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Review

Molecular Basis for the Phenotypic Diversity of Phenylketonuria

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Introduction

Phenylketonuria (PKU) is an autosomal recessive inherited deficiency of hepatic phenylalanine hydroxylase (phenylalanine 4-monooxygenase: EC 1.14.16.1; L-phenylalanine, tetrahydropteridine: oxygen oxidoreductase) which catalyzes the hydroxylation of phenylalanine to tyrosine. When untreated the disease causes mental retardation at a variable degree (1) and all neonates are screened for hyperphenylalaninemia.

A switch in the screening procedure from a test that relies on detection of phenylpyruvate in the urine to a simple and sensitive method for the determination of phenylalanine (phe) in blood (2) converted what had been thought to be a simple clinical and biochemical entity into a spectrum of disorders showing hyperphenylalaninemia (3). The discovery of hyperphenylalaninemic conditions which to a variable degree biochemically mimic the condition described by Følling in 1934 (4) has demanded increased knowledge of the various types of defects in the metabolism of phe (e.g. phenylalanine hydroxylase (PAH) deficiency, tetrahydrobiopterin (BH₄) deficiency) because of the ultimate prognosis of each type in relation to the need for optimal treatment (5).

The different phenotypes of phenylalanine hydroxylase deficiency

Within the first decade of screening for neonatal hyperphenylalaninemia it became clear that the PAH deficiency trait is phenotypically heterogeneous (6). There are two well-defined clinical and biochemical entities of phenylalanine PAH deficiency; a) phenylketonuria (PKU) with pretreatment blood phe levels > 1200 µmol/l, < 1% of normal enzyme activity and a low tolerance for phe (7) and, b) persistent benign hyperphenylalaninemia (non-PKU HPA) with phe levels persistently below 600 µmol/l on a normal diet and with 5–10% of normal enzyme activity (8). In addition to these well-defined phenotypes there are intermediate “variant” or “mild” forms of PKU with pretreatment blood phe levels of 600–1200 µmol/l, 1%–5% of normal enzyme activity, and a relaxed phenylalanine tolerance (Fig. 1). Recommended concentrations of serum phe to be maintained during therapy are 160–300 µmol/l (9, 10, 11). Some centers initiate dietary treatment at phe levels above 1200 µmol/l (10, 12, 13), others start treatment at phe levels above 900 µmol/l (14, 15), and some start dietary treatment when blood phe levels exceed 600 µmol/l (9, 16).

A classification of the different phenotypes of PAH deficiency based on the dietary phe tolerance of the child was proposed in 1980 (8). The phe tolerance is related to the parental phenotype as determined by the tyrosine response to a phe loading test (Table 1) (17). The most obvious explanation would be different mutant alleles (8, 18).

Abbreviations: phenylalanine hydroxylase, PAH; phenylketonuria, PKU; hyperphenylalaninemia, HPA; polymerase chain reaction, PCR; allele-specific oligonucleotide, ASO; phenylalanine, phe.

Figure 1. The different phenotypes of phenylalanine hydroxylase deficiency classified according to pretreatment phenylalanine levels and the amount of phenylalanine tolerated in mg per kg body weight per day at 5 years of age. Most centers start treatment, when phenylalanine levels exceed 600 $\mu\text{mol/l}$ and patients with these levels are classified as having one of the various phenotypes of phenylketonuria (PKU). Individuals with phenylalanine levels below 600 $\mu\text{mol/l}$ show a normal development without treatment and have the non-PKU hyperphenylalaninemia (HPA) phenotype.

Table 1. Relation between the parental phenotype and the combined phenotype of their affected offspring.

Phenotype of parent*	0-2	>2-6	>6
0-2	Classical PKU	Classical PKU	Mild PKU
>2-6	Classical PKU	Mild PKU	Non-PKU HPA
>6	Mild PKU	Non-PKU HPA	Non-PKU HPA

* Phenotype of parent: rate of tyrosine formation times maximum value of serum tyrosine divided with the maximum value of serum phenylalanine observed after a load of 0.1 g phenylalanine/kg body weight.

The association between RFLP halotypes and clinical phenotypes

A partial cDNA of the PAH gene was cloned in 1982 (19) and the first results of segregation analyses published in 1983 (Fig. 2) (20, 21). Haplotype studies were initiated in 1984 (22, 23, 24) and the relationship between the haplotypes of the two inherited mutant alleles and the phenotype of the offspring was demonstrated in 1987 (Table 2) (25), and later confirmed (26).

Figure 2. The segregation of the PKU alleles in a Danish family demonstrated by restriction fragment length polymorphisms at the phenylalanine hydroxylase locus obtained by the restriction enzyme *Hind*III and the pHPAH247 cDNA probe. The PKU-allele segregates with the 4.2 kb fragment from the mother (M) and the father (F) to the PKU-child (P). The normal phenylalanine hydroxylase allele segregates with the 4.0 kb fragment from the parents to the unaffected sibling (S), who has inherited the two normal chromosomes of the family.

Table 2. Haplotype of PKU alleles correlated to phenotypes in 74 Danish patients.

Haplotype of mutant alleles									
		1	2	3	4				
1	Mo								
	Mo								
	Mo								
2	C Mo		C						
	C Mo		C						
	C Mo		C						
3	C Mo Mi	C C C	C C C	C C C					
	C Mo Mi	C C C	C C C	C C C					
	C Mo Mi	C C C	C C C	C C C					
		C C C	C C C	C C C					
4	C Mi		Mi	C	Mo Mi Mi Mi	Mo Mi			
	C Mi		Mi	C	Mo Mi Mi Mi	Mo			
	Mi		Mi		Mo Mi Mi Mi	Mo			
	MI				MI Mi				

C: Classic PKU; <20 mg phe/kg/day
 Mo: Moderate PKU; 20-25 mg phe/kg/day
 Mi: Mild PKU; >25 mg phe/kg/day tolerated at five years of age

PKU-mutations

The correlation between RFLP haplotypes and clinical phenotypes (Table 2) (25) formed a strategy for sequencing the first two PKU-alleles in Danish chil-

dren with severe classic PKU (27). The deficient PAH activity of an allele associated with haplotype 3 was discovered to be a G → A mutation in the obligatory donor splice site in intron 12 (IVS-12). The result is that the coding sequence of exon 12 is spliced off during the transcription (28). The allele responsible for severe classic PKU in Denmark and associated with haplotype 2 was discovered to be a C → T transition in codon 408 of exon 12, which results in a substitution of arginine with tryptophan. Expression studies showed no detectable enzyme activity (29).

During the past four years an increasing number of mutations in the PAH gene have been discovered. In 1989 11 mutations were described (26), and 31 mutations were known in November 1990 (30). In November 1991 the number of known mutations in the PAH gene may have reached more than 50. However, many mutations are rare and some are common only within certain ethnic groups or populations. The codon 408 mutation is common in Eastern European

countries (31, 32) and is probably of Slavic origin. The originally discovered IVS-12 mutation may have a founding population in Northern Europe, perhaps in Jutland of Denmark (33). A substitution in intron 10 is prevalent among PKU patients in Turkey and Bulgaria and the distribution suggests a Middle Eastern origin. A secondary dissemination of this allele may have occurred during the Roman colonization, and the later extent of the Ottoman Empire (34).

Relation of mutation genotype to biochemical and clinical phenotype

In vitro expression studies revealed a spectrum of PAH activities from 0% to 50% of normal activity depending on the mutant allele examined (7). In accordance with the *in vitro* studies there is a relationship between the pretreatment blood phe levels (the biochemical phenotype) and the amount of phe tolerated in the diet to keep blood phe at nearly normal levels (Fig. 3). The result is a relation between the

Phenylketonuria mutations and the associated phenotypes in 59 Danish patients

Figure 3. The relation between the inherited mutant alleles and the pretreatment blood phenylalanine levels (the biochemical phenotype) and the amount of phenylalanine tolerated in the diet to keep blood phenylalanine at nearly normal levels (clinical phenotype). The mutant alleles of the patients were detected by allele-specific oligonucleotide probing after PCR amplification of the appropriate exons of the PAH gene.

Table 3. Correlation between mutation genotype and different phenotypes of PKU and non-PKU hyperphenylalaninemia in 82 Danish patients.

Mutation	R408W IVS-10 IVS-12	R261Q L348V	Y414C	I306W D415N
R408W IVS-10 IVS-12	Severe classic PKU N = 39	Moderate PKU N = 5	Mild PKU N = 22	Non-PKU HPA N = 4
R158Q E280K P281L	Severe classic PKU N = 7	Moderate PKU N = 1	Mild PKU N = 1	
R261Q L348V		Mild PKU N = 2		
Y414C			Mild PKU N = 2	

inherited two mutant alleles and the clinical phenotype of the patient (Table 3). This observation also explains the previously described association between the heterozygous phenotypes of the parents as determined by the tyrosine response to a phe load, and the phenotype of their affected offspring (Table 1).

The genotype-phenotype relation and "danger levels" for mental deficiency

Since the institution of treatment for PKU, the blood level at which dietary restriction of phe intake should be initiated has been debated. A survey of 43 untreated mentally retarded institutionalized patients with PKU revealed that 80% of the patients had two mutations (IVS-12 and R408W), which are common among patients with classic PKU. However, 20% of the patients had mutations (Y414C, R158Q) which cause moderate or mild PKU and blood phe levels of 600–1200 $\mu\text{mol/l}$ (35). The occurrence of mental retardation in patients with serum phe concentrations below 1200 $\mu\text{mol/l}$ (36) and the reported cases of 16 normally intelligent (IQ > 90) people with PKU (37) may have some bearing on the hypothesis that mild PKU may cause a reduction in IQ points which is fatal for some (IQ without PKU added 100), but not for others (IQ without PKU added 140).

So, we believe that we must treat all infants appearing to have PAH deficiency with serum phe levels above 600 $\mu\text{mol/l}$, as these levels of phe may cause mental deficiency. The recent knowledge about the mutations associated with this phe level (7, 35) enables us to distinguish neonates with this biochemical phenotype of the disease by ASO probing of PCR-amplified DNA from the blood spots used for neonatal screen-

ing of hyperphenylalaninemia. Children with this type of PAH deficiency have a relaxed phe tolerance and need less rigid dietary restrictions of phe intake (8).

The molecular basis for non-PKU hyperphenylalaninemia

The question whether dietary management should be initiated in all neonates with PAH deficiency appeared when it was recognized that children with serum phe levels below 600 $\mu\text{mol/l}$ appear to show a normal intellectual and behavioural development without treatment (38, 39). These children have the non-PKU HPA phenotype.

Initiation of dietary therapy soon after birth has important psychological and social implications (40). So, it is imperative as early as possible to distinguish newborn children who require a phenylalanine restricted diet, from those who have the non-PKU HPA phenotype. Recently, Avigad et al. (41) observed that in six of 27 families the non-PKU HPA phenotype resulted from compound heterozygosity for haplotypes associated with a PKU allele and an allele, which might carry a mutation with a milder effect. By ASO probing of PCR-amplified DNA from 17 children with non-PKU HPA we have observed that 12 patients had inherited one of the common mutations associated with PKU and six patients had a mutation found in patients with severe classic PKU (R408W or IVS-12) (42). We identified two new mutations in four of these six patients. It was further demonstrated that the identified mutations (D415N in exon 12 and L306V in exon 9 of the PAH gene) had less impact on the heterozygote's ability to hydroxylate phenylalanine to tyrosine compared to the parent carrying the classic PKU mutation (42). The combined effect of these mutations in their affected offspring is either no detectable increase or a slight increase in serum tyrosine within the first hour after a phenylalanine load (43) which explains the non-PKU HPA phenotype at the molecular level. The observation that PKU mutations combined with less deleterious mutations cause the non-PKU HPA phenotype, and that particular mutation-RFLF haplotype combinations are associated with this phenotype enables us for the first time to distinguish hyperphenylalaninemic neonates who need dietary therapy, from those who do not.

Future studies

A possible relationship between mutation genotype and the ultimate prognosis of the individual with PKU, as well as the question to what extent the mutation genotype of a PKU female and the inherited

mutant allele of her child may influence the outcome following a planned pregnancy, are new challenges for future studies.

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