ASSOCIATION OF MITOCHONDRIAL DNA VARIANTS AND COGNITIVE IMPAIRMENT OF PHENYLKETONURIA PATIENTS

POVEZANOST VARIJANTI U MITOHONDRIJALNOJ DNK I KOGNITIVNOG FENOTIPA KOD PACIJENATA SA FENILKETONURIJOM

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Summary

Background: Phenylketonuria (PKU) is a metabolic disorder caused by phenylalanine hydroxylase gene (PAH) mutations. If left untreated, PKU patients develop severe mental retardation potentially due to neurodegeneration. This is the first study that investigates presence of mitochondrial DNA variants in PKU patients, m.10398A, reportedly associated with neurodegenerative diseases and m.10410T.

Methods: We analyzed 64 PKU patients and 50 healthy controls from Serbian population. PKU patients were categorized into groups according to time of diagnosis and compliance to low-phenylalanine diet. The IQ was determined according to age-appropriate scales.

Results: We detected m.10398A and m.10410T variants by direct sequencing. Frequency of m.10398A was similar in patients and healthy controls (82.81% and 82.00% respectively) suggesting their identical ethnic background. No variation was detected for m.10410. In group with late diagnosis and poorly controlled diet, no statistically significant difference in average IQ was found between patients with m.10398A and m.10398G. The same was shown for PKU patients with higher IQ, diagnosed at neonatal screening and treated with low-phenylalanine diet. However, when patients carrying p.L48S, a PAH mutation with inconsistent effect, were excluded from the study, presence of m.10398A variant was associated with lower IQ.

Kratak sadržaj

Uvod: Fenilketonurija (PKU) je metaboličko oboljenje uzrokovano mutacijama u genu za fenilalanin hidroksilazu (PAH). Ukoliko se ne leće, pacijenti sa fenilketonurijom razvijaju tešku mentalnu retardaciju, koja može biti i posledica neurodegeneracije. Ovo je prva studija koja istražuje prisustvo mitohondrijalnih DNK varijanti kod pacijenata sa fenilketonurijom, m.10398A, za koju je pokazana povezanost sa neurodegenerativnim oboljenjima, kao i m.10410T.

Metode: U ovoj studiji je analizirano 64 pacijenta sa fenilketonurijom i 50 zdravih kontrola iz srpske populacije. Pacijenti su podeljeni u grupe prema uzrastu u kome je postavljena dijagnoza i odnosu prema dijeti sa niskim sadržajem fenilalanina.IQ je određen pomoću odgovarajućih uzrasnih skala.

Rezultati: Mitohondrijalne DNK varijante, m.10398A i m.10410T, su detektovane sekvenciranjem. Učestalost m.10398A je bila jednaka kod pacijenata i zdravih kontrola (82,81% i 82,00%), što ukazuje na isto etničko poreklo ovih ispitanika. U slučaju m.10410, nisu detektovane različite varijante. U grupi pacijenata kojima je kasno postavljena dijagnoza i koji su neadekvatno lečeni, nije pronađena statistička značajnost u srednjoj vrednosti IQ između pacijenata sa m.10398A i m.10398G. Isto je pokazano i za pacijente sa višim IQ koji su otkriveni prilikom neonatalnog

List of Abbreviations: IQ, Intelligence quotient; mtDNA, Mitochondrial deoxyribonucleic acid; PAH, Phenylalanine hydroxylase gene; Phe, Phenylalanine; PCR, Polymerase Chain Reaction; Phe, Phenylalanine; PKU, Phenylketonuria; ROS, Reactive oxygen species.
Conclusions: This study emphasizes the importance of neonatal screening and good control of low-phenylalanine diet in PKU patients. Statistical analysis did not indicate clear impact of mitochondrial DNA variant m.10398A on IQ of PKU patients, except when PAH genotype was also considered. Studies in larger cohorts will elucidate the association between PAH gene mutations, mitochondrial DNA variants and complex PKU cognitive phenotype.

Keywords: cognitive impairment; mitochondrial DNA; modifier genes; phenylalanine hydroxylase; phenotype-genotype correlation; phenylketonuria

Introduction

Phenylketonuria (PKU, MIM#261600) is an inborn error of metabolism caused by a deficiency of the hepatic enzyme, phenylalanine hydroxylase (PAH), due to mutations in the PAH gene (1). Defects in the PAH enzyme result in elevated serum level of phenylalanine (Phe), elevated level of Phe in the brain, and in severe mental retardation. If the disorder is diagnosed shortly after birth and if the proper treatment is initiated early, mental retardation can be prevented. However, even in well treated PKU patients, intelligence quotient (IQ) is several points lower than in healthy controls, and neuropsychological and neuropsychological impairments develop (2–3). To this day, the exact pathophysiological mechanism by which PKU results in cognitive dysfunction remains unclear.

Phenylketonuria is a monogenic disease which is transmitted in an autosomal-recessive pattern. It has been proven that two disease-causing mutations in the PAH gene stand as the main determinant of phenylalanine hydroxylase activity, all of which can in turn induce apoptosis in neuronal cells and lead to neurodegeneration (27). Some of these variations are actually disease-causing mutations that cause several rare mitochondrial diseases, such as Alzheimer’s disease (25, 26). Several mitochondrial DNA variants have been repeatedly associated with these conditions. Variants in mitochondrial DNA can lead to perturbations in calcium level, altered energy production, and neurodegenerative diseases such as Alzheimer’s disease (21, 22), Parkinson’s disease (23, 24), Huntington’s disease (25, 26). Several mitochondrial DNA variants have been repeatedly associated with these conditions. Variants in mitochondrial DNA can lead to increased reactive oxygen species (ROS) production, perturbations in calcium level, altered energy production, all of which can in turn induce apoptosis in neuronal cells and lead to neurodegeneration (27). Having in mind that cognitive dysfunction in PKU comes from neuronal cell death, we focused our lower IQ scores (10). However, significant genotype-cognitive phenotype inconsistencies were observed (11, 12). Complex features, such as extent of cognitive impairment and intelligence, are influenced by numerous factors in addition to the PAH genotype. It was documented that patients with high blood levels of Phe but low levels of Phe in the brain have better cognitive outcome, even when untreated (13, 14). This has drawn attention to genes responsible for transport through the blood–brain barrier. The gene sequence responsible for the 4F2hc/LAT1 complex was investigated, but no pathogenic genetic variants have been found in a group of PKU patients with low brain Phe levels (15, for a review see 16). As a result, the search for new PKU modifier genes continues.

Apart from variants in the nuclear human genome, germline and somatic changes in mitochondrial DNA (mtDNA) have also been frequent throughout human evolution, making the mitochondrial DNA highly variable between different populations. The existence of numerous mitochondrial haplotypes and haplogroups has enabled tracing back of human evolutionary history. Nevertheless, in addition to defining haplogroups, germline variations in mtDNA have often been associated with different human diseases. Some of these variations are actually disease-causing mutations that cause several rare mitochondrial diseases, such as Leber’s hereditary optic neuropathy (17) and Kearns-Sayre syndrome (18, 19). However, mitochondrial involvement is also suggested in other, more common diseases, such as diabetes (20) and neurodegenerative diseases such as Alzheimer’s disease (21, 22), Parkinson’s disease (23, 24), Huntington’s disease (25, 26). Several mitochondrial DNA variants have been repeatedly associated with these conditions. Variants in mitochondrial DNA can lead to increased reactive oxygen species (ROS) production, perturbations in calcium level, altered energy production, all of which can in turn induce apoptosis in neuronal cells and lead to neurodegeneration (27). Having in mind that cognitive dysfunction in PKU comes from neuronal cell death, we focused our studies on PKU patients, except when PAH genotype was included in the analysis. Surveying for mitochondrial DNA variances m.10398A, which PKU results in cognitive dysfunction remains unclear. Studies in larger groups will elucidate the association between metabolic PKU mutations and cognitive phenotype (IQ score) has also been noted. Untreated PKU patients carrying mutations severely affecting PAH enzyme structure and function, generally have lower IQ scores (10). However, significant genotype-cognitive phenotype inconsistencies were observed (11, 12). Complex features, such as extent of cognitive impairment and intelligence, are influenced by numerous factors in addition to the PAH genotype. It was documented that patients with high blood levels of Phe but low levels of Phe in the brain have better cognitive outcome, even when untreated (13, 14). This has drawn attention to genes responsible for transport through the blood–brain barrier. The gene sequence responsible for the 4F2hc/LAT1 complex was investigated, but no pathogenic genetic variants have been found in a group of PKU patients with low brain Phe levels (15, for a review see 16). As a result, the search for new PKU modifier genes continues.

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attention on mitochondrial DNA variants repeatedly associated with neurodegeneration.

One of the most extensively studied mitochondrial DNA variants is m.10398A>G. This variant results in a nonsynonymous amino acid substitution from threonine (encoded by the A variant) to alanine (encoded by the G variant) within the nicotinamide adenine dinucleotide dehydrogenase (NADH) subunit of complex I of the electron transport chain. The association of this variant with impaired neuronal function was first reported when epidemiologic studies showed that m.10398A was associated with bipolar disorder (28, 29). Shortly after, it was reported that m.10398A was associated with Parkinson’s disease (23) and Alzheimer’s disease (21). All of these studies have conferred the m.10398A variant is the disease risk one, whilst m.10398G was considered as the protective one. A recent study using updated calcium imaging techniques has shown that the m.10398A variant alters mitochondrial calcium levels and pH (30). Several mechanisms of how the m.10398A variant might be influencing neurodegeneration have been proposed, and it is probably via increased ROS and inducing apoptosis due to oxidative stress. The m.10398A variant is hypothesized to alter the structure of complex I resulting in increased ROS generation at this site. Increased ROS in cybrid cells harboring the m.10398A variant (compared to m.10398G) was shown (31). These epidemiologic and functional studies have suggested that the m.10398A variant is associated with the degenerative phenotype, whereas the m.10398G variant is usually protective.

Our study is the first one to hypothesize that PKU patients with the m.10398A variant may be more predisposed to neuronal cell death (similar to neurodegenerative diseases), due to the excess of Phe in brain. Those patients would be strongly advised to continue with a restricted Phe diet for life. For that reason, we investigated the association of the m.10398A variant with cognitive phenotype (evaluated as IQ scores) of Serbian PKU patients.

We also analyzed the m.10410T>C variant. Mitochondrial DNA variant m.10410C is one of the variants that define mitochondrial haplogroup D4a and it is located in the tRNAArg gene. This variant was previously thought to have functional significance, but experiments with cybrid cells did not show any significant changes in mitochondrial respiratory function or morphology in cybrids harboring m.10410C (32).

Materials and Methods

Patients with phenylketonuria

In this study we investigated 64 patients with PKU and 50 ethnicity and sex matched healthy controls. This study was conducted according to the guidelines of the Helsinki Declaration and was approved by the Ethics Committee at the Institute for Mother and Child Health Care »Dr Vukan Ćupić«. All parents and/or patients gave informed consent.

Neonatal screening for PKU was established in Central Serbia in 1982, and in 2003 it was expanded to Vojvodina (Serbian Northern province) (8). Although most patients included in this study were detected by the newborn screening program, some patients were diagnosed later in life during evaluation of psychomotor retardation and measurement of blood Phe level at the Institute for Mother and Child Health Care »Dr Vukan Ćupić« in Belgrade. Patient samples were further referred to the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, for molecular genetic analysis. Mutations in the PAH gene of PKU patients were identified by standard methods (Denaturing Gradient Gel Electrophoresis and DNA sequencing) as previously described (7, 8, 33).

Clinical data collected include: pre-treatment and maximal phenylalanine blood concentration, phenylalanine tolerance, IQ, age of diagnosis, application of a low Phe diet, and other relevant data (8).

To date, time of diagnosis and good compliance to a low Phe diet were proven to be crucial for the development of cognitive phenotype (1). Therefore, for the purpose of this study, patients were classified into four categories. Considering the differences regarding the time of diagnosis in our cohort of patients, we distributed patients into groups consisted of patients diagnosed by neonatal screening (DBNS) and patients diagnosed after 6 months of life – late diagnosis (LD). Next, having in mind the important effect of proper treatment to PKU cognitive phenotype, we subdivided these two groups by compliance to PKU diet: patients with good compliance to low Phe diet treatment (LPD), and patients with poorly controlled low Phe diet (PC).

Cognitive phenotype of PKU patients

We have used age-appropriate scales to assess patients’ cognitive phenotype. For children under age 3, we have used Brunet-Lezine scale to determine psychomotor development. For children over 3 and adults we have used Wechsler Scales appropriate to the age of patients to assess cognitive development.

Identification of the m.10398A>G and m.10410T>C mitochondrial DNA variants

Mitochondrial DNA was extracted from peripheral blood by the Qiagen DNA Blood Mini Kit (Qiagen). The mtDNA variants, m.10398A>G and m.10410T>C, were identified by direct DNA sequencing. The mitochondrial DNA region from
9968nt to 10814nt was amplified with KAPA Taq DNA Polymerase (KAPA Biosystems) using forward primer 5’-CTCCATCTATTGATGGGCTT-3’ and reverse primer 5’-GGAAAGTCATGTCAGTGGTAGTA-3. The PCR conditions were as follows: denaturation step at 95 °C for 5 min, 35 cycles of amplification (95 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s) and elongation step for 10 min at 72 °C. The obtained PCR product of 846 bp was sequenced with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Alternatively, identification of m.10398A>G was performed by PCR-RFLP with the DdeI restriction enzyme according to manufacturer’s instruction (New England Biolabs). The DdeI restriction site is present when G is at the m.10398 mtDNA position. The result of the restriction reaction was verified on 4% EtBr stained agarose gel (Serva).

Statistical analysis
The difference in allele distributions between patients and healthy controls was analyzed with the Pearson chi-squared test. The IQ score differences between groups of patients were analyzed using a Student’s t-test. A p-value less than 0.05 was considered statistically significant.

Results
We used the Pearson chi-squared test to compare the frequency of m.10398A versus m.10398G variants between PKU patients and healthy controls. In PKU patients, m.10398A was present in 53 out of 64 cases (82.81%) and in healthy controls the frequency was almost the same: 41 out of 50 individuals (82.00%) had m.10398A. As for the m.10410T>C, only m.10410T variant was detected in the group of PKU patients (100.00% frequency). Therefore, these results were not further subjected to statistical analysis.

It is worth noting that we did not detect heteroplasmy for either m.10398A>G or m.10410T>C variants in any subject.

Although the m.10398A variant was uniformly distributed among PKU patients and the control group, we further analyzed the distribution of the m.10398A variant within the four different groups of patients (DBNS+LPD, DBNS+PCD, LD+LPD and LD+PCD) (Table I). Corresponding to the high frequency of m.10398A in the group of PKU patients, we found that the m.10398A variant is more frequent than m.10398G in each of the PKU patient groups.

Furthermore, we analyzed the association between patients’ IQ scores and the presence of the m.10398A or m.10398G variant in their mitochondrial DNA. We found that in the group of patients diagnosed at neonatal screening and treated with a low Phe diet, no statistically significant difference in average IQ was found between patients with m.10398A and m.10398G (Table I). The same was shown for PKU patients with low IQ due to late diagnosis. However, patients with m.10398A had slightly lower average IQ than patients with m.10398G (Table I).

Considering the phenotypic inconsistency of the p.L48S PAH mutation (8), we have further stratified the group of patients diagnosed at neonatal screening and treated with a low-phenylalanine diet (Table II). We analyzed the association of m.10398A>G mitochondrial DNA variant with the IQ score of PKU patients who were not carriers of p.L48S mutation in the PAH gene. Using Student’s t-test we found that patients carrying the m.10398A variant have significantly lower IQ scores (p=0.045).

Discussion
Variants in mitochondrial DNA can cause inefficient oxidative phosphorylation leading to the accumulation of ROS, mtDNA damage and neuronal cell death, which leads to neurodegeneration (27). Since the m.10398A variant was reported to alter the ND3 subunit of the electron transport chain and cause oxidative stress, we have investigated the association of the m.10398A variant with cognitive impairment in PKU patients.

The m.10398 nucleotide position in the human mitochondrial genome is highly variable, and the frequencies of A and G variants vary significantly among different populations (34). In contrast to African

Table I Allele frequencies and IQ scores distributions within four groups of unrelated Serbian PKU patients (DBNS – diagnosis by neonatal screening, LD – late diagnosis, LPD – good control of low Phe diet, PCD – poorly controlled low Phe diet).

| PKU patients | m.10398A | m.10398G |
|--------------|----------|----------|
|              | Allele Frequency | Percentage | IQ (average) | Allele Frequency | Percentage | IQ (average) |
| DBNS+LPD     | 26/31    | 83.87%   | 97.08   | 5/31    | 16.13%   | 96.60    |
| DBNS+PCD     | 6/6      | 100.00%  | 77.08   | 0/6     | 0.00%    | NA       |
| LD+LPD       | 5/6      | 85.33%   | 74.30   | 1/6     | 16.67%   | 60.00    |
| LD+PCD       | 2/4      | 50.00%   | 47.50   | 2/4     | 50.00%   | 55.00    |
American and Asian populations, the frequency of m.10398A in Caucasian populations is relatively high (close to 80.00%) (34). This is in concordance with findings for the Serbian population, both for the PKU patient group and healthy controls. Uniform distribution of the m.10398A variant in PKU patients and healthy controls from the Serbian population can be explained by mitochondrial haplogroups. The Serbian population has similar frequencies of mitochondrial haplogroups as other European populations, with haplogroup H (which bears the m.10398A variant) being the most frequent (41.00%), followed by U5 (9.40%), J and U4 (6.80%) (35, 36).

We analyzed the cohort comprised of PKU patients with a diverse degree of cognitive impairment, ranging from severe mental retardation (IQ 20–30) to normal intelligence (IQ 110). For the purpose of this study, patients were classified into appropriate groups to better assess cognitive phenotype in regard to the association with the m.10398A mitochondrial DNA variant. They were classified into four categories considering differences regarding the time of diagnosis and compliance to the low Phe therapy (Table I). Corresponding to the high frequency of m.10398A in the whole cohort of PKU patients, we found that the m.10398A variant is more frequent than m.10398G in each of the PKU patient groups.

Furthermore, we analyzed the association between patients’ IQ scores and the presence of the m.10398A or m.10398G variant in their mitochondrial DNA. The group of patients that have been diagnosed later in life and therefore lacked proper treatment had considerably lower IQ scores, which was expected considering the essential role of a low Phe diet for normal cognitive outcome of PKU patients. Also, in LD+PCD group of PKU patients, a statistically significant difference in average IQ was not found between patients with m.10398A and m.10398G.

However, patients with m.10398A had slightly lower average IQ than patients with m.10398G (Table I), implying that a statistically significant difference could be found in a larger cohort. Thus, it still remains unclear whether the m.10398A mitochondrial DNA variant could be of particular importance for the cognitive outcome of untreated patients.

We found that in the group of patients diagnosed at neonatal screening and continuously treated with a low Phe diet, no statistically significant difference in average IQ was found between patients with m.10398A and m.10398G (Table I).

Table II Allele frequencies and IQ scores distributions within the DBNS+LPD group of unrelated Serbian PKU patients (only genotypes without p.L48S PAH mutation).

| PKU patients (only genotypes without PAH p.L48S) | m.10398A | m.10398G |
|------------------------------------------------|----------|----------|
|                                               | Allele   | Percentage | IQ (average) | Allele   | Percentage | IQ (average) |
| DBNS+LPD                                      | Frequency|           |             | Frequency|           |             |
| 16/19                                         | 84.21%   | 95.94     |             | 3/19     | 15.79%     | 101.00      |

Nowadays, PAH genotype is generally considered the main determinant of PKU phenotype. Therefore, we made the attempt to minimize inconsistency that comes from the PAH genotype. It was previously shown that the p.L48S PAH mutation significantly contributes to a genotype-phenotype correlation inconsistency (8). For that reason, we excluded the patients carrying p.L48S, from properly treated group of patients (DBNS+LPD) and we found that patients with the m.10398A variant had significantly lower IQ scores in comparison to m.10398G (Table II). This result should be considered preliminary due to the small sample size, but it could lead to a better understanding of the complex relationship between the PAH genotype, mitochondrial DNA variants and cognitive impairment of PKU patients.

As a result of this study, the importance of neonatal screening and good control of a low-phenylalanine diet for phenotype of PKU patients, were once more emphasized. Additionally, to the best of our knowledge, this is the first study of the association of mitochondrial DNA variants and cognitive impairment in PKU patients. Although our results need to be confirmed in larger groups, they have provided the first clue for the complex relationship between the PAH gene, mtDNA and cognitive phenotype of PKU patients.

The sophisticated interplay between Phe in excess, ROS production, mtDNA variations and mitochondrial impairment in the process of PKU patients’ cognitive phenotype formation is still to be elucidated. There might be even more mitochondrial DNA variants that influence neuronal cell death, as well as variants in nuclear encoded genes that may alter the proper function of mitochondria. Further study of these pathways could reveal other, currently undiscovered, PKU modifier genes.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.
30. Kazuno AA, Munakata K, Nagai T, Shimozono S, Tanaka M, Yoneda M, et al. Identification of mitochondrial DNA polymorphisms that alter mitochondrial matrix pH and intracellular calcium dynamics. PLoS Genet 2006; 2: e128.

31. Kulawiec M, Owens KM, Singh KK. mtDNA Gm.10398A variant in African-American women with breast cancer provides resistance to apoptosis and promotes metastasis in mice. J Hum Genet 2009; 54: 647–54.

32. Hayashi J, Ohta S, Kagawa Y, Takai D, Miyabayashi S, Tada K, et al. Functional and morphological abnormalities of mitochondria in human cells containing mitochondrial DNA with pathogenic point mutations in tRNA genes. J Biol Chem 1994; 269: 19060–6.

33. Majkić-Singh N, Šumarac Z. Quality indicators of the pre-analytical phase. J Med Biochem 2012; 31: 174–183.

34. Torroni A, Lott MT, Cabell MF, Chen YS, Lavergne L, Wallace DC. mtDNA and the origin of Caucasians: identification of ancient Caucasian-specific haplogroups, one of which is prone to a recurrent somatic duplication in the D-loop region. Am J Hum Genet 1994; 55: 760–76.

35. Cvjetan S, Tolk HV, Lauc LB, Čolak I, Đorđević D, Efremovska Lj et al. Frequencies of mtDNA Haplogroups in Southeastern Europe – Croatians, Bosnians and Herzegovinians, Serbians, Macedonians and Macedonian Romani. Coll Antropol 2004; 28: 193–8.

36. Zgonjanin D, Veselinović I, Kubat M, Furac I, Antov M, Loncar E et al. Sequence polymorphism of the mitochondrial DNA control region in the population of Vojvodina Province, Serbia. Legal Medicine 2010; 12: 104–7.

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