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Review article
Pregledni članak

MOLECULAR DIAGNOSIS OF PHENYLKETONURIA: FROM DEFECTIVE PROTEIN TO DISEASE-CAUSING GENE MUTATION

MOLEKULARNA DIJAGNOZA FENILKETONURIJE: OD PROMENA U PROTEINU DO MUTACIJA U GENU

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Summary: Phenylketonuria (PKU) is the most common inborn error of amino acid metabolism, with an average incidence of 1/10000 in Caucasians. PKU is caused by more than 500 mutations in the phenylalanine hydroxylase gene (*PAH*) which result in phenylalanine hydroxylase (PAH) enzyme deficiency. Two approaches, in vitro expression analysis of mutant PAH and genotype-phenotype correlation study, are used for the assessment of severity of *PAH* mutations. It has been shown that there is a significant correlation between mutant *PAH* genotypes and PKU phenotypes. As a result, the molecular diagnosis is completely shifted toward the detection of mutations in the phenylalanine hydroxylase gene. The study of the molecular basis of PKU in Serbia included identification of the spectrum and frequency of *PAH* mutations in Serbian PKU patients and genotype-phenotype correlation analysis. By using both PCR-RFLP and »broad range« DGGE/DNA sequencing analysis, the mutation detection rate reached 97%. Thus, the base for molecular diagnosis, genetic counseling and selection of BH4-responsive PKU patients in Serbia was created.

Keywords: phenylalanine hydroxylase, phenylalanine hydroxylase gene mutations, phenotype-genotype correlation, phenylketonuria, tetrahydrobiopterin

Kratak sadržaj: Fenilketonurija (PKU) najčešći je urođeni metabolički poremećaj u populaciji belaca (1/10000). Fenilketonurija nastaje kao posledica više od 500 mutacija u genu za fenilalanin hidroksilazu (*PAH*) koje dovode do deficijencije u aktivnosti enzima fenilalanin hidroksilaze (PAH). Pomoću in vitro ekspresione analize mutiranog enzima PAH i genotip-fenotip korelacije procenjuje se težina mutacija. Pokazano je da postoji značajna korelacija između genotipova u kojima su prisutni mutirani aleli gena *PAH* i fenotipa PKU. Zbog toga je detekcija mutacija u genu za fenilalanin hidroksilazu sastavni deo moderne dijagnostike PKU. Studija molekularne osnove fenilketonurije u Srbiji obuhvatila je identifikaciju spektra i frekvencije mutacija *PAH* i analizu korelacije genotipa i fenotipa pacijenata. Kombinovanjem metoda, PCR-RFLP, DGGE širokog spektra i DNK sekvenciranja, postignut je nivo detekcije mutacija od 97%. Na taj način je u Srbiji postavljena osnova za molekularnu dijagnostiku, genetsko savetovanje i odabir pacijenata sa fenilketonurijom kojima bi BH4 terapija bila od koristi.

Ključne reči: fenilalanin hidroksilaza, korelacija fenotip-genotip, mutacije u genu za fenilalanin hidroksilazu, fenilketonurija, tetrahidrobiopterin

Introduction

Phenylketonuria (PKU, MIM#261600) is the most common inborn error of amino acid metabolism, with an average incidence of 1/10000 in Caucasians. PKU is caused by a deficiency of the hepatic enzyme, phenylalanine hydroxylase (PAH, EC 1.14.16.1), which fails to catalyze the conversion of phenylalanine (Phe) to tyrosine (Tyr). Normal dietary phenylalanine intake in the presence of compromised enzyme activity results in an elevated serum level of phenylalanine – hyperphenylalaninemia (HPA). The imbalance between an inborn metabolic error and nutrition can have a toxic effect, leading to impaired cognitive development and neurophysiological function in the patient (1).

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Abbreviations: PKU – Phenylketonuria; *PAH* – Phenylalanine hydroxylase gene; PAH – Phenylalanine hydroxylase; HPA – Hyperphenylalaninemia; MHP – Mild hyperphenylalaninemia; BH4 – (6R)-L-erythro-5, 6, 7, 8-tetrahydrobiopterin; Phe – Phenylalanine; Tyr – Tyrosine; IVE – In vitro expression; DNA – Deoxyribonucleic acid; PCR – Polymerase Chain Reaction.

Fortunately, PKU was one of the first genetic disorders with effective medical treatment. Successful treatment is based on early diagnosis followed by lifelong restriction of phenylalanine in the diet. Nowadays the majority of developed countries perform neonatal screening for PKU. The existence of a simple laboratory test (2) and facilitated collection and transport of blood samples from newborns have established PKU screening as the prototype for genetic screening in human populations.

The key marker for neonatal screening is elevated serum concentration of phenylalanine (1). This parameter is also being used for the categorization of patients into three phenotypic categories. The newborns with Phe>1200 μmol/L are considered to have classical PKU, a level of Phe between 600 and 1200 μmol/L corresponds to mild PKU, and Phe lower than 600 μmol/L is marked as mild hyperphenylalaninemia (MHP) (3). Classical PKU, as well as mild PKU, requires well-adjusted restriction of phenylalanine intake to ensure normal physiological, neurological and cognitive development, whereas no dietary correction is required to prevent neurological symptoms in the patients with MHP (1).

PKU is primarily caused by mutations in the phenylalanine hydroxylase gene. Mutations in the genes encoding enzymes for the synthesis or recycling of tetrahydrobiopterin, BH4 (BH4 deficiency), account for less than 2% of patients with HPA (4). Therefore, the key to understanding the variability of the PKU phenotype manly lies in the great number (>500) of different disease-causing mutations detected in the *PAH* gene (5). These mutations differ by their type (missense 63%, small deletions 13%, splicing 11%, nonsense 5%, large deletions 3%, insertions 1%) and by their position in the gene (6). Thus, the effect of a mutation is determined by the severity of introduced change and by the significance of the position of the affected amino acid residue for the phenylalanine hydroxylase structure and function. An impairing effect of nonsense mutations, splicing defects, deletions and insertions on the PAH enzyme is expected. The effect of a single amino acid substitution caused by a missense mutation is unclear and has to be analyzed.

Defective phenylalanine hydroxylase

Phenylalanine hydroxylase is a tetramer, a dimer of homodimers. The molecular mass of each subunit is approximately 50 kDa and it consists of three domains: the regulatory domain (residues 1–142), the catalytic domain (residues 143–410) and the tetramerization domain (residues 411–452). In order to be catalytically active, the enzyme has an absolute need for ferrous iron, tetrahydrobiopterin and molecular oxygen (7).

Mutated phenylalanine hydroxylase is defective and frequently characterized by decreased thermodynamic stability of the native-state tetramer and/or rate of monomer folding (8). Thus, the quality control system within the cell accelerates the degradation of a mutated protein (9). Numerous studies showed that it was the main mechanism of impairment of the PAH enzyme function caused by *PAH* mutations (8, 10–12). On the other hand, a minority of *PAH* mutations change the kinetic properties (V_{max}, K_m for substrate or cofactor) of phenylalanine hydroxylase (8, 13).

How could the defective enzyme be analyzed in order to understand the effect of a *PAH* mutation?

A structural model of phenylalanine hydroxylase was completed (14–16) and the location, along with structural contacts of individual amino acid residues, was documented (7, 17). This provided the base for a molecular modeling of the mutation effect in silico (18). Although molecular modeling represents a valuable approach, it is not informative and predictive in all cases.

Two approaches, in vitro expression analysis and phenotype-genotype correlation study, have been widely used for the analysis of disease-causing *PAH* gene mutations.

In vitro expression systems

Since in vivo PAH protein expression is limited to the liver and kidney, direct study of the mutant enzymes from PKU patients is not feasible (19). An alternative approach for the study of defective phenylalanine hydroxylase, in vitro expression (IVE), has been used for more than two decades (20–22). Approximately 100 variations of phenylalanine hydroxylase, carrying naturally occurring mutations, have been investigated in several expression systems (www.pahdb.macgill.ca). IVE provides a valuable tool for studying structure and function of the mutant enzyme and revealing the mechanism by which a mutation in the *PAH* gene exerts its damaging effect. Since there is no ideal in vitro system, only by comparing results from different types of in vitro expression systems and diverse subsequent analysis, understanding of the mutant PAH characteristics becomes complete (8).

From phenotype to genotype

Genotype-phenotype correlation study has been widely used in order to elucidate the effect of a *PAH* gene mutation.

Pioneering studies on genotype-phenotype correlation were conducted at the beginning of the nineties (23–25). Many other studies (26, 27) as well as two comprising a large number of patients (28, 29) have consequently been conducted.

The most reliable approach for the assessment of the effect of a *PAH* gene mutation is to analyze it in homozygous and functionally hemizygous patients, in whom the mutation acts on its own (26). In functionally hemizygous patients, a missense mutation, whose effect is analyzed, is combined with a null mutation which completely impairs the PAH activity.

Frameshift, splice-site mutations, base substitutions that introduce a premature stop codon are generally considered as null ones. But this should be taken with caution, because, for example, some splicing mutations do not fully abolish enzyme activity. Additionally, there are missense mutations with severe effect. Hence, mutations that have no residual enzyme activity in vitro (R408W, P281L and S231F) are also considered as functionally null (29, 30).

From genotype to phenotype

The analysis of a defective protein from a patient is not reasonable or possible. Therefore molecular diagnosis is completely shifted toward the detection of mutations in the phenylalanine hydroxylase gene.

At first, it appeared that the genotype strongly correlated with the metabolic phenotype (the blood level of phenylalanine). This remains true for the majority of PAH alleles, mainly those that produce extreme effects. These mutations are consistent in their effects on phenotype and considered as generally predictive markers of PKU severity. As for the rest of the mutations that produce partially active enzymes, interactions between monomers in compound hetero-

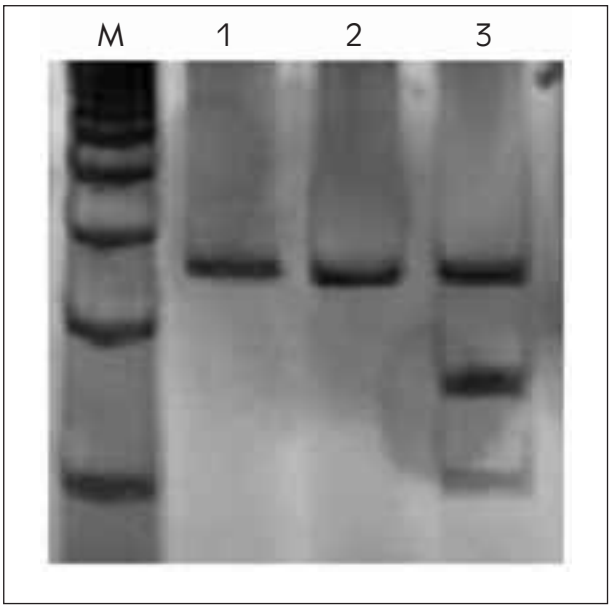

Figure 1 Detection of R408W mutation by PCR-RFLP methodology. PAGE analysis shows normal (1, 2) and a heterozygous carrier (3). M- DNA marker- 100 bp ladder.

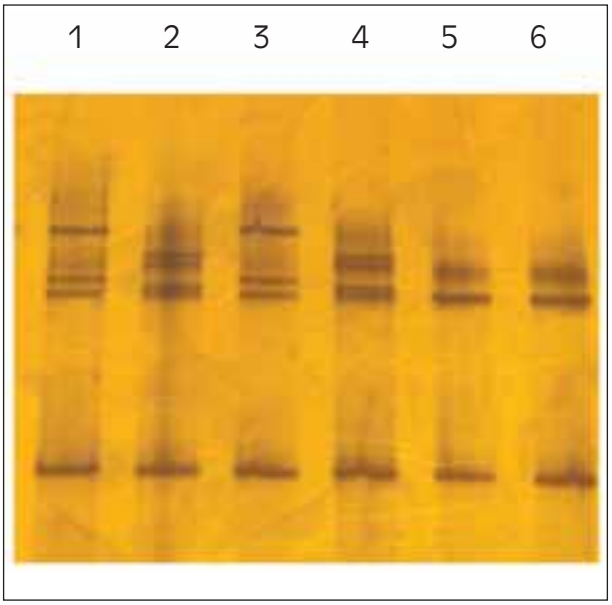

Figure 2 Detection of different changes in *PAH* exons 6 and 10 by multiplex DGGE with broad range gradient (0 – 80%). Heterozygous carriers of different mutations within exon 6 of the PAH gene (1-6) are presented.

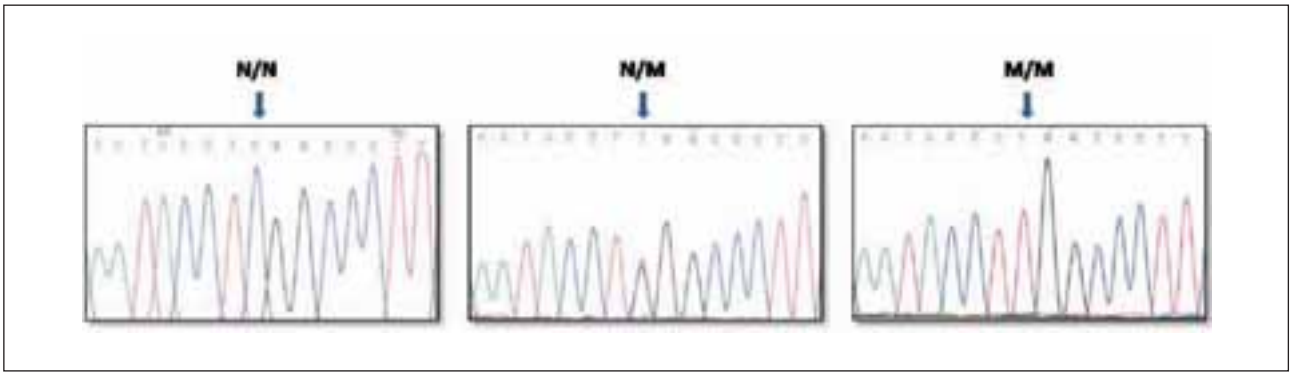

Figure 3 Detection of a mutation by DNA sequencing. Normal (N/N) c.[1222C]+[1222C], heterozygous (N/M) c.[1222C]+[1222C>T] and homozygous mutant (M/M) c.[1222C>T]+[1222C>T] variants of the same nucleotide within the exon 12 of the *PAH* gene are presented.

zygotes, intracellular quality control system, along with other factors, contribute to the variability of the phenotype and make the prediction of PKU severity less precise (31, 32).

However, there are indeed strong correlations between mutant *PAH* genotypes and PKU phenotypes, and the real practical value lies in the counseling and treatment of patients with PKU and their families (28, 29, 33). Unlike for other diseases, such as thalassemia syndromes (34, 35), prenatal diagnosis for PKU is rarely performed. Given that effective treatment for PKU exists, termination of pregnancy, if two mutant *PAH* alleles are detected, is not medically justified.

Recently, the genotyping of PKU patients gained new motivation. Since Kure and coworkers described patients who responded to oral administration of BH4 by demonstrating lower blood phenylalanine levels, several studies reported that BH4 can be successfully used for the long-term treatment of PKU patients as an alternative to dietary treatment (36–40). Additionally, this new alternative treatment has shown to be valuable in the treatment of maternal PKU (41).

Previously, the original use of BH4 was limited to patients suffering from BH4 deficiency (42). Yet, this new group of BH4-responsive patients carried the mutations in the *PAH* gene. Further investigations showed that this response mainly involves a chaperone-like effect on enzyme stability, and in some cases it improves the kinetic response involving binding of BH4 in the catalytic domain of the PAH (43, 44). Thus, the metabolic response to BH4 relies on existing residual PAH enzyme activity and consequent restoration of phenylalanine oxidation by PAH (45). The main criterion for applying the BH4 therapy is the selection of patients on the basis of the BH4 loading test. A generally accepted criterion for the classification of PKU patients as BH4-responsive is their response to oral administration of BH4 (10–20 mg/kg body weight) involving a lowering of the blood phenylalanine level (at least 30%) within 8 to 24 hours (46). Furthermore, the investigations revealed a correlation between the response to BH4 and the patient's genotype (47, 48). Therefore, genotype information becomes important for the selection of patients in whom the BH4-responsiveness is expected, especially in the countries where the BH4 loading test cannot be routinely performed.

Phenylketonuria in Serbia

Neonatal PKU screening has been established since 1982 in Central Serbia and the incidence of PAH deficiency is 1:12300 newborn infants. Each year, approximately 3 to 4 new patients are diagnosed through neonatal screening and genetic counseling programs. The first complete study on the molecular basis of PKU in Serbia included identification of the spectrum and frequency of mutations in the *PAH* gene and the genotype-phenotype correlation analysis of patients with PKU (49). According to the pre-treatment serum phenylalanine level, 34 unrelated patients were assigned to classical PKU (65%) and mild PKU (35%). By combining PCR-RFLP (restriction fragment length polymorphism) and 'broad range' DGGE (denaturing gradient gel electrophoresis)/DNA sequencing analysis (*Figure 1–3*), 19 mutations were identified (13 missense, 3 nonsense, 2 splice and 1 frameshift-del). Mutation detection rate was 97%. The most frequent mutations were: L48S (21%), R408W (18%), P281L (9%), E390G (7%) and R261Q (6%), accounting for 60% of all mutant alleles. Although the frequency of L48S mutation was the highest ever reported, haplotype analysis showed association with at least two different haplotypes and implied that L48S was imported into the Serbian population from populations with different genetic backgrounds (50).

Majority of detected mutations, 14 of them, occurred at the frequency of less than 5%. Therefore it was not surprising that the homozygosity value of the *PAH* locus (0.10), as well as the genotypic homozygosity (8.82%) were low. The heterogeneity of the molecular basis of PKU in the Serbian population was evident. The characterization of *PAH* mutations created the basis for the molecular diagnostics and genetic counseling of patients with phenylketonuria in Serbia.

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