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ORIGINAL ARTICLE

Genotype–phenotype correlations analysis of mutations in the phenylalanine hydroxylase (*PAH*) gene

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Abstract The aims of our research were to define the genotype–phenotype correlations of mutations in the phenylalanine hydroxylase (*PAH*) gene that cause phenylketonuria (PKU) among the Israeli population. The mutation spectrum of the *PAH* gene in PKU patients in Israel is described, along with a discussion on genotype–phenotype correlations. By using polymerase chain reaction/denaturing high-performance liquid chromatography (PCR/dHPLC) and DNA sequencing, we screened all exons of the *PAH* gene in 180 unrelated patients with four different PKU phenotypes [classic PKU, moderate PKU, mild PKU, and mild hyperphenylalaninemia (MHP)]. In 63.2% of patient genotypes, the metabolic phenotype could be predicted, though evidence is also found for both phenotypic inconsistencies among subjects with more than one type of mutation in the *PAH* gene. Data analysis revealed that about 25% of patients could participate in the future in (6*R*)-L-*erythro*-5, 6, 7, 8-tetrahydrobiopterin (BH_4) treatment trials according to their mutation genotypes. This study enables us to construct a national database in Israel that will serve as a valuable tool for genetic counseling and a prognostic evaluation of future cases of PKU.

Keywords Phenylalanine hydroxylase gene · PAH · Phenylketonuria · Mutation analysis · Genotype · Phenotype

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Introduction

Hyperphenylalaninemia (HPA) is a group of diseases characterized by the persistent elevation of phenylalanine (Phe) levels in tissues and biological fluids. The most frequent form is phenylalanine hydroxylase (PAH; E.C. 1.14.16.1) deficiency, causing phenylketonuria (PKU; McKusick MIM 261600) or non-PKU HPA and corresponding to about 98% of all HPA cases. PAH deficiency is an autosomal recessive disorder affecting 1:10,000 individuals in Caucasian populations (Scriver et al. 1995; Dilella et al. 1986), but in some populations, the prevalence much higher (e.g., in the Catalonian population—about 1:6,600 and in about 1:5,000 live births in Ireland and western Scotland and among Yemenite Jews (Avigad et al. 1991). PKU results in mental retardation and other neurological complications that can be avoided by early treatment (Güttler and Lou 1990). It presents a broad phenotype spectrum ranging from classic PKU to moderate HPA depending on the residual enzymatic activity (Okano

et al. 1991). Since the first mutation in the *PAH* gene was identified in a PKU patient (Dilella et al. 1986), almost 500 different mutations have been identified and listed in the *PAH* mutation database (PAHdb; http://www.mcgill.ca/pahdb) with various phenotypic consequences. Most of them are point mutations and microdeletions usually localized to the coding region or the intron–exon boundaries of the *PAH* gene. The mutations are evenly distributed along the *PAH* exons, although they are mainly found in its 3′ region.

Studies of *PAH* 3D structures (Erlandsen and Stevens 1999) reveal that the PAH enzyme is comprised of four monomeric proteins. Each monomer has three structural domains: an N-terminal regulatory domain (residues 1–142), a catalytic domain (residues 143–410), and a C-terminal tetramerization domain (residues 411–452). The studies have provided information on the active site and binding sites for its substrate and cofactor.

The number of different mutations in a given population is usually high, with a few prevalent mutations and a large number of private mutations (Avigad et al. 1991). Moreover, there are substantial differences in the mutational spectra between populations, making it difficult to establish a genotype–phenotype correlation. The associated phenotypes due to PAH deficiency range in severity from classic through mild PKU to mild hyperphenylalaninemia (MHP), as are defined by pretreatment blood phenylalanine levels (Song et al. 2005).

Several reports have been published that describe *PAH* gene mutations and analyses of genotype–phenotype correlations in European (Guldberg et al. 1993a, b; Zschocke and Hoffmann 1999; Kozak et al. 1997; Mallolas et al. 1999; O'Donnell et al. 2002; Guldberg et al. 1993a, b) and Chinese (Song et al. 2005) populations. From 1990 to 1993, there have been four publications on mutation screening in Israel: a report on a relative small number of different patients (27 patients with non-PKU HPA) that found that all of them were compound heterozygote (Avigad et al. 1990); one splice-site mutation in Palestinian Arabs (Kleiman et al. 1992); the S349P mutation found in Jews from Morocco and Tunisia (Weinstein et al. 1993); and a mutation limited to a common deletion mutation of exon-3 in Yemenite Jews (Avigad et al. 1990). Recently, a report describing the *PAH* gene mutation spectrum in the Israeli subpopulations was publish (Bercovich et al. 2008).

PAH is a nonheme iron-dependent enzyme that requires (6*R*)-L-*erythro*-5, 6, 7, 8-tetrahydrobiopterin (BH_4) as an essential cofactor in the hydroxylation of L-Phe and also uses dioxygen as a substrate (Erlandsen et al. 2004). Certain PKU patients have been responsive to BH_4 loading, resulting in a decreased L-Phe level. Several possibilities have previously been put forward to explain the BH_4 response in mild PKU (Erlandsen et al. 2004): (a) decreased affinity of the mutant PAH for BH_4; (b) stabilization of the active tetramer/dimer forms of the mutant proteins and protection from proteolytic cleavage, i.e., BH_4 can act as a chemical chaperone, preventing misfolding and causing subsequent ubiquitin-dependent proteasomal degradation; (c) up-regulation of *PAH* gene expression; (d) BH_4-induced change in BH_4 biosynthesis; and (d) PAH mRNA stabilization, as shown for nitric oxide syntheses. In the international database (Blau 2003) is a list of genotypes that would respond well to BH_4 treatment (BIOPKU; http://www.bh4.org/BH4DatabasesBiopku.asp), as indicated by the lowering of L-Phe levels in patients' blood (Waters 2003). Most of the reported mutations in the *PAH* gene that were found to respond to BH_4 treatment were in the catalytic region of the protein without direct connection to the binding of the cofactor (Blau and Erlandsen 2004). Therefore, identification of mutations responsive to BH_4 treatment can help in the clinical diagnosis and/or the type of alternative treatment of PKU patients (Blau and Erlandsen 2004).

Mutation analysis of a given population is useful for further understanding the structural and functional aspects of the mutant protein and the correlation between genotype and phenotype. It is helpful in facilitating genetic consultation of patients' families. In this paper, we present a comprehensive analysis of mutations and respective genotype–phenotype correlations in 360 independent PKU alleles from 180 patients representing the majority of the different ethnic groups in Israel (Jewish and Arabs).

Methods

Patients

DNA samples were collected in the Metabolic Disease Unit at Safra Children's Hospital, Sheba Medical Center. This unit treats and provides follow-up for 450 patients coming from 200 different families with known ethnic origin. A total of 180 DNA samples (360 independent alleles) were obtained from unrelated patients with PKU.

The PKU phenotypes were determined according to the classification scheme proposed by Guldberg et al. (1998), which subdivides PAH deficiency into four categories: classic PKU, moderate PKU, mild PKU, and mild HPA (MHP). Classic PKU is caused by a complete or near-complete deficiency of *PAH* activity. Affected individuals typically show very high elevation of phenylalanine (>20 mg/dl) and tolerate less than 250–350 mg/day of dietary phenylalanine to keep plasma concentration of phenylalanine at a safe level of no more than 5 mg/dl. Without dietary treatment, most individuals develop profound, irreversible mental retardation. Individuals with

moderate PKU have plasma phenylalanine concentrations of 10–20 mg/dl and tolerate 350–400 mg/day of dietary phenylalanine. Those with mild PKU have plasma phenylalanine concentrations 6.5–10 mg/dl and tolerate 400–600 mg/day of dietary phenylalanine. Infants with MHP have plasma phenylalanine concentrations of less than 6.5 mg/dl on a normal diet. Such individuals may not need dietary treatment and have normal cognitive neuropsychological development.

DNA purification and PCR amplification

Genomic DNA was extracted from ethylenediaminetetraacetic acid (EDTA)-preserved blood samples and isolated according to standard procedures (Bercovich and Beaudet 2003). The 13 exons and their exon-flanking intronic sequences of the *PAH* gene were amplified by polymerase chain reaction (PCR). The sequences of PCR primer were designed in accordance with the literature (Guldberg et al. 1998) and Table 1. PCR reactions were carried out in a volume of 50 µl with 10 mM Tris hydrochloride (HCl), pH 8.3, 1.5 mM magnesium chloride ($MgCl_2$), 50 mM potassium chloride (KCl), 0.2 mM deoxynucleotide triphosphate (dNTP), and 30 nM primers. The PCR conditions were as described (Bercovich and Beaudet 2003): 94°C for 5 min, followed by 30 cycles with annealing temperatures for 1 min, and 72°C for 1 min; the reactions were ended with a final extension step at 72°C for 7 min.

Primers, annealing temperatures, and PCR product sizes for each of *PAH* exon will be provided by the authors upon request.

Mutations screening methods

Genetic diagnosis depends heavily on the availability of efficient and sensitive methods for detecting DNA mutations and sequence variations. With the expansion in our understanding of the human genome and the relevance of mutations to human disease, there is a compelling need for methods of mutation detection having high sensitivity and allowing for high throughput using partial or complete automation. The ideal method to use for mutation analysis, particularly if large numbers of DNA fragments to be analyzed, should be sensitive, nonhazardous, relatively inexpensive, and semi- or fully automated to minimize costs and labor utilization. dHPLC had been shown to meet these criteria for a growing number of applications in disease-related gene analyses (Bercovich and Beaudet 2003). Scanning for DNA mutations and variants using dHPLC involves subjecting PCR products to chromatography using an ion-pair reversed-phase cartridge. PCR products are denatured and allowed to reanneal. Under conditions of partial denaturation with a linear acetonitrile gradient, heteroduplexes from PCR samples having an internal sequence variation display a reduced column retention time relative to their homoduplex counterparts. The elution profile for heterozygous samples is typically quite distinct from that of either homozygous sequence, making the identification of heterozygous mutations relatively straightforward. An analysis for mutations on the X chromosome in males or for homozygous autosomal mutations requires mixing the test sample with the DNA of a known sequence.

Establishing dHPLC conditions

Mutation analysis was performed using the WAVE apparatus from Transgenomic Inc. (Omaha, NE, USA). The PCR products were denatured at 95°C for 5 min and cooled to 65°C down a temperature gradient of 1°C/min. Samples were kept at 4°C until 5 µl was applied to a preheated C18 reversed-phase column based on nonporous poly(styrene-divinylbenzene) particles (DNA-Sep Cartridge, Cat. no. 450181; all dHPLC catalog numbers are from Transgenomic Inc.). DNA was eluted within a linear acetonitrile gradient consisting of buffer A [0.1 M triethylammonium acetate (TEAA; Cat. no. SP5890)]/buffer B [0.1 M TEAA, 25% acetonitrile (Cat. no. 700001)]. The temperature at which heteroduplex detection occurred was deduced from the Transgenomic software (Wavemaker 4.2) and the Stanford dHPLC melting program (http://insertion.stanford.edu/meltdoc.html), which analyzes the melting profile of each specific DNA fragment. Fragments length and melting temperature (TM) for dHPLC analysis are presented in Table 1.

Homozygous mutations

To identify homozygous mutations, 10 µl PCR product of wild-type DNA and 10 µl PCR product of sample DNA were mixed 1.1 and denatured at 95°C. This enabled detection of homozygous mutations by formation of a heteroduplex (Shlush et al. 2002).

DNA sequencing

DNA sequencing was performed on the PCR products after dHPLC analysis, as previously described (Bercovich and Beaudet 2003). Fragments showing an abnormal dHPLC pattern were investigated for identification of sequence variants by automated sequence analysis on the ABI Prism 377 (Applera). This was performed according to the manufacturer's protocol using a reamplified PCR product of the abnormal fragment (forward and reverse). The sequence variants were classified according to international

Table 1 Polymerase chain reaction (PCR) primer sequences, PCR product size, and denaturing high-performance liquid chromatography (dHPLC) gradient temperatures for the 13 coding exons of the phenylalanine hydroxylase (*PAH*) gene

Primer name	Sequence (5′-3′)	Product length (bp)	Annealing temperature for dHPLC (°C)
PAH ex1			
Forward	GTT ACT GTG CGG AGA TGC ACC ACG	282	62
Reverse	CTT CGG ATC TCT TTC TCT GGA GGC		
PAH ex2			
Forward	GGA GGT TTA ACA GGA ATG AAT TGC	225	56.8
Reverse	GGA AGT TTG CTA CGA CAT TAT CC		
PAH ex3			
Forward	CCA GTG ACT GTC TCC TCA CCC TCC	268	57.6
Reverse	ACA GTG TGG AGT TAC TTA TGT TGC		
PAH ex4			
Forward	CAA TCT GTA CTC AGG ACG TTG CC	157	61.5
Reverse	AAA ATC TCA TCC TAC GGG CCA TGG		
PAH ex5			
Forward	GTA CCA GAC CTC TTC CTA TGA AG	195	60.6
Reverse	GAT GAG GGC AAG GGA GAA GCA GGC		
PAH ex6			
Forward	TGC TTG AGA CAC CTA TTT TGT GCC	277	58.9
Reverse	CCT CCC CCA ACT TTC TGC		
PAH ex7			
Forward	GCC TCT GAC TCA GTG GTG ATG AGC	240	61.8
Reverse	AGA TGG CGC TCA TTG TGC CTG GC		
PAH ex8			
Forward	TCA TGT AGA AAG ACT GAG TCT GGC	260	59.8
Reverse	ACT GTA CCT GGT TTC CGC TCT TGC		
PAH ex9			
Forward	ATG GCC AAG TAC TAG GTT GG	203	59
Reverse	GGA AAG TTT CAA AGA CCT GAG GGC		
PAH ex10			
Forward	AGG TAT CCC TTC ATC CAG TCA AGG	238	57.7
Reverse	CAA TAA TGG TTT TCT GTA CCC ACC		
PAH ex11			
Forward	GAT GCA GCA GGG AAT ACT GAT CC	295	60.1
Reverse	GAC ATT GGA GTC CAC TCT CCT GGC		
PAH ex12			
Forward	GAC TAC CTT TCT CCA AAT GGT GC	217	59
Reverse	TCG ATT ACT GAG AAA CCG AGT GGC		
PAH ex13			
Forward	ATC CCC TAG TGC TTT GCA CTG AG	197	58.2
Reverse	GAT GAA AGA AAT AGT TGG ATC TCC		

databases (http://data.mch.mcgill.ca/pahdb_new/). The numbering of nucleotides follows GenBank accession number U49897.1 (http://www.pahdb.mcgill.ca/).

Genotype–phenotype analysis

In the analysis, nonsense, frameshift, and splice-site mutations are counted as null mutations, and four phenotypes are distinguished: classic PKU, moderate PKU, mild PKU, and MHP. For our analysis, genotypes were first divided into two categories: homoallelic mutant *PAH* genotype and heteroallelic mutant *PAH* genotype. Next, the heteroallelic *PAH* genotypes were further listed roughly in the order of increasing predicted residual activity (PRA), showing a transition from null + null through null + missense (functionally heterozygous) and finally to missense + missense (compound heterozygous).

Statistical analysis

Analyses were performed using the EpiInfo 2000 software (http://www.cdc.gov/epiinfo). The odds ratio (OR) and confidence interval (CI) were calculated as an estimation of risk among mutation carriers. Chi-square was used to determine the statistical significance of the different frequencies of genetic variations between patients and controls.

Results

Phenotype determination in the different ethnic groups of Israel

The aims of our research were to define the genotype–phenotype correlations of mutations in the *PAH* gene that causes PKU among the Israeli population. DNA samples were collected in the Metabolic Disease Unit at Safra Children's Hospital, Sheba Medical Center. This unit treats and provides follow-up for 450 patients from 200 different families with known ethnic origin. All samples reported in this paper are PKU patients detected after neonatal screening. The patients were tested for the exclusion of lacking the cofactor BH_4 for the differential characterization of the cause for the absent of PAH. Out of the 180 samples selected for mutations screening, seven patients did not follow through with this test, so it is not completely clear for these patients whether the cause for the diseases was a defective *PAH* gene. Patient DNA was screened for mutations in all 13 exons of the *PAH* gene. Screening was preformed using the dHPLC technique and sequencing DNA alterations for mutation characterization. Of the 180 patients, 114 were Jewish, 58 were Arabs, and eight were European. A total of 49 different mutations were found in our screening: 30 missense, 7 splice, two nonsense, eight deletion, two insertion mutations. Of these, three were only found in a homozygous state (Y198_E205fs, P225T and T117kfs).

PAH mutations spectrum

Mutations were detected in 148 of 154 alleles (96.1%) in classic PKU patients compared with only 55 of 72 alleles (76.4%) in MHP patients (Table 2).

Twenty-two PKU patients that had only one PAH heterozygous mutation were from all the four PKU phenotypes. But this was more frequent in MHP [nine of 36 patients (25%)] in comparison with classic PKU [four of 77 patients (5%)]. Among the 180 patients, the 49 mutations had different frequencies in the four PKU phenotypes (Table 2). In classic PKU patients, 58.4% were homozygous in comparison with only 11.1% in MHP patients. Mild PKU patients were 72.5% compound heterozygous in comparison with only 35.1% in classic PKU. Even though PKU mutations found in Israel are highly heterogeneous and the genotypes of most patients are compound heterozygote (52.2%), classic PKU makes up the predominant type in our sample (42%) in comparison with moderate PKU, mild PKU, and MHP (8.9%, 28.3%, and 20%, respectively, Table 2).

Genotype–phenotype correlation

In our work, nonsense, frame-shift ,and splice-site mutations are calculated as null mutations. Studying genotype–phenotype correlations in homoallelic mutant *PAH* genotypes and in null + null and null + missense (functionally heterozygous) genotypes has enabled us to discover the effect that single mutation exerts on phenotype (Kayaalp et al. 1997; Waters 2003). Seventeen different null mutations were found, which were 30% of patients' alleles (125/360, Table 3). In our research, the genotypes were divided in to PAH homoallele ($n = 21$) and heteroallele ($n = 86$) (Table 3). The heteroallele genotype was divided in to

Table 2 Distribution of patients with heterozygous or homozygous mutations in the phenylalanine hydroxylase (PAH) gene according to the four different patient phenylketonuria phenotypes categories

	Homozygous mutations		One heterozygous mutation		Two heterozygous mutations	
	n	RF (%)	*n*	RF (%)	*n*	RF (%)
Classic PKU ($n = 77$)	45	**58.4***	4	**5.2**	27	35.1
Moderate PKU ($n = 16$)	3	**18.8**	2	12.5	11	68.8
Mild PKU ($n = 51$)	5	**9.8**	7	13.7	37	72.5
MHP ($n = 36$)	4	**11.1**	9	**25^**	19	52.8

RF (%) relative frequencies, parentage of patients with a mutation in the phenotypic category. *PKU* phenylketonuria, *MHP* mild hyperphenylalaninemia

* Significant difference from the MHP phenotype ($P = 0.0000022$)

^ Significant difference from one heterozygous mutation in the classic PKU phenotype ($P = 0.002$)

null + null mutations ($n = 3$), null + missense mutations ($n = 35$) that account as functional-homozygote genotype, and missense + missense ($n = 34$), apparently compound heterozygous (Table 3). Fourteen genotypes did not mach any of these categories. Through this analysis, of the 49 different mutations that found in our project, in 34 we could identify the effect of the PKU phenotypes in cretin (69.4%). Seventeen of them were null mutations, and 17 were missense that, when they appear as homozygous or in combination with a null mutation, give a define phenotype.

Eleven mutations were found in at least two different phenotypes, and five mutations were found in at least three different PKU phenotypes (Tables 2, 3). In six mutations, there were no correlations to any of the PKU phenotypes. In some of the missense mutations, in the *PAH* gene, the PAH PRA in vitro (http://www.pahdb.mcgill.ca) is known. Mutations that cause severe (classic) phenotypes are those in which the PRA is less then 10% (Avigad et al. 1990). The R158Q mutation has a PRA of less then 10%, and so this mutation was found to be associated with the severe phenotype (Erlandsen and Stevens 1999). The PAH enzymatic activity with the R261Q, P281L, S349P and R408W missense mutations are known to be <1%, <1%, <1%, and 30%, respectively. These mutations had a null effect, as can be seen from the fact that when they were in combination with known null mutations, the patient phenotype was always the severe PKU. Two missense mutations (L197F and H271Q) of which there is no knowledge on the influence of PAH activity also showed severe phenotype when they in a homoallelic state (Table 3). In summary, missense mutation genotype–phenotype correlation revealed that eight mutations were associated with the severe phenotype (classic PKU): R252W, S349P, P281L, R158Q, R261Q, L197F, H271Q, and P225T (Tables 3, 4); eight mutations were clearly associated with the mild PKU phenotypes (MHP or mild PKU): V230I, A403V, D415N, A300S, A395G, I174V, I95F, and R261P, and one mutation was associated with the moderate phenotype: R241H. Distribution analysis for the types of missense mutations according to the functional domain in the PAH protein revealed that most were in the catalytic domain, one mutation was found in the tetramerization domain, and none were in the regulatory domain (Table 4). Null mutations were found in all the three domains.

Eight common polymorphic DNA alterations (SNPs) at different frequencies were detected in patients from all ethnic groups and controls group: 5UTR −71 A/C, E56E, IVS2 +19 T/C, Q232Q, V245V, IVS9 +43 G/T, L385L, and IVS12 −35 C/T. Two of the SNPs were found at high frequencies in both Jewish and Arab patients: Q232Q and L385L (66.1% and 81.4%, respectively). The E56E SNP was only found in Arab patients' DNA (13.8%).

Genotypes respond to BH_4 treatment

In the study we found two known mutations that will response well to BH_4 treatment: A300S and IVS4 − 5C/G (allelic frequencies 5.8% and 0.8%, respectively) (Blau and Erlandsen 2004). Eight more mutations that cause minor downregulation of enzymatic activities and therefore are potential to BH_4 treatment were found: L48S, A104D, R158Q, V230I, R241H, R261Q, A403V, and D415N (allelic frequencies 7.8%, 0.3%, 0.6%, 2.5%, 8.9%, and 0.3%, respectively).

Discussion

Genotype–phenotype correlation

Although PKU mutations detected in Israel are highly heterogeneous and the genotypes of most patients are compound heterozygotes (52.2%), classic PKU comprised the predominant type found in our samples (42%) compared with moderate, mild, and MHP (8.9%, 28.3%, and 20%, respectively, Table 2). Studying the genotype–phenotype correlations in homoallelic mutant *PAH* genotypes and in null + null and null + missense (functionally heterozygous) genotypes enabled us to discover the effect that a single mutation exerts on the phenotype (Kayaalp et al. 1997; Waters 2003).

By analyzing homozygous (null + null) and functional heterozygous (null + missense) mutations, the effect of 31 of 49 (63.3%) different mutations on the phenotypes could be determined (Table 3). Seventeen mutations were defined as null (nonsense, frame shift, and splice site), causing severe changes in the PAH protein and are associated with the PKU phenotype (more than 20 mg/dl of phenylalanine) (Waters 2003). When missense mutations are located in a functional codon, which has also been conserved during evolution, it can occasionally be defined as a null mutation. This is true of the well-known mutation R408W located in the region connecting the catalytic and tetramerization domains (Zschocke 2003). In some *PAH* gene missense mutations, the PRA in vitro (http://www.pahdb.mcgill.ca) is known.

Mutations that cause the severe phenotypes are those in which the PRA is less than 10% (Avigad et al. 1990). The R158Q mutation has a PRA of less then 10%, associating this mutation with the severe phenotype (Erlandsen and Stevens 1999). We found that these missense mutations (L197F, P225T, and H271Q) could be identified as null mutations, since they caused the severe phenotype (Table 3). Four mutations were associated with the mild PKU phenotype (I95F, I174V, R261P, and D415N, Table 3). These amino acids are probably located in a

Table 3 Phenotype–genotype correlation of the 49 mutations found in the phenylalanine hydroxylase (*PAH*) gene of 180 phenylketonuria (PKU) patients

Classification	Genotypes	PRA (%)	No. of patients			
			Classic PKU	Moderate PKU	Mild PKU	MHP
Homozygous	IVS4 + 5G > T + IVS4 + 5G > T	Null	3	–	–	–
	IVS10 − 11G > A + IVS10 − 11G > A	Null	13	–	–	–
	F55fsX6 + F55fsX6	Null	3	–	–	–
	T117fsX78 + T117fsX78	Null	1	–	–	–
	Y198_E205 > Sfs + Y198_E205 > Sfs	Null	1	–	–	–
	K363fsX37 + K363fsX37	Null	1	–	–	–
	IVS2 + 1G > A + IVS2 + 1G > A	Null	6	–	–	–
	EX3del + EX3del	Null	4	–	–	–
	P281L + P281L	<1	1	–	–	–
	R408W + R408W	<1	3	–	–	–
	S349P + S349P	<1	1	–	–	–
	R241H + R241H	23	–	1	1	–
	R261Q + R261Q	30	2	–	–	–
	L48S + L48S	39	2	2	2	–
	V230I + V230I	63	–	–	–	1
	A403V + A403V	~100	–	–	2	1
	L197F + L197F	?	2	–	–	–
	P225T + P225T	?	1	–	–	–
	H271Q + H271Q	?	1	–	–	–
	A300S + A300S	?	–	–	–	1
	A395G + A395G	?	–	–	–	1
Heterozygous	IVS4 + 5G > T + P211 > Hfs	Null + null	1	–	–	–
	IVS4 + 5G > T + IVS10 − 11G > A	Null + null	2	–	–	–
	IVS10 − 11G > A + S436 > Pfs	Null + null	1	–	–	–
	IVS10 − 11G > A + R252W	Null + <1	1	–	–	–
	IVS10 − 11G > A + S349P	Null + <1	1	–	–	–
	IVS4 + 5G > T + P281L	Null + <1	1	–	–	–
	IVS10 − 11G > A + R408W	Null + <1	2	–	–	–
	IVS4 + 5G > T + R408W	Null + <1	1	–	–	–
	K363NfsX37 + R408W	Null + <1	1	–	–	–
	IVS4 + 5G > T + R158Q	Null + 10	1	–	–	–
	P211 > Hfs + R241H	Null + 23	–	–	1	–
	EX3del + R241H	Null + 23	–	1	–	–
	IVS2 + 1G > A + R261Q	Null + 30	1	–	–	–
	IVS10 − 11G > A + R261Q	Null + 30	1	–	–	–
	IVS4 + 5G > T + R261Q	Null + 30	1	1	–	–
	R243X + R261Q	Null + 30	–	1	–	–
	IVS10 − 11G > A + L48S	Null + 39	2	1	–	–
	IVS4 − 5C > G + L48S	Null + 39	–	–	1	–
	IVS10 + 1G > T + L48S	Null + 39	–	1	–	–
	D17fsX1 + L48S	Null + 39	1	–	–	–
	R176X + L48S	Null + 39	–	–	1	–
	IVS10 − 11G > A + V230I	Null + 63	–	–	1	–
	EX3del + V230I	Null + 63	–	–	–	1
	IVS10 − 11G > A + D415N	Null + ~100	–	–	1	–
	IVS10 − 11G > A + A403V	Null + ~100	–	–	2	–

Table 3 continued

Classification	Genotypes	PRA (%)	No. of patients			
			Classic PKU	Moderate PKU	Mild PKU	MHP
	IVS2 + 1G > A + A403V	Null + ~100	–	–	1	–
	IVS4 + 5G > T + A403V	Null + ~100	–	–	1	–
	F55LfsX6 + A403V	Null + ~100	–	–	3	–
	IVS2 − 2del + A403V	Null + ~100	–	–	1	–
	EX3del + A403V	Null + ~100	–	–	1	–
	IVS10 − 11G > A + A300S	Null + ?	1	–	4	–
	EX3del + A300S	Null + ?	–	–	1	–
	R243X + A300S	Null + ?	–	–	1	–
	IVS10 − 11G > A + H271Q	Null + ?	1	–	–	–
	IVS2 + 1G > A + L197F	Null + ?	1	–	–	–
	IVS4 + 5G > T + A395G	Null + ?	–	1	–	1
	IVS4 + 5G > T + I174V	Null + ?	–	–	1	–
	IVS4 − 5C > G + R261P	Null + ?	–	–	1	–
	IVS4 + 5G > T + ?	Null + ?	–	–	1	–
	IVS10 − 11G > A + ?	Null + ?	–	–	–	1
	IVS4 − 5C > G + ?	Null + ?	–	1	–	–
	F55LfsX6 + ?	Null + ?	1	–	–	–
	IVS7 + 5G > A + ?	Null + ?	–	–	1	–
	R243X + ?	Null + ?	–	–	–	1
	S349P + A259V	<1 + <1	1	–	–	–
	R408W + R241H	<1 + 23	–	1	1	–
	S349P + A104D	<1 + 26	1	–	–	–
	S349P + L48S	<1 + 39	1	–	–	–
	P281L + L48S	<1 + 39	1	–	–	–
	R408W + L48S	<1 + 39	–	1	–	–
	S349P + V230I	<1 + 66	–	–	–	1
	P281L + A403V	<1 + ~100	–	–	1	–
	R408W + A300S	<1 + ?	–	–	1	1
	S349P + A300S	<1 + ?	–	–	1	–
	P281L + A300S	<1 + ?	–	–	1	–
	R408W + E178G	<1 + ?	–	1	–	–
	P281L + E178G	<1 + ?	1	–	–	–
	R408W + ?	<1 + ?	2	–	–	–
	P281L + ?	<1 + ?	–	–	1	–
	E280K + ?	1 − 3 + ?	1	–	–	–
	R158Q + I95F	10 + ?	–	–	1	–
	F39>Sdel + V230I	20 + 63	–	–	–	1
	R241H + A300S	23 + ?	–	–	–	2
	R241H + T323del	23 + ?	–	1	–	–
	R241H + ?	23 + ?	–	–	–	2
	R261Q + L48S	30 + 39	1	–	–	–
	R261Q + A403V	30 + ~100	–	–	1	–
	R261Q + E178G	30 + ?	–	–	1	–
	R261Q + ?	30 + ?	–	–	1	–
	L48S + A403V	39 + ~100	–	–	1	–
	L48S + E178G	39 + ?	–	–	2	–

Table 3 continued

Classification	Genotypes	PRA (%)	No. of patients			
			Classic PKU	Moderate PKU	Mild PKU	MHP
	L48S + T380M	39 + ?	–	–	–	2
	I306V + F55L	39 + ?	–	–	–	1
	V230I + A403V	63 + ~100	–	–	–	1
	V230I + V230A	63 + ?	–	–	–	1
	V230I + L369V	63 + ?	–	–	–	1
	A403V + R169H	~100 + ?	–	–	–	1
	A403V + I95F	~100 + ?	–	–	1	–
	A403V + T380M	~100 + ?	–	–	–	3
	A403V + A300S	~100 + ?	–	–	2	1
	A403V + ?	~100 + ?	–	–	3	2
	V230A + A300S	? + ?	–	–	1	–
	I95F + T323del	? + ?	–	1	–	–
	L369V + T380M	? + ?	–	–	–	1
	A300S + ?	? + ?	–	1	–	1
	R53H + ?	? + ?	–	–	–	2

PRA predicted residual activity in vitro: according to the PAH enzymatic activity prediction (PRA) in vitro (http://www.pahdb.mcgill.ca) site, *MHP* hyperphenylalaninemia

region that is not critical to PAH activity, and therefore, their substitution did not completely abolish PAH function. Most missense mutations were observed in the catalytic domain in which the functional site of *PAH* is located. One mutation was in the tetramerization domain, and two mutations were discovered in the regulatory region (Table 4). Null mutations were found in all three functional domains (Table 4). It seems that none of these domains plays a roll in the genotype–phenotype correlation. Two known missense mutations (R261Q and L48s), which have been previously reported not to show this correlation, did not demonstrate any such correlation in this study either. The R261Q has a PRA of 30% (Okano et al. 1991), which is probably not enough to completely abolish PAH activity. The amino acid derived from the L48S mutation, which does not show any damaging effect on the monomeric structure or on the specificity of PAH function, is located far from the active site of the protein (Guldberg et al. 1998; Erlandsen and Stevens 1999). This mutation was present in all phenotypes (Table 3). We found six patients who were homozygous to the L48S mutation. Two of them had a polymorphism in the *PAH* gene, which means that the entire gene is not deleted (one had the classic PKU and one with the moderate PKU). In the remaining four patients, polymorphisms were found in three different PKU phenotype categories. Therefore, this mutation has the most unpredictable phenotype outcome and should be investigated further.

In compound heterozygote patients, a wide matching range existed between a *PAH* mutant genotype and a PKU phenotype. When the compound heterozygotes are functional hemizygous (null + missense) genotypes, the less severe mutation of the two is the one that determines the phenotype (Guldberg et al. 1998). Thus, when one of the mutations has a severe effect and the second allows for at least a partially functioning PAH, the PKU metabolic phenotype will be less severe. For example, the IVS10 − 11G > A (null) + A403V (PRA = ~100%) produces the mild PKU phenotype (Table 3). When the two mutations are in a compound heterozygous state and have the same predicted effect on the phenotype, the phenotype resulting from them will be less severe. For instance, when the R241H and A300S mutations are combined with a null mutation, they will confer a mild PKU phenotype, and when they are together, they will impart the MHP phenotype. When the two different mutations (of the two alleles) are null, the phenotype will tend to be severe. Mutations with a severe effect on PAH activity and which cause the classic PKU phenotype are usually in a homozygous state compared with mutations that cause the MHP phenotype (58.4% and 11.1%, respectively). This is probably true, since most MHP cases are results of compound heterozygotes in which one of the mutations is on one of the alleles and the mild mutation is on the second allele. The mild mutation is probably expressed only when it interacts with another severe mutation (Avigad et al. 1991; Kleiman et al. 1994). Furthermore, if one heterozygous mutation in the *PAH* gene does not cause the MHP phenotype to be expressed (Kleiman et al. 1994), probably another mutation exists that has not been detected in these patients in regions

Table 4 Mutation genotypes in clinical phenotypes

Protein domain	Mutation	Classic PKU (154 alleles)		Moderate PKU (32 alleles)		Mild PKU (102 alleles)		MHP (72 alleles)	
		No. of alleles	RF (%)	No. of alleles	RF (%)	No. of alleles	RF (%)	No. of alleles	RF (%)
Regulatory domain	D17fsX1	1	0.6	–	–	–	–	–	–
Nt 1-426	F39 > Sdel	–	–	–	–	–	–	1	1.4
aa 1-142	L48S	10	6.5	7	21.9	9	8.8	2	2.8
	R53H	–	–	–	–	–	–	2	2.8
	F55fsX6	7	4.5	–	–	3	2.9	–	–
	F55L	–	–	–	–	–	–	1	1.4
	IVS2 + 1G > A	14	9.1	–	–	1	1	–	–
	IVS2 − 2del	–	–	–	–	1	1	–	–
	EX3del	8	5.2	1	3.1	2	2	1	1.4
	I95F	–	–	1	3.1	2	2	–	–
	A104D	1	0.6	–	–	–	–	–	–
	T117fsX78	2	1.3	–	–	–	–	–	–
	IVS4 + 5G > T	13	8.4	2	6.3	3	2.9	1	1.4
	IVS4-5C > G	–	–	1	3.1	2	2	–	–
Catalytic domain	R158Q	1	0.6	–	–	1	1	–	–
Nt 427-1230	R169H	–	–	–	–	–	–	1	1.4
aa 143-410	I174V	–	–	–	–	1	1	–	–
	R176X	–	–	–	–	1	1	–	–
	E178G	1	0.6	1	3.1	3	2.9	–	–
	L197F	5	3.2	–	–	–	–	–	–
	Y198_E205 > Sfs	2	1.3	–	–	–	–	–	–
	P211 > Hfs	1	0.6	–	–	1	1	–	–
	P225T	2	1.3	–	–	–	–	–	–
	V230I	–	–	–	–	1	1	8	11.1
	V230A	–	–	–	–	1	1	1	1.4
	R241H	–	–	5	15.6	4	3.9	4	5.6
	R243X	–	–	1	3.1	1	1	1	1.4
	R252W	1	0.6	–	–	–	–	–	–
	A259V	1	0.6	–	–	–	–	–	–
	R261P	–	–	–	–	1	1	–	–
	R261Q	8	5.2	2	6.3	3	2.9	–	–
	H271Q	3	1.9	–	–	–	–	–	–
	E280K	1	0.6	–	–	–	–	–	–
	P281L	5	3.2	–	–	3	2.9	–	–
	IVS7 + 5G > A	–	–	–	–	1	1	–	–
	A300S	1	0.6	1	3.1	12	11.8	7	9.7
	I306V	–	–	–	–	–	–	1	1.4
	T323del	–	–	2	6.3	–	–	–	–
	S349P	6	3.9	–	–	1	1	1	1.4
	IVS10 + 1G > T	–	–	1	3.1	–	–	–	–
	IVS10 − 11G > A	38	24.7	1	3.1	8	7.8	1	1.4
	K363fsX37	3	1.9	–	–	–	–	–	–
	L369V	–	–	–	–	–	–	2	2.8
	T380M	–	–	–	–	–	–	6	8.3
	A395G	–	–	–	–			3	4.2
	A403V	–	–	1	3.1	22	21.6	10	13.9
	R408W	12	7.8	3	9.4	2	2	1	1.4

Table 4 continued

Protein domain	Mutation	Classic PKU (154 alleles)		Moderate PKU (32 alleles)		Mild PKU (102 alleles)		MHP (72 alleles)	
		No. of alleles	RF (%)	No. of alleles	RF (%)	No. of alleles	RF (%)	No. of alleles	RF (%)
Tetramerization domain	D415N	–	–	–	–	1	1	–	–
Nt 1231-1356	S436 > Pfs	1	0.6	–	–	–	–	–	–
aa 411-452	Total	148	96.1	29	90.6	92	90.2	55	76.4

RF (%) relative frequencies, percentages of patients with a mutation in the phenotypic category. *Nt* mRNA nucleotide number, *aa* amino acid number

that have not yet been screened, e.g., the 5′UTR, 3′UTR, promoter, etc.

The main factor that probably influence the sensitivity to BH_4 treatment is the allelic composition in the PAH locus. The level of response to treatment is dependent upon the type of mutant allele and its effect on enzymatic activity and enzyme wholeness (Blau and Erlandsen 2004). Therefore, in a situation in which the two mutations that cause low or absent PAH enzymatic activity are on different alleles, the outcome will mostly be the severe phenotype , and it is expected that these patients would not response well to BH_4 treatment; in previous cases, it was found that some classic PKU did response to this treatment. On the other hand, patients with two mutations that show relatively high enzymatic activities (>30%) probably will have mild PKU or MHP phenotypes, and therefore are expected to response well to BH_4 treatment (Erlandsen et al. 2004). Patients with a combination of a mild mutation and a severe mutation will mostly not response well to this type of treatment, but this response is dependent on the specific genotype (Blau and Erlandsen 2004). The biochemical phenotypes associated with R158Q and R261Q are mostly severe but are inconsistent (Table 4). This fact, combined with reports of response to tetrahydrobiopterin load in patients who carry these mutations (Leuzzi et al. 2006) deserves to be explored more. Response to BH_4 is only limited to the mild mutation, which is still an interesting enigma. In our study, about 25% of patients could be candidates in the future for BH_4 treatment trials according to their mutations genotypes.

Conclusion

The definition of the PKU mutational profile in Israel enables us to construct a national database covering detailed information on genotype–phenotype correlations. This database may serve as a valuable tool in genetic counseling and in prognostic evaluation of future PKU cases.

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