Evidence in Latin America of Recurrence of V388M, a Phenylketonuria Mutation with High In Vitro Residual Activity

Lourdes R. Desviat, Belén Pérez, Marisel De Lucca, Veronica Cornejo, Benjamin Schmidt, and Magdalena Ugarte

Centro de Biología Molecular "Severo Ochoa" CSIC-UAM, Universidad Autónoma de Madrid, Madrid; INTA, Santiago; and APAE, São Paulo

Summary

Phenylketonuria mutation V388M is frequent in the Iberian Peninsula. In vitro, the V388M mutant enzyme has similar immunoreactive protein and phenylalanine hydroxylase mRNA and has 43% residual activity, which correlates well with the mild phenotype exhibited by the homozygous patients. In Spain it has been detected in 5.7% of the mutant alleles and is always associated with haplotype 1.7. This mutation is also present in high frequency in some Latin American countries (Brazil, 9%; Chile, 13%). It is interesting that in Chile most of the alleles bearing this mutation carry haplotype 4.3, although in Brazil it is found only on the background of haplotype 1.7. The origin of V388M in Spain on haplotype 1.7 and in Chile on haplotype 4.3 is clearly different. Recurrence is the most plausible explanation, because the mutation involves a CpG dinucleotide, and a recombination event transferring the mutation from haplotype 1 to 4 is unlikely.

Introduction

Phenylketonuria (PKU) is a recessive metabolic disorder affecting ~1/10,000 individuals in the Caucasian population. The gene responsible for PKU, the phenylalanine hydroxylase (PAH) gene, has been cloned and sequenced (Kwok et al. 1985), and, to date, >170 different mutations have been detected. In many cases, strong associations have been found between mutations and RFLP haplotypes defined by seven diallelic polymorphisms and a VNTR system in the PAH gene (Eisensmith and Woo 1992). A short tandem repeat (STR) polymorphism has also been described in intron 3 of the PAH gene (Goltsov et al. 1993). Some mutations have been observed on different haplotype backgrounds: E280K on haplotypes 1, 4, and 38 (Lyonnet et al. 1989; Okano et al. 1990); IVS10nt546 on haplotypes 6, 10, and 36 (Dworniczak et al. 1991); or R408W on haplotypes 1, 2, and 44 (Tsai et al. 1990; Eisensmith et al. 1995). Possible mechanisms to account for the multiple haplotype associations include recombination, gene conversion, and recurrence.

There has been much speculation about the origin and evolution of the PKU alleles throughout the world. The PAH locus appears to have a low mutation and recombination rate, since most populations present a limited number of prevalent mutant alleles. The strong associations of mutations and RFLP, VNTR, and STR haplotypes in the PAH gene may be the result of specific founding events (Wang et al. 1991; Eisensmith et al. 1992).

In some areas, human migration may have played a role in the distribution of PKU mutations. In Latin American countries, recent studies have shown the presence of Mediterranean PKU mutations introduced there by Southern European settlers (Pérez et al. 1993, 1994a). Latin America is ethnically very heterogeneous, each country arising from a particular mixture of Caucasians from Southern Europe (mainly Spain), Amerindians derived from Mongolian populations, and Negroids from Africa (Arzimanoglou et al. 1995). The analysis of the prevalent PKU mutations in Latin America and their origin will provide information on the genetic basis of PKU in these countries.

Mutation V388M was first detected in a Japanese patient (Takahashi et al. 1992) and has been described as one of the major Portuguese mutations, with a frequency of 19% (Leandro et al., in press). In Spain it has been found associated with haplotype 1.7 (Pérez et al. 1994b). In this study, we have analyzed this mutation and the PAH polymorphisms in Spain, São Paulo (Brazil), and Chile to investigate the origin of V388M in Latin America. We have also analyzed the PAH polymorphisms in the Japanese patient with V388M. We have performed the in vitro expression analysis of V388M in COS cells, correlating the residual activity with the biochemical phenotype in the patients. This work provides a starting point in the understanding of the genetic heterogeneity of PKU in the New World.

Subjects and Methods

This study includes unrelated PKU patients from Spain (234), Chile (73), and Brazil (79). The patients
from Spain come from different screening and follow-up centers all over the country. Among the patients diagnosed in Madrid, there is one of Lebanese origin. The sample from Chile can be considered representative of the whole country, while in Brazil we are analyzing only patients from São Paulo, who may represent a population ethnically different from that found in other Brazilian regions. In both Santiago de Chile and São Paulo, screening is universal, and the PKU cases are representative of the demographic profile.

In some cases, DNA from the family members was also available for the analysis of polymorphisms in the PAH gene. The source of DNA was whole blood or dried blood spots from routine screening and follow-up.

Mutation V388M was detected by digestion of amplified exon 11 with BsaAI (Leandro et al., in press). The BsaAI restriction site (TAC/GTG) is eliminated in the V388M mutation (TACATG). The V388M samples were also digested with NlaIII (restriction site CATG), to confirm the G-to-A substitution in the first nucleotide of codon 388. Primers and PCR conditions for the amplification of DNA from dried blood spots have been described elsewhere (Pérez et al. 1993).

RFLP haplotypes (EisenSmith and Woo 1992) were determined by a combination of Southern blotting and hybridization with a full-length cDNA probe (for EcoRI and EcoRV polymorphisms), PCR and restriction enzyme digestion (for BglII, PvuII(a), PvuII(b), MspI, and XmnI polymorphisms), and PCR and electrophoretic examination of the VNTR alleles in the 3' end of the PAH gene (Goltsov et al. 1992). In some cases, only dried blood spots from the proband and family were available, thus excluding the examination of the EcoRV and EcoRI polymorphisms. To aid in the identification of alleles, ascertaining the presence or absence of haplotype 4.3, the silent polymorphisms Q232Q and V245V were examined by digestion of amplified exons 6 and 7 with DdeI and AulI, respectively (Lichter-Konecki et al. 1994). A Japanese patient, who is a compound heterozygote V388M/R413P, was also included in the study of PAH polymorphisms. Samples from the patient and family were kindly provided by Drs. Narisawa and Kazutoshi.

The alleles in the STR system in the 3' end of the PAH gene (Goltsov et al. 1993) were amplified with a fluorescent primer and were analyzed using A.L.F. DNA Sequencer and Fragment Manager (Pharmacia). Fluorescent internal lane standards were used as size markers. The allele length reflects the true size of the fragments and thus is 2 bp shorter than the allele lengths given by Goltsov et al. (1993), as described elsewhere by Zschocke et al. (1994).

Expression analysis was performed in COS cells, using the human expression vector pRC/CMV. The constructs with the PAH cDNA and the β-galactosidase cDNA inserted in the pRC/CMV vector were kindly provided by Dr. R. C. Eisensmith. The oligonucleotide-directed mutagenesis kit from Amersham was used to introduce the V388M mutation in the PAH sequence, using the mutagenic oligo 5'-CTGTATTACATGGCAGAGAG-3'. COS cells were transfected with the lipofectin reagent (B.R.L.). Determination of the enzymatic PAH activity, Western blotting, and mRNA analysis were performed as described by Pérez et al. (1993). PAH-immunoreactive protein, PAH mRNA, and Phe-to-Tyr conversion were all quantitated by laser densitometry. Three independent experiments were performed.

Results

Expression Analysis

The functional significance of the V388M mutation was assayed by transient expression of the mutant cDNA in COS cells. β-Galactosidase cDNA was cotransfected with the normal and mutant constructs, and transfection efficiency was found to be comparable in both cases, as monitored by β-galactosidase activity. Cells transfected with normal and mutant cDNAs had similar amounts of immunoreactive protein and steady state levels of PAH mRNA (data not shown). The mutant enzyme had 43% residual activity, when compared to the normal enzyme. (fig. 1)

The examination of the biochemical phenotype in patients with defined genotype is shown in table 1. Because of the socioeconomic conditions of some of the PKU families, some patients do not follow treatment, so only the data of Phe at diagnosis was available. The patients homozygous for V388M exhibit a mild phenotype, with Phe levels at diagnosis between 15–20 mg/dl, and a Phe tolerance of 500–600 mg Phe/d.

Mutation Analysis

Mutation V388M was screened in PKU patients from all over Spain and was detected in 5.7% of the mutant alleles (27/468). The only homozygous patient for
Table 1

<table>
<thead>
<tr>
<th>No. of Patients</th>
<th>Mutations</th>
<th>Residual PAH Activity (%)</th>
<th>Phe at Diagnosis (mg/dl)</th>
<th>Tolerance (mg Phe/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>V388M/V388M</td>
<td>43/43</td>
<td>15–20</td>
<td>500–600</td>
</tr>
<tr>
<td>1</td>
<td>V388M/V388M</td>
<td>43/43</td>
<td>20</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>V388M/IVS10nt546</td>
<td>43/0</td>
<td>20–30</td>
<td>250–400</td>
</tr>
<tr>
<td>1</td>
<td>V388M/IVS10nt546</td>
<td>43/0</td>
<td>&gt;20</td>
<td>ND</td>
</tr>
<tr>
<td>1</td>
<td>V388M/Y414C</td>
<td>43/43</td>
<td>15.2</td>
<td>850</td>
</tr>
</tbody>
</table>

NOTE.—Table shows the Phe at diagnosis and Phe tolerance in patients with both PKU mutations characterized. Residual PAH activity corresponds to the mutant enzyme activity in vitro compared with the wild-type PAH: IVS10nt546 (Dworzanski, 1991) and Y414C (Okano et al., 1991). ND = not determined.

V388M was of Lebanese origin. In Latin America, V388M was found in high frequencies in Chile (19 [13%] of 146 mutant alleles) and Sao Paulo (14 [8.8%] of 158 mutant alleles). In Chile and in Sao Paulo, there are two homozygous patients for V388M, and the rest of the alleles correspond to heterozygous patients.

Analysis of Polymorphisms in the PAH Gene

In the patients with V388M we have examined the RFLP, VNTR, and STR haplotypes. In some cases, complete haplotyping was not possible, but the presence of the V245V and Q232Q polymorphisms confirms with high probability the identification of haplotype 4.3 (Lichter-Konecki et al., 1994). In Spain, V388M has been found associated exclusively with haplotype 1.7. The patient with Lebanese origin homozygous for V388M was also homozygous for haplotype 4.3. In Japan, V388M is associated with haplotype 4.3. In Sao Paulo, V388M is associated with haplotype 1.7, while in Chile, V388M is present in the background of haplotypes 1.7 and 4.3. The results are shown in table 2.

Table 3 summarizes the results obtained from the analysis of the HindIII VNTR alleles in all the chromosomes bearing V388M. In Spain and in Sao Paulo, V388M was always found on the background of the 7-repeats VNTR allele. In Chile, 79% (14/19) of the alleles with V388M are associated with haplotype 4.3, and only 21% (4/19) are associated with the VNTR of 7 repeats. This would correspond to an overall frequency of 9.5% (14 of 146 total alleles) of V388M on haplotype 4.3 and 3.4% (5/146) of V388M associated with the VNTR of 7 repeats. The ethnic origin of the Chilean patients with V388M was investigated. The maternal grandparents of one patient carrying haplotype 4.3 were from the Middle East, while one patient with haplotype 1.7 had Spanish grandparents. In the rest of the patients, both parents and grandparents were Chileans, with no known European ancestors.

The analysis of the STR alleles could be performed in 24 alleles with V388M (9 from Chile, associated with both haplotypes 4.3 and 1.7; 6 from Brazil; 6 from Spain; 2 from Lebanon; and 1 from Japan). One allele from Spain and one allele from Japan presented the 242-bp STR allele. The rest of the chromosomes had the 238-bp STR allele. These correspond to the allele lengths of 244 and the 240 bp as defined by Goltsov et al. (1993), which are the most common STR alleles in Caucasian and Oriental chromosomes.

Discussion

The V388M mutation, elsewhere described in Japan and Portugal (Takahashi et al., 1992; Leandro et al., in press) has been expressed in COS cells, revealing that it is a disease-causing mutation. The mutant enzyme has 43% of residual activity. Secondary structure predictions do not indicate a major change in the conformation of the molecule in the region with the valine-to-methionine substitution. The data are compatible with the mild phenotype of the homozygous patients, who present a relatively high Phe tolerance (500–600 mg Phe/d).

The V388M mutation is relatively frequent in the Latin American countries analyzed (Sao Paulo, 9% and Chile, 13%). In the samples analyzed from Sao Paulo, V388M is exclusively associated with the 7-repeats VNTR allele, and the study of RFLP polymorphisms reveals a concordance with haplotype 1.7. The origin can thus be traced to the Iberian Peninsula, where this mutation is prevalent, specially in Portugal (19%; Leandro et al., in press). The haplotype association of V388M in Portugal has not yet been described, but it will probably be haplotype 1.7, as in Spain.

It is interesting that, in Chile, where there has been practically no Portuguese influence, V388M is present in much higher frequency than in Spain (13% versus 5.7%, respectively). RFLP analysis revealed that most of the alleles bearing V388M are haplotype 4.3. In one patient, the origin of V388M on haplotype 4.3 could be traced to the Middle East, as seen from the genealogical data. The ethnic background in the remaining patients provides no information on the origin of the mutant allele. The V388M mutation on haplotype 1.7 has presumably a Spanish origin. Haplotypes 1 and 4 differ at five individual RFLP sites, located at both sides of the mutation, so a recombination transferring the mutation from haplotypes 1 to 4 is unlikely. The fact that V388M involves a CpG dinucleotide, which sustains a higher mutation rate (Cooper and Youssoufian, 1988), makes recurrence the most plausible explanation. Haplotypes 1 and 4 are considered the most ancient and are well represented among normal chromosomes (Konecki and Lichter-Konecki, 1991), thus having a higher probability of sustaining random mutational events.
Table 2

Association of Mutation V388M with Different Polymorphic Markers in the PAH Gene

<table>
<thead>
<tr>
<th>PAH POLYMORPHISMS</th>
<th>HindIII</th>
<th>EcoRV</th>
<th>Q232Q</th>
<th>V245V</th>
<th>HAPLOTYPE</th>
<th>ORIGIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Alleles</td>
<td>BglII</td>
<td>PvuIIa</td>
<td>PvuIIb</td>
<td>EcoRI</td>
<td>MspI</td>
<td>XmnI</td>
</tr>
<tr>
<td>4 ......</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>2 ......</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1 ......</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>5 ......</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>2 ......</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>1 ......</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>8 ......</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

NOTE.—Plus (+) and minus (−) signs indicate, respectively, absence or presence of a polymorphic restriction site. Numbers in the HindIII VNTR correspond to the numbers of repeated units present. ND = not determined.

* Most plausible haplotype, taking into account the PAH polymorphisms analyzed.

Haplotype 4.3 is prevalent among Orientals, and V388M has also been found in a Japanese patient homozygous for haplotype 4.3 (the patient is a compound heterozygote V388M/R413P) (Takahashi et al. 1992). The presence of V388M on haplotype 4.3 in Chile could be explained by an introduction of Oriental PKU chromosomes into the native population of Latin America. Amerindians are believed to be descendants of small migratory Asian populations who crossed the Bering land bridge. Gene frequency data on blood-group markers, HLA types, and mtDNA markers strongly support this hypothesis (Williams et al. 1985; Szathmary 1993), although the mtDNA polymorphisms present in Amerindian tribes actually are rare in Asia (Schurr et al. 1990). This same hypothesis could be applied to mutation V388M associated with haplotype 4.3 present today in Chile and infrequent in Asia. The Chilean population arose predominantly from a mixture of Caucasians and Amerindians. The percentage contribution of Amerindian genes to the Chilean population is 30% (Rios et al. 1994). In contrast, in São Paulo, the population is prevalently Caucasian (of western European descent), which explains the presence of V388M on haplotype 1.7, of Portuguese and Spanish origin. Another fact in support of this view is the finding, after a preliminary analysis in a small number of PKU samples from Mexico, of mutation V388M associated with the 3-repeats VNTR allele (data not shown). This would indicate the presence in Central America of V388M on haplotype 4.3. It is extremely unlikely that Spanish settlers introduced this mutation. A more detailed study of haplotypes and mutations in different Latin American countries could help to clarify this view. Figure 2 shows a summary of the location of the V388M mutation and the haplotype backgrounds studied in this work.

The study of the STR alleles has not provided any additional information, because all the V388M alleles have the 238-bp or the 240-bp STR allele common in Caucasian and Oriental chromosomes. The data have been included for comparison with other studies.

The presence of V388M on haplotype 4.3 in the Lebanese patient can be explained once more by recurrence, recombination, or gene conversion. The parents are not consanguineous, the mother is Jordanian and the father Lebanese, and both populations are predominantly Caucasoid (Cavalli-Sforza et al. 1994). There is no obvious relationship between Lebanon and Japan that could indicate the source of this allele. Although recurrence appears to be the most likely explanation, we cannot completely dismiss other mechanisms, because we have no data available on the frequency and haplotype association of V388M in Middle East. Recurrence may indeed be a common phenomenon in some human disorders. Recently, Morral et al. (1994) showed that some cystic fibrosis mutations have appeared three or four times in different genetic backgrounds.

The results obtained in this study show once more the

Table 3

Analysis of the VNTR Alleles and V245V and Q232Q Polymorphisms in All the Chromosomes with V388M Included in This Study

<table>
<thead>
<tr>
<th>No. of Alleles</th>
<th>HindIII VNTR</th>
<th>V245V</th>
<th>Q232Q</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 .....</td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>Spain</td>
</tr>
<tr>
<td>2 .....</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>Lebanon</td>
</tr>
<tr>
<td>1 .....</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>Japan</td>
</tr>
<tr>
<td>14 .....</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>Chile</td>
</tr>
<tr>
<td>5 .....</td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>Chile</td>
</tr>
<tr>
<td>14 .....</td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>Brazil</td>
</tr>
</tbody>
</table>

NOTE.—Plus sign (+) indicates the presence of a polymorphism, and minus sign (−) indicates the absence of a polymorphism.
utility of analyzing polymorphic markers, to provide insights into the origin and subsequent distribution of disease-causing mutations. V388M is a mild mutation frequent in Latin American countries, where this information may aid in the correct diagnosis and treatment of the PKU patients.

Acknowledgments

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