Mutations of the phenylalanine hydroxylase gene in Iranian patients with phenylketonuria

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Abstract
Phenylketonuria (PKU) is an autosomal recessive disease which results from mutations in the phenylalanine hydroxylase (PAH) gene. The aim of this study was the identification of sixteen different mutations in Iranian patients with hyperphenylalaninemia. The mutations were detected during the characterization of PAH genotypes of 39 PKU patients from Qazvin and Zanjan provinces of Iran. PAH mutations have been analyzed by PCR and direct sequencing of PCR products of the promoter region and all 13 exons of PAH gene, including the splicing sites. A mutation detection rate of 74.3% was realized. Two mutations were found at high frequencies: R176X (10.25%) and p.P281L (10.25%). The frequencies of the other mutations were: IVS2+5G>A (2.56%), IVS2+5G>C (2.56%), p.L48S (2.56%), p.R243Q (2.56%), p.R252Q (5.12%), p.R261Q (7.69%), p.R261X (5.12%), p.E280K (2.56%), p.I283N (2.56%), IVS9+5G>A (2.56%), IVS9+1G>A (1.28%), IVS11+1G>C (1.28%), p.C357R (1.28%), c.632delC (2.56%). The present results confirm the high heterogeneity of the PAH locus and contribute to information about the distribution and frequency of PKU mutations in the Iranian population.

Keywords: Phenylketonuria, PAH gene, Iranian population, Mutation detection

Background
Deficiency of hepatic phenylalanine hydroxylase (PAH) [EC.1.14.16.1], which converts phenylalanine to tyrosine, is the major frequent cause of hyperphenylalaninemia (Guldberg et al. 1998). This enzyme defect, causes toxic accumulation of phenylalanine in the body fluids and damage to the nervous system that can result in growth failure, microcephaly, mental retardation and neurobehavioral abnormalities (Zhang et al. 2005). Phenylketonuria (PKU) is one of the most common inborn disease of amino acid metabolism, characterized by mutation of the PAH gene (Williams et al. 2008). According the levels of phenylalanine, they are four categories: mild hyperphenylalaninemia (HPA), mild PKU, moderate-PKU, and classic-PKU. Classical PKU is the most severe form of this disorder. A phenylalanine restricted diet, can be useful to prevent the neurotoxic complication of Phe and its metabolites (Olsson et al. 2007). The prevalence of PKU varies worldwide. In Caucasians, the prevalence is about 1/10,000 live births (Olsson et al. 2007), while that in Iranian population was 1/3627 (Koochmeshgi et al. 2002). In fact, the high rate of consanguineous marriages in Iran may be a contributing factor to the high incidence. The human PAH gene is located on chromosome 12q23.2 and is 90 kb in size with 13 exons and 12 introns (Santos et al. 2010). So far, several hundred different mutations in this gene have been identified in PKU patients and listed in the PAH mutation Analysis Consortium database (http://www.pahdb.mcgill.ca). The most frequently occurring type of these mutations are missense mutations (Scriver 2007). The PAH gene mutations demonstrate considerable ethnic groups and geographic areas variation (Zschocke 2003). Previous studies have shown a correlation between PAH genotype and metabolic phenotype in PKU and have suggested the phenotypic relations of particular mutation combinations (Desviat et al. 1997; Kayaalp et al. 1997; Romano et al. 1996). Mutation...
analysis of a given population can be useful for the better understanding functional aspects of mutant protein and the relationship between genotype/phenotype.

Objectives
The purpose of this study was to identify the molecular basis of PKU in Iranian Patients. In addition, we examined the variation in all 13 exons of the PAH gene in 39 patients, predominantly from Qazvin and Zanjan provinces of Iran.

Patients and methods
Thirty-nine unrelated children with PAH deficiency were enrolled for this study after obtaining informed consent from the parents. A total of 39 patients, 24 cases were from the province of Qazvin and 15 cases from the Zanjan region. The PAH activity was measured by conventional biochemical methods. Most of the patients were identified when they showed mental retardation and few patients were identified during neonatal screening. The subjects were with ages ranging from 1 month to 10 years old. The clinical criteria were classical PKU with blood phenylalanine concentrations >20 mg/dl (>1200 µm/L) (Guttler 1980). The study was approved by the ethics committee of Qazvin University of medical sciences.

DNA analysis
Genomic DNA was isolated from the leukocytes in blood samples using a DNA purification Qiagen kit (Valencia, CA, USA). All 13 PAH exons and their flanking intronic sequences were amplified by PCR using primers designed by primer 3 software. The primers sequences can be provided upon request. PCR reaction were performed on the Gene AMP PCR System Verity, (Foster City, CA, USA). The PCR condition were 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 45–60 °C for 30 s, 72 °C for 30 s followed by 72 °C for 5 min. Samples were electrophoresed in 2 % agarose gel. The PCR products were sequenced by ABI prism 3130 genetic analyzer (Applied Biosystems, USA) and compared with the human genomic DNA sequence in GenBank to identify the mutations.

Results
In this study, we detected causative mutations on 49 of the 78 mutant alleles (diagnostic efficiency 74.3 %) (Table 1). These included: eight missense mutations (50 %), five splice mutations (31 %), two nonsense mutations (12.5 %) and one deletion (6.25 %) (Table 1). Exon 7, 6, 2 and the flanking intronic regions include 85.5 % of the mutant alleles. The p.R176X and p.P281L mutations were the most frequent (10.5 %) followed by p.R261Q (7.69 %), p.R261X and p.R252Q (5.12 %), accounting for nearly 40 % of all mutations. Mutations p.R261X and p.R252Q were less frequent. All other mutations had frequencies less than 3 %. Among the 39 unrelated families studied, 20 (51.2 %) were homozygote, 6 (15.3 %) heterozygote and 2 (5.12 %) were compound heterozygote and 11 (28.2 %) were no PKU- causing mutations. The following polymorphisms were detected in the PAH gene: p.L385L, p.Q232Q and p.V245V with the frequency of 84, 51 and 17 % respectively were shown the highest prevalence among the other polymorphisms (Table 2). Genotypes of 39 PKU patients are shown in Table 3.

Discussion
In this study, we identified mutations in the PAH gene and to evaluate the genetic heterogeneity of PKU disease in 39 unrelated Iranian patients who had been referred to Qazvin and Zanjan provinces. From this experiment, 28 of 39 PKU patients were found to contain the mutation. Our analysis of the homozygosity of the mutations were nearly similar to that observed in northwestern Iranian populations (Bonyadi et al. 2010). The majority of the recognized mutations are situated in the catalytic domains (143–410 amino acid), and some of them (p.P281L, R252W) are located in the cofactor binding regions. The most common mutation in our samples is PP281L. This data agreement with what was found in other group Iran (Bonyadi et al. 2010; Hamzehloei et al. 2012). The PP281L mutation in exon 7 with a relative frequency of 10.5 % is C → T substitution lead to conversation of Pro to Leu at codon 281 of PAH. The another major mutation in our study was p.R176X (10.25 %), which is similar to the data obtained in population of Khorasan Razavi origin (Hamzehloei et al. 2012).Previous study on the genotype/phenotype association demonstrated generally a positive correlation between R176X mutation and classic phenotype(Acosta et al. 2001; Bueno et al. 2013). Several studies reported that the IVS10-11G>A mutation, a splice mutation in the end of intron 10, observed with a high incidence among in the Mediterranean region, Brazil and some parts of Iran including: East Azerbaijan, Semnan, Khorasan Razavi, Hamedan (Dianzani et al. 1995; Kleiman et al. 1994; Rivera et al. 1998; Zare-Karizi et al. 2011), however this mutation was not found in the present study. The virtual absence of this mutation in our study may reflect the regional variability of populations. The second most frequent mutation identified in present study, R261Q (7.69 %) occurs on a CpG mutation hotspot on exon 7, leads to the conversion of Arg to Gln at codon 261 of PAH. This mutation is a common mutation in the Mediterranean and southern Europe but has a very low incidence in Spain (Couce et al. 2013; Loebel 2007; Perez et al. 1994; Rivera et al. 1998; Rivera et al. 2011). Furthermore, the frequency of R243Q mutation has been reported to be 18.2 % in Chinese and 12 % in
Koreans, whiles in the present study the frequency of this mutation was found to be 2.5% (Song et al. 2005). Most mutant PAH alleles probably with influencing on PAH gene transcription and translation can decrease the intracellular stability of protein and finally reduce enzyme function completely. In this study, we also analyzed the association between mutations and polymorphisms. The c.755G>A mutation located on the same allele with the c.168+19T>C polymorphism. Also, we observed association between the p.Q232Q polymorphism and c.843T>A, c.1199+1G>C. c.969+1G>A, c.1069+1G>C mutations occurred on the same allele in cis form, that particularly in the final case, similar associations have been reported in previous study (Hamzehloei et al. 2012). The majority of mutant alleles (73%) were located on exon 7 and 6 that is in agreement with previous studies in Iranian populations (Hamzehloei et al. 2012; Zare-Karizi et al. 2011). In addition, the novel gene variant c.1069T>C (p.C357R) has been seen in Iranian population for the first time. Thereby to plan detection strategy; the samples will be screened first for mutations in these regions. If mutations were not identified, the other exons and their adjacent will be tested.

**Conclusion**

Our results of Iranian individuals with PKU confirm a heterogeneous spectrum of mutations, displaying different ethnic and geographical origins. Moreover, our

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### Table 1  Spectrum and frequency of PAH mutations identified in 39 patients

| Systematic name (DNA level) | Trivial name (protein effect) | Location | Mutation type | Number of alleles | Frequency (%) |
|-----------------------------|-------------------------------|----------|---------------|-------------------|---------------|
| c.168+5G>A                  | IVS2+5G>A                     | Intron 2 | Splicing      | 2                 | 2.56          |
| c.168+5G>C                  | IVS2+5G>C                     | Intron 2 | Splicing      | 2                 | 2.56          |
| c.143T>C                    | p.L48S                        | Exon 2   | Missense      | 2                 | 2.56          |
| c.526C>T                    | p.R176X                       | Exon 6   | Nonsense      | 8                 | 10.25         |
| c.632delC                   | p.P211>Hfs                    | Exon 6   | deletion      | 2                 | 2.56          |
| c.838G>A                    | p.E280K                       | Exon 7   | Missense      | 2                 | 2.56          |
| c.782G>A                    | p.R261Q                       | Exon 7   | Missense      | 6                 | 7.69          |
| c.842C>T                    | p.P281L                       | Exon 7   | Missense      | 8                 | 10.25         |
| c.781C>T                    | p.R261X                       | Exon 7   | Nonsense      | 4                 | 5.12          |
| c.755G>A                    | p.R252Q                       | Exon 7   | Missense      | 4                 | 5.12          |
| c.728G>A                    | p.R243Q                       | Exon 7   | Missense      | 2                 | 2.56          |
| c.848T>A                    | p.I283N                       | Exon 8   | Missense      | 2                 | 2.56          |
| c.969+1G>A                  | IVS9+1G>A                     | Intron 9 | Splicing      | 1                 | 1.28          |
| c.969+5G>A                  | IVS9+5G>A                     | Intron 9 | Splicing      | 2                 | 2.56          |
| c.1199+1G>C                 | IVS11+1G>C                    | Intron 11| Splicing      | 1                 | 1.28          |
| c.1069T>C                   | p.C357R                       | Exon 11  | Missense      | 1                 | 1.28          |
| Total (number of alleles identified) |                               |          |               | 49                | 74.3          |

**Table 2  PAH polymorphisms identified in 39 patients**

| Systematic name (DNA level) | Trivial name (protein effect) | Location | Number of alleles | Frequency (%) |
|-----------------------------|-------------------------------|----------|-------------------|---------------|
| c.696G>G                    | p.Q232Q                       | Exon 6   | 40                | 51.28         |
| c.735G>A                    | p.V245V                       | Exon 7   | 14                | 17.9          |
| c.912G>A                    | p.Q304Q                       | Exon 8   | 2                 | 2.56          |
| c.1155G>G                   | p.L385L                       | Exon 11  | 66                | 84.61         |
| c.168+19T>C                 | IVS2+19T>C                    | Intron 2 | 5                 | 6.4           |
| c.-71A>C                    | S-UTR                         | S-UTR    | 4                 | 5.1           |
| c.843T>A                    | p.P281P                       | Exon 8   | 2                 | 2.56          |
| IVS3-22C>T                 | c.353-22C>T                  | Intron 3 | 2                 | 2.56          |
| Total (number of alleles identified) |                               |          | 135              |               |
findings were slightly different from other ethnic groups. These findings can be useful to genotype/phenotype relationship in patients and provide future some ability to confirmatory diagnostic testing, prognosis and predict severity of PKU patients.

Authors’ contributions
AB: project development, data collection. FS: project development, data collection. ZR: data collection, data analysis. SA: data collection, data analysis. RN: project development, data collection, data analysis, manuscript writing and editing. All authors read and approved the final manuscript.

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Table 3 Distributional genotypes in 39 PKU patients

| Genotype | Polymorphism | Number of patients |
|----------|--------------|--------------------|
| u/u      | c.168+19T>C, c.1155G>C, c.696A>G | 1 |
| c.838G>A,p.E280K/c.838G>A,p.E280K | c.735G>A, c.912G>A, c.1155C>G | 1 |
| u/u      | c.1155G>G   | 1 |
| c.782G>A,p.R261Q/c.782G>A,p.R261Q | c.1155G>G | 1 |
| u/u      | c.735G>A, c.1155C>G | 1 |
| u/u      | c.168+19T>C, c.1155G>G, c.696A>G | 1 |
| c.842C>T;p.P281L/c.842C>T;p.P281L | c.696A>G, c.1155C>G | 2 |
| u/u      | c.168+19T>C, c.1155G>G, c.696A>G | 1 |
| c.781C>T;p.R261X/c.781C>T;p.R261X | c.1155G>G | 1 |
| c.755G>A,p.R252Q/U | c.696A>G, c.1155C>G | 1 |
| c.842C>T;p.P281L/c.842C>T;p.P281L | c.696A>G, c.1155C>G | 1 |
| u/u      | c.696A>G, c.1155C>G | 1 |
| c.755G>A,p.R252Q/U | c.735G>A, c.1155C>G | 1 |
| IVS9+1G>T/U | c.696A>G, c.1155C>G | 1 |
| c.781C>T;p.R261X/C781C>T;p.R261X | c.696A>G, c.1155C>G | 1 |
| c.782G>A,p.R261Q/c.782G>A,p.R261Q | c.696A>G, c.1155C>G | 2 |
| c.755G>A,p.R252Q/c.755G>A,p.R252Q | c.696A>G, c.1155C>G, c.168+19T>C | 1 |
| c.526C>T;p.R176X/c.526C>T;p.R176X | c.-71A>C | 1 |
| c.526C>T;p.R176X/c.526C>T;p.R176X | c.1155G>G | 1 |
| c.143T>C, p.L48S/c.143T>C, p.L48S | c.1155G>G | 1 |
| u/u      | c.1155G>G | 1 |
| IVS2+5G>A/IVS2+5G>A | c.848T>A/p.I283N/c.848T>A/p.I283N | 1 |
| c.526C>T;p.R176X/c.526C>T;p.R176X | c.696A>G, c.1155C>G | 1 |
| c.842C>T;p.P281L/IVS11+1G>C | c.696A>G, c.1155C>G | 1 |
| c.842C>T;p.P281L/c.842C>T;p.P281L | c.696A>G, c.1155C>G | 1 |
| c.632delC/p.P211>HSfs/c.632delC/p.P211>HSfs | c.1155G>G, c.735G>A | 1 |
| u/u      | c.735G>A, c.1155C>G | 1 |
| c.728G>A,p.R243Q/U | c.735G>A, c.1155C>G | 1 |
| IVS9+5G>A/IVS9+5G>A | c.735G>A, c.1155C>G | 1 |
| IVS9+5G>A/IVS9+5G>A | c.696A>G, c.1155C>G | 1 |
| c.728G>A,p.R243Q/c.1069T>C-p.C357R | c.735G>A | 1 |
| u/u      | c.696A>G, c.1155C>G | 1 |
| IVS2+5G>C/IVS2+5G>C | c.696A>G, c.843T>A, c.1155C>G | 1 |
| u/u      | c.735G>A, IVS3-22C>T | 1 |

U: unidentified

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Compliance with ethical guidelines
Competing interests
The authors declare that they have no competing interests.
Ethical statement
All human studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in Declaration of Helsinki and its later amendments.

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References
Acosta A, Silva W Jr, Carvalho T, Gomes M, Zago M (2001) Mutations of the phenylalanine hydroxylase (PAH) gene in Brazilian patients with phenylketonuria. Hum Mutat 17:122–130
Bonyadi M, Omrani O, Moghanjoghi SM, Shiva S (2010) Mutations of the phenylalanine hydroxylase gene in Iranian Azeri Turkish patients with phenylketonuria. Genet Test Mol Biomarkers 14:233–235
Bueno MA, Gonzalez-Lamuno D, Delgado-Pecellin C, Aldamiz-Echevarria L, Perez B, Desviat LR, Couce ML (2013) Molecular epidemiology and genotype-phenotype correlation in phenylketonuria patients from South Spain. J Hum Genet 58:279–284
Couce ML, Boveda MD, Fernandez-Marmiesse A, Miras A, Perez B, Desviat LR, Fraga JM (2013) Molecular epidemiology and BH4-responsiveness in patients with phenylalanine hydroxylase deficiency from Galicia region of Spain. Gene 521:100–104
Desviat LR, Perez B, Garcia MJ, Martinez-Pardo M, Baldellou A, Arena J, Sanjurjo P, Campistol J, Couce ML, Fernandez A et al (1997) Relationship between mutation genotype and biochemical phenotype in a heterogeneous Spanish phenylketonuria population. Eur J Hum Genet 5:196–202
Dianzani I, Giannattasio S, de Sanctis L, Alliaudi C, Lattanzio P, Dionisi Vici C, Burlina A, Burroni M, Sebastio G, Carnevale F et al (1995) Characterization of phenylketonuria alleles in the Italian population. Eur J Hum Genet 3:294–302
Guldberg P, Rey F, Zschocke J, Romano V, Francois B, Michiels L, Ullrich K, Hoffmann GF, Burgard P, Schmidt H et al (1998) A European multicenter study of phenylalanine hydroxylase deficiency: classification of 105 mutations and a general system for genotype-based prediction of metabolic phenotype. Am J Hum Genet 63:71–79
Guttler F (1980) Hyperphenylalaninemia: diagnosis and classification of the various types of phenylalanine hydroxylase deficiency. Acta Paediatr Scand Suppl 280:1–80
Hamzehloei T, Hosseini SA, Valili R, Mojarad M (2012) Mutation spectrum of the PAH gene in the PKU patients from Khorasan Razavi province of Iran. Gene 506:230–232
Kayaalp E, Treacy E, Waters PJ, Byck S, Nowacki P, Scrivcr CR (1997) Human phenylalanine hydroxylase mutations and hyperphenylalaninemia phenotypes: a metaanalysis of genotype-phenotype correlations. Am J Hum Genet 61:1309–1317
Kleiman S, Avigad S, Vanagaste L, Shiruvellevitz A, David M, Eisensmith RC, Brand N, Schwartz G, Rey F, Munnich A et al (1994) Origins of hyperphenylalaninemia in Israel. Eur J Hum Genet 2:24–34
Koochmeshgi J, Bagheri A, Hosseini-Mazinani SM (2002) Incidence of phenylketonuria in Iran estimated from consanguineous marriages. J Inherit Metab Dis 25:80–81
Loeber JG (2007) Neonatal screening in Europe; the situation in 2004. J Inherit Metab Dis 30:430–438
Olsson GM, Montgomery SM, Alm J (2007) Family conditions and dietary control in phenylketonuria. J Inherit Metab Dis 30:708–715
Perez B, Desviat LR, De Luca M, Ugarte M (1994) Spectrum and origin of phenylketonuria mutations in Spain. Acta Paediatr Suppl 407:34–36
Rivera I, Leandro P, Lichter-Konecki U, Tavares de Almeida I, Lechiner MC (1998) Population genetics of hyperphenylalaninemia resulting from phenylalanine hydroxylase deficiency in Portugal. J Med Genet 35:301–304
Rivera I, Mendes D, Afonso A, Barros M, Ramos R, Janeiro P, Oliveiera A, Gaspar A, Tavares de Almeida I (2011) Phenylalanine hydroxylase deficiency: molecular epidemiology and predictable BH4-responsiveness in South Portuguese PKU patients. Mol Genet Metab 104(Suppl) S86–S92
Romano V, Guldberg P, Guttler F, Meli C, Mollica F, Pavone L, Giovannini M, Riva E, Basucci G, Luotti D et al (1996) PAH deficiency in Italy: correlation of genotype with phenotype in the Sicilian population. J Inherit Metab Dis 19:15–24
Santos LL, Fonseca CG, Starling AL, Januario JN, Aguilar MJ, Peixoto MG, Carvalho MR (2010) Variations in genotype-phenotype correlations in phenylketonuria patients. Genet Mol Res 9:1–8
Scrivcr CR (2007) The PAH gene, phenylketonuria, and a paradigm shift. Hum Mutat 28:831–845
Song F, Ou YJ, Zhang T, Jin YW, Wang H, Zheng XY (2005) Phenylketonuria mutations in Northern China. Mol Genet Metab 86(Suppl 1):S107–S118
Williams RA, Mamatte CD, Burnett JR (2008) Phenyketonuria: an inborn error of phenylalanine metabolism. Clin Biochem Rev 29:31–41
Zare-Karizi S, Hosseini-Mazinani SM, Khazaee-Koohpar Z, Seifi SM, Shahsavann-Behebodi B, Akbari MT, Koochmeshgi J (2011) Mutation spectrum of phenylketonuria in Iranian population. Mol Genet Metab 102:29–32
Zheng H, Jiang J, Zhai X, Fang G, Gao J, Shi M, Wang Y (2005) Identification of novel mutations in the phenylalanine hydroxylase gene of classical phenylketonuria. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 22:134–137
Zschocke J (2003) Phenylketonuria mutations in Europe. Hum Mutat 21:345–356