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Genetic and phenotypic aspects of phenylalanine hydroxylase deficiency in Spain: molecular survey by regions

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We present an extensive study of the genetic diversity of phenylalanine hydroxylase deficiency in the Spanish phenylketonuria population. We have analysed 195 PKU patients by DGGE analysis identifying 67 different mutations which represent 89% of the total mutant chromosomes. Seventeen mutations first described in Spain have not yet been detected elsewhere; ten of these are reported here for the first time. The clinical significance of this high genetic heterogeneity was examined by analysing the genotype–phenotype correlations, mainly focusing on the mild hyperphenylalaninaemia (MHP) phenotype. The genotypes found in a group of 93 MHP patients, the largest analysed so far, are described in detail, as well as the relative frequencies of the MHP mutations identified. From the total pool of mutations, 27 can be considered severe, 18 can be defined as mild and 13 as associated with MHP. The prevalent mutations correspond to one severe mutation (IVS10nt-11), one MHP mutation (A403V) and two mild mutations (I65T and V388M). The high frequency of mutations with a low degree of severity can explain the relatively higher prevalence of MHP and mild PKU phenotypes in Spain compared with Northern European populations. We have looked at the geographical distribution in Spain of the more common mutations, finding evidence of local mutation clustering, which could be the result of differences in the ethnic background and/or of genetic drift within each region.

Keywords: PKU mutations; geographic distribution; MHP genotypes; phenotype–genotype correlations

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Introduction

Mutations in the phenylalanine hydroxylase (*PAH*) gene are responsible for phenylketonuria (PKU), an autosomal recessive disorder that affects 1:10 000 Caucasians. As a consequence of the *PAH* enzyme impairment, phenylalanine (Phe) accumulates in physiological fluids and, if left untreated, neurological damage occurs. The analysis of the molecular basis of PKU worldwide has led to the identification of more than 350 different *PAH* gene mutations which have been systematically compiled by the *PAH* Mutation Analysis Consortium (<http://www.mcgill.ca/PAHdb>). The populations analysed present great variability in the mutational spectrum, according to their past genetic history. In Northern European populations, two mutations (IVS12nt1 and R408W) account for 60% of the total PKU chromosomes,¹ whilst in Southern European populations there is a greater genetic heterogeneity, eg in Spain, the three prevalent mutations (IVS10nt-11, I65T and V388M) are responsible for only 30% of the mutant alleles.²

The underlying genetic heterogeneity is the basis of the biochemical and clinical diversity of the disorder. It is now evident, from *in vitro* expression studies and from the increasing knowledge obtained in the study of the genotype–phenotype correlations, that the different mutations exert different effects on the *PAH* enzyme activity and stability, allowing prediction of the outcome in each patient depending on the combination of mutations present. An important issue derived from these results is the definition of MHP mutations which always confer the MHP phenotype, regardless of the mutation present on the other allele, which is particularly useful for distinguishing patients requiring dietary treatment from those who do not.

In previous studies of the Spanish PKU population 40 different mutations were detected after DGGE gene scanning of 129 mutant chromosomes.² In this report we present an update on the spectrum of PKU mutations in the Spanish population from a study of 390 independent alleles. We have looked at the geographic backgrounds of the mutations in order to map the distribution of each PKU mutation in Spain. The examination of patients' phenotypes expands previous work,³ allowing estimation of the severity of most of the mutations detected. In particular, an extensive molecular study of 93 MHP patients further delineates the genotypes responsible for this phenotype.

Patients and Methods

The study includes whole blood samples from 195 patients, referred to Madrid for genotype analysis, from different hospitals and follow-up centres in central Spain (mainly the Madrid area), the north (Basque country), north-west (Galicia), south and east of Spain. In the analysis of the distribution of the PKU mutations, we have defined geographical subsets after investigating the origins of parents and grandparents, taking into account also the strong association between surnames and defined Spanish regions. In the central region of Spain we have included patients living in Madrid but whose grandparents have different origins, as is the general rule in big capital cities, so as to define a geographical region representative of the overall demographic profile in Spain.

Patients were considered as having hyperphenylalaninaemia when the serum phenylalanine levels at diagnosis were higher than 120 µmol/L and after exclusion of a defect in tetrahydrobiopterin metabolism. Among the patients, 28 (14.4%) had classical PKU (tolerance < 400 mg Phe/day), 16 (8.3%) had moderate PKU (tolerance 400–500 mg Phe/day), 41 (21%) had mild PKU (tolerance 500–900 mg Phe/day) and 93 (47.6%) corresponded to mild hyperphenylalaninaemia (MHP, without dietary restriction), as defined previously.³ For 17 patients, most of them not compliant to diet or lost to follow-up, phenotype assignment was not possible.

Genomic DNA from patients and parents was extracted from whole blood using standard procedures.⁴ In some cases, PCR amplification was performed directly using dried blood spots as source of DNA.⁵ The 13 genomic fragments covering the entire coding region and splice junctions of the *PAH* gene were amplified and analysed by simultaneous 'broad-range' DGGE.⁶ All fragments displaying an aberrant migrating band pattern in the DGGE gel were subjected to direct sequencing using the *fmol* sequencing kit (Promega, Madrid, Spain) and analysed on an automated DNA sequencer (ALF express, Pharmacia, Barcelona, Spain). When possible, the identified mutations were confirmed by restriction analysis (either directly or after creating the restriction site in the amplification, ACRS) of PCR products amplified again in affected subjects and their parents. The Mendelian inheritance was confirmed when parental samples were available by DGGE or restriction enzyme digestion.

In the estimation of mutation severity, after examining the observed phenotype for homozygous and functionally hemizygous patients, we have also taken into account the residual activity from those mutations expressed *in vitro* (data accessible at the *PAH* Mutation Analysis Consortium web site, <http://www.mcgill.ca/PAHdb>) and the nature of the mutation, regarding stop, nonsense and splicing mutations as severe.

Results

Mutational Spectrum

In a previous study, 63 PKU families representing 129 independent chromosomes were analysed by DGGE and direct sequencing.² Most of the families lived in Madrid, having diverse geographical origin due

to immigration and admixture, and the sample was considered representative of the Spanish population. To validate and extend these results we have now incorporated into the *PAH* gene analysis patients from different follow-up centres in Spain. A total of 195 PKU patients have now been studied and we have increased the number of mutations detected from 40 to 67 (Figure 1), ten of them are novel. From the 67 identified mutations, 39 are disease causing, proved by *in vitro* expression or deduced from the nature of the mutation. The remaining mutations are unlikely to be neutral polymorphisms, following the criteria defined,⁷ although expression analysis is necessary to confirm this.

We have characterised 89% of the total mutant alleles. The four most frequent mutations (IVS10nt-11, A403V, V388M and I65T), with frequencies < 6%, account for only 31% of the total PKU chromosomes. From the remaining mutations 39 are very rare, present only on one or two mutant chromosomes and the others have frequencies between 0.8–4.5%.

Novel Mutations

The ten previously unreported mutations correspond to eight missense mutations (D59Y, N61K, E76G, R169H, R176Q, P275R, A313V, I406T), one deletion causing a frameshift (G352fsdelT) and one splicing mutation (IVS3nt-1) (Table 1). The N61K mutation was detected on two alleles from two heterozygous patients and the remaining mutations were present on one allele.

Geographic Distribution

We have examined the relative frequencies of the more common mutations in defined geographical subsets, the central region of Spain (mainly the Madrid area), Galicia (north-west Spain), Andalusia (south Spain),

and the Mediterranean area (east coast). Another population subset within Spain with a clear ethnic origin, pre-Indoeuropean, are the Basques.⁸ However, PKU patients referred from the centres in the Basque

Figure 1 Schematic representation of the *PAH* gene with the location of the 67 mutations and 7 neutral polymorphisms detected in the Spanish PKU population

Table 1 Novel mutations identified in this study in Spanish PKU patients, showing the observed genotype-phenotype correlations

Mutation	Nucleotide change	Codon N/M	Genotypes	Phenotypes
D59Y	c.175 G→T	GAT/TAT	D59Y/A403V	MHP
N61K	c.183 C→G	AAC/AAG	N61K/IVS1nt5	MHP
			N61K/R176L	MHP
E76G	c.227 A→G	GAG/GGG	E76G/P122Q	MHP
IVS3nt-1	c.353-1 g→c	353-1 g→c	IVS3nt-1/S349P	classic PKU
R169H	c.506 G→A	CGC/CAC	R169H/P281L	MHP
R176Q	c.527 G→A	CGA/CAA	R176Q/R158Q	MHP
P275R	c.824 C→G	CCC/CGC	P275R/L348V	mild PKU
A313V	c.938 C→T	GCA/GTA	A313V/nd	MHP
G352fsdelT	c.1056delT	1056delT	G352fsdelT/I306V	MHP
I406T	c.1217 T→C	ATA/ACA	I406T/I65T	mild PKU

Nucleotide numbering starts at the ATG translation initiation codon, the corresponding GenBank accession number is U49897; nd: not detected after DGGE analysis

country had, in most cases, different origins and have been included in other geographical subsets. In the only patient with clear Basque ancestry, up to four generations, and a mild PKU phenotype, no mutations were found after DGGE and subsequent sequencing of the coding regions and known intronic sequences of the *PAH* gene.

When we analyse the Spanish PKU mutations by geographical region we observe a uniform distribution of IVS10nt-11, the prevalent mutation in Spain, but some of the other common mutations, although present overall, are clustered in specific regions (Table 2). Studies with less frequent mutations did not give any significant values (data not shown).

In Galicia, the predominant mutation (after IVS10nt-11) is R261Q, and the A300S mutation is also clustered in this region, with very few alleles found elsewhere. However, I65T and A403V are clearly less frequent than in other regions, or compared with the overall frequency.

The Spanish subpopulation in the south of Spain (Andalusia) has received influences from all the Mediterranean cultures, as well as a strong Arab influence. We have found an extremely high frequency of A403V and D415N mutations in this region (Table 2), which also reflects the predominating MHP phenotype present in this region (approximately 50% of the total cases). In contrast, the I65T mutation is not frequently detected here, and R261Q is totally absent.

The Mediterranean subset, which has received influence from different Mediterranean cultures (Romans, Greeks, Phoenicians), shows high frequencies of the mutations A403V and R243Q and a low frequency of V388M.

As expected, in central Spain the mutation frequencies correspond largely to the overall frequencies. This probably reflects the heterogeneous origins of the

population around a large capital city, due to immigration and admixture.

Genotype–Phenotype Correlations: Mutation Severity

Detailed genotype–phenotype correlations of the Spanish population have been documented elsewhere.³ The data recently obtained with the patients incorporated into this study further expand the pattern of associated phenotypes with each mutation, compiled by the *PAH* Analysis Consortium Database (<http://www.mcgill.ca/PAHdb>). To date, 137 different genotypes have been identified in Spanish patients. From these, those cases corresponding to I65T or R261Q combined with null mutations (functionally hemizygous patients), have no clear association with a single phenotype, confirming previous results.^{3,9,10} Thus, I65T combined with P281L results in classical PKU, but with S196fs or IVS7nt1 it results in moderate PKU, and with either IVS10nt-11 or R111X it results in mild PKU. R261Q with S196fs was found in a classic PKU patient, but it was also detected in moderate PKU patients combined with IVS10nt-11, R408W or P281L. Both I65T and R261Q (25% and 30% residual activity, respectively) combined with mild mutations always result in mild PKU. Another mutation which associated with null mutations results in different phenotypes is R176L. It is present always in MHP patients, even combined with IVS10nt-11, Y204X or R243X, but with Q304Q, a mutation presumably affecting splicing, it results in mild PKU. These cases represent the only inconsistencies in the genotype–phenotype correlations in our population.

After examining the phenotype in homozygous and functionally hemizygous Spanish patients, and taking into account the nature of the mutation and the residual *in vitro* activity (available for 23 of the

Table 2 Relative frequencies of PKU mutations in geographical subsets within Spain

Mutation	Central Spain <i>n</i> =154	Galicia (north-west) <i>n</i> =78	South of Spain <i>n</i> =96	Mediterranean (east) <i>n</i> =40	Overall <i>n</i> =390
	Mutation frequency (%)				
IVS10	9	11.5	8.3	12.5	9.8
V388M	4.5	6.4	10.4	2.5	6.2
I65T	9.5	3.8	3.1	10	6.7
A403V	7	1.3	14.6	12.5	8
R261Q	2	10.3	0	5	4
R243Q	3.2	3.8	2	7.5	3.6
A300S	0.6	6.4	1.2	0	1.8
D415N	3.2	0	7.3	0	3.3
Identified (%)	91	86	93	90	89

n: number of alleles

mutations present in Spain), we have deduced the mutation severity for 58 (86%) of the mutations (Table 3). The prevalent mutations in Spain correspond to one severe mutation (IVS10nt-11), one MHP mutation (A403V) and two mild mutations (I65T and V388M). The estimation of the mutation severity coincides with results in other Caucasian populations.⁹⁻¹¹

MHP Patients

We have studied the molecular basis of the MHP phenotype in 93 patients, defining 13 different mutations associated with MHP (Table 3). In this group of patients the prevalent MHP mutations are A403V (16.7%), R176L (8.6%) and D415N (6.5%). T380M, A300S and V230I are present in 5%, 3.7% and 3.2%, respectively, of the alleles, and the remaining mutations have frequencies < 1.5%.

In the MHP patients we have detected 68 different genotypes (Table 4), which correspond to combinations of

Table 3 Classification of PKU mutations detected in Spain, based on the estimation of their severity

Severe	Mild	MHP	Undefined
IVS1nt5	F39L	N61K	L48S
G46S ^a	I65T ^a	S87R	D59Y
IVS3nt-1	R68S	R169H	E76G
R111X	A104D ^a	R176L	P147S
D151G	P122Q	V230I	R176Q
A165T	D129G	V245A	P275R
R176X	R158Q ^a	A300S	R297C
S196fs	N167I	I306V	A313V
Y198fs	E178G ^a	A322G	I406T
Y204X	G218V ^a	T380M	
P211fsdelC	R243Q ^a	E390G	
R243X ^a	P244L ^a	A403V ^a	
R252W ^a	R261Q ^a	D415N	
Y277D	L348V ^a		
E280K ^a	V388M ^a		
P281L ^a	P407S		
IVS7nt1	R408Q ^a		
IVS7nt3	Y414C ^a		
Q304Q			
IVS8nt-7			
A309V			
L311P ^a			
S349P ^a			
G352fs			
IVS10nt-11 ^a			
R408W ^a			
IVS12nt1 ^a			

^amutations expressed *in vitro*

Table 4 Genotypes observed in Spanish MHP patients

Allele 1	Null mutations	Allele 2 Missense mutations ^a	MHP mutations
A403V	E280K IVS10nt-11 IVS8nt-7 R111X S349P	R68S D59Y I65T G218V P122Q P147S E178G R243Q R158Q V388M	R176L A300S A403V
R176L	IVS10nt-11 R243X Y204X P281L	R243Q R261Q V388M Y414C	D415N E390G N61K A403V
D415N	E280K IVS10nt-11 R243X S349P	I65T L348V R261Q V388M	R176L T380M
T380M	IVS1nt5	I65T R243Q	T380M A300S D415N
V230I	IVS10nt-11 L311P R158Q Y198fs	V388M	
A300S		I65T R243Q V388M R243Q	A403V T380M
V245A	IVS1nt5 R243X		
E390G	IVS12nt1		R176L
S87R	IVS10nt-11		
N61K	IVS1nt5		
R169H	P281L		
A322G	P211fsdelC		
I306V	G352fsdelT		
E76G		P122Q	
R176Q		R158Q	
V388M		N167I R68S	

^acorresponding to either mild mutations or mutations of undefined severity, see Table 3.

- a) a MHP mutation with either a severe or a mild mutation,
- b) two MHP mutations, or
- c) two mild mutations.

In the course of this study, four individuals with very slight hyperphenylalaninaemia (125–180 μ M) were referred to us for genetic analysis. DGGE gene scanning ruled out any mutations in the coding and adjacent intronic regions of the *PAH* gene, finally discarding a *PAH* deficiency.

Discussion

A total of 390 independent Spanish PKU chromosomes has been found to carry 67 different mutations, among them 17 are at present unique to our population and 10 of them are described here for the first time. Increasing the number of analysed alleles with respect to earlier studies has resulted in the detection (on one or two alleles) of mutations prevalent in Northern Europe (IVS12nt1 and R408W) and which were considered before to be totally absent in our population.² In this update on the spectrum of PKU mutations in Spain, there is a significant increase (8% versus 3.1%) in the relative frequency of A403V respect to earlier studies, where the MHP phenotype was under-represented in the total patient sample. In the present work, 47.6% of the analysed patients exhibit the MHP phenotype, more in agreement with the pattern of phenotype distribution observed in Spain (according to the 1996 census of the National Screening Programme, 54% of the total positive cases, 1:9500, corresponded to MHP patients). A403V appears now as the second most frequent mutation in Spain (8%), after IVS10nt-11 (10%). The relative frequencies of the remaining mutations basically resemble those previously described.²

Overall, the sample of 195 patients analysed by DGGE represents different geographical subsets, ascertained by genealogical studies and origin of the surnames, which are related to specific regions of Spain. Patients in central Spain (Madrid area) represent all the different regions, corroborated by the similarities in the mutation distribution of this sample and the total one. In the remaining geographical subsets (Table 2), we observe patterns of mutation clustering, as has been reported for CF.¹² In particular, in Galicia there was an unexpected distribution of CFTR alleles, analogous to what we have observed in the study of PKU mutations. This is probably due to genetic drift and the relative isolation of this population, both geographically and migrationally (practically no immigration). Although Galicia seems to have received Celtic influences from the North Atlantic coast during the first millennium BC, the present study reveals a lower frequency in this region of I65T, a mutation of proposed Celtic origin.¹³ A recent analysis of mtDNA of the Galician population demonstrates that the referred contact with the Celts had little demographic effect and places Galicians at the genetic edge of European variation.¹⁴

It can be assumed that the clustering of R261Q mutation in Galicia and in other regions^{15,16} is caused

by a local increase through genetic drift. In Portugal, R261Q is the second most frequent mutation (10.4%),¹⁷ which could also account for its elevated frequency in Galicia, geographically close.

At present, genetic differences tend to disappear with migration and admixture between regions. However, stratification of PKU mutations is evident in Spain and in other countries,^{18–20} reflecting the maintenance of population subsets. In this way, the various demographic histories of the Spanish population may account for the non-uniform distribution of PKU mutations other than IVS10nt-11.

In the analysis of the genotype–phenotype correlations we have used functionally hemizygous genotypes to evaluate the effect of missense alleles. Our findings have clinical relevance, as the estimation of the severity of most of the mutations in Spain confirm data from other populations,^{9–11} allowing an increasingly reliable prediction of the biochemical phenotype. In the case of I65T and R261Q, it is worth noting that, when expressed *in vitro*, these two mutations as well as others for which discordant genotype–phenotype associations have been referred (V388M, Y414C, R158Q), all show immunoreactive protein. In medium chain acyl-CoA (MCAD) deficiency, the amount of stable mutant protein and the residual activity can be modulated changing the cultivation temperature and the levels of chaperonins, providing clues to explain the variable phenotypical manifestations of the disorder.^{21,22} In PKU, where most expression studies have shown decreased stability of the mutant proteins, such mechanisms involving the protein quality control system²³ could also underlie the variable phenotypic expression of unstable mutant PAH proteins.

The high frequency of mutations with a low degree of severity can explain the relatively higher prevalence of MHP and mild PKU phenotypes in Spain compared with Northern European populations. In this work we detail the genotypes found in a group of 93 MHP patients, the largest analysed so far. Overall, there are 13 mutations which are unambiguously associated with the MHP phenotype (Table 3). The pattern of MHP mutations, with a prevalence of A403V (16.7%), differs from that in other populations; in Denmark the predominant MHP mutation is D415N,²⁴ in Sicily the highest frequency corresponds to A300S¹¹ and in Ireland to T380M.²⁵

In the course of this study, four patients were found to present no mutations in the analysed regions of the *PAH* gene. Although mutations elsewhere in the gene

could not be definitely ruled out, a *PAH* deficiency was finally discarded, considering that the phenylalanine levels were borderline (125–180 μ M) with the generally accepted threshold for dietary intervention. This extends the usefulness of DGGE analysis to distinguish patients with phenylalanine levels close to the cut-off from the normal range.

Although one might suppose, in view of the multiple mutations identified at present in the *PAH* gene, that the detection of novel mutations is becoming rare, it is evident that in the total pool of PKU chromosomes there is a very high number of rare mutations which are being discovered progressively as more alleles are studied worldwide.

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