Discordant phenylketonuria phenotypes in one family: the relationship between genotype and clinical outcome is a function of multiple effects

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Abstract

Four members spanning three generations of one family have phenylketonuria of varying degrees of severity. Two first cousins were screened in the neonatal period and have had dietary phenylalanine restriction since diagnosis, the older patient having been classified as having more severe PKU and the younger one as having mild PKU. Their mutual grandfather and his older brother also have a significant hyperphenylalaninaemia and are of normal intelligence despite never having had restricted phenylalanine intake. Mutation analysis of the phenylalanine hydroxylase (PAH) gene has established that there are four different mutations, two in exon 2 (F39L and L48S) and two in exon 3 (R111X and S67P), which give rise to PKU in this family. In order to establish their relative severity, we screened the PKU populations of western Scotland and the south west of England for these mutations. The exon 3 mutations are rare; however, F39L is relatively common in Scotland and L48S in England. A comparison of diagnostic blood phenylalanine concentrations in subjects carrying L48S/null or F39L/null mutations with those carrying two null mutations suggest that these exon 2 mutations are less deleterious. Thus, in this family, the different biochemical phenotypes can be explained, in part, by different genotypes at the PAH locus but our results show that the relationship between genotype and clinical outcome is more complex and is a function of multiple effects.

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It is clear now that for any genetic disorder, except sickle cell disease, genetic heterogeneity is the rule rather than the exception. The relationship between variation at the level of the gene and phenotypic variability can be apparently direct, as in some single gene disorders, or it can be more complex, involving gene/gene or gene/environment interactions. Of the single gene disorders, it is tempting to relate clinical variability to different combinations of mutations at a single locus, particularly if it is known that individual mutations have different quantitative effects on protein structure or function.

Hyperphenylalaninaemia is usually caused by mutations at the phenylalanine hydroxylase (PAH) gene. To the end of October 1994, 206 different mutations have been reported by the PAH Mutation Analysis Consortium.¹ In vitro expression analysis has shown that some result in a complete loss of enzyme activity whereas others are associated with residual activity ranging anywhere from 3 to 75%.²⁻⁶ It would be anticipated that various combinations of mutations would result in a full spectrum of biochemical phenotypes ranging from "classical" phenylketonuria (PKU) requiring strict dietary management to mild non-PKU hyperphenylalaninaemia in which dietary restriction of phenylalanine is not necessary.

In general, there appears to be a correlation between genotype and biochemical phenotype as determined by phenylalanine load studies or blood phenylalanine concentrations at diagnosis.⁴ However, large variation in intellectual outcome of untreated subjects with the same genotype has been observed both within and between families⁷ and this suggests that the relationship between genotype and clinical effect is probably more complex.

We previously reported a family in which there are four affected members spanning three generations.⁸ This included two first cousins who had different biochemical presentations in the neonatal period and their mutual grandfather and his older brother who had previously undiagnosed significant hyperphenylalaninaemia but were of apparently normal intelligence. Haplotype analysis made it likely that there were three, or possibly four, different mutations giving rise to PKU in the family.

In this paper we report the existence of four mutations at the PAH locus in this family, two in exon 2 (F39L and L48S) and two in exon 3 (R111X and S67P). We have established the relative frequency of these mutations in the PKU populations of south west England and western Scotland. In addition, diagnostic blood phenylalanine concentrations in subjects carrying L48S or F39L suggest that they are of a less deleterious type. S67P is a private mutation and also almost certainly associated with residual enzyme activity in vivo. Finally, although the different biochemical presentations of the affected subjects in this family can be explained, in part, by different genotypes, we believe that the clinical outcome of the untreated subjects provides additional evidence that there is not necessarily a simple correlation between phenotype and genotype at the PAH gene.

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Figure 1 A concise family pedigree and the mutations that family members carried. Altogether there were five offspring in generation II. Only those with affected children are included.

Subjects and methods

THE FAMILY

Altogether there are five offspring in generation II.⁸ For simplicity only two who are themselves parents of affected children are shown in fig 1. The affected subjects in generation III were identified on neonatal screening. III-1 has been on a restricted phenylalanine intake since diagnosis. III-4 has had some dietary restriction although there is some doubt about compliance. Details of the clinical histories have been reported previously,⁸ and are given briefly in table 1. I-2 and I-3 are both of apparently normal intelligence. They were unaware of their significant hyperphenylalaninaemia until they were investigated as part of this study.

MUTATION ANALYSIS

DNA was prepared from peripheral leucocytes using a phenol/chloroform extraction procedure according to standard protocols.

Initially affected subjects were screened for mutations in exons 3, 7, and 12 using heteroduplex analysis generated by synthetic PCR amplifiable DNA. Constructs were made for these exons to identify the more common mutations which were known to occur in the PKU population of the United Kingdom.⁹ Details of the heteroduplex method used to detect mutations in exon 12 have been published elsewhere.¹⁰

Denaturing gradient gel electrophoresis (DGGE) of all amplified 13 exons was undertaken on subjects I-2, I-3, and III-1 (in whom no mutation had been identified by heteroduplex analysis) according to the method of Guldberg *et al.*¹¹

Direct sequencing of amplified products using the ABI 373a automatic sequencer, as described elsewhere,¹² or using a Cycle Sequencing kit (Pharmacia) was undertaken on those exons which showed banding patterns indicating the presence of an altered sequence.

Subsequently a synthetic construct was made to identify mutations in exon 2, and a total of 308 independent alleles from the PKU populations of the south west of England and Scotland were screened for the four mutations that were identified in this family.

STATISTICAL METHODS

A comparison of blood phenylalanine concentrations at diagnosis was made for PKU subjects carrying F39L/null, L48S/null, and null/null mutations. Because there were regional differences in the relative frequencies of F39L and L48S (see Results), comparisons were made originally with subjects within the same population carrying two null mutations. A Kruskal-Wallis one way analysis of variance was used to see if there were differences among the four groups overall. Further comparisons between pairs of groups were made using two tailed Mann-Whitney U tests.

Results

Heteroduplex analysis showed that only III-4 had a banding pattern in exon 3 which suggested the presence of an altered sequence. Direct DNA sequencing established that this was a previously undescribed mutation, consisting of a T to C transition at codon 67 (TCT to CCT), which substitutes a proline residue for serine in the PAH protein. Genetic analysis of the parents, II-3 and II-4, confirmed that this base change was inherited from II-4 and is associated with haplotype 4. This mutation is also detectable by single stranded conformational polymorphism (SSCP) analysis (results not shown).

DGGE analysis established the presence of base changes in exons 2 and 3 in samples from I-2 and III-1. Direct sequencing of these exons showed them to be F39L (exon 2), L48S (exon 2), and R111X (exon 3). PCR amplification of these exons and restriction enzyme digestion with *Mae*III, *Msc*I, and *Bsp*HI respectively showed that L48S was the mutation that all affected subjects had in common and was thus associated with haplotype 4. R111X, inherited from II-1, was associated with haplotype 5 and F39L with haplotype 1.

A compilation of various biochemical and

Table 1 Summary of genetic and biochemical parameters of key subjects in the pedigree

	I-2	I-3	II-1	II-2	II-3	II-4	III-1	III-4
Blood phenylalanine at diagnosis ($\mu\text{mol/l}$)	1020	860	—	—	—	—	2500	650
Age at diagnosis	63 y	65 y	—	—	—	—	7 d	7 d
Phenotype	Untreated PKU, normal intelligence	Untreated PKU, normal intelligence	Carrier	Carrier	Carrier	Carrier	Treated PKU, normal intelligence	Treated PKU, normal intelligence
PAH haplotypes	1/4	1/4	5/5	7/4	4/4	4/4	4/5	4/4
PAH genotype	F39L/L48S	F39L/L48S	R111X/normal	L48S/normal	L48S/normal	S67P/normal	L48S/R111X	L48S/S67P

Table 2 Relative frequencies and haplotype/VNTR associations of the four mutations in the PKU populations of south west England and western Scotland

Place	No of alleles	Mutation	Rel frequency	Haplotype/VNTR
SW England	103	F39L	<1%	1.8
		L48S	7%	4.3
		R111X	<1%	5.8
		S67P	<1%	4.3
W Scotland	206	F39L	6%	2.8
		L48S	<1%	2.3
		R111X	0	
		S67P	0	

genetic parameters for key family members is given in table 1.

Two mutations, L48S and F39L, were found on additional PKU chromosomes in south west England and western Scotland. L48S was found more frequently in the south west of England whereas in Scotland it was F39L. Table 2 shows their relative frequencies and haplotype/VNTR associations in the two populations.

In order to ascertain the relative quantitative effects of these mutations on phenylalanine hydroxylase activity *in vivo* we compared blood phenylalanine concentrations at diagnosis in subjects whose genotypes were L48S/null or F39L/null with the values in subjects from the same population who carried two null mutations (fig 2). The null mutation in the English group was principally IVS12nt1g→a and in the Scottish group it was R408W. There were significant differences among the four groups overall ($p=0.003$). There was also a suggestion that the English and Scottish null/null groups were different but the difference did not achieve statistical significance ($p=0.053$). Comparison between children with an L48S/null or F39L/null genotype and children with both null mutations were therefore made separately for each country. In the Scottish series, the children with the single F39L mutation had lower blood phenylalanine concentrations at diagnosis than those with two null mutations ($p=0.003$) (fig 2). In the English series there was a corresponding difference in that children with the single L48S mutation had lower values than the children with both null mutations ($p=0.048$). There was a suggestion that the English children with the single L48S gene had lower values than the Scottish children with F39L, but the difference did not achieve statistical significance ($p=0.095$).

Discussion

In the family reported here, four mutations in different combinations have given rise to hyperphenylalaninaemia. Three of these (L48S, F39L, and R111X) have been found in the PKU populations in other parts of the world including Germany,¹³ Turkey,¹³ Italy,¹⁴ Sicily,¹⁵ Australia,¹⁶ and the Orient.^{17,18} The fourth, S67P, has not been reported previously and appears to be a private mutation. In the south west of England F39L is relatively rare whereas L48S is the fourth most common mutation, after IVS12nt1g→a, R408W, and I65T.¹⁹ In

Figure 2 Phenylalanine concentrations in subjects carrying F39L or L48S mutations. Cross bars represent the median value. S1: Scottish patients carrying F39L/null mutations. S0: Scottish patients carrying two null mutations. E1: English patients carrying L48S/null mutations. E0: English patients carrying two null mutations.

western Scotland, on the other hand, the relative frequencies are virtually reversed where L48S is rare and F39L, along with IVS12nt1g→a, is the third most common after R408W and I65T.¹⁹ This pattern parallels that which is observed for the exon 12 mutations (R408W and IVS12)¹⁹ and is further evidence of the different ancestries of the two populations. R111X has not been found on any other mutant chromosome in this study. It was previously reported in the PKU populations of the Orient^{17,18} on haplotype 4. In this family it is on a haplotype 5 which bears little similarity to haplotype 4 using eight RFLP markers.²⁰ Since a CpG dinucleotide is involved²¹ the mutation could have arisen independently on these two haplotype backgrounds.

Direct evidence of the quantitative effects on PAH activity in *in vitro* expression systems is not available for the mutations found in this family. However, a non-parametric statistical comparison of the blood phenylalanine concentration at diagnosis in subjects who carry L48S and F39L in combination with known null mutations suggests that both are less deleterious than null mutations. The results on L48S are compatible with those of Konecki *et al*¹³ who have reported that patients homozygous for L48S manifested a less severe phenotype and were classified as having mild PKU.

In this family the diverse biochemical presentations in affected subjects can be explained, in part, by their different combinations of mutations. The most severe hyperphenylalaninaemia which has required dietary phenylalanine restriction since infancy is seen in III-1 who has L48S in combination with R111X, a mutation which creates a premature stop codon. Although we have not been able to establish the relative quantitative effect of the private mutation, S67P, the child's phenylalanine concentration at diagnosis and virtual absence of dietary phenylalanine restriction make it almost certain that this is also a less deleterious one.

The older men appear to have two milder mutations, and despite having moderately raised blood phenylalanine concentrations they showed no overt sign of impaired intellectual development or neurophysiological dysfunction. However, detailed psychological assessment was not undertaken, and so it is possible that there has been some mild impairment of specific areas of cognitive function. Nevertheless, I-1 held desk jobs requiring arithmetical skills during his working life.

It has been recognised for some time that not all people with untreated PKU will suffer mental handicap.²² Equally, mutation analysis at the PAH gene now shows that not all subjects with the same genotype will have the same biochemical phenotype, a variability which can be seen both within and between families.⁷ The precise biological mechanisms by which phenotypic variation occurs are not known but it is certain that the contributing factors are multiple and varied. Treacey *et al.*,²³ for example, have shown that whereas there is identical hydroxylation of phenylalanine to tyrosine in affected sibs with the same genotype, there can be variation in phenylalanine use through additional pathways involved in phenylalanine homeostasis. Ramus *et al.*⁷ invoke the possibility of a gene which modifies the effect of high phenylalanine concentrations on the brain.

Thus, it is clear that for the disorders of metabolism in vitro expression systems are useful laboratory tools for establishing the more proximate effects of a particular mutation, such as the effects on mRNA synthesis, protein synthesis, or enzyme activity. This allows a relative order of severity of various mutations to be established. However, the more ultimate effects in affected subjects (ease of dietary biochemical control or clinical outcome in treated or untreated patients) must take into account additional complementary influences that are involved both in phenylalanine metabolism in particular and in intellectual and neurophysiological development in general. Ultimately, a simple correlation may not always be apparent between genotype at the PAH gene and biochemical and clinical phenotype.

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