Incidence of Neonatal Hyperphenylalaninemia Based on High-performance Liquid Chromatography Confirmatory Technique in Mazandaran Province, Northern Iran (2007–2015)

Abstract

Background: Classic phenylketonuria (PKU) is a metabolic disorder. The purpose of this study was to assess epidemiological factors of PKU phenotypes in a neonatal screening program for Mazandaran, Iran. Methods: In this descriptive-retrospective study from 2007 to 2015, neonates PKU level was conducted by phenylalanine level based on a biochemical technique by ELISA and then by confirmatory methods high performance liquid chromatography. Results: Of the 407,244 screened newborns (48.7% girls and 51.3% boys), 14 girls and 13 boys were diagnosed definitely from 465 suspicious cases of PKU. The incidence of PKU was 0.66 in 10,000, which was noted in different severity (severe PKU - 1:67,874, mild PKU - 1:45,249, and HPA - 1:33,937). In addition, we did not detect any cases of nonclassic PKU. Conclusions: Although the consanguineous marriage pattern is a major cause of hyperphenylalaninemia (HPA) particularly in Iranian, there was no significant difference between groups in this study. Now, screening should be executed for all of the family that they have the familial history of PKU in Iran. According to varies actual of prevalence and incidence rate of PKU reported a real patient and taking PKU with mild PKU and HPA, it is recommended, the will provide the PKU reports based on the severity of the disease.

Keywords: Chromatography high-pressure liquid, Iran, neonatal screening, phenylketonurias

Introduction

Phenylalanine (Phe) is an essential amino acid. The phenylalanine hydroxylase (PheOH or PHA) enzyme and its cofactor tetrahydrobiopterin (THB) catalyze the conversion of phenylalanine to tyrosine, and their deficiency results in accumulation of phenylalanine in body fluids and central nervous system.[1-2] In affected patients, excessive phenylalanine is metabolized to phenyl ketones that are excreted in the urine.[3,4] The PAH gene is located on the long arm of chromosome 12 in the region q22-q24. In 98% of PKU patients, defects of the PAH enzyme are due to mutations in the PAH gene on chromosome 12q23.2.[5,6] In 98% of cases, the damages of central nervous system are due to mutations in the gene encoding the enzyme L-PHA (EC 1.14.16.1).[7-8]

Elevated phenylalanine, if not treated in the 1st days of the life, causes irreversible brain and mental damages (microcephaly and seizures).[9] Deficiency of PHA causes severe phenylketonuria (PKU) and deficiency of its cofactor THB causes malignant PKU.[10] If the serum tyrosine and urine THB levels were normal and sera phenylalanine levels were ≥20 mg/dl, between 10 and 20 mg/dl, and between 2 and 10 mg/dl, the newborns were diagnosed as having severe PKU, mild PKU, and hyperphenylalaninemia (HPA), respectively.[4]

There are several methods to PKU identify in blood-dried specimens (DBS) screening laboratory; such as fluorometric,[11] enzymatic, colorimetric,[8,12] high-performance liquid chromatographic (HPLC),[8,13] and more recently tandem mass spectrometric methods.[14,15]

The first pilot study for the assessment of neonatal HPA in Iran was started in Tehran from 1982,[16] and the first National Neonate Screening Program (NNSP) in Iran was begun from 2002 in Fars province[17] and afterward in Mazandaran Province in 2007, based on the law; all infants should be screened for three diseases
including hypothyroidism, PKU, and glucose-6-phosphate dehydrogenase deficiency.

The purpose of this survey was to evaluate the development and organization of phenylalanine level in newborn screening programs and their limitations and expectations in Mazandaran Province in the northeast of Iran (Sari) based on biochemical ELISA and HPLC technique. Its geographical coordinates are 36° 34’ 4” North and 53° 3’ 31” East and its original name (with diacritics) is Sari. Mazandaran is located in the Northern Iran and the southern coast of the Caspian Sea [Figure 1]. It is bordered clockwise by Golestan, Semnan, and Tehran Provinces. This province also borders Qazvin and Gilan to the west,[18] Mazandaran province with an area equivalent to 23,842 km², about 1.46% of the Iran country’s area and eighteenth considered in these respect 31 provinces in the country[19] and with around 3.1 million inhabitants seventh considered in these respect 31 provinces in the country.[20] The probable correlation between the PKU values in newborn in positive samples and with related potential risk factors and environmental factors was evaluated.

Methods
Sample collection
In this descriptive-retrospective study, total newborns population were screened through an NNSP, in all 21 cities of Mazandaran in 2007–2015. During the days 3–5 after birth, heel blood samples were taken based on Schleicher and Schuell 903 (Bioscience, Germany) papers with Clinical Laboratory Standards Institute[21] and Ministry of Health of Iran newborn PKU Screening Program protocols by an experienced technician, and the samples were sent to the special screening laboratory in the Referral Laboratory Mazandaran University Medical Science.

Analysis phenylketonuria in neonatal samples by ELISA technique

The valid DBS specimens were punched at least 5 mm in diameter, and the phenylalanine was measured quantitatively by the colorimetric method (NEO-PKU kit, Kimia Pajouhan, Iran). All unsuitable DBS specimens were evaluated on a new prepared sample. Standard curve values were obtained based on kit protocol with six blood spot standards 0, 2, 4, 8, 16, 32 concentrations in duplicate form in every test plate. Acceptable range (optical density) for standard 0 concentration was <0.08 and for standard 32 concentration was >0.240. As well as, we used blood spot for controls (low and high level) in duplicate form. Acceptable ranges were for low control spot 1.5–3.4 mg/dl and high control spot 6.5–12.3 mg/dl. Normally (reference limit), DBS phenylalanine concentrations in neonates ranged from ~1.8 mg/dl to ~3.9 mg/dl. In referral laboratory, a phenylalanine cutoff point of <4 mg/dl (0.24 mmol/l) was used. This cutoff level increases the sensitivity of the screening test and reduces the risk of missing the diagnosis of disorders of the phenylalanine metabolism in subjects with less pronounced phenylalanine-circulating levels at the first investigation.

All the newborn’s cases with phenylalanine levels >3.9 mg/dl initially an additional sample were retested in parallel with the first DBS suspect sample, then reported immediately was re-tested at the time off between the 9th and 16th day of life with another DBS specimen collection. Subjects with normal phenylalanine concentrations (<3.9 mg/dl) in this second DBS specimen were classified as false positives and those with a phenylalanine concentration >3.9 mg/dl as positives. Afterward, they were referred to the pediatric endocrinologist, the scientific adviser of this plan in Mazandaran University of Medical Sciences for further evaluation.

Phenylketonuria analysis in neonatal samples by high-performance liquid chromatography technique

About 3 mL of the venous blood was obtained in the heparinized tube from these newborns. Their sera were sent for the evaluation of phenylalanine and tyrosine by HPLC method. If the serum phenylalanine level was ≥10 mg/dl and tyrosine level was normal, the newborns were diagnosed as having HPA. In those with serum phenylalanine levels between 7 and 9.9 mg/dl, another blood sample was checked 1 week later, and if the serum phenylalanine levels were equal or >7 mg/dl, they were diagnosed definitely as having HPA. The newborns with serum phenylalanine levels between 2 and 6.9 mg/dl were considered healthy, and they were only scheduled for visit by a pediatric endocrinologist. All children with a positive screening test for HPA or PKU in Mazandaran Province (other than Babol city) were referred for free diagnosis, treatment, and follow-up to the metabolic center at the pediatrics subspecialty ward of Bou Ali Sina Hospital, which is the largest-oldest center of children in Northern Iran, to visit via a pediatric endocrinologist.

HPLC technique is the most widely used quantitative screening method for different and particular chemical materials, especially diagnosing inborn metabolism disorders, chosen for its speed and specificity, and because it permits simultaneous quantification of several biochemical markers using small sample volumes. This method, using assessment phenylalanine level and phenylalanine/tyrosine ratio, reduces false-positive results.[7,24]
Bioperin and neopterin analysis in urine neonatal samples by high-performance liquid chromatography technique

To differentiate between classic and nonclassic PKU in authenticated cases, the bioperin and neopterin tests in urine samples were performed by HPLC method in Pasteur Institute of Iran in Tehran.

If a newborn was diagnosed as having PKU, but in a future visit, transient HPA was confirmed, then he/she would be excluded from the study. If, in the repeated visits, investigators confirmed that the newborns actually had PKU, then they were included in the study.

Quality control

All neonatal screening laboratories (primary and final centers) participated in the External Quality Assessment Program by the National Neonatal Screening Quality Control, Iranian Association of Clinical Laboratory Doctors.

Data analysis

The correlation between the PKU values in the newborn in positive specimens with related potential risk factor had consanguinity between parents and other factors such as geographic origin (town, rural), gender, the age of parents, neonates term (mature, premature), neonates weight (normal, low weight, high weight), underlying disease of parents, underlying disease of neonates, number of neonates per birth (single, twin, and multifetal), and number of the affected sibling were evaluated. The collected data were analyzed by SPSS software [IBM Corp. Released (2010). IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.]. Data were reported by descriptive statistics. 99% confidence interval was noted, and $P < 0.01$ was considered statistically significant.

Results

During the 9 years of study, a total of 407,244 live infants were born in the Mazandaran Province, of whom 198,240 (48.7%) were girls and 209,204 (51.3%) were boys. Mothers aged 13–49 years (mean; 26.3 years). Blood testing by DBS showed 465 suspected samples with serum phenylalanine levels $\geq$4 mg/dL (recall samples). They were referred to the pediatric endocrinologist for further evaluation. Finally, according to the screening blood test, by HPLC method, and future outpatient visits, HPA was confirmed in 27 newborns (0.66 in 10,000) including 14 girls and 13 boys. In other words, the incidence of PKU in female and male newborns was 0.67 and 0.65 in 10,000, respectively. There was no significant statistical difference in the incidence rate in male and female infants ($P < 0.0001$).

Table 1 was shown neonatal PKU screening according to assessment methods (ELISA and HPLC).

Parental age was obtained in positive cases in mothers of 18–37 years (average 27.7 years) and in fathers of 21–50 years (average 32.7 years). All positive PKU neonates were determined with the mature term, normal weight, without underlying disease of parents or newborns term of residence, and single per birth (except a baby was born in Babol at 2012 in twin form, which was accompanied with a healthy baby).

Classification and geographic origin distribution of HPA phenotypes of neonates screening by HPLC confirmed method according to gender, year, and accommodation city are summarized in Tables 2, 3 and Figure 2.

Should be noted, don’t observe any positive Severe PKU confirmed cases in four cities Ramsar, Nor, Savadkoh, and Behshahr.

The highest number of severe PKU phenotype with two cases at Chalus and mild PKU phenotype in Sari and Ghaemshahr each one with three cases was determined, which all cases had consangunility. In addition, the

Table 1: Neonatal phenylketonuria screening by enzyme-linked immunosorbent assay and high-performance liquid chromatography methods in Mazandaran Province, Iran (2007-2015)*

<table>
<thead>
<tr>
<th>Year</th>
<th>Total; female/male</th>
<th>Incidencea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number neonatal screened</td>
<td>Number recall cases identified of screened by ELISA</td>
</tr>
<tr>
<td>2007</td>
<td>42,328; 20,759/21,569</td>
<td>254; 112/142</td>
</tr>
<tr>
<td>2008</td>
<td>45,703; 22,506/23,197</td>
<td>66; 31/35</td>
</tr>
<tr>
<td>2009</td>
<td>45,442; 22,050/23,392</td>
<td>92; 45/47</td>
</tr>
<tr>
<td>2010</td>
<td>44,420; 21,447/22,973</td>
<td>19; 13/6</td>
</tr>
<tr>
<td>2011</td>
<td>44,342; 21,168/23,374</td>
<td>11; 5/6</td>
</tr>
<tr>
<td>2012</td>
<td>44,352; 21,719/22,633</td>
<td>7; 2/5</td>
</tr>
<tr>
<td>2013</td>
<td>45,994; 22,738/23,256</td>
<td>5; 1/4</td>
</tr>
<tr>
<td>2014</td>
<td>47,220; 22,672/24,548</td>
<td>6; 1/5</td>
</tr>
<tr>
<td>2015</td>
<td>47,443; 23,181/24,262</td>
<td>5; 3/2</td>
</tr>
<tr>
<td>Total</td>
<td>407,244; 198,240/209,204</td>
<td>465; 214/251</td>
</tr>
</tbody>
</table>

*Yearly percentage incidence per 10,000 neonates. ELISA=Enzyme-linked immunosorbent assay, HPLC=High-performance liquid chromatography
In one family, two affected sibling daughters with phenotype PHA were born in Chalus, at an interval of 6 years (2009 and 2015 years).

**Discussion**

Screening for congenital metabolic disease is an important form of prevention in pediatrics. This activity is very useful for the error detection of many inborn errors. It should be noted that many kinds of congenital disorders can be successfully treated if early detection is obtained. Many metabolic disorders can be stopped from further progression to permanent damage in patients if the specific biochemical supplementation is done in the early phase. The three screenings became the main neonatal screening practices. They were run under the National Public Health Policies. Metabolic diseases of the nervous system vary considerably in their clinical and pathological aspects. In these disorders, mental retardation and epileptic syndrome are the prominent presentations.

The incidence of newborn PKU is varied in different populations. The lowest incidence was in Thailand, <1 in 220,000,[15,25,27] 1:161,748 in Mexico,[28] and 1:143,000 in Japan.[29] Among European countries, the incidence in Ireland and Western Scotland is unusually high (1 in 4500), which is one of the highest incidences in the world,[30] in other countries, very different ranges were reported between 1:5,000 and 1:15,000,[31] Sweden from 1:18,300 to 1:14,200 into two periods of before and after 1990[32] and Germany with

### Table 2: Classification and geographic distribution of hyperphenylalaninemia phenotypes of neonates screening by high-performance liquid chromatography confirmed method according to gender, year, and family relationship, in Mazandaran Province, Iran (2007-2015) (n=27)

<table>
<thead>
<tr>
<th>Year</th>
<th>Total cases; female/male</th>
<th>Severe PKU</th>
<th>Mild PKU</th>
<th>PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>2007</td>
<td>2; 2/0</td>
<td>Chalus*</td>
<td></td>
<td>Ghaemshahr*</td>
</tr>
<tr>
<td>2008</td>
<td>2; 0/2</td>
<td></td>
<td></td>
<td>Galugah*</td>
</tr>
<tr>
<td>2009</td>
<td>7; 5/2</td>
<td>Chalus*</td>
<td></td>
<td>Ghaemshahr*</td>
</tr>
<tr>
<td>2010</td>
<td>3; 3/0</td>
<td>Tonekabon</td>
<td></td>
<td>Babolsar</td>
</tr>
<tr>
<td>2011</td>
<td>1; 1/0</td>
<td>Sari</td>
<td></td>
<td>Babolsar</td>
</tr>
<tr>
<td>2012</td>
<td>2; 1/1</td>
<td>Babol</td>
<td></td>
<td>Babolsar</td>
</tr>
<tr>
<td>2013</td>
<td>3; 0/3</td>
<td>Babol*</td>
<td></td>
<td>Babolsar</td>
</tr>
<tr>
<td>2014</td>
<td>4; 1/3</td>
<td>Ghaemshahr*</td>
<td></td>
<td>Amol*</td>
</tr>
<tr>
<td>2015</td>
<td>3; 1/2</td>
<td>Noshahr</td>
<td></td>
<td>Amol*</td>
</tr>
<tr>
<td>Total</td>
<td>27;</td>
<td>14/13 (100%)</td>
<td>6 (22.2%)</td>
<td>9 (33.3%)</td>
</tr>
</tbody>
</table>

*Family relationship between parents, †Family relationship between neonates, ‡Family relationship between 2 sister baby. Severe PKU=Serum phenylalanine ≥20 mg/dL, Mild PKU=Serum phenylalanine 10-20 mg/dL, PHA=Serum phenylalanine ≥2-10 mg/dL. PKU=Phenylketonuria, HPLC=High-performance liquid chromatography, PHA=Phenylalanine hydroxylase.

In one family, two affected sibling daughters with phenotype PHA were born in Chalus, at an interval of 6 years (2009 and 2015 years).
In Asian countries, in China, the incidence which assessed in a very wide range between 1:12,473 in Brazil to 1:161,748 in Mexico,

In the United States, the PKU Incidence is from 1:19,000 to 1:13,500. In Latin American countries (a region confirmed by 20 countries), neonatal PKU incidence was assessed on a very wide range between 1:12,473 in Brazil to 1:161,748 in Mexico, and in Cuba, it was obtained as 1 in 52,590 in newborns' lives. In some studies conducted in regional countries, Turkey with 1:6,697 and Sulaimani city in Iraq with 1–8333 can also be mentioned.

In Iran, there have been limited studies on the incidence of PKU. In a pilot study performed by Kabiri and Farhud on 8633 newborns born in different hospitals in Tehran which was the first survey on this issue, the incidence rate was calculated as 1.1 in 10,000 or 1:9091. In Habib et al.'s study from 2004 to 2007, the incidence of PKU in Fars Province, Southern Iran, was 1.6 in 10,000 (1:6250). Considering the epidemiological study of PKU in Khorasan Province, Northeastern Iran, on 69,347 newborns in 2013, four positive cases (1 in 17,335 living babies) were detected. During the 1-year descriptive cross-sectional study, all newborns for the measurement of serum phenylalanine were diagnosed as 8 in 76,966 cases (1:10,000) and 4 out of 22,131 cases (1:5532) in Fars Province (2007–2008) and Yazd Province (2010–2011), respectively.

In another study in Shiraz, Fars Province (2001), by Goblahar et al. conducted on 1544 children with signs and symptoms of metabolic diseases, the incidence of PKU was calculated to be 1 in 3672. In another study in Isfahan (2001), the incidence rate among 1611 mentally disabled institutionalized patients was 20% (36 in 1611). In our survey, the incidence of PKU was 0.66 in 10,000 in 407,244 cases; in other words, equally one case per 15,083 lives neonatal per year, which is similar to several previous reports such as Brazil and Mexico. Except from a proportional similarity with their study, this investigation can not be compared with any other studies. Of interest, the infants PKU incidence in Mazandaran Province was significantly higher than those that were previously reported which were compared to the other settings implementing the universal screening such as Mexico and other reports; however, it is less than the other above-mentioned studies. The newborn sample size collection in our survey with 407,244 cases corresponded to the results carried out in Germany (423,773 cases), but more than other studies performed in Iran and other countries, except the studies in Thailand and China which assessed whole newborns in their countries. In the study conducted in Isfahan, the mentally disabled institutionalized patients were studied, whereas in our study, all the newborns were studied. Our study like Habib’s...
In other words, in our survey, the incidence rate was in severity; severe PKU of 1:67,874, mild PKU of 1:45,249, and HPA of 1:33,937 on the living births (this proportion in both severe and mild PKU forms was 1:27,150), which was much less than the assessment performed in Turkey with 1:5049 and 1:4172 for PKU and HPA and in Yazd Province of Iran with severe PKU of 1:1383 and mild PKU of 1:4149 and also less than Cuba with 1:38577 and 1:22503 for PKU and HPA, respectively, but more than that of Mexico with PKU incidence of 1:161748 and without any case of HPA.\[8,28,33,42\] Unfortunately, other epidemiological severity did not provide classification of the disease based on the phenotype which is a major distinction of our study compared to similar studies.

Besides, PKU cases in Iranian and other children had consanguineous parents.\[33,39,42,44\] However, in this study, results obtained no significant differences between the parents’ family relationship. While some European countries have announced, it might be due to the increased incidence as well as creating new mutations in this disease, migration of people with different races to their country.\[32\]

Despite the fact that the greatest number of positive cases in this study was observed in Sari, Chalus and Kelardasht, Ghaemshahr and Simorgh, Babol and Tonekabon, and Abasabab towns with 6, 5, 4, 2, and 2 cases, respectively; however, considering the birth rate per city, the most abundant was determined with incidences of 2.86%, 2.41%, 1.60%, 1.05%, 1%, 0.98%, 0.92%, 0.84% and Sari belonging to Chalus and Kelardasht, Galogah, Fereidonkenar, Mahmodabad, Ghaemshahr and Simorgh, Joybar, Tonekabon, Noshahr, and Sari, respectively [Table3]. Furthermore, were diagnosed the most incidences with 1.54% in 2009 and 0.85% in 2014 and the lowest incidences with 0.23% in 2011 and 0.44% in 2008 [Table1]. In addition, results of the present study had not involved any positive cases of nonclassic PKU.

The main causes which make a lot of false positives (recall test) by fluorimetric technique in 3 years from 2007 to 2009 can be mentioned for the new setting up method, improper DBS sampling, unsuitable DBS samples storage, card contamination, inappropriate DBS transporting (temperature and time), high temperature in ELISA work room (above 25°C), and low threshold cutoff point chosen for ELISA technique.\[8,45,46\] For these suspected samples (recall test), the items fell by in the following years to keep track and manage theoretical and practical training in addition to the, Performing a correct and coherent documentation of the information are discussed above so that, for recall case, the positive case ratio from 254:2 in 2007 reduced to 19:3 in 2010, 6:4 and 5:3 in 2014 and, 2013 and 2015 years, respectively [Table1].

Conclusions

According to the national estimation of PKU in Iran, as performed by the Ministry of Health, one in a population of 800 is at the risk of the disease. Comparing our findings, we realize that the prevalence rate is considerably lower in our study 1 in 15,083 than the actual incidence rate. Moreover, the theoretical and practical training management is the most important cause for decreasing working errors (preanalytical, analytical, and postanalytical errors).

Accordingly, Iran has a very wide geographical area along with cultural and genetic situation which is entirely different, and taking this point into consideration on the situation of incidence and phenotypes neonatal PKU in Iran, there is no comprehensive census; therefore, it is recommended that more comprehensive research is done in this regard.

Regarding the actual difference between the prevalence and incidence rate of PKU reported by the actual PKU and considering the mild-PKU and HPA, it is suggested that the Ministry of Health reports on PKU be presented based on the severity of the disease.

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Conflicts of interest

There are no conflicts of interest.

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