Research Article

Özlem Öz*, Emiş Deniz Akbulut, Müjgan Ercan Karadağ, Ataman Gönel and İsmail Koyuncu

Amino acid metabolism disorders and PAH gene mutations in Southeastern Anatolia Region

[Güneydoğu Anadolu Bölgesinde Aminoasid Metabolizma Bozuklukları ve PAH Gen Mutasyonları]

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Abstract

Objectives: Inborn errors of metabolism are generally autosomal recessive inherited disorders. The incidence and genetic features of neonatal metabolic disorders vary significantly by regions and populations. In this study, we aimed to determine the amino acid metabolism disorders and evaluate the genetic test results of these patients retrospectively.

Methods: The blood samples collected from heel blood and dried on filter cards in the neonatal screening program, were analyzed for amino acid metabolism disorders by (LC)-MS/MS method. Patients with suspected metabolic diseases were diagnosed with NGS method.

Results: Amino acid metabolism disorders were detected in 66 of 2,104 patients who were screened for suspected neonatal metabolic disorders. Sixty-two of 66 patients were diagnosed with phenylketonuria, the rest of them were diagnosed with tyrosinemia type I, arginosuccinate lyase deficiency, citrullinemia type 1 and Maple Tree syrup disease. The most common PAH gene mutations were c.1208C>T (A403V).

Conclusion: Phenylketonuria was the most common disease among amino acid metabolism disorders in Şanlıurfa. There were different allele frequencies compared to the PAH mutations reported in previous studies. This may be due to the different characteristics of the populations and also the high rate of consanguineous marriage in our region.

Keywords: aminoacid metabolism disorders; LC-MS/MS; PAH gene; phenylketonuria; phenylalanine hydroxylase.

Öz

Amaç: Doğumsal metabolizma bozuklukları genellikle otozomal resesif geçiş gösteren hastalıklardır. Yenidoğan metabolik bozukluklarının insidansı ve genetik özellikleri bölgelere ve popülasyonlara göre önemli ölçüde değişkenlik gösterir. Bu çalışmada Şanlıurfa'da görülen amino asit metabolizması bozukluklarının belirlenmesi ve bu hastaların genetik test sonuçlarının retrospektif olarak incelenmesi amaçlandı.

Gereç ve Yöntemler: Yenidoğan tarama programında, topuk kanından toplanmış ve filtre kartlarında kurutulmuş kan örnekleri amino asit metabolizması bozuklukları

***Corresponding author:** Dr. **Özlem Öz**, Department of Medical Genetics, Faculty of Medicine, Harran University, Osmanbey Campuss, 63300 Şanlıurfa, Turkey, Phone: +90 414 318 30 00, Fax: +90 414 318 3192, E-mail: ozlemdroz1@gmail.com. https://orcid.org/0000-0002-5533-6025

Emiş Deniz Akbulut, Department of Medicinal Biochemistry, Ankara City Hospital, Ankara, Turkey

Müjgan Ercan Karadağ, Department of Medicinal Biochemistry, Faculty of Medicine, Afyon University of Health Sciences, Afyon, Turkey. https://orcid.org/0000-0002-9291-4197

Ataman Gönel and İsmail Koyuncu, Department of Medicinal Biochemistry, Faculty of Medicine, Harran University, Şanlıurfa, Turkey

açısından LC-MS/MS yöntemiyle analiz edildi. Metabolik hastalık şüphesi olan hastalara NGS yöntemi ile tanı konuldu.

Bulgular: Doğumsal metabolizma bozukluğu ön tanısı ile taranan toplam 2,104 hastanın 66'sında aminoasit metabolizma bozukluğu bulundu. 66 hastanın 62'sinde fenilketonüri tanısı, geri kalanında ise Tirozinemi tip I, Arjininosukinat liyaz eksikliği, Sitrullinemi tip 1 ve Akça Ağaç şurubu hastalığı tanısı konuldu. En sık gözlenen PAH gen mutasyon c.1208C>T (A403V) idi.

Sonuç: Şanlıurfa'da amino asit metabolizma bozuklukları arasında en sık görülen hastalık fenilketonüri olarak gözlendi. Sonuçlarımıza göre, önceki çalışmalarda bildirilen PAH mutasyonlarına göre farklı alel frekansları mevcuttu. Bu durum, popülasyonumuzun farklı özelliklere sahip olması ve bölgemizdeki akraba evliliği oranının yüksek olmasından kaynaklanabilir.

Anahtar sözcükler: aminoasit metabolizma bozuklukları; Fenilketonüri; Fenilalanin hidroksilaz; PAH geni; LC-MS/MS.

Introduction

Hereditary metabolic diseases are rare disorders of single genes coding for enzymes that convert substrates into specific products. These diseases usually emerge with the accumulation of toxic substrates or the deficiency of essential metabolites. Despite their rare incidence, these disorders constitute an important public health problem, totally [1, 2]. Inborn errors of metabolism (IEM), generally occurring in 1 per 1,000 births, are mostly inherited in a recessive fashion [3]. Today, newborn screening programs are implemented in many countries around the world to diagnose and treat IEM as early as possible before irreversible damage occurs. Newborn screening test panels and methodologies vary substantially from one country to another. Tandem mass spectrometry (MS/MS) is being used increasingly in newborn screening because this analysis method significantly increases the number of metabolic disorders that can be detected from dried blood-spot specimens.

One group of these disorders is amino acid metabolism disorders which include phenylketonuria (PKU), maple syrup urine disease, tyrosinemia, and homocystinuria [4]. PKU is the most common hereditary metabolic disease which [5] develops due to the deficiency of phenylalanine hydroxylase (PAH) and when left untreated results in growth failure and severe intellectual disability and so on. Additionally, the incidence and genetic features of neonatal metabolic disorders vary significantly by regions and populations.

In this retrospective study, it was aimed to determine the amino acid metabolism disorders in Şanlıurfa and to evaluate the genetic test results of these patients.

Material and methods

Samples

Patients who were found suspicious as a result of screening within the scope of the neonatal screening program and who were referred to the pediatric nutrition-metabolism laboratory from family health centers were included in the study. In the study plasma amino acid results of 2,104 newborn screening samples sent to Harran University Metabolism Laboratory during 01.01.2018 and 01.01.2020 were evaluated. Sixty-six patients with suspected amino acid metabolism disorders detected during newborn screening and requested for genetic analysis were included in the retrospective study (66/2,104). This study was approved by the ethical committee of Harran University Faculty of Medicine on 24.02.2020 with number 04-07.

Amino acid analysis by LC-MS/MS

In the newborn screening program blood specimens were collected by trained nurses via heel-stick and spotted on filter paper cards. Amino acid metabolism disorders in dried blood spots (DBS) was measured on liquid chromatography with tandem mass spectrometry (LC-MS/MS) method (Shimadzu LCMS-8040, Kyoto, Japan) by the use of a multiple reaction monitoring (MRM) approach. Patients who were evaluated with a pre-diagnosis of amino acid metabolism disease and whose results were suspicious after three measurements were further examined by genetic analysis.

Genetic analysis

Blood samples were taken into ethylenediaminetetraacetic acid (EDTA) containing tubes from 66 subjects who had suspected amino acid metabolism disorders sent to Harran University Genetic Laboratory. Genomic DNA was isolated according to the kit protocol (EZ1 Advanced Instruments, Qiagen, Hilden, Germany). Amplification was performed with primer pairs containing exon regions of the PAH, fumarylacetoacetate hydrolase (FAH), argininosuccinate lyase (ASL), argininosuccinate synthase 1 (ASS1) and branched chain keto acid dehydrogenase E1 subunit alpha (BCKDHA) gene according to the kit protocol (NEXTflex™ DNA Sequencing Kits, Bioo Scientific Corporation, Texas, USA) by polymerase chain reaction (PCR). Next-generation DNA sequencing data was evaluated using Mutation Surveyor program.

Statistical analysis

SPSS (IBM SPSS Statistics 24) program was used in the statistical evaluation of all results obtained. Numerical values were calculated as

result mean ± standard deviation (mean ± SD), nominal values as percentage (%).

Results

There were a total of 2,104 patients with a suspected diagnosis of amino acid metabolism disorder, of which 54.8% were male and 45.2% were female. After evaluation of the patients according to their laboratory results, amino acid metabolism disorders were detected in 66 of these patients. Phenylketonuria was determined in 62 patients of 66 patients; Tyrosinemia type I in one patient; argininosuccinate lyase deficiency in one patient; Citrullinemia type 1 in one patient; Maple syrup urine disease in one patient was detected who underwent genetic examination for suspected congenital metabolic disease. The numbers of mutations and alleles detected in the patients were given in Table 1. Also we identified a novel mutation (c.1324G>A) in ASL gene which was not reported before in the literature with the patient diagnosed with Argininosuccinate lyase deficiency. The most common PAH gene mutations were c.1208C>T (A403V) with a rate of 18/130, c.898G>T (A300S) with a rate of 17/130 and c.688G>A (V230I) with a rate of 15/130, respectively (Table 1).

According to serum enzyme levels of 62 patients with phenylketonuria were divided into three groups. According to this, those with enzyme levels >1,200 μM (>20 mg/dL) have phenylketonuria (PKU, HPA I); those between 600 and 1,200 μM (10–20 mg/dL) have mild PKU (HPA II); those between 120 and 600 μM (2–10 mg/dL) have mild hyperphenylalaninemia (HPA III) were grouped. In our study, four patients diagnosed with PKU, 45 patients diagnosed with mild PKU and 21 patients diagnosed with mild HPA were identified. Homozygous mutations in the PAH gene were detected in 33 of the patients; three patients diagnosed with FKU, four patients diagnosed with mild PKU, 26 patients diagnosed with mild HPA. The most common mutation in patients diagnosed with PKU and mild PKU was c.1066-11G>A, while c.1208C>T and c.1139C>T mutations were detected in mild HPA patients. Serum phenylalanine levels of patients are summarised in Table 2.

Table 1: Mutations detected in patients with inborn error of metabolism identified by expanded newborn screening.

Disease	Gene	Mutation alleles numbers	Nucleotide variant	Amino acid variant	Pathogenicity	Reported	Cases
Phenylketonuria	**PAH**	**130**					**103**
		7	c.782G>A	p.R261Q	P	Y	6
		10	c.441+5G>T	-	P	Y	6
		15	c.688G>A	p.V230I	P	Y	13
		12	c.1066-11G>A		P	Y	9
		5	c.842C>T	p.P281L	P	Y	3
		17	c.898G>T	p.A300S	P	Y	14
		5	c.1097C>A	p.P366H	P	Y	3
		18	c.1208C>T	p.A403V	P	Y	13
		4	c.1169A>G	p.E390G	P	Y	3
		9	c.1139C>T	p.T380M	P	Y	7
		1	c.1181A>C	p.D394A	P	Y	1
		1	c.331C>T	p.R111*	P	Y	1
		2	c.529G>A	p.V177M	P	Y	1
		1	c.1099delC	p.L367fs	P	Y	1
		5	c.1097C>A	p.P366H	P	Y	3
		1	c.143T>C	p.L48S	P	Y	1
		1	c.165T>G	p.F55L	P	Y	1
		1	c.47-48delCT	p.S16*	P	Y	1
		2	c.281T>G	p.I94S	P	Y	1
		2	c.969+5G>A	-	P	Y	2
Tyrosinemia type I	**FAH**	**2**	c.437_444delATGCGTTG	p.L148Kfs*33	P	Y	**1**
Argininosuccinate lyase deficiency	**ASL**	**2**	c.1324G>A	p.G442S	P	N	**1**
Citrullinemia type 1	**ASS1**	**2**	c.1085G>T	p.G362V	P	Y	**1**
Maple syrup urine disease	**BCKDHA**	**2**	c.890G>A	p.R297H	VUS	Y	**1**

PAH, phenylalanine hydroxylase; FAH, fumarylacetoacetate hydrolase; ASL, argininosuccinate lyase; ASS1, argininosuccinate synthase 1; BCKDHA, branched chain keto acid dehydrogenase E1 subunit alpha; P, pathogenic; VUS, variant of uncertain significance; Y, yes; N, none.

Table 2: Serum phenylalanine levels in patients bearing homozygote genotypes.

Serum Phe levels, μmol/L (mean ± SD)	Metabolic phenotype	Homozygous mutation	Number of patients
1,594.35 ± 214.93	FKU	c.1066-11G>A	2
2,217.22	FKU	c.441+5G>T	1
325.80 ± 110.58	HPA	c.782G>A	2
329.47± 74.20	HPA	c.441+5G>T	2
407.95 ± 0.54	HPA	c.1097C>A	2
201.61 ± 51.73	HPA	c.1208C>T	5
452.44	HPA	c.1169A>G	1
262.74 ± 148.97	HPA	c.1139C>T	5
282.41± 53.31	HPA	c.898G>T	3
169.89	HPA	c.1181A>C	1
289.02 ± 55.87	HPA	c.688G>A	2
94.11	HPA	c.529G>A	1
144.02	HPA	c.158G>A	1
277.28	HPA	c.281T>G	1
722.97 ± 114.7	MFKU	c.842C>T	2
822.26	MFKU	c.441+5G>T	1
706.8	MFKU	c.1066-11G>A	1

Phe, phenylalanine; PKU, phenylketonuria; HPA, hyperphenylalaninemia; MFKU, mild phenylketonuria.

Discussion

In this study, 62 and four infants detected through newborn screening were diagnosed to have PKU and tyrosinemia type I, argininosuccinate lyase deficiency, citrullinemia type 1 and Maple syrup urine disease, respectively with genetic analysis. Besides, a novel mutation (c.1324G>A) was identified in the ASL gene that was not previously reported in the literature in patient with argininosuccinate lyase deficient. This mutation type was found to be pathogenic on silico analyses using SIFT (sorting intolerant from tolerant), PolyPhen2 (polymorphism phenotyping v2) and mutation taster programs.

The global incidence of PKU is estimated to be one in 10,000 births while it is one in 4,500 births in our country especially because of high rates of consanguineous marriages [6, 7]. Phenylketonuria is an autosomal recessive inherited disease that occurs due to mutations in the PAH gene located on chromosome 12q22-q24.1. Up to now more than 800 mutations related with this gene have been described. Generally, compound heterozygous mutations affecting two different alleles are detected. Different mutations may be observed in different populations owing to the hotspots in the PAH gene [8, 9].

The most commonly reported mutation for PKU in the USA was c.1222C>T; while it was IVS10-11G>A (c.1066-11G>A) in Iran and Spain; c.1238G>C in Japan; c.728G>A in China; c.781C>T in North Caucasus; c.1068C>A and c.728G>A in Southern Korea; c.1162G>A in Brazil; c.782G>A and c.1222C>T in Syria [10].

The frequency of c.1066-11G>A mutation was 30% in Turkey, 25% in Bulgaria, 19% in Italy, 15% in Spain and Sicily, 13% in Greece, and 11% in Portugal [11, 12]. It has been reported c.1066-11G>A was the most common PKU mutation in different studies conducted in Turkey and Mediterranean region. Ozguc et al. [13] investigated the mutation analysis in 44 classical PKU Turkish patients and they identified c.1066-11G>A was found in 32% of the mutant alleles and comprises 74–75% of the mutations. Luleyap et al. [14] searched the PAH gene mutations in 23 unrelated PKU patients in Cukurova region. They reported their finding with c.1066-11G>A mutation was found with a frequency rate of 58.7%. However, in our study the frequency of c.1066-11G>A mutation was found to have a fourth frequency with a 9.2% rate, and this was lower than previous reports. The possible reason of the high prevalence of this allele in our country and the Mediterranean countries may be the geographic starting point and dispersion of this allele via migration. The origin of this mutation was thought to be Neolithic farmers living in 8,000 years before Christ who migrated to western regions across Mediterranean [12, 15].

In the present study the major mutation was c.1208C>T (A403V). The second and third most common mutations were c.898G>T (A300S) and c.688G>A (V230I), respectively. We think that these differences in the frequency of mutations are due to different ethnic groups and populations.

R261Q mutation is one of the mis-sense mutations affecting the catalytic subunits of the PAH enzyme and frequently detected in Mediterranean countries. In our country this is the second most common mutation type reported in PKU patients. In a study from the Mediterranean region of our country its detection frequency was 15.2% and was defined as the second most common mutation [14]. In addition to this, in a study investigating PAH gene mutations in Turkish phenylketonuria patients, it was found that p.R261Q mutation was the second most common mutation with a frequency of 8.7% [16]. Also similar findings to the same study were observed to be consistent with two studies involving fewer Turkish patients [13, 17]. Whereas, in our study R261Q mutation was detected in 5.4% of the patients with a seventh order of frequency.

A300S and A403V are two of the other frequent mutations in PKU patients. Individuals carrying the A300S mutation were reported to present with a milder type of the disease. In a study from Southern Italy, allele frequency of A300S and A403V were determined as 12.4 and 20.0%, respectively. It has been reported that the disease

progresses more mildly in patients with this mutation, and that often mild phenylketonuria accompanies this mutation [18]. In our study detection rates of these mutant alleles were similar which was with a rate of 17/130 (13.1%) and with a rate of 18/130 (13.8%) for A300S and A403V, respectively. However, in a study investigating the phenylalanine hydroxylase activity on tetrahydrobiopterin responsiveness in Turkish population, it was found that the A300S mutation was detected in the sixth frequency with 5% allele frequency and A403V mutation was not observed in Turkish population [16].

In another study investigating the molecular epidemiology of phenylalanine hydroxylase deficiency in Southern Italy; V230I is one of the rare mutations seen in PKU patients with a previously reported frequency of 0.2% in Slovenia, 5.6% in Italy and lower than 4% in Iran [19–21]. V230I mutation was not observed previously in studies reported from Turkey. On the contrary, an increased frequency (11.5%) of this mutation was detected in our study. Additionally, a rare splice site mutation c.441+5G>T which was reported with a rate of 1.2% in a multicentric population study [22] while it was more common with a rate of 7.7% in our study. Otherwise Dobrowolski et al. [16] reported this mutation with a %3 allele frequency in Turkish population.

Mutations lead to alterations in enzyme activity and clinical phenotypes vary according to mutation types. In a study from Spain p.E280K, p.R243X, p.R243Q, p.R158Q, p.R252W, IVS12+1G>A, p.S349P and p.R408W mutations were presented with near-complete deficiency of PAH activity, p.R261Q, p.V388M, p.R176L and p.L348V mutations were with moderately deficient enzyme activity and p.E390G, p.V230I, p.P244L and p.P211T mutations were reported to be with higher PAH activity [23]. Unlike this study, c.1066-11G>A, c.441+5G>T mutations caused HPA I (>1,200 μM), c.842C>T (p.Pro281Leu) mutation was with HPA II (600–1,200 μM) and c.1208C>T, c.1139C>T, c.898G>T mutations were related with HPA III (100–600 Mm) in our study. Also in a study investigating the PAH activity in Turkish population, the patients with c.1066-11G>A,c.441+5G>T and c.842C>T mutations had classic PKU metabolic phenotype and PAH activity was not determined. Similar results to our study, the patients with c.898G>T mutation had mild HPA with 31% PAH activity in the same research [16].

As a conclusion, determination of the incidence of amino acid metabolism disorders detected through newborn screening and allelic distributions of these individuals with genetic testing in a center from the southeastern region of Turkey were presented in this study. We concluded that the first three mutations seen in our region is different from the mutations previously reported in Turkish population. In addition, frequent mutations related with PKU and their relations with the PAH activity were reported which may contribute to the literature in terms of genetic variability. According to the results of this study different allele frequencies from the previous reports for PAH mutations were observed which may be due to the population differences and the higher rates of consanguinity marriages in this region.

This study has some limitations; one major limitation was that we couldn't analyze the phenylalanine hydroxylase levels and detect the dihydropteridine reductase deficiency in all patients because of the study design as retrospective manner.

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