Am. J. Hum. Genet. 43:914–921, 1988

Clinical and Molecular Heterogeneity of Phenylalanine Hydroxylase Deficiencies in France

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Summary

RFLPs of 68 normal and 74 mutant alleles at the phenylalanine hydroxylase (PAH) locus were determined in 37 French kindreds. A total of 23 haplotypes, including 18 normal and 16 mutant alleles, were observed. Two-thirds of all mutant alleles were confined within only four haplotypes, while the last third was accounted for by 12 haplotypes, including eight haplotypes absent from Caucasian pedigrees reported thus far. Several mutant haplotypes were present in typical phenylketonuria only, others were present in variants only, and some were present in both. In addition, a particular mutant haplotype (haplotype 2) was found to harbor different mutations in our series, resulting in either typical phenylketonuria or in mild hyperphenylalaninemias. The diploid combination of so many mutant haplotypes in PAH-deficient patients and of compound heterozygosity at the PAH locus in southern Europe might account for the broad spectrum of individual phenotypes observed in France.

Introduction

Phenylketonuria (PKU) is an autosomal recessive disease due to a deficiency of hepatic phenylalanine hydroxylase (PAH; phenylalanine-4-mono oxygenase, E.C.1.14.16.1). PAH, which catalyzes the hydroxylation of phenylalanine to tyrosine, is a mixed-function oxidase that requires tetrahydrobiopterin as a cofactor for activity. Mass neonatal screening for PKU has led to the discovery of variant forms of the disease which differ by their residual PAH activity. The absence of PAH activity results in typical PKU with a low tolerance for dietary phenylalanine, while children with residual PAH activity have a higher tolerance in phenylalanine and do not require any dietary treatment.

Using a full-length cDNA clone complementary to PAH mRNA, S. L. Woo's group performed extensive RFLP haplotype analysis at the PAH locus. They showed that 90% of PAH alleles in northern-European Caucasian kindreds are confined to only four RFLP haplotypes (Chakraborty et al. 1987; Güttler et al. 1987). In the present study, which concerned a mixed population of southern- and northern-European ancestry, 74 mutant alleles were associated with 16 RFLP haplotypes. It is interesting that the RFLP haplotypes at the PAH locus in PKU patients differed from that found in children with residual PAH activity. Some RFLP haplotypes (e.g., haplotype 38) were associated with variants only, and others (e.g., haplotypes 1 and 3) were associated with classical PKU. Finally, some RFLP haplotypes (e.g., haplotype 2) were found in both types of variants. In the latter cases, the molecular heterogeneity of the mutant haplotypes could be demonstrated. Therefore, the diversity of mutant haplotypes strongly suggests that several mutations at the PAH locus exist in southern Europe, resulting in either typical PKU or in variants forms of PAH deficiency.

Received March 21, 1988; final revision received August 8, 1988.

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0002-9297/88/4306-0012$02.00

Methods

Patients

Patients with permanent hyperphenylalaninemia and

belonging to 50 unrelated families and followed at the Hôpital des Enfants-Malades, Paris, were classified according to the following four criteria: (1) dietary tolerance for phenylalanine, defined as the highest phenylalanine intake (in milligrams per day) consistent with acceptable phenylalanine levels in plasma (0.2–0.4 μmol/ml); (2) plasma phenylalanine levels after a standard phenylalanine loading test at 1 mo and 12 mo of age (Rey et al. 1987); (3) in vivo estimation of the residual hydroxylating system calculated on the disappearance rate constant of phenylalanine following an intravenous load (Rey et al. 1979); and (4) in vitro measurement of PAH activity assayed by needle biopsy of the liver (Bartholomé et al. 1975).

Thus, the following two groups were arbitrarily defined using the above criteria: (1) typical PKU, characterized by a dietary phenylalanine tolerance of approximately 250 mg/day, plasma phenylalanine levels above 1.5 mmol/liter following oral loading tests, and an in vivo and/or in vitro PAH activity below 1% of normal controls and (2) variant forms of hyperphenylalaninemia characterized by the persistence of a residual PAH activity. This latter group includes (*a*) the mild permanent hyperphenylalaninemias with plasma phenylalanine levels below 0.9 mmol/liter following oral loading tests, a dietary tolerance averaging 750 mg/day, and a residual PAH activity of 4%–6% of normal controls and (*b*) the "Mediterranean form" originally individualized by Mary Efron (1967) and characterized by intermediary values of dietary phenylalanine tolerance (500 mg/day) and a residual PAH activity of 1%–3% of that in normal controls.

Determination of Haplotypes and Their Association with Hyperphenylalaninemias

Genomic DNA was isolated from peripheral blood leukocytes, digested using seven enzymes—including *Pvu*II (two separate polymorphisms), *Bgl*II, *Xmn*I, *Msp*I, *Hind*III, *Eco*RV, and *Eco*RI + *Bam*HI—for determination of RFLP genotypes. The cDNA probe was the recombinant plasmid ph PAH 247, which contains a 2.4-kb DNA insert encoding the entire PAH mRNA (Kwok et al. 1985). RFLP genotypes were established for each nuclear family member, and haplotypes at the PAH locus were determined by comparing RFLP genotypes of parents and sibs. Since hyperphenylalaninemias are autosomal recessive disorders, four independent haplotypes were established in each family, including two normal and two mutant alleles. Therefore, the complete study of the 50 nuclear families provided information on French DNA haplotypes in both normal and mutant PAH genes. Unambiguous determination of haplotypes was possible in 37 of 50 families, for a total of 142 chromosomes (68 normal alleles and 74 mutant alleles). Comparison of RFLP and haplotype frequencies between normal and mutant alleles was made using the χ^2 test. Yates's correction was introduced when necessary.

It must be noticed that in some comparisons the sample size might be too small to observe a statistically significant difference. Indeed, with a false-positive probability of 5% ($\alpha = 5\%$) and a false-negative probability of 10% ($\beta = 10\%$), the sample size required to observe a difference of frequencies of 20% is about 130 (the frequencies being near to 40%–60%).

Results

Frequency of RFLPs at the PAH Locus

Ten RFLPs have been described at the PAH locus (Lidsky et al. 1985). These RFLPs served as markers in following the segregation of normal and mutant PAH genes in 37 families living in France.

Seventy percent of the families studied were indigenous, originally from Britanny, Burgundy, Picardy, Alsace, Jura, and the Paris area. In other cases, at least one grandparent was an immigrant from either south Europe (Spain, Italy, or Portugal) or northern-European countries. Finally, 10% of the families studied were Arab families from northern Africa (Algeria, Tunisia, and Morocco) and were frequently consanguineous.

Table 1 shows the allele frequencies for the RFLP sites at the PAH locus in the population studied. No association between hyperphenylalaninemias and a particular RFLP was apparent at the population level, except for the restriction enzyme *Eco*RI. When the group of mutant alleles was split into alleles from PKU patients or variants, a significant difference appeared between normal alleles and variants for restriction enzyme *Hind*III (table 1). However, since the number of the variant alleles studied remains low, more data are required to confirm this possible association.

RFLP Haplotypes and Frequencies in Normal PAH Genes

The combination of the 10 RFLPs along the PAH genes determines a particular haplotype at this locus. For the 10 RFLPs described, there can theoretically be more than 1,000 different haplotypes at the PAH locus. Nevertheless, a total of 18 RFLP haplotypes (referred to here as described in table 2) were observed among normal PAH genes (table 3). Among them, eight haplo-

Table 1

Allele Frequencies for the RFLP Sites at the PAH Locus in France

RFLP Site	Allele (kb)	1 Normal Allele (N = 68)	2 Mutant Allele (N = 74)	3 PKU (N = 51)	4 Variants (N = 23)	Statistical Comparison 1–2	1–3	1–4	3–4
*Pvu*a	6	69.1	79.7	86.2	65.2	NS	$P < .05$	NS	$P < .05$
*Bgl*II	1.7	32.4	27.0	23.5	34.8	NS	NS	NS	NS
*Pvu*b	9.1	11.8	13.5	7.8	26.1	NS	NS	NS	NS
*Eco*RI	11.0	55.9	37.8	43.1	26.1	$P < .05$	NS	$P < .05$	NS
*Xmn*I	6.5	32.4	24.3	25.5	21.7	NS	NS	NS	NS
*Msp*I	19	60.3	72.0	70.6	78.3	NS	NS	NS	NS
*Hin*dIII	4.0	29.4	28.4	19.6	47.8	NS	NS	NS	$P < .05$
	4.2	64.7	62.1	78.4	26.1	NS	$P < .05$	$P < .01$	$P < .01$
	4.4	5.9	9.5	2.0	26.1	NS	NS	$P < .05$	$P < .01$
*Eco*RV	25.0	47.1	36.5	31.4	47.8	NS	NS	NS	NS

Note.—The frequencies (%) of RFLPs at the PAH locus in normal parental alleles (1) are compared with those of all mutant alleles (2), to alleles from PKU patients (3), or to alleles from variants (4), classified as described in Methods. NS = not significant.

Table 2

RFLP Haplotypes of PAH Locus from Parental Chromosomes in French Families with PAH Deficiencies

	Restriction Enzyme							
	*Pvu*a	*Bgl*II	*Pvu*b	*Eco*RI	*Xmn*I	*Msp*I	*Hin*dIII	*Eco*RV
Haplotype:								
1	+	−	−	−	−	+	−	−
2	+	−	−	−	−	+	+	+
3	+	−	−	+	+	−	−	−
4	+	−	−	+	+	−	+	+
5	−	+	+	+	−	+	−	+
6	−	+	+	+	−	+	−	−
7	−	+	−	+	+	−	−	−
9	+	+	−	+	−	+	−	+
11	−	+	−	+	−	+	−	+
12	+	−	−	−	−	+	=	+
16	+	−	−	+	−	−	+	+
21	−	+	+	+	−	+	=	+
24	+	−	−	−	−	+	+	−
28	+	−	−	−	+	−	+	+
29	−	+	−	−	−	−	−	−
34	−	+	−	+	−	+	−	−
36	−	+	+	−	−	+	−	−
37	−	−	−	−	−	+	−	−
38	−	+	+	−	−	+	=	−
39	+	−	+	+	−	+	−	−
41	+	−	−	+	−	+	+	+
42	−	+	−	+	−	−	−	−
43	+	−	−	−	+	+	−	−
Symbol:								
+	6.0	1.7	9.1	11.0	6.5	19.0	4.0	25.0
−	19.0	3.6	11.5	17.0	9.4	23.0	4.2	30.0
=							4.4	

types were not represented in Caucasian pedigrees reported thus far (Chakraborty et al. 1987; also see table 3).

Although 18 different haplotypes were observed for the normal genes, more than 60% of normal alleles were confined to only four haplotypes (haplotypes 1, 4, 5, and 7), while haplotypes 1–4 accounted for only 50% of normal genes in France (instead of 75% of normal genes in Denmark; $P = .05$).

RFLP Haplotypes and Frequencies in Mutant PAH Genes

The RFLP haplotypes of 74 mutant PAH genes were determined in the 37 families, and a total of 16 different haplotypes were observed. Among them, eight haplotypes not reported so far in Caucasian pedigrees accounted for 19% of mutant genes in the present study (table 3). Although 16 haplotypes were observed among mutant alleles, more than 80% of mutant PAH genes were confined within only six RFLP haplotypes, namely, haplotypes 1–4, 9, and 38. Haplotypes 1–4 alone accounted for 66% of mutant PAH genes instead of the 50% in normal PAH genes ($P = .05$). It is worth noting that the same haplotypes accounted for about 90% of all mutant alleles in Denmark ($P < .001$).

Finally, of the 23 haplotypes detected in the sample, 11 occurred on both normal and mutant chromosomes, seven on normal chromosomes only, and five on mutant chromosomes only. In three cases, abnormal fragments generated by the restriction enzyme *Msp*I (two cases) and *Eco*RI (one case) precluded the determination of the complete haplotype, as recently reported (Litchmer-Konecki et al. 1987). No recombination at the PAH locus was noted in the 142 meioses presented in the present study.

Correlation of Clinical Phenotypes with RFLP Haplotypes at the PAH Locus

Table 3 compares the haplotype frequencies, observed in France, for both normal and mutant alleles at the PAH locus. The table shows that the haplotype fre-

Table 3

Haplotypes Frequencies at the PAH Locus in France

Haplotype	1, Normal Allele ($N = 68$)	2, All Mutant Alleles ($N = 74$)	3, PKU ($N = 51$)	4, Variants ($N = 23$)	Statistical Test 1–2	1–3	1–4	3–4
1	26.5	31.1	39.2	13.0	NS	NS	NS	$P < .05$
2	5.9	17.6	11.8	30.4	$P < .05$	NS	$P < .01$	5%–10%
3	2.9	9.5	13.7	0	NS	NS	NS	NS
4	14.7	8.1	3.9	17.4	NS	NS	NS	NS
5	8.8	0	0	0	$P < .05$	NS	NS	NS
6	1.5	2.7	3.9	0	NS	NS	NS	NS
7	11.8	4.1	5.9	0	NS	NS	NS	NS
9	2.9	6.8	9.8	0	NS	NS	NS	NS
11	1.5	0	0	0	NS	NS	NS	NS
12	4.4	1.4	2.0	0	NS	NS	NS	NS
16	5.9	1.4	2.0	0	NS	NS	NS	NS
21	1.4	0	0	0	NS	NS	NS	NS
24	1.4	0	0	0	NS	NS	NS	NS
28	0	1.4	2	0	NS	NS	NS	NS
29	1.4	0	0	0	NS	NS	NS	NS
34	0	1.4	0	4.3	NS	NS	NS	NS
36	0	1.4	2	0	NS	NS	NS	NS
37	1.4	0	0	0	NS	NS	NS	NS
38	0	8.1	0	26.1	$P < .05$	NS	$P < .001$	$P < .001$
39	0	1.4	2	0	NS	NS	NS	NS
41	1.4	0	0	0	NS	NS	NS	NS
42	2.9	2.7	2.0	4.3	NS	NS	NS	NS
43	2.9	1.4	0	4.3	NS	NS	NS	NS

Note.—The frequencies of RFLP haplotypes at the PAH locus in normal parental chromosomes (1) are compared to those of all mutant chromosomes (2), to chromosomes from typical PKU patients (3), or to chromosomes from variants (4), classified as described in Methods. NS = not significant.

quency of normal genes was largely similar to that of mutant genes, except for haplotype 2 ($P < .05$) and haplotype 38 ($P < .05$).

However, when the group of "mutant genes" was split in the two groups as described in Methods, it appeared that the significance of the statistical tests was greatly reinforced. First, 100% of haplotype 38 genes ($P < .001$) and 75% of mutant haplotype 2 genes ($P < .01$) were found to be carried by patients with residual activity. Second, 86% of the mutant genes in variants were confined within only four haplotypes—namely, haplotypes 1, 2, 4, and 38—while these haplotypes accounted for only 47% of normal genes ($P < .001$). Third, several haplotypes, including haplotype 3, were encountered in PKU patients only and were totally absent in variants (haplotypes 3, 6, 7, 9, 12, 16, 28, and 39). Finally, 75% of the PKU genes were confined within only four haplotypes—namely, haplotypes 1, 2, 3, and 9—while these four haplotypes accounted for 38% of normal genes ($P < .001$).

Homozygosity and Heterozygosity of RFLP Haplotypes at the PAH Locus and Their Relationship with the Clinical Heterogeneity of Hyperphenylalaninemias

Table 4 shows that two-thirds of the children reported in the present study were heterozygous for their RFLP haplotypes at the PAH locus. Only one-third were homozygous, which of course does not mean homozygosity for a particular mutation at this locus. Homozygosity of RFLP haplotypes concerned only four haplotypes: haplotypes 1, 2, 7, and 38.

Homozygosity for mutant haplotype 1 genes constantly resulted in typical PKU. Heterozygosity for mutant haplotype 1 genes resulted in either typical PKU or mild hyperphenylalaninemia. When the children heterozygous for haplotype 1 had typical PKU, the other gene was a mutant allele constantly absent from mild variants (table 4).

Homozygosity for mutant haplotype 2 genes resulted in either typical PKU or mild variants in our series. The point mutation at codon 408 of the PAH cDNA, which is in linkage disequilibrium with haplotype 2 PKU genes in Denmark (DiLella et al. 1987), was sought in our patients by using polymerase chain-reaction amplification of their genomic DNAs (Saiki et al. 1985). We found that mild hyperphenylalaninemia in our patients homozygous for haplotype 2 was not due to this particular mutation (C. Caillaud, unpublished data). Heterozygosity for mutant haplotype 2 genes resulted in either typical PKU or in mild hyperphenylalaninemia (table 4). When the children heterozygous for haplotype 2 had typical PKU, the point mutation at codon 408 was indeed involved (C. Caillaud, unpublished data), the other mutant haplotype being usually associated with a typical form of the disease (table 4). Similarly, heterozygosity for haplotype 4 usually resulted in typical PKU when associated with haplotypes 1 or 6 but in mild variants when associated with mutant haplotype 2.

The mutant haplotype 3 gene, the main mutant allele in northern Europe (38%), was much less represented in our series (9.5% of all mutant alleles, 13.7% of mutant alleles in PKU patients). Heterozygosity for mutant haplotype 3 genes constantly resulted in typical PKU, regardless of the haplotype of the other mutant allele. The splicing mutation specifically associated with mutant haplotype 3 genes in Denmark (DiLella et al. 1986) was present in all our PKU patients heterozygous for haplotype 3 (S. L. C. Woo, personal communication). None of the patients was homozygous for haplotype 3.

Finally, homozygosity for haplotype 38 constantly resulted in a variant form of the disease in three families. We have shown that this haplotype is in tight linkage disequilibrium with a point mutation at nucleotide 1065 of the PAH cDNA (Lyonnet et al., submitted). This mutation, which was initially found in northern

Table 4

Correlation of Phenotypes with Mutant RFLP Haplotypes at the PAH Locus

	1	2	3	4	7	38	39	43
1	5T							
2	1T	1T						
	1V	3V						
3	3T	2T						
4	1T							
	2V	1V						
6				1T				
7					1T			
9	1T	1T	1T		1T		1T	
12	1T							
16	1T							
28	1T							
35				1V				
38						3V		
42			1T					1V

NOTE.—This table correlates the clinical phenotypes with the diploid haplotype combinations at the PAH locus. Listed on the abscissa and ordinate are mutant RFLP haplotypes at the PAH locus, as described in table 2. T = typical PKU; V = variants.

Africa, was also found in old families from Burgundy and might therefore correspond to the Saracen invasion of southern Europe during the seventh century. We have not found normal haplotype 38 genes to date (table 4).

Discussion

Using a full-length cDNA clone as the hybridization probe, the Woo group first performed extensive RFLP haplotype analysis of the PAH locus. The RFLP haplotypes of 66 normal and 66 PKU alleles in 33 Danish kindreds were determined, and a total of 12 RFLP haplotypes were observed (Chakraborty et al. 1987; Guttler et al. 1987). Although nine different haplotypes were described for the PKU chromosomes, about 90% of all mutant alleles were associated with only four haplotypes (haplotypes 1–4). Almost 38% of mutant alleles in Denmark had a single haplotype (haplotype 3) that was relatively rare among the normal gene pool (3%). This mutant haplotype 3 is in linkage disequilibrium with a point mutation at the 5′ splice donor site of intron 12 (DiLella et al. 1986). In addition, Woo and his colleagues showed that another mutant haplotype (haplotype 2) is in linkage disequilibrium with a point mutation at codon 408 of the mRNA (DiLella et al. 1987). In both cases, the mutant alleles resulted in typical PKU.

We show here that the number of RFLP haplotypes at the PAH locus is much higher in France than in the northern-European population reported elsewhere (Woo et al. 1986; Chakraborty et al. 1987). A total of 23 RFLP haplotypes (including 18 normal and 16 mutant alleles) were observed in the present study. The four haplotypes that account for 88% of normal genes in Denmark (haplotypes 1, 4, 5, and 7) accounted for only 60% of normal genes in France, the other 40% being evenly distributed among 12 haplotypes. Similarly, the number of mutant RFLP haplotypes proved to be particularly high as well. The four RFLP haplotypes (haplotypes 1–4) that account for 90% of all mutant alleles in Denmark accounted for only 66% of mutant PAH genes in France. The other 34% were accounted for by 12 haplotypes, including eight haplotypes (19% of all mutant alleles) absent from Caucasian pedigrees reported thus far. This feature suggests that a certain heterogeneity at the PAH locus exists in France. However, it is worth noting that a bias of ascertainment might have occurred between the French and the Danish studies, expecially as the authors of the Danish study considered their reported patients as being typical PKU only (DiLella et al. 1986; Chakraborty et al. 1987; Güttler et al. 1987).

In spite of the particular heterogeneity of RFLP haplotypes at the PAH locus in both normal and mutant alleles, a significantly different distribution of RFLP and haplotypes appeared when the group of mutant alleles was split into two groups according to the clinical phenotypes of the patients. Several mutant RFLP haplotypes were present in typical PKU only, others were present in variants only, and others were present in both classes of patients. Indeed, homozygosity for mutant haplotype 1, the most frequent haplotype in France, constantly resulted in typical PKU, while homozygosity for haplotype 38 resulted in variant hyperphenylalaninemias (Lyonnet et al., submitted). Moreover, heterozygosity for mutant haplotype 1 resulted in typical PKU when associated with mutant haplotypes absent from mild variants, while it resulted in mild hyperphenylalaninemias when associated with haplotypes 2 or 4. Similarly, a child heterozygous for haplotype 42 and haplotype 3 mutant alleles had typical PKU, while his first-cousin, heterozygous for the same haplotype 42 and another mutant haplotype (haplotype 43), had a mild variant. These data suggest that some mutant haplotypes (e.g., haplotype 3 in Denmark) are associated with completely inactive gene products, resulting in typical PKU, while others are associated with mutant alleles that retain a significant residual activity (leading to variant hyperphenylalaninemias). Thus, the combination of many different mutant haplotypes in PAH-deficient patients and of compound heterozygosity at the PAH locus results in the broad spectrum of individual phenotypes observed in France. This heterogeneity probably hampers a clear-cut classification of hyperphenylalaninemias.

In fact, many other lines of evidence suggest that multiple mutations account for PAH deficiencies. Biochemical studies have identified several kinetic and immunoreactive phenotypes (Friedman et al. 1973; Bartholomé et al. 1984). Northern blot analysis of mRNAs from needle biopsies of the liver derived from PKU patients showed the presence of PAH mRNAs in some samples and no detectable mRNAs in others (DiLella et al. 1985). Molecular cloning and expression studies revealed the presence of several mutations resulting in PAH deficiencies. Three mutations were found to be associated with particular RFLP haplotypes, namely, haplotypes 2, 3, and 38 (DiLella et al. 1986, 1987; Woo et al. 1986; Lyonnet et al. 1988). Mutant alleles associated with haplotypes 3 and 2 have been analyzed by hybridization with oligonucleotides specific for the

splicing mutation (DiLella et al. 1986) and the Arg^{408}-Trp^{408} missense mutation (DiLella et al. 1987). Seven of eight mutant haplotype 3 alleles and five of five mutant haplotype 2 alleles bore the same mutations, respectively (DiLella et al., manuscript in preparation). Our data, however, show that different mutations of the PAH genes occur on mutant alleles with haplotype 2. The point mutation associated with mutant haplotype 2 in Denmark was indeed detected in our patients with typical PKU but not in children with mild variants (C. Caillaud, unpublished data). Ledley et al. (1986) have already shown that different mutations can occur on alleles with the same haplotypes at the PAH locus. Similarly, molecular genetic analysis of the β-globin locus in β-thalassemias demonstrated that, although a strong correlation exists between RFLP and specific mutations, all mutations within an RFLP haplotype are not of one single type (Orkin and Kazazian 1984).

In the case of PAH deficiencies in southern Europe, the multiplicity of mutant haplotypes at the PAH locus strongly suggests that, in contrast with cystic fibrosis (Beaudet et al. 1988) or sickle-cell anemia (Orkin and Kazazian 1984), the hyperphenylalaninemia syndromes are determined by a number of as yet uncharacterized mutations in various diploid combinations. Cloning and characterizing the different mutations in the most prevalent haplotypes at the PAH locus will probably help us to understand the clinical heterogeneity of hyperphenylalaninemias in southern Europe.

Acknowledgments

We gratefully thank S. L. C. Woo (Howard Hughes Medical Institute) for his helpful discussion and comments and for giving us the PAH cDNA probe for RFLP studies. We thank Monique Poussière for typing the manuscript and Alan Strickland for his help in preparing it.

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