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The analysis of using a panel of the most common variants in the *PAH* gene for the newborn screening in Ukraine

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ABSTRACT

Phenylketonuria (PKU) is hyperphenylalaninemia that develops due to a deficiency of the phenylalanine hydroxylase enzyme (PAH). Identification of variants in the *PAH* gene is necessary for verification of the diagnosis, choice of treatment tactics, detection of heterozygous carriers. The aim of the study was to analyze the effectiveness of identification of selected pathological variants in the *PAH* gene during the newborn screening program. This study relied on the results of the examination of 257 patients (138 boys and 119 girls) with hyperphenylalaninemia from different regions of Ukraine. Genotyping was performed on nine pathogenic variants in *PAH* gene: I65T, R261Q, G272*, R252W, R261*, R408W, IVS12 + 1G > A, Y414C, IVS10-11G > A. According to the results of the study, variants R408W (AF = 52.7%), R252W (AF = 3.5%) and Y414C (AF = 1.8%) were the most common. More than half of the examined patients (51.7%) had a compound genotype with a major variant of R408W in one allele. Approximately a quarter of the examined patients (26.8%) had the R408W/R408W genotype. In 12.1% of patients, the applied panel of variants of the *PAH* gene did not allow us to identify the pathogenic variant in any allele. We conclude that the selected panel allowed us to identify the presence of variants in 87.9% of patients with PKU. The panel of genetic testing in the *PAH* gene for the newborns that we used for the study allows accurate prediction of some phenotypes for therapy planning. But in-depth analysis of pathological gene variants may be necessary for unclear and difficult cases of the disease, and for genetic counseling of patients families.

Abbreviations: PAH, phenylalanine hydroxylase; PKU, phenylketonuria.

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1. Introduction

Phenylketonuria (PKU) is hyperphenylalaninemia that develops due to a deficiency of the phenylalanine hydroxylase enzyme (PAH, EC 1.14.16.1). In classical PKU, there is an accumulation of phenylalanine in the blood and intermediates of its metabolism (phenylpyruvate, phenylacetate, phenyl lactate, etc.) in the urine, reduced tyrosine levels, impaired metabolism of other amino acids [1]. These disorders lead primarily to damage of the central nervous system.

Back in 1988, Scriver et al. [2] found that the human genotype is an important determinant of the amino acid phenotype in blood plasma. To date, it is well known that PKU occurs due to variants of the *PAH* gene, which significantly impair the function of PAH or even lead to its absence. The *PAH* gene was identified in 1985, it is located on the long arm of chromosome 12 in the region q22–24.1 [3]. It has a length of about 90 kbp, includes 13 exons and encodes the PAH protein, consisting of 451 amino acid residues [4]. To date, 1553 variants in the *PAH* gene are known, and their spectrum and prevalence have population features [5]. Identification of variants in the *PAH* gene is necessary for verification of the diagnosis, choice of treatment tactics, detection of heterozygous carriers [6,7]. On the other hand, different populations of the world have a pronounced genetic heterogeneity, therefore, the analysis of the frequency and spectrum of variants of the *PAH* gene in our country are necessary to develop the most effective algorithms for molecular and genetic research in patients with PKU.

It should be noted that in developed countries, clinical manifestations of classical PKU are rarely registered as of today [8]. This is facilitated by the detection of patients in the early preclinical stages of the disease during biochemical screening, which determines the level of phenylalanine in the newborn's blood. Further prescription of diet therapy minimizes damage to the nervous system. In Ukraine, nationwide newborn screening for PKU has been conducted since 2004. The procedure for screening, confirming the diagnosis, treatment and monitoring of identified patients is determined by the clinical protocol "Phenylketonuria and other hyperphenylalaninemias", approved by the Order of the Ministry of Public Health of Ukraine. The introduction of this procedure allowed us to ensure the appropriate level of early presymptomatic diagnostics and modern treatment of PKU in Ukraine and led to the absence of registered cases of the natural course of this severe and debilitating disease in children.

The aim of the study was to analyze the effectiveness of identification of selected pathological variants in the PAH gene during the newborn screening program.

2. Materials and methods

2.1. Study population

This study relied on the results of the examination of 257 patients (138 boys and 119 girls) with hyperphenylalaninemia, detected as a result of newborn screening (confirmed phenylalanine level >120 μmol/L or 2 mg/dL) from different regions of Ukraine, who were referred for molecular and genetic testing at SI "Reference-centre for molecular diagnostic of Public Health Ministry of Ukraine" (SI "RCMD") during 2011–2020.

The study was conducted according to the criteria set by the declaration of Helsinki. The study was approved by the Ethics Committee of Shupyk National Healthcare University of Ukraine (protocol No.2 from April 28, 2015). All parents of the infants gave informed consent for participation in the study.

3. Methods

In compliance with the clinical protocol, blood for the screening test was taken no earlier than 48 h and no later than the 5th day after the birth of the child. Capillary blood was used for the examination, which was taken from the heel of the newborn on filter paper. The level of phenylalanine was determined by the standard method – fluorometric analysis using a set of reagents "Neonatal PHENYLALANINE" (Labsystems Diagnostics Oy, Finland) on analyzers "Victor" (Wallac Oy, Finland). When elevated phenylalanine levels were detected, blood resampling was performed with the following clarifying diagnostics: quantitative amino acid analysis was determined, and when phenylalanine levels were detected >120 μmol/L, studies were conducted to determine the *PAH* gene variants.

Genotyping was performed on nine pathogenic variants: I65T, R261Q, G272*, R252W, R261*, R408W, IVS12 + 1G > A, Y414C, IVS10-11G > A (Table 1).

The genomic DNA for molecular genetic studies was isolated from peripheral blood or from dry blood spots on filters papers using a commercial "Quick-DNA Miniprep Plus Kit" (Zymo Research, USA) according to the manufacturer's protocol. Molecular genetic studies of variants I65T, R252W, R261Q, R261*, G272*, R408W, Y414C, IVS10-11G > A, IVS12 + 1G > A of *PAH* gene were carried out using polymerase chain reaction-restriction fragment length polymorphism method according to protocols [9,10]. The studied gene regions were amplified using a commercial kit "DreamTaq Green PCR Master Mix" (Thermo Scientific, USA) and specific oligonucleotide primers (Metabion, Germany). Appropriate restriction enzymes (ThermoScientific, USA) were used for restriction analysis. Digested products were separated using agarose gel electrophoresis and visualized on a UV transilluminator. A protocol of genetic research is shown in Table 2.

3.1. Statistical analysis

Statistical analysis was performed using Microsoft Excel 2016 Pro Plus and SPSS v.26 software packages. To compare the frequency distribution of alleles/combination of alleles in groups of the study used descriptive statistics and calculation of of Pearson's χ^2 criteria (Pearson's χ^2 with Yates correction). Differences were considered significant for all types of analysis at a level of $p < 0.05$.

4. Results

As of 2020, there were 12 regional medical and genetic centers in Ukraine, where newborn screening and further diagnostics of PKU were conducted.

Analyzing the data of official statistics, it was found that during 2011–2020, newborn screening for PKU in Ukraine covered, on average, 89.2% of living newborns (Table 3) [11,12].

The average incidence of hyperphenylalaninemia in the screened newborns was 1:880, and the average incidence of PKU was 1:7110.

Since 2011, 257 children were genotyped at SI "RCMD" using our proposed panel of *PAH* variants, which was 49.8% of all newborns with confirmed presence of PKU (Table 3). The analysis of the conducted research allowed us to determine the prevalence of the spectrum of *PAH* variants in children diagnosed with PKU living in Ukraine (Table 4).

As in the vast majority of European countries, the R408W variant of the *PAH* gene is the most common among patients with PKU in Ukraine (its allele frequency (AF) is 52.7%). The remaining eight studied variants comprise only 10.1%, and 37.2% are unidentified options.

The frequency of the R408W variant of the *PAH* gene in patients from Ukraine did not differ significantly from the frequencies determined in patients from Russia (Moscow region), Moldova, Hungary and Slovakia (Table 5). In Belarus and Poland, the frequency of detection of the R408W variant of the *PAH* gene was significantly higher, and in Romania, on the contrary, it was lower.

At the next stage of the research, the frequencies of genotypes were analyzed in terms of the *PAH* gene variants in the study group of patients (Table 6).

More than half of the examined patients (51.7%) had a compound genotype with a major variant of R408W in one allele and another

Table 1
Genetic characteristics of the studied variants of *PAH* gene (according to BIOPKU [5]).

Trivial name	Protein variant	DNA change, accession number	Gene region	Protein domain	**Phenotype** (for patients with homozygous variant)	**BH4 Responsiveness** (for patients with homozygous variant)
R408W	p.Arg408Trp	c.1222C > T, rs5030858	exon 12	catalytic	Classic PKU (in 99.3% cases)	No (in 96.9% cases)
Y414C	p.Tyr414Cys	c.1241A > G, rs5030860	exon 12	oligomerization	Mild PKU (in 89.7% cases)	Yes (in 100% cases)
R252W	p.Arg252Trp	c.754C > T, rs5030847	exon 7	catalytic	Classic PKU (in 98.9% cases)	No (in 100% cases)
R261Q	p.Arg261Gln	c.782G > A, rs5030849	exon 7	catalytic	Mild PKU (in 67.9% cases), classic PKU (in 32.1% cases)	Yes (in 73.3% cases), no (in 22.7% cases)
R261*	p.Arg261Ter	c.781C > T, rs5030850	exon 7	catalytic	Classic PKU (in 100% cases)	No (in 80% cases), slow (in 20% cases)
G272*	p.Gly272Ter	c.814G > T, rs62514952	exon 7	catalytic	Classic PKU (in 100% cases)	Not tested
I65T	p.Ile65Thr	c.194 T > C, rs75193786	exon 3	regulatory	Mild PKU (in 71.7% cases), classic PKU (in 28.3% cases)	Yes (in 84.6% cases), no (in 15.4% cases)
IVS12 + 1G > A	–	c.1315 + 1G > A, rs5030861	intron 12	–	Classic PKU (in 98.9% cases)	No (in 86.7% cases), slow (in 13.3% cases)
IVS10-11G > A	–	c.1066-11G > A, rs5030855	intron 10	–	Classic PKU (in 98.4% cases)	No (in 94.0% cases)

Table 2
Summary of PCR-RFLP analysis.

Variants	Primer sequence	Amplicon (bp)	Restriction enzyme	Size of restriction fragments (bp)
I65T	AACGAGAAGGTCTAGATTC GTTAGGTTTTCCTGTTCTGG	132	TaqI	N: 18 + 114 M: 132
R252W	CAAACCTCATTCTTGCAGCAGG ACTACCAAAGGTCTCCTAGTGC	285	*Ava*I	N: 124 + 161 M: 285
G272*	CAAACCTCATTCTTGCAGCAGG ACTACCAAAGGTCTCCTAGTGC	285	BamHI	N: 99 + 186 M: 285
R261Q	CAAACCTCATTCTTGCAGCAGG ACTACCAAAGGTCTCCTAGTGC	285	*Hin*fI	N: 30 + 123 + 132 M: 30 + 255
R261*	CAAACCTCATTCTTGCAGCAGG ACTACCAAAGGTCTCCTAGTGC	285	*Dde*I	N: 32 + 253 M: 32 + 119 + 134
Y414C	AGTCTTCGATTACTGAGAAA TCGGCCCTTCTCAGTTCGGT	147	*Rsa*I	N: 20 + 127 M: 147
R408W	CTCGTAAGGTGTAAATTACGTA CCAAATGGTGCCCTTCACTCAAGCC	181	*Sty*I	N: 181 M: 66 + 115
IVS12 + 1G > A	CTCGTAAGGTGTAAATTACGTA CCAAATGGTGCCCTTCACTCAAGCC	181	RsaI	N: 21 + 160 M: 181
IVS10-11G > A	TAGACATTGGAGTCCACTCTC TGCAGCAGGGAATACTGATC	295	DdeI	N: 295 M: 52 + 243

Note: N – ancestral allele, M – derived allele.

Table 3
Characteristics of the results of newborn screening for PKU in Ukraine during 2011–2020 years.

Year	Living newborns	Examined newborns	Hyper-phenylalaninemia was detected	Confirmed diagnosis PKU	Research of genotype in our center
2011	502,595	461,328	305	55	13
2012	520,705	459,920	382	62	26
2013	503,657	505,091	400	60	26
2014*	465,882	372,470	1167	51	26
2015*	411,781	362,242	630	52	32
2016*	397,037	357,647	343	54	31
2017*	363,987	310,876	305	50	29
2018*	335,874	292,915	243	48	37
2019*	308,817	296,654	200	41	25
2020*	293,457	248,938	196	43	12
Total	4,103,792	3,668,081	4171	516	257

Note * excluding temporarily occupied territories in Luhansk and Donetsk regions, the Autonomous Republic of Crimea, city Sevastopol.

variant in the second allele. In most cases (42.8%), the second allele reliably had a rare variant that was not included in our panel of variants of the *PAH* gene, which did not allow us to identify it in this study. Approximately a quarter of the examined patients (26.8%) had the R408W/R408W genotype, most common in terms of the homozygous variant. Homozygous genotypes by R252W, R261Q and Y414C variants, respectively, were detected in three patients. In 12.1% of patients, the applied panel of variants of the *PAH* gene did not allow us to identify the pathogenic variant in any allele.

5. Discussion

In 1934, Fölling A and Über A [20], when examining children with mental retardation, suggested that there was a link between imbecility and metabolic disorders – phenylpyruvic acid was found in patients' urine, in contrast to the urine of healthy people. Penrose L (1935) [21] continued this research and suggested the term «phenylketonuria». Later, Bickel H et al. (1953) [22] reported the effectiveness of a diet low in phenylalanine in children with PKU and emphasized the need for further controlled studies in young children. In the 1960s, Guthrie R

Table 4
Distribution variants of *PAH* gene in 514 chromosomes of PKU patients from Ukraine.

Variants	Number of patients	Number of homozygotes	Number of compound heterozygotes	Number of alleles
R408W	202	69	133	271 (52.7%)
R252W	17	1	16	18 (3.5%)
Y414C	8	1	7	9 (1.8%)
R261Q	7	1	6	8 (1.6%)
IVS10-11G > A	8	0	8	8 (1.6%)
IVS12 + 1G > A	6	0	6	6 (1.2%)
G272*	2	0	2	2 (0.4%)
I65T	1	0	1	1 (0.2%)
R261*	0	0	0	0 (0.0%)
Total identified	–	–	–	323 (62.8%)
Total unidentified	–	–	–	191 (37.2%)

Table 5
Frequency variants of *PAH* gene in the countries bordering Ukraine.

Population PKU	Number of investigated chromosomes	2 most frequent variants	Reference
Ukraine	514	R408W – 52.7%, R252W – 3.5%	Present study
Russia	142	R408W – 47.9%, R261Q – 9.1%	Nikiforova (2017) [13]
Belarus	510	R408W – 66.5%, R158Q – 6.7%	Cukerman (2008) [14]
Poland	134	R408W – 68.0%, IVS10-11G > A – 6.0%	Dobrowolski (2009) [15]
Moldova	182	R408W – 50.6%, P281L – 5.5%	Badicean (2015) [16]
Romania	162	R408W – 37.7%, L48S – 9.3%	Gemperle-Britschgi (2016) [17]
Hungary	70	R408W – 48.6%, 2nd – not avaliable	Zschocke (2003) [18]
Slovakia	414	R408W – 47.3%, R158Q – 5.3%	Polak (2013) [19]

Table 6
Frequency of genotypes by variants of *PAH* gene.

Genotype	Study group ($n = 257$)
R408W/R408W	69 (26.8%)
R252W/ R252W	1 (0.4%)
R261Q/R261Q	1 (0.4%)
Y414C/Y414C	1 (0.4%)
R408W/R252W	5 (1.9%)
R408W/ IVS12 + 1G > A	5 (1.9%)
R408W/Y414C	5 (1.9%)
R408W/ IVS10-11G > A	4 (1.6%)
R408W/R261Q	2 (0.8%)
R408W/I65T	1 (0.4%)
R408W/G272*	1 (0.4%)
IVS12 + 1G > A/IVS10-11G > A	1 (0.4%)
R261Q/Y414C	1 (0.4%)
R408W/X	110 (42.8%)
R252W/X	11 (4.3%)
IVS10-11G > A/X	3 (1.2%)
R261Q/X	3 (1.2%)
G272*/X	1 (0.4%)
Y414C/X	1 (0.4%)
X/X	31 (12.1%)

Note X – unidentify pathogenic variant

(1961) [23] developed a test to determine phenylalanine in a dry blood spot. It was the development of this method that became a revolutionary breakthrough, which in a few years enabled the introduction of newborn screening to determine the level of phenylalanine.

According to national statistics in Ukraine's neighboring countries, the following PKU frequency was established: Russia – 1:7714, Belarus – 1:7309, Poland – 1:8068, Hungary – 1:12689, Slovakia – 1:5229 [24]. Thus, the frequency of PKU detection in Ukraine determined by us did not differ significantly from the neighboring countries. Therefore, when forming the panel of the most common variants in the *PAH* gene, we used information about the variants that are the most common in Europe [25]. The results of our analysis showed that in Ukraine among patients with PKU, the three most common variants are R408W, R252W and Y414C – their total frequency was 58%.

Missense replacement R408W is the most common in our country and is found in 52.7% of mutant chromosomes in both hetero- and homozygous states. This variant is caused by a CGG-to-TGG transition in exon 12, resulting in an amino acid substitution (Arg-to-Trp) at residue 408 (R408W) of *PAH* gene and is a null mutation associated with <0.3% of normal activity and a severe PKU phenotype [26].

In 1991 Okano et al. [27] described a CGG-to-TGG transition at the first base of codon 252 of the *PAH* gene, which resulted in the substitution of tryptophan for arginine (R252W). Further analysis of expression vectors containing the mutant cDNA and transfected into mammalian cells revealed negligible enzyme activity (below 1% of normal levels) and undetectable levels of immunoreactive PAH protein. The frequency of the R252W variant, determined in our study, was 3.5%.

The tyrosine414-to-cysteine (Y414C) variant in exon 12 of *PAH* gene caused TAC-to-TGC transition at the second base of codon 414 [5]. In vitro expression studies showed that the Y414C variant produced a protein with a significant amount of PAH enzyme activity, i.e., approximately 50% of normal steady-state levels [28]. Sweden had the highest number of cases PKU with Tyr414Cys variant in the world – with an AF 23.6% [29]. The frequency of this variant in patients with PKU from Ukraine is 1.8%.

Analysis of the results of our study showed that the selected panel of variants in the *PAH* gene enables us to identify their presence in 87.9% of patients with PKU, which allows, in most cases, to definitively confirm the patient's diagnosis and plan further tactics to determine sensitivity to cofactor therapy. In 12.1% of cases, we did not identify the genotypes of patients – these cases require additional genetic tests, primarily in-depth study of the *PAH* gene, as well as differential diagnosis with *PAH*-independent forms of hyperphenylalaninemia.

6. Conclusions

A retrospective analysis of ten-year results of molecular and genetic testing of the *PAH* gene covered almost 50% of children from Ukraine who were later diagnosed with PKU. The selected panel allowed us to identify the presence of variants in 87.9% of patients with PKU. R408W (AF = 52.7%), R252W (AF = 3.5%) and Y414C (AF = 1.8%) were the most common among them. The panel of genetic testing in the *PAH* gene for the newborns that we used for the study allows accurate prediction of some phenotypes for therapy planning. But in-depth analysis of pathological gene variants may be necessary for unclear and difficult cases of

the disease, and for genetic counseling of patients families.

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CRediT authorship contribution statement

Liliya Fishchuk: Formal analysis, Writing – original draft. **Zoia Rossokha:** Conceptualization, Writing – review & editing. **Natalia Olkhovich:** Data curation, Supervision. **Nataliia Pichkur:** Resources, Data curation. **Olena Popova:** Methodology, Validation. **Nataliia Medvedieva:** Methodology, Validation. **Viktoriia Vershyhora:** Methodology, Investigation. **Olha Dubitska:** Methodology, Investigation. **Tetiana Shkurko:** Investigation, Data curation. **Larysa Popovych:** Resources, Data curation. **Olga Bondar:** Resources, Data curation. **Irina Morozuk:** Resources, Data curation. **Svitlana Onyshchenko:** Resources, Data curation. **Lyubov Yevtushok:** Resources, Data curation. **Oksana Tsizh:** Resources, Data curation. **Iryna Bryl:** Resources, Data curation. **Olena Tul:** Resources, Data curation. **Svitlana Kalynka:** Resources, Data curation. **Iryna Zinkina:** Resources, Data curation. **Svitlana Matviiuk:** Resources, Data curation. **Yulianna Riabova:** Resources, Data curation. **Nataliia Gorovenko:** Project administration, Supervision.

Declaration of Competing Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability

The authors do not have permission to share data.

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