Characterization of Phenylalanine Hydroxylase Gene Mutations in Chilean PKU Patients

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Abstract Phenylketonuria (PKU, OMIM 261600) is an autosomal recessive disease, caused by mutations in the Phenylalanine Hydroxylase (*PAH*) gene situated in chromosome 12q22-q24.2. This gene has 13 exons. To date, 991 mutations have been described. The genotype is one of the main factors that determine the phenotype of this disease. **Objective:** Characterize PKU genotype and phenotype seen in Chilean PKU patients. **Methods:** We studied the *PAH* gene by restriction fragment length polymorphism (RFLP) and/or sequencing techniques to identify pathogenic mutations in 71 PKU subjects. We classified the phenotype according to Guldberg predicted value. **Results:** We identified 26 different mutations in 134 of the 142 alleles studied (94.4%), 88.7% of the subjects had biallelic pathogenic mutations while 11.3% had only one pathogenic mutation identified. Compound heterozygous represented 85.9% of the cases. Exon 7 included the majority of mutations (26.9%) and 50% of mutations were missense. The most frequent mutations were c.1066-11G > A, c.442-?_509+?del and p.Val388Met. The majority of subjects (52.3%) had the classic phenotype. **Conclusions:** The most frequent mutations in our Chilean PKU population were p.Val388Met, c.442?_509+?del and c.1066-11G > A. It is possible to predict phenotype by detecting the genotype, and use this information to determine disease prognosis and

adjust patient's medical and nutritional management accordingly.

Highlights

- A strong correlation between genotype and phenotype in PKU has been described.
- For the first time in Chile, we analyzed a large number of mutations in the *PAH*, in order to characterize genotype and phenotype.
- In our PKU population the three most frequent mutations were p.Val388Met (17.2%), c.442-?_509+?del (14.9%), and IVS10-11G > A (12.7%).
- Because of the shared history of Spanish colonization, these results could be extrapolated to the rest of Latin America, assisting other countries in a strategy to direct the study to common mutations.
- There was a correlation between predicted phenotype and observed phenotype, except in subjects with p.Val388Met mutation.
- The complete categorization of patients is essential for our center to identify the most vulnerable patients (classic PKU) and create strategies to prevent any difficulties in long-term nutritional treatment.

Introduction

Phenylketonuria (PKU) is an autosomal recessive disease, caused by a total or partial deficit in the phenylalanine hydroxylase activity, which converts phenylalanine (Phe) to tyrosine (Tyr) (Mitchell 2011). This enzyme deficiency causes Phe accumulation (hyperphenylalaninemia) damaging the nervous system, leading to intellectual impairment,

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unless treated opportunely (Greene and Longo 2014). Newborn screening (NBS) for PKU in Chile started in 1992, establishing an incidence of 1:18,916 newborns (Cornejo et al. 2010). The Chilean cohort of PKU cases is one of the largest cohorts followed in a single Metabolic Center.

The PAH gene is located on chromosome 12q22-q24.2, is 90 kb in size with 13 exons and 12 introns (Scriver 2007). PKU is a heterogeneous inborn error of metabolism and to date, 991 mutations have been reported in databases (www.pahdb.mcgill.ca and <http://www.biopku.org/pah/home.asp>) (Wettstein et al. 2015). Almost 60% are missense mutations, mostly located on catalytic domain (Waters 2003; Blau 2016).

Researchers, especially Guldborg, have demonstrated a strong correlation between the genotype and phenotype in PKU (Guldborg et al. 1998; Pey et al. 2003). In addition, genotype can be used to predict the response to sapropterin dihydrochloride, BH4 the synthetic cofactor of the enzyme, necessary to activate PAH activity (Werner et al. 2011).

The NBS program in Chile does not include molecular testing to confirm the diagnosis, like screening programs in other countries (Gizewska et al. 2016). In Chile, PKU patients are diagnosed using Phe level in the newborn period, confirmed with tandem mass spectrometry (MS/MS) and Phe/Tyr ratio at 17.5 ± 8.7 days of age. For the first time in Chile, we characterized the genotype and phenotype of a large cohort of patients. We planned to use this information to personalize treatment for each patient.

Subjects and Methods

A total of 71 Chilean PKU patients that comply with the follow-up program from our Metabolic Center at INTA, University of Chile, were included in this study from a total of 288 patients diagnosed in the NBS program since 1992. The subjects for this study were between 3 months and 32 years of age. The sample was 59.2% male and 40.8% female. Every patient signed the informed consent approved by our institution ethics committee. The samples of 32 PKU patients were analyzed for mutations in Chile and the rest were studied in the Center of Diagnosis of Metabolic Diseases, Madrid, Spain as part of a previous study (Trujillano et al. 2014).

Genetic Analysis

Genomic DNA was isolated from the leukocytes of blood samples using Wizard[™] Genomic DNA purification kit (Promega), following supplier instructions. All 13 exons with their flanking intronic sequences were amplified by PCR (primers sequence provided upon request). PCR conditions were 95°C for 5 min and 36 cycles of 95°C

for 25 s to denature DNA, 25 s at 55°C for annealing and 40 s at 72°C to elongate, and the last cycle for elongation lasted 7 min. For PCR, FastStart Taq DNA Polymerase from Roche[®] was used, which is highly specific on rich GC sequences, adjusting the supplier protocol.

Data from the previous study in Spain determined the most common mutations. Thus, for an early and fast screening for these mutations (for exon 11: p.Val388Met, intron 10: c.1066-11G > A and exon 7: p.Arg261Gln) we used a Restriction Fragment Length Polymorphism (RFLP) protocol (Desviat et al. 1995), that use BsaAI (p.Val388-Met), DdeI (c.1066-11G > A), and HinfI (p. Arg261Gln) restriction enzymes, respectively. These mutations were also confirmed by sequencing.

All PCR products were sequenced by Macrogen, Korea. The obtained sequences were compared with the wild type sequence of the human PAH (NM_000277.1).

Multiplex ligation-dependent probe amplification (MLPA) was performed to detect large genomic deletions in patients without identified mutations on one or both alleles, by the commercially available SALSA MLPA kit P055-D1 PAH (MRC-Holland[®]).

Mutation nomenclature followed the guidelines and recommendations of the Human Genome Variation Society (<http://varnomen.hgvs.org/>).

Genotype–Phenotype Correlation

To predict phenotype we assigned all mutations an arbitrary value (AV) by Guldborg [Classic PKU = 1, Moderate PKU = 2, Mild PKU = 4 and Mild Hyperphenylalaninemia (MHP) = 8] obtained from BIOPKU database. Guldborg phenotype classification is the same as the one published in Phenylketonuria Scientific Review Conference (Camp et al. 2014). This classification separates mild PKU into two different phenotypes: moderate PKU (900–1,200 $\mu\text{mol/L}$) and mild PKU (600–900 $\mu\text{mol/L}$). We used this classification so we could have four different phenotypes to compare them with Guldborg AV.

Phenotypes resulting from a combination of the two mutant alleles were expressed as the sum of the two AVs (Guldborg et al. 1998). These phenotypes were classified according to the predicted residual enzymatic activity based on in vitro expression studies according to PAH database (<http://www.pahdb.mcgill.ca>). We correlated the predicted phenotype with the observed phenotype for patients with complete mutation analysis. Additionally, we searched the BIOPKU database for the BH4 response for each patient.

Statistical Analysis

To correlate the predicted phenotype with the observed phenotype, the Fisher exact test was used. Significance

level was set at $p < 0.05$. Statistical analysis was performed using STATA 13.

Results

We identified 26 different mutations in 134 of the 142 alleles studied (94.4%), completing the analysis in 88.7% of the subjects. Biallelic mutations were detected in 63 cases and only one mutation was identified in 8 (11.3%). Twenty-eight alleles were identified by RFLP as a first screening. The rest of the mutations were identified by sequencing analysis. Most of the subjects were compound heterozygous (85.9%). The exon containing more mutations was exon 7 (26.7%) (p.Arg243Gln, p.Leu249-Phefs*92, p.Arg252Trp, p.Arg261Gln, p.Pro275Arg, p.Thr278Asn, p.Glu280Lys or p.Pro281Leu), followed by 7.7% on exon 5 (c.442-?-509+?del and p.Arg158Gln), 7.7% on exon 11 (p.Val388Met and p.Glu390Gly), and 7.7% on intron 10 (c.1066-11G > A and c.1066-3C > T).

The mutational spectrum included 50% missense, 34.6% splicing, 11.5% deletions, and 3.8% nonsense mutations. Twenty-one mutations were located on the catalytic domain, two on regulatory domain and three on the oligomerization domain (Table 1).

Table 2 shows the subjects with biallelic mutations and their phenotype, based on Guldberg prediction, which showed that 52.4% of our subjects had classic PKU, 28.6% had mild PKU, 4.8% had an undefined phenotype due to no phenotype information on mutation p.Pro275Leu, and the rest had MHP and Mild/MHP PKU.

Overall there was a correlation between predicted phenotype and observed phenotype ($p < 0.007$), however in the subjects with the p.Val388Met mutation we observed discordance between phenotypes ($p = 0.569$) (see Table 3).

Discussion

Genotype characterization allowed us to predict the phenotype in our Chilean patients, which could guide medical and nutritional management. Knowing the exact phenotype could help estimate Phe tolerance and metabolic control throughout life, individualizing treatment (Singh et al. 2016).

With the available data of the mutational spectrum in countries like Brazil, Mexico, and Chile (with this study), it can be concluded that p.Val388Met is one of the most common mutations (21.2%, 8.3%, and 17.3%, respectively). This concordance in mutations could relate to the common origins of these populations traced to the Iberian Peninsula (Santos et al. 2008; Vela-Amieva et al. 2015).

The Spanish colonization of our region may allow for extrapolation of results to the rest of Latin America and guide the search to the specific mutations, which may lower costs for neighboring countries.

It has been shown that the allele which confers a residual enzymatic activity is pseudo-dominant, meaning that this mutation grants the final phenotype (Zschocke 2008; Guldberg et al. 1998). In our patients, this theory is not supported as the p.Val388Met mutation correlated with the observed phenotype in only 26.3% of the population (Table 3). The p.Val388Met mutation affects protein folding and the active tetramer formation (Aldamiz-Echevarria et al. 2016; Gamez et al. 2000) and retains 28% of enzyme activity leading to a mild to moderate phenotype. Most of our group had a more severe phenotype, especially when combined with a null variant (p.Thr278Asn, p.Glu280Lys, p.Arg252Trp, p.Leu249Phefs*92, c.442-?-509+?del, c.441 + 5G > A, c.1066-11G > A and c.1066-3C > T). The destabilizing mutations could be responsible for the phenotype-genotype inconsistencies. Reports indicate that when p.Val388Met is associated with a null mutation, the enzyme activity reduces by 12–15%, causing a classic phenotype (Leandro et al. 2000), which can be explained by the existence of a negative interallelic complementation on proteins expressed from two different pathogenic variants (Leandro et al. 2006; Vela-Amieva et al. 2015).

Other reason for why there is not always a correlation is that single gene disorders are not simple “traits,” but rather complex “traits,” because of modifier genes and epigenetic factors, such as a function of the blood-brain barrier, intestinal absorption of Phe, and Phe hepatic uptake among others that affect phenotype (Scriver and Waters 1999; Aldamiz-Echevarria et al. 2016).

Genotype can also be used to predict the response to sapropterin dihydrochloride (BH4), the synthetic cofactor of the enzyme and necessary to activate PAH activity. BH4 has been used in Europe and North America for more than 10 years, as monotherapy or together with a Phe-restricted diet, depending on drug response per patient (Blau et al. 2011; Werner et al. 2011). Recent reports have revealed that 60–80% of patients with mild PKU and hyperphenylalaninemia (HPA) would benefit from using this drug for treatment (Blau et al. 2011; Wettstein et al. 2015). Mutation analysis in Chilean PKU patients showed that 20% of our analyzed sample could use BH4 as part of their treatment. This information is important for choosing patients for future clinical studies with this drug in our country.

A phenylalanine-restricted diet is the most effective treatment for PKU demonstrated to date. With the diet, it is possible to avoid intellectual disability caused by neurotox-

Table 1 *PAH* gene mutations from PKU patients in Chile

Systematic name (DNA)	Trivial name (protein)	Location	Mutation type	Domain	Alleles number	Alleles frequency
c.1162G > A	p.Val388Met	Exon 11	Missense	Catalytic	23	17.2
c.442-?_509+?del	Ex5del	Exon 5	Deletion	Catalytic	20	14.9
c.1066-11G > A	IVS10-11G > A	Intron 10	Splicing	Catalytic	17	12.7
c.782G > A	p.Arg261Gln	Exon 7	Missense	Catalytic	10	7.5
c.913-7A > G	IVS8-7A > G	Intron 8	Splicing	Catalytic	8	6.0
c.1066-3C > T	IVS10-3C > T	Intron 10	Splicing	Catalytic	7	5.2
c.838G > A	p.Glu280Lys	Exon 7	Missense	Catalytic	7	5.2
c.754C > T	p.Arg252Trp	Exon 7	Missense	Catalytic	6	4.5
c.441 + 5G > A	IVS4 + 5G > T	Intron 4	Splicing	Catalytic	4	3.0
c.824C > G	p.Pro275Arg	Exon 7	Missense	Catalytic	4	3.0
c.842C > T	p.Pro281Leu	Exon 8	Missense	Catalytic	4	3.0
c.728G > A	p.Arg243Gln	Exon 7	Missense	Catalytic	4	3.0
c.1169A > G	p.Glu390Gly	Exon 11	Missense	Catalytic	3	2.2
c.707-7A > T	IVS6-7A > T	Intron 6	Splicing	Catalytic	2	1.5
c.833C > A	p.Thr278Asn	Exon 7	Missense	Catalytic	2	1.5
c.842 + 1G > A	IVS7 + 1G > A	Intron 7	Splicing	Catalytic	2	1.5
c.1045 T > C	p.Ser349Pro	Exon 10	Missense	Catalytic	2	1.5
c.745del	p.Leu249Phefs*92	Exon 3	Deletion	Catalytic	1	0.7
c.331C > T	p.R111*	Exon 3	Nonsense	Regulatory	1	0.7
c.527G > T	p.Arg176Leu	Exon 6	Missense	Catalytic	1	0.7
c.1241A > G	p.Tyr414Cys	Exon 12	Missense	Oligomerization	1	0.7
c.473G > A	p.Arg158Gln	Exon 5	Missense	Catalytic	1	0.7
c.168 + 5G > A	IVS2 + 5G > A	Intron 2	Splicing	Regulatory	1	0.7
c.1315 + 1G > A	IVS12 + 1G > A	Intron 12	Splicing	Oligomerization	1	0.7
c.1199 + 5G > A	IVS11 + 5G > A	Intron 11	Splicing	Oligomerization	1	0.7
c.1200-?_1315+?Del	p.Asn401_Ser439del	Exon 4	Deletion	Catalytic	1	0.7

icity of phenylalanine (Singh et al. 2016). In Chile, the diet is the only treatment available. Identifying patient phenotype allows for early categorization of disease severity and identification of the classic PKU patients, which are the most vulnerable. Many barriers have been described for treatment compliance, such as the Phe-free substitute's palatability and difficulties in food preparation, among others. These factors make optimal metabolic control difficult, especially in classic PKU patients because they tolerate less daily Phe intake and have higher Phe levels, therefore monitoring of these variables should be more meticulous. This study helps to complete all the information with respect to our PKU patients and allows for personalized treatment and follow-up.

Conclusions

Chilean genotype characterization allowed us to identify the predicted phenotype in our patients, which could guide medical and nutritional management.

We completed mutation analysis for the majority of pathogenic variant alleles in the *PAH* gene for our Chilean patients. The most common mutations in our patients were p.Val388Met, c.442-?_509+?del and c.1066-11G > A. Because of the shared history of Spanish colonization these results may be extrapolated to the rest of Latin America, assisting other countries to direct study to the most common mutations lowering costs and time.

There was a correlation between predicted phenotype and observed phenotype in our PKU patients. However cases with the p.Val388Met mutation have a more severe phenotype than expected – an important consideration for treatment.

The complete categorization of patient's phenotype is essential for our center to identify the most vulnerable patients (classic PKU) and create strategies to prevent any difficulties in long-term nutritional treatment.

Table 2 Genotype and phenotype of PKU patients in Chile

Mutations	# of patients	AV1	AV2	SUM AV	Predicted phenotype	BH4
p. Val388Met/p. Val388Met	4	4	4	8	Mild/MHP	Yes
IVS10-11G > A/Ex5del	3	1	1	2	Classic	Undefined
Ex5del/Ex5del	3	1	1	2	Classic	Undefined
IVS10-11G > A/IVS10-11G > A	2	1	1	2	Classic	No
IVS10-11G > A/IVS8-7A > G	2	1	1	2	Classic	Undefined
IVS10-11G > A/p. Val388Met	2	1	4	5	Mild	No
IVS10-3C > T/p. Val388Met	2	1	4	5	Mild	Undefined
IVS4 + 5G > T/p. Val388Met	2	1	4	5	Mild	No
IVS8-7A > G/Ex5del	2	1	1	2	Classic	Undefined
IVS8-7A > G/p. Ser349Pro	2	1	1	2	Classic	Undefined
p. Val388Met/p. Glu280Lys	3	4	1	5	Mild	No
p. Arg243Gln/p. Glu280Lys	2	1	1	2	Classic	Undefined
p. Arg252Trp/Ex5del	2	1	1	2	Classic	Undefined
p. Arg261Gln/p. Val388Met	2	4	4	8	Mild/MHP	Yes
IVS10-11G > A/IVS10-3C > T	1	1	1	2	Classic	Undefined
IVS10-11G > A/p. Pro275Arg	1	1	U	U	U	Undefined
IVS10-11G > A/p. Arg158Gln	1	1	1	2	Classic	No
IVS10-3C > T/IVS4 + 5G > T	1	1	1	2	Classic	Undefined
IVS10-3C > T/IVS7 + 1G > A	1	1	1	2	Classic	Undefined
IVS4 + 5G > T/IVS10-11G > A	1	1	1	2	Classic	No
IVS6-7A > T/IVS6-7A > T	1	1	1	2	Classic	Undefined
IVS8-7A > G/IVS10-11G > A	1	1	1	2	Classic	No
IVS8-7A > G/IVS7 + 1G > A	1	1	1	2	Classic	Undefined
p. Glu280Lys/IVS12 + 1G > A	1	1	1	2	Classic	No
p. Glu280Lys/p. Asn401_Ser439del	1	1	1	2	Classic	Undefined
p. Glu390Gly/IVS2 + 5G > A	1	4	1	5	Mild	Yes
Ex5del/IVS11 + 5G > A	1	1	1	2	Classic	Undefined
p. Leu249Phefs*92/p. Val388Met	1	1	4	5	Mild	Undefined
p. Pro275Arg/Ex5del	1	U	1	U	U	Undefined
p. Pro275Arg/p. Pro275Arg	1	U	U	U	U	Undefined
p. Pro281Leu/Ex5del	1	U	1	U	U	Undefined
p. Arg111*/p. Thr278Asn	1	1	1	2	Classic	Undefined
p. Arg176Leu/p. Pro281Leu	1	8	1	9	MHP	Yes
p. R252W/IVS10-3C > T	1	1	1	2	Classic	Undefined
p. Arg252Trp/p. Pro281Leu	1	1	1	2	Classic	No
p. Arg252Trp/p. Val388Met	1	1	4	5	Mild	No
p. Arg261Gln/p. Glu390Gly	1	4	8	12	MHP	Yes
p. Arg261Gln/Ex5del	1	4	1	5	Mild	Undefined
p. Arg261Gln/p. Pro281Leu	1	4	1	5	Mild	No
p. Arg261Gln/p. Arg243Gln	1	4	1	5	Mild	Yes
p. Arg261Gln/p. Arg261Gln	1	4	4	8	Mild/MHP	Yes
p. Thr278Asn/p. Val388Met	1	1	4	5	Mild	Undefined
p. Val388Met/Ex5del	1	4	1	5	Mild	Undefined
p. Tyr414Cys/Ex5del	1	4	1	5	Mild	Undefined
Total	63					

AV1 arbitrary value by Guldberg to mutation in allele 1. AV2 arbitrary value by Guldberg to mutation in allele 2. SUM AV sum of both AV to predict phenotype. BH4 BH4 response searched in BIOPKU database for each genotype. MHP mild hyperphenylalaninemia. U undefined

Table 3 Genotype and phenotype correlation in patients with the p. Val388Met mutation

Mutations	Expected phenotype	Observed phenotype
p. Val388Met/p. Val388Met	Mild/moderate	Mild
p. Val388Met/p. Val388Met	Mild/moderate	Mild
p. Val388Met/p. Val388Met	Mild/moderate	Classic
p. Val388Met/p. Val388Met	Mild/moderate	Mild
p. Thr278Asn/p. Val388Met	Mild	MHP
p. Glu280Lys/p. Val388Met	Mild	Classic
p. Glu280Lys/p. Val388Met	Mild	Classic
p. Glu280Lys/p. Val388Met	Mild	Moderate
p. Arg252Trp/p. Val388Met	Mild	Classic
p. Leu249Phefs*92/p. Val388Met	Mild	Classic
p. Arg261Gln/p. Val388Met	Mild/MHP	Mild
p. Arg261Gln/p. Val388Met	Mild/MHP	Classic
IVS4 + 5G > T/p. Val388Met	Mild	Classic
IVS4 + 5G > T/p. Val388Met	Mild	Classic
IVS10-11G > A/p. Val388Met	Mild	Classic
IVS10-11G > A/p. Val388Met	Mild	Classic
IVS10-3C > T/p. Val388Met	Mild	Classic
IVS10-3C > T/p. Val388Met	Mild	Classic
Ex5del/p. Val388Met	Mild	Classic

MHP mild hyperphenylalaninemia

Compliance with Ethics Guidelines

Conflict of Interest

Valerie Hamilton, Lorena Santa María, Karen Fuenzalida, Paulina Morales, Verónica Cornejo, Belén Pérez, Magdalena Ugarte, and Lourdes Desviat declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). The IRB and the Institute of Nutrition and Food Technologies (INTA) approved a waiver of consent for registries and chart reviews.

Details of the contributions of individual authors:

Valerie Hamilton contributed to the study conception and design; acquisition, analysis and interpretation of the data, drafting of the manuscript and patient follow-up.

Karen Fuenzalida contributed to the study conception and analysis of the data.

Paulina Morales contributed to the study conception.

Lorena Santa María contributed to the study conception and design, acquisition, analysis and interpretation of the data, and revising the manuscript.

Juan Francisco Cabello contributed to revising the manuscript and patient follow-up.

Verónica Cornejo contributed to acquisition of the study and patient follow-up.

Belén Pérez contributed to the study conception, analysis of the data, and revising the manuscript.

Magdalena Ugarte contributed revising the manuscript.

Lourdes Desviat contributed revising the manuscript.

All authors gave approval of the final manuscript.

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