MARIA SCURTUL, CHIRIL BOICIUC*, DANIELA BLĂNIȚĂ,
VICTORIA SACARĂ, NATALIA UȘURELU*

PHENOTYPE PREDICTION IN PHENYLKETONURIA PATIENTS FROM MOLDOVA BASED ON GENOTYPE DATA

Institute of Mother and Child, Chisinau, Republic of Moldova

REZUMAT

PREDICȚIA FENOTIPULUI LA PACIENȚII CU FENILCETONURIE DIN MOLDOVA ÎN BAZA GENOTIPULUI

Introducere: Fenilcetonuria (PKU) este o boală metabolică autozomal recesivă, cauzată de deficienţa enzimei fenilalaninhidroxilaza ca urmare a mutaţiilor din gena *PAH*. Luând în considerare, că celulele nervoase sunt cele mai sensibile la efectele toxice ale nivelelor ridicate ale fenilalaninei serice, retardul mental este unul din cele mai proeminente manifestări ale bolii. Tipul mutaţiei din gena PAH influenţează activitatea metabolică restantă a enzimei fenilalaninhidroxilaza.

Scopul: Estimarea fenotipului prezis în raport cu genotipul la pacienţii cu PKU din Republica Moldova, pe baza diferitor programe de predicţie.

Material şi metode: În prezentul studiu au fost folosite datele a 9 pacienţi cu PKU, diagnosticaţi prin screeningul neonatal (>3 mg/dL) în perioada 2018-2019, la care s-au analizat mutaţiile în gena PAH. Pentru estimarea fenotipului acestor pacienţi a fost folosită metoda valorii arbitrare (AV). A doua metodă a constat în utilizarea instrumentului online BIOPKU (*BIOPKU;* http://www.biopku.org). Identificarea mutaţiilor frecvente s-a realizat prin metoda *PCR/RFLP* (*p.R408W, p. P281L, p.L48S, p.R252W, p.R158Q, p.R261Q*), iar pentru detectarea mutaţiilor mai rare s-a efectuat secvenţierea unor exoni din gena PAH.

Rezultate: Utilizând datele despre genotipurile patologice identificate la cei 9 pacienți cu PKU investigaţi, a fost estimat fenotipul lor. Metoda de predicţie AV a dat rezultate în care 66.7% din pacienţi aveau forma clasică a PKU, pe când în 33.3% de cazuri a fost detectat fenotipul cu PKU moderată, nefiind prezise fenotipuri cu hiperfenilalaninemie (HPA). Cea de-a doua metodă, ce implica utilizarea bazei de date *BIOPKU*, a fost folostă în cazul a 8 genotipuri din 9 din cauza lipsei informaţiei despre o combinaţie de alele. Genotipurile celor 8 pacienţi au fost estimate ca tipice PKU clasice. Nu au fost înregistrate cazuri de formă moderată a PKU sau hiperfenilalaninemie. Comparând datele de la instumentele de predicţie cu fenotipurile observate, s-a constatat că există o diferenţă la nivelul genotipului *p.R408W/p.L48S*, analizat mai profund din perspectiva heterogenităţii sale. Prin acestea concludem că în Republica Moldova prevalează formele clasice de PKU înregistrate în ultimii 2 ani.

Concluzii: Metodele de predicţie a fenotipului precum AV sau BIOPKU au punctele lor forte. În cazul completării bazelor de date internaţionale cu cazuri proprii, va spori calitatea şi varietatea programelor de predicţie..

Cuvinte-cheie: Fenilcetonurie, predicţia genetică, genotip, fenotip, BIOPKU.

РЕЗЮМЕ

ПРОГНОЗИРОВАНИЕ ФЕНОТИПА У ДЕТЕЙ С ФЕНИЛКЕТОНУРИЕЙ ИЗ МОЛДОВЫ НА ОСНОВЕ ГЕНЕТИЧЕСКОГО АНАЛИЗА

Введение: Фенилкетонурия (ФКУ) - аутосомно рецессивная наследственная болезнь обмена веществ, вызванная нарушением активности печёночного фермента фенилаланин-гидроксилазы. Учитывая тот факт, что нервные клетки самые уязвимые к токсичному эффекту повышенных уровней фенилаланина в крови, наиболее явное проявление данной болезни — это задержка умственного развития. Мутации, отвечающие за фенилкетонурию происходят в гене фенилаланингидроксилазы (PAH). Тип мутации влияет на активность фермента и соответственно на фенотип.

Цель: Прогнозирование фенотипа у больных ФКУ из Молдовы на основе генотипирования с помощью прогнозирующих программ.

Материалы и методы: В качестве материала были использованы данные 9 молдавских пациентов с ФКУ, диагностированных неонатальным скринингом (>3 мг/дл), в период 2018-2019, с полным выявленным генотипом гена *PAH*. Для предсказания фенотипа был использован метод произвольного значения (AV). Второй метод включает в себя работу с программой *BIOPKU* (*BIOPKU*; http://www.biopku.org). Идентификация общих мутаций (*p. R408W, p. P281L, p. L48S, p. R252W, p. R158Q, p. R261Q*) была осуществлена с помощью ПЦР/ПДРФ, а также выявление других редких мутаций с помощью секвенирования кодирующей части гена *PAH*.

Результаты: Используя данные о патологических генотипах, выявленных в период 2018- 2019 были предсказаны фенотипы 9 пациентов с ФКУ. Полученные результаты при использовании системы (AV) показали преобладание классической формы ФКУ в 66,7%, исключение было представлено средними формами ФКУ (33,3%). При использовании *BIOPKU,* было проанализировано 8 из 9 генотипов пациентов с ФКУ. В данном случае, все генотипы были обозначены как классическая форма ФКУ. Учитывая тот факт, что расчетный фенотип генотипа *p.R408W/p.L48S* отличается от такового в наблюдаемых случаях, данная комбинация была изучена подробнее из перспективы неоднородности фенотипов среди разных случаев. Как следствие, можно утвердить что в Молдове зарегистрированы классические формы ФКУ за последние 2 года.

Заключение: Системы предсказания фенототипа, такие как метод AV и *BIOPKU,* могут быть полезными в исследовательской деятельности, каждая система, в свою очередь, имеют свои преимущества, пополняя интернациональные базы генетических данных, при этом качество прогнозов фенотипов значительно улучшится.

Ключевые слова: Фенилкетонурия, генетический прогноз, генотип, фенотип, BIOPKU.

Introduction

Phenylketonuria (PKU) is an inborn metabolic error, with an autosomal recessive type of inheritance. The biochemical cause of PKU is phenylalanine hydroxylase (PAH) deficiency. The given hepatic enzyme, combined with Tetrahydrobiopterin (BH4) cofactor contributes to degradation of phenylalanine (*Phe*) that is one of the essential amino acids. In case of a metabolic error due to a mutation, PAH enzyme is partially inactive or not functional that leads to accumulation of Phe blood level in body tissues with a toxic effect on them[1].

The genetic cause of the disease are the mutations in the PAH gene located on chromosome 12q23.2. The activity of phenylalanine hydroxylase directly depends on the mutation type- nonsense mutations, frameshift mutations, splicing mutations, deletions and insertions lead to an inactive enzyme, weather missense mutations lead to a more variable outcome regarding PAH enzymatic activity[2]. In case of poor effectiveness of the enzyme (PAH), the nerve cells in the brain that are the most sensitive to high *Phe* level suffer the most, that leads to brain damage [1]. Also, amongst phenylketonuria manifestations could be mentioned poor pigmentation, growth failure, microcephaly, skin rashes, seizures, and others. There are several types of PKU based on phenotype manifestations and severity of the diagnosis: classic PKU and variant PKU, including moderate PKU and mild PKU, mild hyperphenylalanemia (HPA) or non-PKU HPA, and BH4- responsive PKU [3]. BH4 responsive PKU is a type of PAH deficiency that can be treated by BH4 endogenic cofactor that lowers *Phe* level in plasma as an addition to the diet [3].

Prediction of the phenotype based on the genotype may be useful in further studies of the disease and considerably improve the understanding of genotype - phenotype relationship based on biostatistical data. Although prediction programs cannot be used in diagnostics and are not precise enough, they can be improved and applied in research field. Those programs use records of medical cases, analyze them and attribute them values that characterize the severity of the condition, based on the reported cases. In addition, we can obtain supplementary valuable information, such as BH_4 responsiveness in patients with PKU. Thus, we can already use the prediction tools for preventive analysis and correlation.

Aim: Evaluation of the expected phenotype in Moldovan PKU patients based on their genotype, according to various prediction methods.

Materials and methods:

The data of 9 Moldovan PKU patients diagnosed by neonatal screening (>3 mg/dL) during 2018- 2019 with fully- identified genotype of *PAH* gene were used.

Genotyping Methods

Identification of six common mutations (*p.R408W, p. P281L, p.L48S, p.R252W, p.R158Q, p.R261Q*) was performed by PCR/RFLP method as described in the previous research by K. Boiciuc [2]. In case a pathological genotype was not identified by the given

method, *PAH* gene exons were sequenced using capillary electrophoresis and chemistry from Applied Biosystems (USA) as described [4].

Arbitrary Value Method

For prediction of the patients' phenotype the arbitrary value system was used, accordingly to LL Wang [5]. Based on the residual activity of phenylalanine hydroxylase (PAH) in vitro, the mutations were classified in four categories and for each there was attributed an arbitrary value (AV), in dependence with the out coming phenotypes: for the null mutations AV= 1, with unnoticeable in vitro residual activities (<10%); AV= 2 for 10%-30% activity of wild type *PAH*; AV=4 for 30%-70%; AV=8 with residual activities greater than 70%. The phenotype of an individual is expressed as a sum of both mutant alleles' AVs. The total AV is used in prediction of the PKU type: 2 is for classic PKU; 3-4 for moderate PKU; 5-8 for mild PKU and 9-16 for mild HPA [5]. In order to homogenize the results, moderate PKU will be integrated into mild PKU category. Thus, the range for mild PKU will be AV= 3-8.

BIOPKU Method

The second prediction system involved *BIOPKU* program (*BIOPKU; http://www.biopku.org*). The given software attributes each pathologic mutation Allelic Phenotype Value (APV) that is graded on a scale. Genotypic Phenotype Value (GPV), also called APV*max*, is the highest APV value from the two alleles. There are three types of PKU differentiated in the following system, based on APV values: 0-2.7 is classic PKU, 2.8-6.6 is mild PKU, and 6.7-10.0 is mild HPA. It is important to mention that there is always overlapping between classical PKU and mild PKU, as well as between mild PKU and mild HPA.

Results

Molecular genetic analysis revealed six distinct pathological genotypes in *PAH* gene in nine Moldovan PKU patients that were used for prediction tools to estimate the out coming phenotypes.

Arbitrary Value Method

There were analyzed 6 genotypes of 9 Moldovan PKU patients. It can be observed that the majority of the genotypes lead to AV=2, it equals to 66.7% (n=6) of patients with classic PKU identified by the given approach. Therefore, there was a single genotype associated with mild PKU detected in 3 patients with an AV=4, representing 33.3% of all (Tab. 1).

Table 1. Results from arbitrary value method phenotype prediction on Moldovan PKU patients 2018-2019

Genotype	Number of patients	AV Total	Phenotype
p.R408W/p.L48S	3	4	Mild PKU
p.R408W/p.P281L	1	1	Classic PKU
IVS7+4A>G/IVS11+7T>C	1	1	Classic PKU
p.P281L/IVS12+1G>A	1	1	Classic PKU
p.R408W/p.R408W	2	1	Classic PKU
p.R408W/IVS12+1G>A	1	1	Classic PKU

Considering that the AV depends on residual enzymatic activity of PAH, as a consequence most of mutations lead to null activity of the enzyme. Regarding those facts, we can assume that classic PKU considerably predominates in Moldovan patients registered last years, accordingly to arbitrary value method.

BIOPKU Results

Using *BIOPKU* program, 5 genotypes from 8 of Moldovan PKU patients were analyzed out of 9, given that there was no data about IVS7+4A>G/IVS11+7T>C genotype due to absence of records related to this particular genotype. The missing genotype makes up 11.11% from the total number. The table 2 indicates that 89.9% (n=8) of processed genotypes in the given study fall within classic PKU. In contrast, there were no cases with mild PKU or mild HPA detected using this prediction method (Table 2).

Table 2. Results from BIOPKU phenotype prediction system in Moldovan PKU patients.

Genotype	Number of patients	GPV (APV total)	Phenotype
p.R408W/p.L48S	3	2	Classic PKU
p.R408W/ p.P281L	1	0	Classic PKU
IVS7+4A>G/IVS11+7T>C	1	Absent	Absent
p.P281L/IVS12+1G>A	1	0	Classic PKU
p.R408W/ p.R408W	2	0	Classic PKU
p.R408W/IVS12+1G>A	1	0	Classic PKU

The results show little diversity in phenotypes due to a small number of cases of fully genotyped patients and absence of information about some of the mutations in *BIOPKU* database.

Discussions

Based on early diagnosis and continuous treatment with clinical child monitoring and metabolic control we could decide about type of PKU and in our study group all patients were categorized as classic PKU beside that having the until present, there were registered 7 cases of PKU with the p.R408W/p.L48S genotype. The given genotype was observed to be very heterogeneous in manifestations. For instance, in this there was a patient that was missed out from neonatal screening, diagnosed at 23 years old with a 1293.4

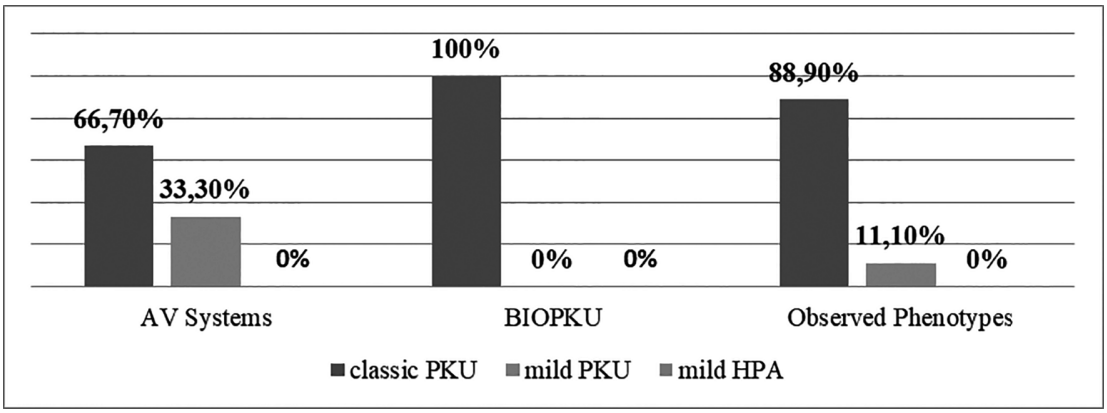

Fig. 1 Differences between predicted and observed phenotypes in Moldovan PKU patients 2018-2019

p.R408W/p.L48S genotype which shows a mild evolution. We could mention the fact that classical form of PKU caused by those five different genotypes of *PAH* gene there were exactly predicted by both AV system and *BIOPKU*. However, the difference between observed and predicted phenotype was detected in patients with the *p.R408W/p.L48S* genotype. Due to enzyme activity up to 30%, for *L48S* mutation and its combination with a null mutation μmol/L blood *Phe* level and escaped mental retardation [6],[7]. In addition, there was another patient with the same genotype diagnosed by neonatal screening and early treated with a good metabolic control that led to normal development of the child. On the other hand, in the same group there is a case, of a patient that was late diagnosed, at three years old and had severe mental retardation with a characteristic phenotype, even was tested as BH4-responsive.

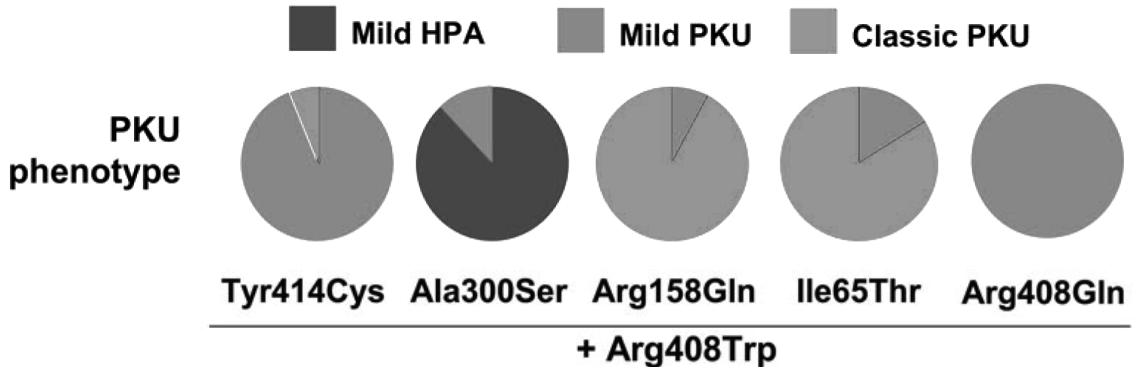

Fig. 2 Variation in Correlation of Genotype and Phenotype Reported by N. Blau 2016

(p.R408W), AV system predicted a milder phenotype. On the other hand, *BIOPKU* predicted a classic phenotype on the same genotype, according to its database and prediction algorithms.

In the study group, there were present three patients with the p.R408W/p.L48S genotype. In relation to these cases, there was observed no homogeneity at the phenotype level. Considering that, two patients had classical form of PKU as predicted by *BIOPKU* tool and one patient with Mild form, accordingly to AV system calculation.

At the same time, it is worth mentioning that from the beginning of the molecular genetic diagnostic of PKU As we know, the phenotype is predicted by genotype, being established by a correlation between them. However, in the study group there was noticed phenotype variability, caused by the same genotype. The following tendency was not only described in our group, similar cases being reported. One of the most significant review on this phenomenon was made by Prof. N. Blau [8], that analyzed the statistical and analytic power of large mutational databases such as *BIOPKU*. According to this, there was observed that patients that share a common mutation may have different phenotypes, as long as it is a complex and multifactorial correlation between the genotype and phenotype.

Conclusions

As prediction methods, both AV system and *BIOPKU* have advantages. *BIOPKU* for instance, is more informative because it offers data about BH4 responsiveness of a patient with a certain genotype[3]. This tool has a large database with lots of records of real cases that enhance the accuracy of predictions. The great advantage of the AV system is that if the type of the mutation is known as one causing null activity of PAH, its AV is constant, AV=1. This fact offers more possibilities because even if a new mutation is detected and its type is one of those leading to PAH effectiveness lower than 10%, the AV will be known (AV=1). [5]

The results returned by prediction programs, such as AV system and *BIOPKU*, do not always correspond with the results from real clinical cases. Thus, the data obtained can be only considered as provisory because the accuracy of predictions is not satisfactory in order to be applied in clinical cases. It is necessary to mention that there are other factors that influence the phenotype and its severity.

This type of research is benefic in the way that international databases such as *BIOPKU* can be completed with records of Moldovan PKU patients. This helps to improve the quality of phenotype prediction in PKU cases and add new mutations that are characteristic to certain populations. As a result, the database becomes numerous that allows more possibilities and enlarges the range of information that it can offer, based on the genotype.

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