Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ DERGİSİ

Cukurova Med J 2016;41(4):702-708
DOI: 10.17826/cutf.254199

ARAŞTIRMA/RESEARCH

The analysis of the phenylalanine hydroxylase gene mutations by sequencing and ARMS techniques in Turkish patients

Türk hastalarda fenilalanin hidroksilaz gen mutasyonlarının dizileme ve ARMS teknikleri ile incelenmesi

Ümit Lüleyap[1], Perçin Pazarcı[1], Gamze Cömertpay[1], Halise Neslihan Önenli[2], Ayfer Pazarbaşı[1], Davut Alptekin[1], Halil Kasap[1], Ursula Froster[3]

[1]Cukurova University, Faculty of Medicine, Department of Medical Biology and Genetics, [2]Department of Pediatric Metabolism and Endocrinology, Adana, Turkey
[3] University of Leipzig Faculty of Medicine, Institute of Human Genetics, Leipzig, Germany

Cukurova Medical Journal 2016;41(4):702-708.

Abstract

Purpose: Phenylketonuria is an autosomal recessive deficiency of the hepatic enzyme phenylalanine hydroxylase. With this study, detection of the most frequent phenylalanine hydroxylase gene mutations in Turkish population is aimed.

Material and Methods: 23 unrelated phenylketonuria patients (46 alleles) who are living in Cukurova region, Turkey were investigated.First, all exons were screened by using DHPLC method then the direct sequencing technique and ARMS methods were used to analyze mutation suspected samples.

Results: IVS10-11g→a splicing mutation in 27 alleles (58.7%), R261Q mutation in 7 alleles (15.2%) and E178G, P281L, R243X, R243Q, R408W, Y386C mutations, all in a frequency of 2/46 (4.3%) is found.

Conclusion: The arginine amino acid, accounting for 68.4% of changes in exon sites 7 and 12, where R243X, R243Q, R261Q and R408W mutations occur, is thought to be important for amino acid changes in phenylalanine hydroxylase gene among phenylketonuria patients in Çukurova region. Single-base mutations like IVS10-11g→a and P281L could be detected with an accuracy of 100% by the use of specifically designed primers by authors according to ARMS technique and it is relatively cheaper and requesting less technical equipment.

Key words: ARMS, DHPLC, Phenylketonuria, sequencing, Turkish population

Öz

Amaç: Fenilketonüri, hepatik bir enzim olan fenilalanin hidroksilazın otozomal resesif bozukluğu sonucu ortaya çıkan bir durumdur. Bu çalışmada, Türk popülasyonunda en sık görülen fenilalanin hidroksilaz geni mutasyonlarını belirlemek amaçlandı.

Gereç ve Yöntem: Öncelikle tüm ekson bölgeleri DHPLC metodu kullanılarak tarandı. Ardından mutasyon şüphesi taşıyan örnekler sekanslama ve ARMS yöntemleri kullanılarak analiz edildi.

Bulgular: IVS10-11g→a splayzing mutasyonu 27 allelde (%58,7), R261Q mutasyonu 7 allelde (%15,2) ve E178G, P281L, R243X, R243Q, R408W, Y386C mutasyonları ise 2'şer allelde (%4,3) tespit edildi.

Sonuç: R243X, R243Q, R261Q ve R408W mutasyonlarının oluştuğu 7 ve 12'inci ekson bölgelerinde arginin aminoasidinin değişimi Çukurova bölgesindeki fenilketonüri hastalarında fenilalanin hidroksilaz geni mutasyonlarında önemli bir yer tutmaktadır (%68,4). IVS10-11g→a ve P281L gibi tek baz mutasyonları bizim tarafımızdan dizayn edilmiş primerler ve ARMS tekniği kullanılarak daha ucuza, daha az teknik ekipmanla ve %100 doğrulukla tespit edilebilmektedir.

Anahtar kelimeler: ARMS, DHPLC, Fenilketonüri, dizileme, Türk popülasyonu

Yazışma Adresi/Address for Correspondence: Dr. Ümit Lüleyap, Cukurova University, Faculty of Medicine, Department of Medical Biology and Genetics, Adana, Turkey. E-mail: umitluleyap01@gmail.com
Geliş tarihi/Received: 02.05.2016 Kabul tarihi/Accepted: 07.06.2016

INTRODUCTION

Phenylketonuria (PKU) is caused by the deficiency of the phenylalanine hydroxylase enzyme (PAH) and is an autosomal recessive disorder. Unless the intake of phenylalanine is restricted, it results in severe hyper-phenylalaninaemia and mental retardation1. More than 950 different mutations in PAH gene have been defined up to date (PAHdb; http://www.pahdb.mcgill.ca/) since the identification of the PAH gene in 19862.

Classical methods such as SSCP (Single Strand Conformational Polymorphism) and DGGE (Denaturing Gradient Gel Electrophoresis) are used for screening of mutations in genes that consist many exons[3]. Recently, DHPLC (Denaturing High-Performance Liquid Chromatography Technique) has been the preferred method for the analysis of genes such as; BRCA1[4], BRCA2[5] and CFTR[6] because of its advantages in comparison to these classical methods. The same is valid for screening the mutations of the PAH gene. The detection of mutations is possible by different molecular techniques like ARMS (Amplified Refractory Mutation System), ASO (Allele Specific Oligo-nucleotide), RFLP (Restriction Fragment Length Polymorphism) and sequencing where preference is made according to the type of mutation, the study target, the sensitivity and specificity of the methods used and the capability of each laboratory. We aimed to detect the most frequent PAH gene mutations in Turkish population, firstly for-screening the mutated exons by DHPLC then determining the exact nature of mutations by ARMS technique, which is relatively cheaper and not requesting a large technical capability.

MATERIALS AND METHODS

The DNA samples were collected from 23 unrelated patients (46 independent alleles), that are living in Çukurova Region, Adana /Turkey, with classical PKU, diagnosed on the basis of the initial plasma phenylalanine (PA) concentrations and positive urine ferric chloride test in the University of Çukurova, Faculty of Medicine, Department of Pediatric Endocrinology and Metabolic Diseases (Adana/Turkey), and the samples transported to Germany and stored at the Institute of Human Genetics in Leipzig.

Study is approved by the human studies ethical committee of the Cukurova University.

PCR amplification

Blood samples were preserved in EDTA and Genomic DNA was extracted from samples by using standard procedures7. 50 µl reaction volumes containing approximately 200 ng DNA in KCL (10mM), 50 µmol of each primer, Tris-HCL (1.5 mM), MgCl2 (200 µm) of each dNTP and IU TaqGold-Polymerase (PE) were used for amplification of samples. For PCR reaction, denaturation was done at 95°C for 10 min, 30 cycles of 1 min. at 95°C, 1 min at the fragment–specific annealing temperature and 1 min at 72°C. After 30 cycles another extension step of 10 min at 72°C was done.

Primers were designed to obtain fragments between 150 and 300 bp by using the Gene-Runner software (www.generunner.net). Table 1 shows the primers, length of PCR products and the annealing temperatures for each of the PAH exons and also the primers used for ARMS method for IVS10-11g→a and P281L. Additionally; three primers were designed as "normal", "mutant" and "common" to determine the allele status of each specific mutation to detect single base substitution i.e. IVS10-11g→a and P281L mutations by ARMS technique. Normal primers were designed with a change pertaining only to previous nucleotide in 3' end (C highlighted in Table 2) and this substitution detects with the aim of detecting the normal alleles.

In the detection of mutant alleles, changes comprised the last two nucleotide in 3' end (C and A highlighted in Table 2) corresponding to the mutation point of the mutant primers, considering the g→a (IVS10-11g→a) and C→T (P281L) base substitutions with necessary changes to increase the specificity of the amplification of the last nucleotide[8] (Table 1). Primers were designed using Gene Runner program. DHPLC analysis was done by using an automated DHPLC device (WAVE: Transgenomic, San Jose, CA, USP) according to manufacturer's manual. PCR products (5-15 µl and 10-100 ng DNA each) were denaturized at 95°C for 5 min and re-annealed gradually by decreasing the sample temperature from 95 to 65°C over a period of 30 min[3].

Sequence analysis

Abnormal DHPLC patterns were noted and these samples were examined to identify sequence variants by using automated sequence analysis on ABI Prism 377 (Applera). Afterwards, sequence variants were classified according to international databases (http://www.pahdb.mcgill.ca/) by using the re-amplified PCR products of these fragments.

Statistical analysis

Descriptive analysis is used to evaluate the results.

Table 1. The list of the primers of PAH gene used in sequencing and ARMS techniques

Type of Technique	Primer No	Sequence 5' → 3'	Product Length (bp)	Annealing Temperature
DHPLC and Sequencing	PAH For Ex2	TTCATGCTTGCTTTGTCC	299	50
	PAH Rev Ex2	CTGTTCCAGATCCTGTGTTC		
	PAH For Ex3	TGTGACTGTCTCCTCACC	265	50
	PAH Rev Ex3	GACATGTGAGTTACTTATGTTG		
	PAH For Ex4	TGTACTCAGGACGTTGCCTTC	146	54
	PAH Rev Ex4	CTCATCTACGGGCCATGGAC		
	PAH For Ex5	AAGCATTCATAAAGGTACCAG	202	52
	PAH Rev Ex5	AAGGGAGAAGCAGGCTAG		
	PAH For Ex6	TAACCTGCATTCTGCTGTG	317	53
	PAH Rev Ex6	TCCTCTGCCTCAATCCTC		
	PAH For Ex7	TGCCTCTGACTGAGTGGTG	242	55
	PAH Rev Ex7	AAGATGGCGCTCATTGTG		
DHPLC and Sequencing	PAH For Ex8	CTGCCCATTCCTCATGTAGA	226	56
	PAH Rev Ex8	CTGGGCTCAACTCATTTGAG		
	PAH For Ex9	ATGGCCAAGTACTAGGTTG	199	54
	PAH Rev Ex9	AGTTTCAAAGACCTGAGGGC		
	PAH For Ex10	TCCCAGTCAAGGTGACAC	260	50
	PAH Rev Ex10	GGATACAAATAGGGTTTCAAC		
	PAH For Ex11	TGCAGCAGGGAATACTGATC	293	56
	PAH Rev Ex11	TAGACATTGGAGTCCACTCTC		
	PAH For Ex12	ATGCCACTGAGAACTCTCTT	245	50
	PAH Rev Ex12	AGTCTTCGATTACTGAGAAA		
	PAH For Ex13	TCACTAGGACACTTGAAGAG	163	48
	PAH Rev Ex13	TCTCCATCAACAGATTCAC		
ARMS	IVS10-11g→a Normal For	AGTGATAATAACTTTTCACTCG	265	60
	IVS10-11g→a Mutant For	ACAGTGATAATAACTTTTCACTCA		
	IVS10-11g→a Common Rev	ATAGACATTGGAGTCCACTCTC		
ARMS	P281L Normal Rev	GCTGGAGGACAGTACTCATG	211	60
	P281L Mutant Rev	GCTGGAGGACAGTACTCATA		
	P281L Common For	GTGCCTCTGACTGAGTGGTG		

Table 2. The ARMS primers designed for the detection of IVS mutation

Primer (5' → 3')	Function
CAGTGATAATAACTTTTCACTTG	DNA Sequence
CAGTGATAATAACTTTTCACTC[1]G	Normal Forward primer
ACAGTGATAATAACTTTTCACTC[1]A[2]	Mutant Forward primer
[1]Nucleotide substitution in normal and mutant primers [2]Nucleotide substitution in mutant primer specific to the g→a mutation	

Table 3. The distribution of mutations and polymorphisms in PAH gene detected by DHPLC and sequencing analysis techniques

Gene : PAH	Amino acid change	Nucleotide change	Nucleotide position (cDNA)
Exon 6	E178G	GAA/GGA	533A>G
Exon 7	R243X	CGA/TGA	727C>T
	R243Q	CGA/CAA	728 G>A
	R261Q	CGA/CAA	728G>A
	P281L	CCG/CTG	842V>T
Intron 10	IVS10-11g→a	1066-11g→a	1066-11g→a
Exon 11	Y386C	TAT/TGT	1157>G
Exon 12	R408W	CGG/TGG	1222 C>T
Polymorphism	Q232Q	-	-
	V245V	-	-

Table 4. Allelic distribution of PAH gene mutation and its frequency

Number of Patient	Polymorphism	Allele 1	Allele 2	%	Frequency
10	1 V245V	IVS10	IVS10	58.7	(27/46)
3		IVS10	R261Q	15.2	(7/46)
2		IVS10	P281L	4.3	(2/46)
2		IVS10	E178G	4.3	(2/46)
1	1 V245V	R243X	R243X	4.3	(2/46)
1		R243Q	R243Q	4.3	(2/46)
2	1 V245V	R261Q	R261Q	4.3	(2/46)
1		Y386C	Y386C	4.3	(2/46)
1		R408W	R408W	4.3	(2/46)
				7	
Total 23 patients					

RESULTS

Mutations and polymorphisms that are found by sequencing the mutation suspected samples determined by DHPLC analysis are shown in Table 3. The IVS10-11g→a splicing mutation was observed in 17 patients, 10 of them have homozygous form and 7 of them have heterozygous form, with a total of 27 (58.7%) of 46 alleles (Table 3, Table 4). The R261Q mutation was the second most frequent mutation observed in 5 patients, 2 of them have homozygous form and 3 of them have heterozygous form with IVS mutation, with a total of 7 (15.2%) alleles out of 46. Both P281L and E178G mutations were seen in 2 patients respectively in a heterozygous form combined with IVS mutation and the frequency was 4.3% (2/46), while R243X, R408W, Y386C and R243Q mutations were seen in homozygous form each in one patient with a frequency of 4.3% (2/46) (Table 3, Table 4).

Samples, determined to possess homozygous or heterozygous IVS mutation by sequencing analysis, were re-analyzed by ARMS technique using newly designed primers. The results were 100% in accordance with the results of the sequencing

analysis, without any non-specific bands; hence they indicated that ARMS technique could be used with sufficient accuracy (Figure 1-a and Figure 2-a). The detection of one patient with P281L mutation presented in heterozygous form revealed the ability of newly designed ARMS primers to detect the related mutation with the same accuracy of sequencing analysis technique (Figure 1-b and Figure 2-b).

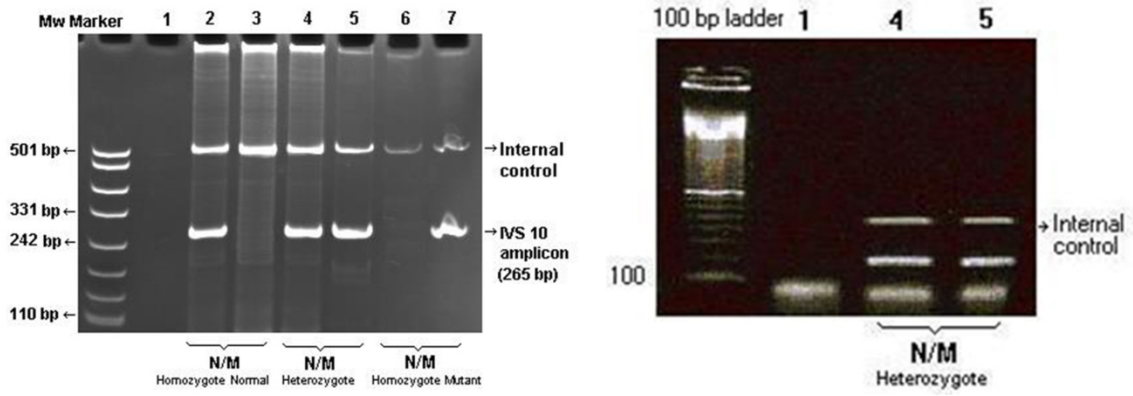

Figure 1. a-b. Gel slides of IVS10-11g→a and P281L mutations detected by ARMS. IVS10-11g→a mutation (a). P281L mutation (b). (Mw marker: pUC18/19 Msp I, Line 1=Negative control, Lines 2, 3= Homozygous Normal allele, Lines 4, 5=Heterozygous allele, Lines 6, 7=Homozygous mutant allele (N=Normal, M=Mutant), Internal controls: TNFα primers; RDN-010-025)

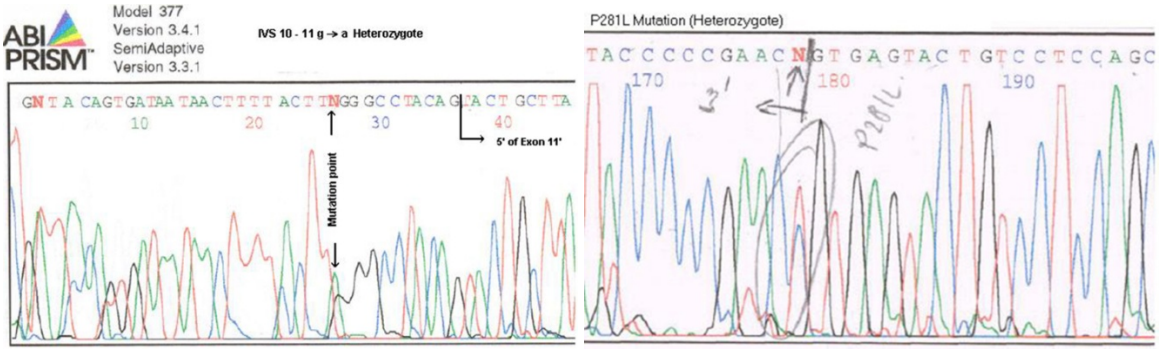

Figure 2. a-b. Electropherograms of IVS10-11g→a and P281L mutations detected by sequencing. IVS10-11g→a mutation (a). P281L mutation (b).

DISCUSSION

Since PAH deficiency is an inherited disorder seen in Caucasians frequently, the population genetics of disease has taken a lot interest from researchers. Extensive mutation data have been published for most countries as a result of researches over the last decade. Spectrum of mutations and the degree of heterogeneity differ distinctively between regions[9].

In a recent review, Blau[10] states that "Most PAH patients have compound heterozygous PAH gene variants and one mutated allele may markedly influence the activity of the second mutated allele, which in turn may influence either positively or

negatively the activity of the biologically active heterotetrameric form of the PAH".

Most common PAH mutation causing PKU is IVS 10-11g→a in the Mediterranean region, particularly in Turkey where its frequency is found to be more than 30% of PKU alleles[11] and also in a study made on patients of Turkish descent living in Germany, a similar frequency of 38% is obtained[12]. IVS 10-11g→a is also found to be the most common PKU mutation in several countries such as; Southern Italy (12.1%)[13], Sicily (15%)[14], Spain (13.8%)[15] and Portugal (14.6%)[16]. Our finding of the frequency for IVS10-11g→a mutation may suggest a selection due to the high frequency of different ethnic groups and consanguineous marriages in Çukurova region[17].

IVS10-11g→a is probably an ancient mutation that originated long before the end of the last ice age and separated into different alleles and subsequently spread with Neolithic farmers through the Mediterranean in early prehistory (in the Middle East by 8.000 BC)[18,19]. Thus looking at the earlier frequency (1/34) records for the heterozygous form of PAH gene in Turkey[11], the frequency of this ancient gene mutation seems to continue increasingly since our findings are considerably higher than the previous records.

Our finding of R261Q mutation as the secondly most frequent mutation is concordant with the allelic frequency found in Turkey by Turkish researchers and among Turkish immigrants living in Germany as detected by German researchers[12]. This important mutation observed in a frequency of 13% in European population is another point of interest. The arginine amino acid, accounting for 68.4% of changes in exon sites 7 and 12 where R243X, R243Q, R261Q and R408W mutations occur, is thought to be important for amino acid changes in PAH gene among PKU patients in Çukurova region. However, this result is concordant with ethnicity/region findings for Turkish population presented at pahdb web site, revealing arginine as the most frequent amino acid change with 31.5% (18/57).

Of mutations including IVS10–11g→a in intron 10 which is particularly important for Turkish population, almost 87% occur in between exons 7 to 11. This high frequency may in-turn indicate that the active domain of the enzyme may be coded at this region[20].

Although the frequency of the R408W mutation in Turkey is lower than that in European populations, the similarity of frequency for R261Q mutation and V254V polymorphisms in Turkish and European populations can be explained by the gene flow in the geographical area.

ARMS technique, generally considered among conventional techniques used for the detection of single base mutations, it seems to be advantageous in comparison to ASO requiring radioactive chemicals, to RFLP requiring pure DNA for a clean cut, and to sequencing techniques that are expensive in countries like Turkey, however it also has some disadvantage, due to the problems arising from the design of primers.

Provided that primers are carefully designed, ARMS technique can be successfully used for the verification of other molecular techniques or applied alone for the molecular diagnosis of diseases like phenylketonuria by the detection of IVS10-11g→a PAH gene mutation with a frequency of 38% to 59% in Turkish population, FMF (Familial Mediterranean Fever) which occurs in the same region and Mediterranean countries by the detection of major mutations in 85% of cases; Thalassemia by detection of IVS1-110g→a mutation in 55 to 60% of the cases and CF (cystic fibrosis) with ΔF508 mutation in 80% of cases.

REFERENCES

1. Williams RA, Mamotte CD, Burnett JR. Phenylketonuria: an inborn error of phenylalanine metabolism. Clin Biochem Rev. 2008;29:31-41.
2. DiLella AG, Kwok SC, Ledley FD, Marvit J, Woo SL. Molecular structure and polymorphic map of the human phenylalanine hydroxylase gene. Biochemistry. 1986;25:743-9.
3. Brautigam S, Kujat A, Kirst P, Seidel J, Luleyap HU, Froster UG. DHPLC mutation analysis of phenylketonuria. Mol Genet Metab. 2003;78:205-10.
4. Krivokuca A, Yanowski K, Rakobradovic J, Benitez J, Brankovic-Magic M. RAD51C mutation screening in high-risk patients from Serbian hereditary breast/ovarian cancer families. Cancer Biomark. 2015;15:775-81.
5. Hadiji-Abbes N, Trifa F, Choura M, Khabir A, Sellami-Boudawara T, Frikha M et al. A novel BRCA2 in frame deletion in a Tunisian woman with early onset sporadic breast cancer. Pathol Biol (Paris). 2015;63:185-9.
6. Sorio C, Angiari C, Johansson J, Verze G, Ettorre M, Buffelli M et al. Impaired CFTR function in mild

cystic fibrosis associated with the S977F/T5TG12complex allele in trans with F508del mutation. J Cyst Fibros. 2013;12:821-5.
7. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988;16:1215.
8. Eisenberg S, Aksentijevich I, Deng Z, Kastner DL, Matzner Y. Diagnosis of familial Mediterranean fever by a molecular genetics method. Ann Intern Med. 1998;129:539-42.
9. Zhu T, Qin S, Ye J, Qiu W, Han L, Zhang Y et al. Mutational spectrum of phenylketonuria in the Chinese Han population: a novel insight into the geographic distribution of the common mutations. Pediatr Res. 2010;67:280-5.
10. Blau N. Genetics of Phenylketonuria: Then and Now. Hum Mutat. 2016;37:508-15.
11. Ozguc M, Ozalp I, Coskun T, Yilmaz E, Erdem H, Ayter S. Mutation analysis in Turkish phenylketonuria patients. J Med Genet. 1993;30:129-30.
12. Zschocke J, Hoffmann GF. Phenylketonuria mutations in Germany. Hum Genet. 1999;104:390-8.
13. Trunzo R, Santacroce R, D'Andrea G, Longo V, De Girolamo G, Dimatteo C et al. Phenylalanine hydroxylase deficiency in south Italy: Genotype-phenotype correlations, identification of a novel mutant PAH allele and prediction of BH4 responsiveness. Clin Chim Acta. 2015;450:51-5.
14. Guldberg P, Romano V, Ceratto N, Bosco P, Ciuna M, Indelicato A et al. Mutational spectrum of phenylalanine hydroxylase deficiency in Sicily: implications for diagnosis of hyperphenylalaninemia in southern Europe. Hum Mol Genet. 1993;2:1703-7.
15. Couce ML, Boveda MD, Fernandez-Marmiesse A, Miras A, Perez B, Desviat LR et al. Molecular epidemiology and BH4-responsiveness in patients with phenylalanine hydroxylase deficiency from Galicia region of Spain. Gene. 2013;521:100-4.
16. Rivera I, Mendes D, Afonso A, Barroso M, Ramos R, Janeiro P et al. Phenylalanine hydroxylase deficiency: molecular epidemiology and predictable BH4-responsiveness in South Portugal PKU patients. Mol Genet Metab. 2011;104:86-92.
17. Ulusoy M, Tuncbilek E. [Consanguineous marriage in Turkey and its effects on infant mortality]. Nufusbil Derg. 1987;9:7-26.
18. Dianzani I, Giannattasio S, de Sanctis L, Marra E, Ponzone A, Camaschella C et al. Genetic history of phenylketonuria mutations in Italy. Am J Hum Genet. 1994;55:851-3.
19. Eisensmith RC, Woo SL. Molecular genetics of phenylketonuria: from molecular anthropology to gene therapy. Adv Genet. 1995;32:199-271.
20. Luleyap HU, Alptekin D, Pazarbasi A, Kasap M, Kasap H, Demirhindi H et al. The importance of arginine mutation for the evolutionary structure and function of phenylalanine hydroxylase gene. Mutat Res. 2006;601:39-45.