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RESEARCH REPORT

Four Years of Diagnostic Challenges with Tetrahydrobiopterin Deficiencies in Iranian Patients

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Abstract Hyperphenylalaninemia (HPA) is a condition caused by tetrahydrobiopterin (BH_4) and phenylalanine hydroxylase (PAH) deficiencies. It is essential that differential diagnosis be conducted to distinguish these two causes of HPA, because BH_4 deficiency is a more severe disease involving progressive neurologic deterioration. Based on the biological findings, HPA is defined as a plasma phenylalanine level of >2.0 mg/dl (>120 μmol/l). The National Biochemistry Reference Laboratory at the Pasteur Institute of Iran initiated BH_4 deficiency screening tests for the first time during the implementation of a nationwide phenylketonuria (PKU) screening program. Measurement of blood phenylalanine and urinary neopterin and biopterin was conducted by high-performance liquid chromatography in 617 patients with HPA. Dihydropteridine reductase (DHPR) activity was measured in all patients by kinetic spectrophotometry. Differential diagnosis was conducted for PKU, transient HPA, and BH_4 deficiencies.

Our results indicated that out of 76 cases involving BH_4 deficiencies, 37 had 6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiency, 35 had DHPR deficiency, 1 case had pterin-4a-carbinolamine dehydratase (PCD) deficiency, and 3 cases had GTP cyclohydrolase I (GTPCH) deficiency. In this study, 1 novel deletion mutation and 18 novel missense mutations were reported in addition to mutations that had previously been identified and registered in BIOMDB. At present, the screening program for PKU in Iran includes tests that detect different forms of BH_4 deficiency presenting with HPA. Newborns that are BH_4-deficient benefit from the availability of the tests because they can receive necessary care before being clinically affected.

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Introduction

The fully functional phenylalanine hydroxylase (PAH) system for phenylalanine (Phe) metabolism consists of the PAH (EC 1.14.16.1) enzyme, the cofactor tetrahydrobiopterin (BH_4), and two regenerating enzymes: pterin-4a-

carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) (Blau et al. 2010). PAH deficiency and BH_4 deficiency result in hyperphenylalaninemia (HPA). Elevated Phe is a marker of this disorder; thus, a commonly used plasma Phe cutoff level for HPA diagnosis is >120 μmol/l (>2 mg/dl) (Blau et al. 2010).

BH_4 is an essential cofactor for aromatic amino acid hydroxylases, including phenylalanine, tyrosine, and tryptophan hydroxylases (Arai et al. 1982). Defects in the enzymatic conversion of phenylalanine to tyrosine, tyrosine to L-Dopa, and tryptophan to 5-hydroxytryptophan cause HPA and reduce dopamine and serotonin levels in the central nervous system (Arai et al. 1982).

The classical pathway for the de novo biosynthesis of BH_4 from guanosine triphosphate (GTP) requires three enzymes: GTP cyclohydrolase I (GTPCH; EC 3.5.4.16), 6-pyruvoyl-tetrahydropterin synthase (PTPS; EC 4.6.1.10), and sepiapterin reductase (SR; EC 1.1.1.153) (Blau et al. 2010). In addition, dihydropteridine reductase (DHPR; EC 1.6.99.7) and pterin-4a-carbinolamine dehydratase (PCD; EC 4.2.1.96) are critical for the regeneration of BH_4.

BH_4 deficiency is a severe disease involving progressive neurologic deterioration despite adequate dietary control of blood phenylalanine levels (Arai et al. 1982). Therefore, differential diagnosis to distinguish BH_4 deficiencies from PAH gene deficiencies is very important.

In Iran, screening for phenylketonuria (PKU) began as a pilot program in three provinces in 2006 and was expanded nationwide in 2011 under the direction of the Genetics Office of the Ministry of Health.

For a complete study, DHPR enzyme activity measurement and urinary neopterin and biopterin analyses are required to rule out BH_4 deficiencies. For this purpose, the National Biochemistry Reference Laboratory (NBRL) at the Pasteur Institute of Iran began to administer tests and serve patients in 2010. This program allows for the early identification and timely intervention to reduce morbidity and mortality rates, thus increasing the chances for healthy patient outcomes.

At present, NBRL is the only referral center in Iran to diagnose these disorders by urinary neopterin and biopterin analyses and DHPR enzyme activity determination. The purpose of this paper is to report on the implementation of these tests in Iran and present results from 4 years (2010–2014) of diagnosing autosomal-recessive BH_4 deficiencies at our laboratory.

Materials and Methods

Patients

463 patients diagnosed with HPA (blood phenylalanine >120 μmol/l) and 154 patients with HPA and neurological signs referred by physicians nationwide between 2010 and 2014 were investigated for possible BH_4 deficiencies at NBRL.

Selective tests conducted for all patients included blood phenylalanine measurement by high-performance liquid chromatography (HPLC), the DHPR activity test, and urinary neopterin and biopterin analyses by HPLC (Atherton 1989; Ponzone et al. 2004; Blau et al. 2008). Patients were on unrestricted diets for 3 days before sampling. Medications such as multivitamins, BH4, ferrous sulfate, and folic acid were not taken prior to before sampling. Dried blood samples (DBS) on filter paper cards were prepared by blood sampling, as the DHPR enzyme in DBS is stable enough for the sample to be mailed to a central laboratory (Ponzone et al. 2004).

For a complete evaluation to establish the exact nature of the genotype, blood samples from BH_4 deficient patients and their parents were sent to the genetic laboratory for molecular analysis. Out of 76 patients, gene mutation analysis was only performed for 44 cases because some patients did not consent to the molecular study.

Neopterin and Biopterin Measurement

Fresh clean-catch urine specimens were used to determine pterin profile levels. All urine samples were diluted 100-fold in normal saline (Ribeiro de Castro et al. 2004). Acid oxidation of reduced pterin was conducted according to the protocol of Blau et al. (2008). A Knauer HPLC system and Waters fluorimeter, equipped with a primary filter exciting at 350 nm and a secondary filter emitting wavelengths at 450 nm, and Tracer Excel 120 reversed-phase columns ODSB 5 μm 25 × 0.46 cm, part number: TR-016345, serial number: NF-31639, (Teknokroma) were used for pterin analysis.

Quality assurance of the neopterin and biopterin measurements was obtained by testing standard solutions and monitoring the inter-assay variation of urine precision control prepared in our lab.

DHPR Activity Assay

Enzyme activity was assayed based on spectrophotometric monitoring of the formation of ferrocytochrome C in a coupled reaction, according to the protocols of Arai et al. (1982) and Blau et al. (2008). Quality assurance of the DHPR assay was obtained by testing and monitoring the inter-assay variation of blood spot elutes from a normal adult and a DHPR-deficient patient.

Genetics

DNA was extracted from 5 ml peripheral blood collected from each patient and their parents. Direct sequencing of

PCR products was accomplished using a BigDye Terminator kit (Thermo Fisher Scientific, Life Technologies, USA) according to the manufacturer's protocol, using an ABI3130XL Genetic Analyzer for mutation detection in the Dr. Zeinali Human Genetics Laboratory, Tehran, Iran. Mutations in genomic DNA in GCH1 (OMIM: 600225), PCBD1 (OMIM: 126090), PTS (OMIM: 612719), and QDPR (OMIM: 612676) genes were studied (http://www.dnalc.org).

The bioinformatics analysis of these genes was performed using Gene Runner version 3.05, and mismatches were compared using an in-house MS Word file containing full details of exons (6 in GCH1, 4 in PCBD1, 6 in PTS, and 7 in QDPR genes), intron numbers, codon numbers, and details of known mutations.

Statistical Analysis

Statistical analysis of the results was performed in Microsoft Excel 2010. Median, minimum, and maximum values were extracted for all variables.

Results

In this cross-sectional study, 617 hyperphenylalaninemic cases were tested for DHPR activity and urinary neopterin and biopterin profiles. According to the data, of 617 HPA patients, 76 cases exhibited BH_4 deficiency.

The results, including the median, minimum, and maximum values of the 541 HPA cases, were shown in Table 1. PAH gene mutations were searched in some patients but are not reported in this article.

In the remaining cases, 76 individuals had BH_4 deficiencies with the following subtypes: DHPR deficiency (35 patients), PTPS deficiency (37 patients), PCD deficiency (1 patient), and GTPCH deficiency (3 patients). The results, including the median, minimum, and maximum values for laboratory data in these groups are shown in Table 2.

Out of 76 patients, 4 patients were Afghan and 6 patients had Arab ethnic origins. All 10 of these patients suffered from PTPS deficiency. For 11 patients with DHPR deficiency and 16 patients with PTPS deficiency, diagnosis

Table 1 Biochemical data according to age and phenylalanine level for 541 hyperphenylalaninemic cases

Phenylalanine 120–600 μmol/L				
	<1 Year	1–4 Years	4–10 Years	>10 Years
Phe (B) (μmol/L)	276 (127; 589)	262 (126; 583)	398 (177; 586)	223 (121; 570)
Neo (U) (mmol/mol Creat.)	3.54 (0.39; 10.10)	2.42 (0.40; 15.13)	0.99 (0.76; 2.51)	0.62 (0.46; 1.15)
Bio (U) (mmol/mol Creat.)	1.65 (0.30; 8.83)	3.04 (0.63; 9.06)	1.43 (0.76; 3.77)	1.68 (0.83; 2.49)
DHPR activity (mU/mg Hb)	2.80 (1.40; 4.70)	2.80 (1.40; 4.70)	2.20 (1.80; 4.60)	2.00 (1.40; 2.60)
No. of cases	149	35	12	4
Phenylalanine 600–1,200 μmol/L				
	<1 Year	1–4 Years	4–10 Years	>10 Years
Phe (B) (μmol/L)	863 (600; 1,199)	1,020 (600; 1,193)	1,055 (631; 1,182)	986 (606; 1,185)
Neo (U) (mmol/mol Creat.)	4.68 (1.25; 9.69)	2.66 (1.18; 5.43)	1.77 (0.74; 4.36)	1.23 (0.64; 2.61)
Bio (U) (mmol/mol Creat.)	3.84 (0.43; 8.55)	3.52 (0.80; 6.78)	2.66 (1.43; 5.20)	2.43 (1.17; 5.19)
DHPR activity (mU/mg Hb)	2.60 (1.60; 4.10)	2.40 (1.30; 3.50)	2.00 (1.40; 3.80)	2.10 (1.60; 2.80)
No. of cases	56	30	25	14
Phenylalanine >1,200 μmol/L				
	<1 Year	1–4 Years	4–10 Years	>10 Years
Phe (B) (μmol/L)	1,778 (1,204; 3,039)	1,600 (1,206; 2,707)	1,500 (1,212; 2,100)	1,551 (1,220; 1,961)
Neo (U) (mmol/mol Creat.)	4.43 (1.47; 21.05)	3.52 (1.36; 9.54)	1.95 (0.19; 8.13)	1.10 (0.41; 2.84)
Bio (U) (mmol/mol Creat.)	4.29 (0.81; 13.50)	4.06 (0.97; 15.54)	3.67 (1.00; 10.56)	1.76 (0.44; 4.80)
DHPR activity (mU/mg Hb)	2.60 (1.40; 4.40)	2.30 (1.40; 4.90)	2.00 (1.30; 3.60)	1.85 (1.30; 3.10)
No. of cases	88	45	55	28

Phe (B) phenylalanine in blood, *Neo (U)* neopterin in urine, *Bio (U)* biopterin in urine, *Creat*. urine creatinine, *Hb* hemoglobin

Table 2 Laboratory data of patients with BH_4 deficiency at the time of diagnosis

BH_4 Deficiencies Groups	PTPS Deficiency Group Median (Min; Max)	DHPR Deficiency Group Median (Min; Max)	PCD Deficiency Group Median	GTPCH Deficiency Group Median (Min; Max)	Reference range in normal group
Phenylalanine (B) (μmol/L)	864 (60; 2,064)	264 (49; 1,702)	1,327	1,248 (827; 1,747)	<1 month: 0–124 <16 years: 26–86 ≥16 years: 41–68
Neo (U) (mmol/mol Creat.)	24.08 (4.09; 57.15)	2.49 (0.74; 21.82)	1.52	<0.2	1 day to 10 years: 1.1–4.0 >11 years: 0.2–1.7
Bio (U) (mmol/mol Creat.)	0.11 (0.02; 0.64)	8.54 (2.87; 23.03)	–	<0.2	1 day to 10 years: 0.5–3 >11 years: 0.5–2.7
% Biopterin	0.49 (0.06–4.82)	77.50 (31.46-91.46)	–	–	
DHPR activity (mU/mg Hb)	3.8 (1.70; 4.60)	0.0 (0.0; 0.0)	–	4.20 (3.40–4.50)	1.8–3.8
No. of female cases	18	12	1	2	–
No. of male cases	19	23	0	1	–
No. of total (Percentage; %)	37 (49%)	35 (46%)	1 (1%)	3 (4%)	–
Ages	1 month to 12 years	1 month to 20 years	13 years	5 month to 4 years	–

Phe (B) phenylalanine in blood, *Neo (U)* neopterin in urine, *Bio (U)* biopterin in urine, *%Biopterin* calculated by ((biopterin/(biopterin + neopterin))*100); *Creat*. urine creatinine, *Hb* hemoglobin

occurred prior to one year of age. Consanguineous marriage existed in 64 (84.2%) families. In terms of gender, there was an early equal distribution of PTPS deficiency between females (49%) and males (51%), but in the DHPR deficiency group, the number of males (66%) was twice that of females (34%). Four of the reported cases of DHPR-deficient patients were siblings.

Also, decreases or absences of both neopterin and biopterin were observed in GTPCH deficiency group profile.

In PTPS-deficient patients, neopterin concentrations were significantly increased while biopterin levels were low. The data from this group imply that 37 PTPS-deficient patients (100%) had neopterin >4.0 mmol/mol creatinine, while 35 patients (94.6%) had biopterin ≤0.5 mmol/mol creatinine.

In PCD-deficient patients, elevated neopterin and primapterin levels were observed.

According to the past research, DHPR-deficient subjects showed slightly elevated amounts of biopterin combined with minor increases in neopterin (Blau et al. 2008). In our study, the data showed that 32 DHPR-deficient patients (91.4%) had biopterin >3.0 mmol/mol creatinine. In this group, only 2 cases (5.7%) of 35 DHPR-deficient patients exhibited normal levels of both neopterin and biopterin simultaneously.

Statistical analysis of the phenylalanine results determined that 29 DHPR-deficient patients (82.9%) and 13 PTPS-deficient patients (35.1%) had phenylalanine <600 μmol/l, which demonstrated that most of the BH_4-deficient patients have mild HPA.

The genomic structures of identified mutations for 44 cases in *GCH1*, *PCBD1*, *PTS*, and *QDPR* genes, their inheritance conditions, and other information including biochemical data are shown in detail in Table 3.

In our study, we found that mutations more frequently involved DHPR (46%) and PTPS (49%) than GTPCH (4%) and PCD (1%) (Table 3). All BH_4-deficient patients showed homozygous mutations, except one patient in the PTPS deficiency group, who had c.297C>A and c.84-3C>G in a compound heterozygous form.

Most detected mutations had been previously reported (http://www.biopku.org/BioPKU_DatabasesBIOMDB.asp), but 18 missense mutations (16 in exons and 2 in introns) and 1 deletion mutation were novel and had not been registered in the BioPKU_Databases BIOMDB yet. They are demonstrated by asterisks in Table 3 and included: c.163 + 2 T>C, c.217del(c.217delA), c.265G>A, c.266G>A, c.267A>G, c.673G>A, c.710C>T, c.49G>T, c.68G>A, c.488G>C, and c.344C>T in *QDPR* gene; c.281A>T,c.164.36A>G,c.331G>A,c.400G>A,

Table 3 Gene mutation analysis results and laboratory data for patients with BH_4 deficiencies

Row	Status	ID number	Date of birth	Age at diagnosis	Gender	Consanguinity	Ethnic origin	Phe (B) at birth µmol/l	Phe (B) at diagnosis µmol/l	Coding DNA reference Sequence	NCBI reference sequence for genes	Exon	Inheritance condition	Neo (U) mmol / mol Creat.	Bio (U) mmol / mol Creat.	% Biopterin	DHPR activity
1	GCTPH Def.	P.1030	13.06.2011	3.5 years	M	Yes	Fars	972	1,747	c.551G>A*	NM_000161.2	5	Homozygote	<0.2	<0.2	nd	4.2
2	GCTPH Def.	P.2137	16.12.2014	1.5 months	F	Yes	Fars	900	1,248	c.551G>A*	NM_000161.2	5	Homozygote	<0.2	<0.2	nd	3.4
3	GCTPH Def.	P.2149	12.08.2014	5 months	F	Yes	Azari	1,500	827	nd	–	–	–	<0.2	<0.2	nd	4.5
4	PCD Def.	P.1017	11.08.2000	12.5 years	M	Yes	Fars	–	1,116	c.313 T>C*	NM_000281.3	4	Homozygote	1.52	–	nd	nd
5	DHPR Def.	P.432	28.12.2003	7 years	F	No	Azari	–	220	c.53G>A	NM_000320.2	1	Homozygote	1.54	9.05	85.46	0
6	DHPR Def.	P.223	25.05.2000	10 years	M	Yes	Fars	–	–	c.49G>T*	NM_000320.2	1	Homozygote	1.7	5.28	75.64	nd
7	DHPR Def.	P.1477	25.05.2013	1.5 months	M	Yes	Fars	690	184	c.68G>A*	NM_000320.2	1	Homozygote	3.83	3.89	50.39	0
8	DHPR Def.	P.2104	09.07.2014	6 months	M	Yes	Azari	635	244	c.190 G>A	NM_000320.2	2	Homozygote	1.56	6.88	81.52	0
9	DHPR Def.	P.800	13.07.2011	3.5 years	F	Yes	Fars	360	48.6	c.265G>A*	NM_000320.2	3	Homozygote	1.79	5.08	73.94	0
10	DHPR Def.	P.2195	25.11.2008	6 years	M	Yes	Talesh	–	245	c.266G>A*	NM_000320.2	3	Homozygote	1.98	6.1	75.50	0
11	DHPR Def.	P.1172	06.05.2010	2.5 years	F	Yes	Fars	257	143	c.267A>G*	NM_000320.2	3	Homozygote	3.15	11.28	78.17	0
12	DHPR Def.	P.232	03.04.2009	1.5 years	M	Yes	Lor	–	228	c.341C>T*	NM_000320.2	4	Homozygote	8.47	21.53	71.77	0
13	DHPR Def.	P.935	12.10.2009	2.5 years	M	Yes	Fars	282	714	c.344C>T*	NM_000320.2	4	Homozygote	1.76	10.13	85.20	0
14	DHPR Def.	P.386	29.07.2010	4 months	F	Yes	Fars	–	240	c.449A>G	NM_000320.2	5	Homozygote	6.14	8.03	56.67	0
15	DHPR Def.	P.892	29.01.2004	8 years	M	Yes	Fars	–	506	c.449A>G	NM_000320.2	5	Homozygote	0.74	6.16	89.28	0
16	DHPR Def.	P.1019	22.01.2008	5 years	M	No	Azari	900	300	c.449A>G	NM_000320.2	5	Homozygote	4.72	11.16	70.28	0
17	DHPR Def.	P.1027	23.04.1999	13 years	M	No	Azari	–	228	c.449A>G	NM_000320.2	5	Homozygote	1.05	5	82.64	0
18	DHPR Def.	P.94-89	25.12.2005	9.5 years	F	Yes	Fars	440	593	c.470 T>G	NM_000320.2	5	Homozygote	4.08	22.25	84.50	0
19	DHPR Def.	P.94-124	03.08.2014	1 year	M	Yes	Azari	600	142	c.472 C>T	NM_000320.2	5	Homozygote	1.86	4.25	69.56	0
20	DHPR Def.	P.1524	16.06.2013	3 months	F	Yes	Fars	360	551	c.488G>C*	NM_000320.2	5	Homozygote	4.53	5.26	53.73	0
21	DHPR Def.	P.586	03.11.2008	2.5 years	M	Yes	Azari	–	389	c.217del (c.217delA)*	NM_000320.2	7	Homozygote	2.64	13.1	83.23	0
22	DHPR Def.	P.1117	23.12.2011	6 months	M	Yes	Fars	660	174	c.661C>T	NM_000320.2	7	Homozygote	4.75	6.58	58.08	0
23	DHPR Def.	P.366	26.02.2002	9 years	M	Yes	Azari	–	525.61	c.673G>A*	NM_000320.2	7	Homozygote	2.3	18.34	88.86	0
24	DHPR Def.	P.1274	31.12.1996	16 years	F	Yes	Azari	–	400.88	c.673G>A*	NM_000320.2	7	Homozygote	nd	nd	nd	0
25	DHPR Def.	P.1289	28.11.2012	8 months	M	No	Kord	312	175	c.710C>T*	NM_000320.2	7	Homozygote	1.13	2.88	71.82	0
26	DHPR Def.	P.94-17	29.03.2015	1 month	F	Yes	Azari	600	1,702	c.295 + 1 G>A	NM_000320.2	–	Homozygote	10.59	4.86	31.46	0
27	DHPR Def.	P.925	01.12.2008	3 years	F	Yes	Fars	540	534	nd	–	–	–	3.33	13.9	80.67	0
28	DHPR Def.	P.504	05.06.2008	3 years	F	Yes	Fars	–	626	nd	–	–	–	3.73	12.37	76.83	0
29	DHPR Def.	P.1571	10.11.2012	1 year	M	Yes	Azari	312	840	nd	–	–	–	2.87	15.98	84.77	0
30	DHPR Def.	P.582	05.08.2008	6 years	M	Yes	Azari	–	241	nd	–	–	–	2.34	10.83	82.23	0
31	DHPR Def.	P.211	27.03.2001	11 years	M	Yes	Fars	–	199	nd	–	–	–	1.34	6.95	83.84	0
32	DHPR Def.	P.1738	14.08.2013	6 months	M	Yes	Azari	–	161	nd	––	–	–	1.13	2.87	71.75	0
33	DHPR Def.	P.500	09.01.2010	15 months	M	No	Baloch	–	405	nd	–	–	–	4.72	23.03	82.99	0
34	DHPR Def.	P.1377	18.07.2009	3.5 years	M	Yes	Lor	–	240	nd	–	–	–	1.19	4.86	80.33	0
35	DHPR Def.	P.1383	18.05.2008	5 years	M	Yes	Bakhtiari	–	120	nd	–	–	–	1.77	4.36	71.13	0
36	DHPR Def.	P.1885	07.12.2013	9 months	M	Yes	Lor	432	387	nd	–	–	–	2.7	11.21	80.59	0
37	DHPR Def.	P.1948	08.09.1994	20 years	M	Yes	Tork	–	283	nd	–	–	–	1.59	17.02	91.46	0
38	DHPR Def.	P.94.145	14.06.2014	2 months	F	Yes	Fars	240	1,503	nd	–	–	–	11.04	10.87	49.61	0

(continued)

Table 3 (continued)

Row	Status	ID number	Date of birth	Age at diagnosis	Gender	Consanguinity	Ethnic origin	Phe (B) at birth µmol/l	Phe (B) at diagnosis µmol/l	Coding DNA reference Sequence	NCBI reference sequence for genes	Exon	Inheritance condition	Neo (U) mmol / mol Creat.	Bio (U) mmol / mol Creat.	% Biopterin	DHPR activity
39	DHPR Def.	P.1917	22.05.2014	1.5 months	F	Yes	Fars	570	1,020	nd	–	–	–	21.82	11.38	34.28	0
40	PTPS Def.	P.1160	14.04.2011	1.5 years	M	No	Afghan	–	940	c.155A>G	NM_000317.2	2	Homozygote	48.18	0.35	0.72	nd
41	PTPS Def.	P.247	21.06.2008	2.5 years	F	Yes	Fars	–	672	c.155A>G	NM_000317.2	2	Homozygote	42.9	0.55	1.27	nd
42	PTPS Def.	P.246	21.01.2008	2.5 years	F	Yes	Fars	–	766.02	c.155A>G	NM_000317.2	2	Homozygote	51.49	0.44	0.85	nd
43	PTPS Def.	P.2030	27.08.2014	4.5 months	F	Yes	Azari	630	807	c.199 C>T	NM_000317.2	2	Homozygote	36.57	0.23	0.63	4.3
44	PTPS Def.	P.1816	18.04.2011	3 years	M	Yes	Fars	180	124	c.259 C>T	NM_000317.2	5	Homozygote	7.65	0.03	0.39	1.9
45	PTPS Def.	P.227	30.01.2009	6 months	F	Yes	Kord	–	947	c.281A>T*	NM_000317.2	5	Homozygote	54.23	0.25	0.46	nd
46	PTPS Def.	P.238	18.08.2009	11.5 months	M	Yes	Kord	–	800.55	c.281A>T*	NM_000317.2	5	Homozygote	13.31	0.02	0.15	nd
47	PTPS Def.	P.1157	26.04.2012	4.5 months	F	No	Fars	794	1,353	c.297C>A & c.84-3C>G	NM_000317.2	5	Compound Heterozygote	35.5	0.25	0.70	nd
48	PTPS Def.	P.2001	04.03.2015	7 months	F	Yes	Fars	1,440	1,852	c.317 C>T	NM_000317.2	6	Homozygote	34.9	0.08	0.23	2.6
49	PTPS Def.	P.2194	17.01.2015	1.5 months	F	No	Azari	1,560	1,305	c.317 C>T	NM_000317.2	6	Homozygote	35.99	0.15	0.42	4.6
50	PTPS Def.	P.207	05.03.2001	12 years	F	Yes	Azari	–	2,064	c.331G>A*	NM_000317.2	6	Homozygote	11.87	0.04	0.34	nd
51	PTPS Def.	P.1551	04.08.2013	2 months	M	Yes	Afghan	841	596	c.351C>A*	NM_000317.2	6	Homozygote	12.57	0.16	1.26	nd
52	PTPS Def.	P.236	10.05.2005	5 years	M	Yes	Arab	–	480	c.373A>T	NM_000317.2	6	Homozygote	31.72	0.04	0.13	nd
53	PTPS Def.	P.94-26	19.12.2014	6 months	F	Yes	Arab	1,230	1,616	c.373A>T	NM_000317.2	6	Homozygote	57.15	0.19	0.33	3.8
54	PTPS Def.	P.1124	06.11.2008	4 years	M	Yes	Arab	–	420	c.373A>T	NM_000317.2	6	Homozygote	24.08	0.07	0.29	nd
55	PTPS Def.	P.1344	15.09.2011	1.5 years	M	Yes	Fars	660	1,331	c.373A>T	NM_000317.2	6	Homozygote	22.06	0.11	0.50	nd
56	PTPS Def.	P.1268	08.12.2012	1 month	F	Yes	Azari	1,020	1,241	c.400G>A*	NM_000317.2	6	Homozygote	33.13	0.02	0.06	nd
57	PTPS Def.	P.229	03.07.2009	1 year	M	Yes	Kord	480	90	c.84-3C>G	NM_000317.2	–	Homozygote	6.31	0.32	4.83	nd
58	PTPS Carrier	P.1918	05.06.2014	1 month	F	Yes	Fars	270	244	c.163 + 2 T>C*	NM_000317.2	–	Heterozygote	10.01	3.14	23.88	2.7
59	PTPS Def.	P.1122	19.02.2012	6.5 months	M	Yes	Arab	1,920	1,200	c.164.36A>G*	NM_000317.2	–	Homozygote	48.85	0.04	0.08	nd
60	Probably PTPS Def.	P.878	06.12.2010	1 year	M	Yes	Kord	–	60	nd	–	–	–	11.1	0.12	1.07	nd
61	Probabely PTPS Def.	P.1105	12.04.2012	4 months	F	Yes	Fars	864	894	nd	–	–	–	25.83	0.08	0.31	nd
62	Probabely PTPS Def.	P.622	01.10.2010	9 months	M	No	Fars	–	–	nd	–	–	–	20.4	0.06	0.29	nd
63	Probabely PTPS Def.	P.1213	23.04.2012	1.5 years	M	Yes	Fars	–	1,410	nd	–	–	–	29.48	0.15	0.51	4.1
64	Probabely PTPS Def.	P.260	02.12.2007	5 years	F	No	Fars	–	791	nd	–	–	–	16.27	0.1	0.61	nd
65	Probabely PTPS Def.	P.563	26.08.2007	3 years	M	Yes	Fars	–	1,129	nd	–	–	–	18.06	0.1	0.55	nd
66	Probabely PTPS Def.	P.596	31.10.2005	5 years	M	Yes	Azari	–	426	nd	–	–	–	4.09	0.08	1.92	nd
67	Probabely PTPS Def.	P.1141	16.08.2006	7 years	M	Yes	Kord	–	1,156	nd	–	–	–	22.82	0.11	0.48	nd
68	Probabely PTPS Def.	P.1375	23.08.2006	6.5 years	F	Yes	Arab	–	540	nd	–	–	–	13.81	0.09	0.65	nd
69	Probabely PTPS Def.	P.357	07.06.2010	4.5 months	F	Yes	Azari	–	1,890	nd	–	–	–	55.08	0.64	1.15	nd
70	Probabely PTPS Def.	P.1591	20.09.2012	13 months	F	Yes	Azari	510	1,080	nd	–	–	–	16.05	0.07	0.43	1.8
71	Probabely PTPS Def.	P.1717	15.08.2013	5 months	F	No	Afghan	–	1,573	nd	–	–	–	30.23	0.21	0.69	1.7
72	Probabely PTPS Def.	P.1736	23.03.2009	5 years	M	Yes	Azari	1,320	834	nd	–	–	–	16.62	0.2	1.19	1.8
73	Probabely PTPS Def.	P.1813	27.11.2011	2 years	F	Yes	Fars	–	1,251	nd	–	–	–	19.09	0.16	0.83	nd
74	Probabely PTPS Def.	P.94-128	23.08.2014	1 year	M	Yes	Afghan	–	585	nd	–	–	–	22.43	0.1	0.44	4.3
75	Probabely PTPS Def.	P.94-48	30.01.2014	1.5 years	M	Yes	Arab	–	579	nd	–	–	–	24.63	0.11	0.44	3.9
76	Probabely PTPS Carrier	P.94-95	11.06.2015	1 month	M	No	Azari	360	488	nd	–	–	–	45.62	2.03	4.26	4

Phe (B) phenylalanine in blood, *Neo (U)* neopterin in urine, *Bio (U)* biopterin in urine, *%Biopterin* calculated by ((biopterin/(biopterin + neopterin))*100), *nd* not detected

c.351C>A, and c.163 + 2 T>C in *PTS* gene; c.313 T>C in *PCBD1* gene; c.551G>A in *GCH1* gene.

Moreover, the diagnosis for one of the DHPR-deficient patients with DHPR activity equal to zero was performed by the University Children's Hospital of Zürich, Switzerland. All exons of the *QDPR* gene plus flanking intronic regions, and the complete coding sequence of the cDNA, were tested in this case. The new homozygous alteration c.267A>G, which seems to be synonymous for coding on the protein level (p. Gly89Gly) was found. The program used was "Human Splicing Finder," version 2.4.1 (http://www.umd.be/HSF/). Mutations in c.265G>A which coded (p.Gly89Arg) on the protein level and c.266G>A, coding (p.Gly89Gln) on the protein level, were observed in two other DHPR-deficient patients. All three mutations are novel on Cd89 *DHPR* gene. Phenylalanine levels were <250 μmol/: 49 μmol/l for patient with mutation in c.265G>A, 245 μmol/l for patient with mutation in c.266G>A, and 143 μmol/l for patient with mutation in c.267G>G.

Discussion

BH_4 deficiencies are a very heterogeneous group of diseases (Blau et al. 2001). Every newborn with even slight but persistent HPA should be tested for BH_4 deficiency. Such tests have been introduced in many developed countries, but even today, older children are more commonly diagnosed after the appearance of clinical symptoms, such as hypotonia of the trunk, hypertonia of the extremities, and often, myoclonic seizures unresponsive to a low-phenylalanine diet.

Because classical BH_4 deficiencies are a group of diseases that can be detected but not identified through neonatal mass screening for HPA, selective screening for a BH_4 deficiency is essential for every newborn with slightly elevated phenylalanine levels (Dhondt 1991).

Our findings show that there is no predominant mutation and that the majority of these mutations are isolated in families and scattered throughout the genes of the Iranian population. This finding aligns with a prior Iranian study about the *QDPR* gene (Foroozani et al. 2015). Furthermore, eight of the eleven new mutations reported in this article on the *QDPR* gene were previously reported by Foroozani et al. (2015).

It is interesting that in the DHPR deficiency group, the new homozygous alteration c.267A>G in the *QDPR* gene reported in this paper as a novel mutation, which seems to be synonymous coding on the protein level (p.Gly89Gly), is not an SNP. This mutation generates a new 9G8 (or SR-protein SFRS7) exon-splice-enhancer, which might alter pre-mRNA splicing.

The observation of two other new mutation on codon 89 (c.265G>A and c.266G>A) is a more interesting note that should be reported about Iranian DHPR-deficient patients. All mutations on this codon were novel, and all three patients had mild HPA. Therefore it will be interesting if a phenotype–genotype relation study were carried out.

In the PTPS deficiency group, there were no genetic study data or enzyme activity to confirm disease existence for 17 (46%) patients. According to biochemical findings and clinical signs, they were suspected of having PTPS deficiency. Despite the lack of confirmation for these patients, according to physicians' comments, they were treated with BH_4 tablets.

For a more complete diagnosis it is necessary to measure PTPS activity and assess 5-hydroxyindoleacetic acid, homovanillic acid, and 5-methyl tetrahydrofolic acid in the cerebrospinal fluid. The results in this article show gaps in the prevention and control programs for the diseases. These gaps can often be eliminated by using existing capacities in a standardized and systematic approach. If all newborns with HPA are screened to rule out BH_4 deficiencies within the first month of life, we would be able to report a precise incidence of BH_4 deficiencies per 100,000 live births in Iran in the future.

Under the present circumstances, BH_4-deficient newborns are benefiting from the availability of these tests in the context of a screening program in Iran, because they have the chance to be diagnosed and receive necessary care before clinical damage occurs.

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Compliance with Ethics Guidelines

Consortium

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Conflict of Interest

Shohreh Khatami, Soghra Rouhi Dehnabeh, Sirous Zeinali, Beat Thöny, Mohammadreza Alaei, Shadab Salehpour, Aria

Setoodeh, Farzaneh Rohani, Fatemeh Hajivalizadeh, and Ashraf Samavat declare that they have no conflict of interest.

Informed Consent

The project was supported financially with research grants for implemented programs by the Ministry of Health of Iran. Informed consent was obtained from all patients for inclusion in the study.

Animal Rights

This article does not reference any studies with animal subjects performed by any of the authors.

Details of the Contributions of Individual Authors

Shohreh Khatami, as member of the PKU national scientific committee, contributed to the planning, conducting, and reporting of the BH_4 screening tests described in the article.

Soghra Rouhi contributed to the conception and design of the DHPR test and peterin analysis setup and to drafting the article.

Mohammadreza Alaei, Shadab Salehpour, Aria Sotoudeh, and Farzaneh Rohani contributed as members of the PKU national scientific committee and introduced patients for sample gathering.

Sirous Zeinali, Sarah Azadmeh, Tina Shirzad, Leyli Rejali, Mahbobeh Masoodifarand, and Beat Thöny contributed to the genetics study.

Soghra Khani, Rogiyeh Mirzazadeh, Sedigheh Sadeghi, Somayeh Mahmoudi Baram, Elham Farhangara, Arezu Asgari, Ghazaleh Dadashizadeh, Rayhaneh Hasanzaeh, Mina Barzegari, Parinaz Saeedi, Parastoo Bayat, Robabeh Ahadi, Hamid Mohammadaliha, and Saeedeh Saeedi contributed to the setup and performing of the tests.

Ashraf Samavat (Head of the Genetics Office, Ministry of Health of Iran) and Fatemeh Hajivalizadeh contributed to the design of the PKU screening program in Iran and to revising the article critically for important intellectual content.

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Competing Interests Statement

None

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