance of mutations identification for the precise determination of disease type and nature as well as the most effective treatment strategy.

Therefore, we can divide the studied cohort into three groups according to mutation types and PKU symptoms. The first group consists of patients with very high blood Phe levels and classic PKU symptoms. As to genotypes, they have homozygous p.R408W or p.R408W in compound with one of the following: IVS12+1G>A, IVS4+5G>T, IVS2+5G>A, IVS10-11G>A, p.L48S or p.R158Q. The second group with much lower blood Phe levels and mild PKU manifestations have genotypes p.R408W/p.R158Q, p.R408W/p.R261Q, p.R408W/p.P281L and p.R408W/IVS10-11G>A. The third group is represented by four patients with very low blood Phe levels and faint PKU symptoms. Those are the patient No. 33 with genotype p.R408Q/p.R408W, two patients (No. 34, No. 35) with only one PKU-associated mutation identified in the *PAH* gene and the patient No. 36 without mutations in the *PAH* gene but with a homozygous p.Y150C mutation in the *QDPR* gene.

Although PKU is one of the most thoroughly studied metabolic disorders, the present study indicates that contemporary knowledge of the correlations between *PAH* gene mutation types and possible disease symptoms needs further enhancement before it could be effectively used in clinical practice. This study also illustrates severe limitations of the simple screening diagnostic methods and the critical importance of knowing the exact type of the disorder causing the mutations for precise diagnostics, individual symptoms prediction and effective individual treatment of a particular patient.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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