Phenylketonuria in Iranian population: a study in institutions for mentally retarded in Isfahan

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Abstract

Phenylalanine hydroxylase (PAH) deficiency is caused by mutations in the PAH gene (12q22-q24) resulting in a primary deficiency of the PAH enzyme activity, intolerance to the dietary intake of phenylalanine (Phe) and production of the phenylketonuria (PKU) disease. To date there have been no reports on the molecular analysis of PKU in Iranian population. In this study, the states of the PKU disease in terms of prevalence and mutation spectrum among patients reside in the institutions for mentally retarded in Isfahan was investigated. In the first step, 611 out of 1541 patients with PKU phenotype or severe mental retardation were screened for the PKU disease using the Guthrie bacterial inhibition assay (GBIA) followed by HPLC. Among the patients screened 34 (5.56%) were found positive with abnormal serum Phe of above 7 mg/dl. In the next step, the presence of 18 common mutations of the PAH gene in 26 of the patients with classical PKU (serum Phe above 20 mg/dl) was investigated, using the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). Of the 52 independent mutant alleles that were analyzed, 34 (65.38%) were genotyped showing 8 mutations as follows: R252W (15.38%), Q232Q (13.46%), R261Q (7.69%), delL364 (7.69%), IVS10-11g > a (5.77%), L333F (5.77%), V245V (5.77%) and S67P (3.85%). The results from this study may serve as a reference to analyze the PKU mutations in other part of Iran, and to establish diagnostic tests for carrier detection and prenatal diagnosis of the PKU disease in Iranian population.

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Keywords: Phenylalanine hydroxylase; Phenylketonuria; Mutation; Screening; Iranian population

1. Introduction

Phenylalanine hydroxylase (PAH) is a hepatic enzyme involved in the metabolism of the essential amino acid phenylalanine (Phe) [1]. Deficiency of the enzyme due to mutations in the PAH gene (chromosomal locus 12q22-q24) results in hyperphenylalaninemia which is associated with the phenylketonuria (PKU) disease [2]. This disease is caused by a complete or near-complete deficiency of PAH activity, and is associated with profound and irreversible mental retardation [3,4]. If the disease is diagnosed early at birth and the patient maintained on a strict low-phenylalanine diet, the symptoms of the disease can be prevented [5–7].

PAH deficiency can be diagnosed by detecting an elevation of serum phenylalanine concentrations. Individuals with PAH deficiency show plasma phenylalanine concentrations that are persistently above...
2 mg/dl (120 µmol/l) in the untreated state [8,13]. On one classification, based on the plasma concentrations of phenylalanine in the untreated state and tolerance for dietary phenylalanine, the PAH deficiency can be categorized as: phenylketonuria (classical PKU), moderate PKU, mild PKU, and non-PKU hyperphenylalaninemia (non-PKU HPA) [9]. Once the diagnosis is established by biochemical testing, mutation analysis of the PAH gene can be used in some cases to identify the disease-causing alleles which is useful for genetic counseling purposes and prenatal diagnosis [10].

The number of mutations identified for the PAH gene is dramatically increasing. So far, over 400 different disease-causing mutations have been reported for the PAH gene [11,20]. The various PAH mutations reduce the enzyme activity to different degrees, explaining the broad phenotypic heterogeneity of this disease from severe PKU to mild hyperphenylalaninemia [11]. Correlation between mutations in both PAH alleles and clinical phenotypes have been demonstrated, and several mutations have been related to mild PKU phenotype [12]. Therefore, predicting the clinical severity of PKU from genotype can be an important purpose of molecular analysis in PKU patients.

Molecular genetic testing of the PAH gene is used primarily for genetic counseling purposes to determine carrier status of at risk relatives and for prenatal testing. Prenatal diagnosis of PKU deficiency is possible in pregnancies at risk when mutation analysis has revealed the disease-causing mutations in the PAH gene in an affected family member or when linkage analysis has identified informative markers [15]. When mutation analysis is not available or disease-causing mutations are not identified in a family, linkage analysis can be considered for couples who have a child affected with PKU deficiency. Linkage studies are based upon diagnosis of PAH deficiency in the affected family member and accurate understanding of the genetic relationships in the family [16]. Two intragenic markers to the PAH locus have been used for linkage analysis in PKU: a variable number of tandem repeats (VNTR) and a simple tandem repeat (STR) [16,17]. These markers have shown to be very informative which can be used in many families with PAH deficiency with great accuracy.

Molecular characterization of the PKU disease has been performed in most of the countries around the world. To date, there have been no report on the exact status of the PKU disease and the mutation spectrum of the PAH gene in Iranian population. However, based on the reports from other middle eastern countries and a short report from a study on consanguineous marriages in Iran as well as the absence of a PKU screening program in Iran, it seems that the frequency of the disease to be relatively high in this country [18].

In the present study, the aim was to study the prevalence of the PKU disease among the institutionalized patients suffering from mental retardation, and to characterize the mutations underlying the PKU disease in these patients. In an attempt, 34 patients with PKU were identified among the patients screened for the disease. Analysis of the mutations of the PAH gene resulted in the identification of eight mutations which are discussed in this report.

2. Material and methods

2.1. Patients screening

A total of 1541 patients reside in different institutions for mental retardation in the central province of Iran, Isfahan, was involved in this study. Patients with a known genetic syndrome (e.g. Down syndrome) were excluded from the study. The remaining 611 patient with either PKU phenotype or with unknown reason for their mental retardation were subjected to blood sampling on Guthrie cards. The patients were all born in the province of Isfahan. Before sampling, informed consent was obtained from their parents, and in a number of cases the parents were present at the time of blood sampling. The cards were analyzed using the Guthrie bacterial inhibition assay (GBIA) for serum Phe [8,13]. Among the patients examined 34 were found to be positive for the Guthrie test (serum Phe above 7 mg/dl). The serum Phe was quantitatively measured using the high pressure liquid chromatography (HPLC) assay. Mutation analysis was performed on 26 patients with serum phenylalanine above 20 mg/dl.

2.2. Mutation analysis

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) provides a simple, fast and powerful method in identifying the PAH
mutations, which in some studies using this procedure nearly 80% of the PAH mutations in the PKU patients had been identified [15,19,22]. This is because a large number of the PAH mutations either produce or abolish a restriction site. In this study, 18 mutations of this kind which already identified in the middle east and Mediterranean regions were investigated. In the first step, the genomic DNA was extracted from the leukocytes of the patients using a standard salting out procedure [14]. The quality of the genomic DNA was examined by analysis on agarose gel. In the second step, different exons of the PAH gene containing the mutations under investigation were amplified using PCR with the specific primers. PCR reactions were performed using Eppendorf Gradient Master Cycler thermal cycler (Eppendrof Inc., Germany). Reaction mixtures were made in volume of 25 μl containing 0.2–1 μg of genomic DNA, 5–10 pmol of each primer and 200 μM of each dNTP and the reaction buffer supplied with the Taq DNA polymerase (Cinagene, Tehran, Iran). The sequence of the primers used and the PCR conditions for each pair of the primers were as previously described [19]. PCR primers were made by MWG Inc., Germany. The mutations were analyzed by enzymatic digestion of the PCR products with the appropriate restriction enzymes, followed by gel electrophoresis. The PCR products were analyzed on either 2% agarose gels or 10% polyacrylamide gel depending on the size of the DNA fragments after digestion. The DNA on the agarose gels was visualized by staining with ethidium bromide, and on polyacrylamide with ethidium bromide or silver stating (for some mutations that a small amount of DNA was available or bands were below 100 bp). The condition of gels and electrophoresis were as described [14].

3. Results and discussion

3.1. Screening the institutionalized patients for PKU

The patients reside in the institution for mentally retarded were screened for the PKU disease by measuring their serum phenylalanine using the standard Guthrie test, followed by HPLC. The reports from different countries around the world show that 1–3% of the patients in the institutions for mentally retarded suffer from the PKU disease [21]. In this study 611 patients from eight different institutions for mentally retarded in the province of Isfahan were screened for the PKU disease (see Section 2). Of the patients examined, 34 showed positive Guthrie test with serum Phe above 8 mg/dl. This shows that the prevalence of the disease among the patients screened is about 5.5%. Therefore, considering total number of patients reside in the institutions (see Section 2), the total frequency of PKU including mild, moderate and classical type in patients in institutions for mentally retarded in Isfahan could be estimated as 2.2%, which is relatively high in comparison with other countries [21]. Quantitative measurement of the Phe in the serum of the positive patients allowed the classification of the patients. As shown in Table 1, of the patients, 76.47% had classical type of PKU. These patients were chosen in the next step for molecular analysis of the PAH mutations.

The screening data clearly indicated the relatively high prevalence of the disease among the patients reside in Iranian institutions for mentally retarded. This highlights the necessity of a neonatal screening program for PKU in Iranian population to prevent the incidence of the PKU phenotype in this population. Furthermore, screening the residents of the institutions for mentally retarded and identification of the PKU patients could be useful in determination of carrier states of the disease among the family member of the PKU patients.

3.2. Identification of the PAH mutations

The mutation spectrum of the PAH gene was investigated in the patients with classical PKU showing the serum Phe above 20 mg/dl (see Table 1). To our knowledge, this study was the first work on the analysis of the mutation spectrum of the PAH gene in Iranian PKU patients. Therefore, as the first venue to begin with, analysis of 18 mutations of the PAH

Table 1 Different types of PKU in the patients with positive Guthrie test

<table>
<thead>
<tr>
<th>PKU type</th>
<th>No. of patients</th>
<th>Phe concentration (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild PKU</td>
<td>1 (2.94%)</td>
<td>8–12</td>
</tr>
<tr>
<td>Moderate PKU</td>
<td>7 (20.59%)</td>
<td>12–16</td>
</tr>
<tr>
<td>Classical PKU</td>
<td>26 (76.47%)</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>
gene which were previously identified in the middle eastern and Mediterranean populations were examined using the PCR-RFLP procedure [24]. One reason for this selection was the demonstrated similarity between these populations (especially the Mediterranean one) and the Iranian population in terms of gene flow and disease-causing mutations as reported in studies on a number of genetic diseases such as glucose-6-phosphate dehydrogenase (G6PD) deficiency [25]. Moreover, all the selected mutations create or abolish a restriction site, which make their identification possible using the PCR-RFLP procedure. The mutations were as follows: R252W, Q232Q, R261Q, delL364, IVS10-11g > a, L333F, R408Q, V245V, R408W, R261X, F299C, S273F, G272X, D338Y, A309D and IVS3nt-6t > a.

In order to analyze these mutations, the corresponding exons were PCR amplified in all the 26 patients (52 chromosomes) with classical PKU, using the specific primers. The amplified exons were then examined for the known mutations using the appropriate restriction enzyme(s). As shown in Table 2, of the mutations studied, eight were found on 34 out of 52 independent PAH chromosomes (alleles). The mutation detection rate in this study was, therefore, 65.38%. In Figs. 1 and 2 analysis of some of the mutations is shown. Among the mutations identified, 17 (50%) were missense, 10 (29.4%) silent, 3 (8.8%) splice-site, and 4 (11.8%) deletion (Table 2).

The mutation S67P, which is a missense mutation, showed the lowest frequency (3.85%), but the mutation R252W seemed to be the predominant one with the frequency of 16%. The S67P also shows very low frequency (0.5–3%) in other populations and accounts for the rare mutations of the PAH gene that have been identified [11,23,26]. As shown in Fig. 2A, the S67P mutation results in the loss of an Xba I restriction site. The mutation R252W is a missense mutation, that involves the catalytic domain of the PAH enzyme (amino acids 143–410). In vitro expression analysis showed that this mutation almost fully affected the activity of the enzyme, <1% [11,27]. The prevalence of this mutation varies from 2–8% in different countries [11]. Normal individuals produce two bands of 164 and 121 base pair, but in homozygous affected individuals, one band of 285 base pair is produced in the presence of the Ava I enzyme (Fig. 1B). The mutation R261Q with the frequency of 7.1% in our population is another missense mutation, which occurred in the catalytic domain of the PAH enzyme. The frequency of this mutation is almost similar to the frequency reported from Turkey, which may help the identification of the origin of the mutation in the middle east region [11,27]. This mutation results in loss of a Hind I restriction site in exon 4 (Fig. 1C).

Table 2

<table>
<thead>
<tr>
<th>Exon (E)/Intron (I)</th>
<th>Mutation</th>
<th>Allele frequency (%)</th>
<th>Restriction enzyme</th>
<th>Mutation type</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7</td>
<td>R252W</td>
<td>8 (15.38)</td>
<td>– Ava I</td>
<td>Missense</td>
</tr>
<tr>
<td>E6</td>
<td>Q232Q</td>
<td>7 (13.46)</td>
<td>+ Dde I</td>
<td>Silent</td>
</tr>
<tr>
<td>E7</td>
<td>R261Q</td>
<td>4 (7.69)</td>
<td>– Hind I</td>
<td>Missense</td>
</tr>
<tr>
<td>E11</td>
<td>delL364</td>
<td>4 (7.69)</td>
<td>– Hind III</td>
<td>Deletion</td>
</tr>
<tr>
<td>E10</td>
<td>IVS10-11g &gt; a</td>
<td>3 (5.77)</td>
<td>+ Dde I</td>
<td>Splice-site</td>
</tr>
<tr>
<td>E10</td>
<td>L333F</td>
<td>3 (5.77)</td>
<td>– Ban II</td>
<td>Missense</td>
</tr>
<tr>
<td>E7</td>
<td>V245V</td>
<td>3 (5.77)</td>
<td>+ Ali I</td>
<td>Silent</td>
</tr>
<tr>
<td>E3</td>
<td>S67P</td>
<td>2 (3.85)</td>
<td>– Xba I</td>
<td>Missense</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>34 (65.38)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The percentage was calculated based on total number of 52 alleles in 26 patients.
Fig. 1. (A) A PCR amplification of exons 3, 6, 7, 8, 10, 11 and 12. The PCR products were analyzed on 2% agarose gel; (B) analysis of mutation R252W in exon 7 using AvaI restriction enzyme; (C) analysis of mutation R261Q in exon 7 using HinfI enzyme; (D) analysis of mutation delL364 in intron 11 using enzyme HindIII. The restriction fragment bands for each mutation is indicated as horizontal line below each figure.

from Sweden [11,27]. The mutation results in the loss of a HindIII site in exon 11 (Fig. 1D). For this mutation no heterozygous was identified in this study. The mutation L333F in exon 10 was analyzed using the BanII restriction enzyme (Fig. 2C). This mutation accounted for 5.77% of mutations identified in this study (Table 2).

Mutations Q232Q and V245V are silent mutations that result in the polymorphism in the PAH gene. These mutations occur with various frequencies from 2–45% in different population [11]. A number of silent mutations (e.g. V399V) of PAH gene have been found to involve in so called exon skipping phenomenon which results in creation of new splice-sites [28]. Therefore, these mutations may contribute to the PKU phenotype. Although the role of Q232Q and V245V in PKU is not clear, there might be other mutations associated with these mutations, which
play a critical role in the appearance of the PKU phenotype.

Analysis of the homozygosity of the mutations indicated that 68.4% of the patients were homozygous and 31.6% heterozygous for the mutations that they carry. This is consistent with the high rate of consanguineous marriages in the family of the patients. Interestingly, investigation of the consanguinity in the patient’s family showed that in 68% of the cases, the parents were first cousin (data not shown). This was consistent and clearly explained the homozygosity rate of the mutations that identified in this population.

Although this was a preliminary study on the molecular analysis of PKU in Iranian population, but the data obtained could be used as a base for further investigation of the disease in this population. Furthermore, the mutations that were identified in mentally retarded patients in different institutions of the province of
Isfahan may represent the prevalence of the PAH mutations in the whole Isfahan population. This is supported by the fact that the record on the place of birth of the patients indicated that the patients were virtually randomly distributed among the institutions (data not shown). Obviously, identification of the remaining PAH mutations, and mutations in patients in which, none of the above mutations were present, requires further investigation. This could be achieved by employment of other techniques such as denaturing gradient gel electrophoresis (DGGE) followed by direct sequencing, and the use of polymorphic short tandem repeat and variable number of tandem repeats associated with the PAH gene and the common mutations.

Acknowledgements

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References