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REVIEW

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Mutation analysis anticipates dietary requirements in phenylketonuria

Abstract Phenylalanine hydroxylase (PAH) deficiency is inherited as an autosomal recessive trait and the associated hyperphenylalaninaemia phenotype is highly variable, primarily due to a great allelic heterogeneity at the PAH locus (approximately 400 disease-associated mutations are known). The arbitrary classification of PAH deficiency on the basis of clinical parameters has been complicated by the lack of international guidelines, leading to a wide confusion in both methodology and terminology. Recently, significant improvements in methods for detection of mutations have paved the way for an alternative system for classification of PAH deficiency, which is based solely on PAH genotypes. This paper gives a summary of the recent progress made in establishing a direct correlation between individual PAH mutations and biochemical and metabolic phenotypes, including the use of ''functionally hemizygous'' patients to classify both common and rare mutant alleles, and a simple and general model to predict the combined phenotypic effect of two mutant PAH alleles.

Conclusion Genotype-based prediction of the biochemical phenotype is now feasible in the majority of newborns with hyperphenylalaninemia, which may be useful for refining diagnosis and anticipating dietary requirements. A recent observation suggests that the genotype also determines cognitive development if dietary therapy is discontinued at 6 years of age.

Key words Functional hemizygosity · Genotype-phenotype correlations · Mutation · Phenylalanine hydroxylase · Phenylketonuria

Abbreviations *AV* assigned value · *MHP* mild hyperphenylalaninaemia · *PAH* phenylalanine hydroxylase · *Phe* phenylalanine · *PKU* phenylketonuria

Introduction

Deficiency of phenylalanine hydroxylase (PAH, EC 1.14.16.1) impairs hepatic conversion of phenylalanine (Phe) to tyrosine and is the most common of the hyperphenylalaninaemias with an incidence of approximately 1:10,000 in most Caucasian populations [9]. Within the first decades of population screening for neonatal hyperphenylalaninaemia, it became evident that PAH deficiency is a highly heterogeneous trait showing a broad continuum of phenotypes. At the one end of the scale are conditions characterised by serum concentrations of Phe higher than 20 times the normal level, which usually result in profound and irreversible mental impairment unless the dietary intake of Phe is drastically reduced. At the other end of the scale are conditions characterised by only marginally elevated levels of Phe (3–5 times higher than normal), which have

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no detectable effects on physical, neurological, or cognitive development [4, 9].

PAH deficiency is caused by PAH gene mutations that segregate in a typical autosomal recessive pattern. Substantial work made since the cloning of the human PAH gene in 1983 has suggested that the genotype of an individual, i.e. the precise allelic composition in terms of disease-causing PAH mutations, may be a major determinant of the phenotype. In a previous review [5], we described the many different types of gene alterations harboured by mutant PAH alleles, and discussed how each individual mutation may have a particular quantitative effect on PAH activity. This paper gives a summary of the substantial progress that has been recently made in establishing the correlation between specific PAH mutations and clinical outcome.

Criteria for phenotype classification

PAH deficiency is a ''multifactorial'' disorder in the sense that it has both an inherited component, given by the mutant PAH genotype, and an environmental component, given by the dietary intake of Phe. Because direct determination of PAH activity in liver biopsies is not a feasible approach for diagnosis and classification, a wide variety of indirect methods have been developed to assess the phenotype and the inherent dietary requirements [4, 9]. The most commonly used methods for evaluating the degree of PAH impairment in vivo are based on the determination of pretreatment serum levels of Phe (biochemical phenotype), Phe tolerance, i.e. the dietary intake (per day or per kg body weight per day) tolerated to keep blood Phe levels within the desired therapeutic range at a particular age (metabolic phenotype) and Phe elimination following an oral or intravenous dose of Phe or an oral protein challenge (metabolic phenotype).

Centres throughout the world use different methods and different parameters according to tradition, expertise and resources, and no international guidelines exist at present. This lack of consensus makes comparisons between patients treated at different centres difficult, in particular when attempts are made to correlate the metabolic phenotype with the mutant PAH genotype or other parameters, such as IQ, neurological signs, MRI changes, brain Phe levels, and offspring outcome in maternal phenylketonuria (PKU). We have used for more than 25 years a system that is based on (1) determination of either Phe tolerance at 5 years of age (in patients who require dietary treatment) or serum Phe levels (in patients who require no treatment), and (2) subsequent assignment of patients to one of four arbitrary phenotype categories; ''classical PKU'', ''moderate PKU'', ''mild PKU'', and ''mild hyperphenylalaninaemia (MHP)''. This classification system has been detailed elsewhere [3, 5], and will be referred to throughout this paper.

An infinite number of PAH mutations and genotypes?

The ascertainment of PAH mutations is now nearly complete in many patient populations in Europe and the New World. The combined achievements made by numerous members of the PAH Mutation Analysis Consortium have raised the number of known PAH mutations to approximately 400 (a database collation [8] of mutations is accessible via the internet at http://www.mcgill.ca/pahdb). The observed spectra of PAH mutations closely reflect the impact of population history on genetic variation. The pattern of a typical contemporary European population, as given by frontiers rather than by history, encompasses a high number (20–50) of rare mutations in addition to a few mutations that have reached appreciable frequencies (in the range of 5%–30%) through recurrence, genetic drift, or founder effects. The approximately 400 pathogenic PAH alleles known to date could form more than 80,000 heteroallelic genotypes, i.e. genotypes composed of two different mutant alleles. However, only a minority of these possible genotypes will occur in any population, primarily due to the remarkably skewed relative allele frequencies in most populations (see above). This particular distribution of specific mutant PAH alleles is one of the foundations on which the establishment of genotype-phenotype correlations rests.

The concept of ''functional hemizygosity''

Soon after the identification of two of the world's most common PAH mutations, R408W and IVS12nt1G- > A, the phenotypes associated with these mutations could be established because a great number of homozygous patients were available for classification. However, many of the mutations identified subsequently were relatively rare and appeared in a variety of heteroallelic genotypes, which would mask the contributions from the individual alleles. Considerable heed has therefore been paid to the prospect of accurately determining the enzyme activity associated with individual mutations in artificial cell systems. Whereas it appeared, at first, that in vitro activities correlated broadly with hyperphenylalaninaemia phenotypes, substantial work in the field has now clearly demonstrated that this is not always the case [10]. These observations have led to our present recognition that mutations should be categorised by their effects on enzyme activity as assessed in PAH-deficient patients, at least until more sophisticated expression systems become available.

The major constraints encountered during previous attempts to establish the correlation between individual mutations and a PAH-deficiency phenotype have been that patients with homoallelic genotypes could be found for only a minority of mutations, and that patients with heteroallelic genotypes were not informative. To avoid some of these difficulties, we and others have conducted

large-scale studies on the basis of "functionally hemizygous" patients [2]. These patients carry on one chromosome one of several "null" mutations, i.e. mutations that produce undetectable levels of PAH activity and in combination cause classical PKU in vivo. The functionally hemizygous constellation is equivalent to the homozygous constellation in the sense that only enzyme encoded by the non-null allele contributes to the Phe-hydroxylating capacity. The usefulness of studying functionally hemizygous patients to generate data on genotype-phenotype correlations in PAH deficiency is due to the high relative frequencies of certain null mutations. In two recent large-scale studies, a meta-analysis [7] and a European multicentre study [3], around 35 null mutations were identified, and more than 100 different mutations could be assigned to particular metabolic phenotypes on the basis of functionally hemizygous patients.

Genotype-based prediction of metabolic phenotype

Early studies of the molecular basis of MHP had suggested that the milder of two PAH mutations is "quasi-dominant", i.e. it determines the phenotypic outcome irrespective of the mutation on the other allele [1]. To take this observation into a simple system for expressing the correlation between genotype and phenotype, each category of mutation severity was given an arbitrary value, termed "assigned value" (AV); AV = 1, for classical PKU mutations; AV = 2, for moderate PKU mutations; AV = 4, for mild PKU mutations; and AV = 8, for MHP mutations. This classification system provides a simple numerical parameter, the sum of the two mutations' AVs, that discriminates between the possible combinations of mutation severities (Table 1).

The degree of concordance between predicted versus observed phenotypes was tested in 651 patients with classified genotypes and phenotypes who were enrolled in the European multicentre study (Table 1). The observed phenotype matched the predicted phenotype in 562 of the cases (86%), and in only ten of the cases (1.5%) was the observed phenotype more than one phenotype category away from that expected (Table 1). Notably, there was virtually complete association between genotype and phenotype in the group of individuals with MHP. This group of patients is particularly informative because phenotype classification has been made solely on the basis of serum Phe values with no influence of dietary therapy regimens. The European multicentre study [3] revealed that some genotype-phenotype inconsistencies may be due to phenotype "misclassifications" related to differences in criteria and methods used for phenotype assessment. However, there are also well-documented examples of significant inconsistencies between different patients with identical PAH genotypes. Notably, certain mutations, e.g. I65T, R158Q and R261Q, seem to appear relatively often in these inconsistent cases. We recently proposed [3] that the activities of these mutant enzymes may be subject to regulation by substrate concentrations, implying that the Phe tolerance in patients carrying these mutations may depend on the target plasma Phe levels during treatment.

Genotype related to dietary requirements and outcome

The approach for classification of mutations and genotype-based phenotype prediction was used to study the relation among genotype, biochemical phenotype and cognitive performance in 199 PAH-deficient females enrolled in the Maternal PKU Collaborative Study [6]. Based on their genotype, the subjects were assigned to one of the four classes of severity. The "genotype severity" was significantly related to the untreated blood Phe levels, however with overlaps between the different categories of mutation combinations. Cognitive development in terms of IQ was also significantly related to genotype. Most of the females studied were treated only for the first 6 years of life, and in subjects with genotypes indicating classical or moderate PKU, the mean IQ scores were 83 and 84, respectively, whereas the mean IQ score was 96 in females with a mild genotype. Those who were treated for more than 6 years showed IQ scores 10 points above average for their group [6]. We are cur-

Table 1 Observed versus expected metabolic phenotypes in 651 individuals with PAH deficiency. The expected phenotype was determined for each patient on the basis of the assigned phenotype values (AVs) of the two PAH mutations. The AVs of 105 PAH mutations are listed in [3]

AV1 + AV2	Expected phenotype	*N*	Observed phenotype				Proportion of cases with perfect match between observed and expected phenotype
			Classical PKU	Moderate PKU	Mild PKU	MHP	
2	Classical PKU	321	292	23	6	–	91.0%
3	Moderate PKU	85	27	49	9	–	57.6%
[a]4	Moderate/mild PKU	13	1	6	6	–	92.3%
5–6	Mild PKU	117	4	11	99	3	84.6%
[a]8	Mild PKU/MHP	20	–	–	10	10	100%
9–16	MHP	95	–	–	5	90	94.7%
Total		651	324	89	135	103	

[a] Two mutations with similar severities may confer a milder phenotype than either of the mutations would do if it acted alone. Accordingly, two moderate-PKU mutations (AV1 + AV2 = 2 + 2 = 4) may result in either moderate PKU or mild PKU, and two mild-PKU mutations (AV1 + AV2 = 4 + 4 = 8) may result in either mild PKU or MHP

rently investigating the relation between genotype and cognitive development in 97 Danish PKU patients who have been on diet for 10–14 years. Our preliminary results demonstrate that median IQ is normal and not dependent on genotype. The results from these two studies demonstrate the importance of maintaining dietary therapy, at least until the age of 10–14 years.

Conclusion

Neonatal screening for hyperphenylalaninaemia remains the most effective strategy for detection of new cases of PAH deficiency. DNA diagnostics (Fig. 1) may provide a new and powerful tool for refining the diagnosis, i.e. for anticipating the dietary requirements. Analysis of PAH mutations may be performed directly on the small amount of blood deposited on a Guthrie card, and a genetic diagnosis may therefore be made immediately after birth with no further examination of the child. The ability to predict the phenotype in a newborn with PAH deficiency not only enables the design and early implementation of an optimal dietary regimen, it also greatly improves counselling of the patient's family.

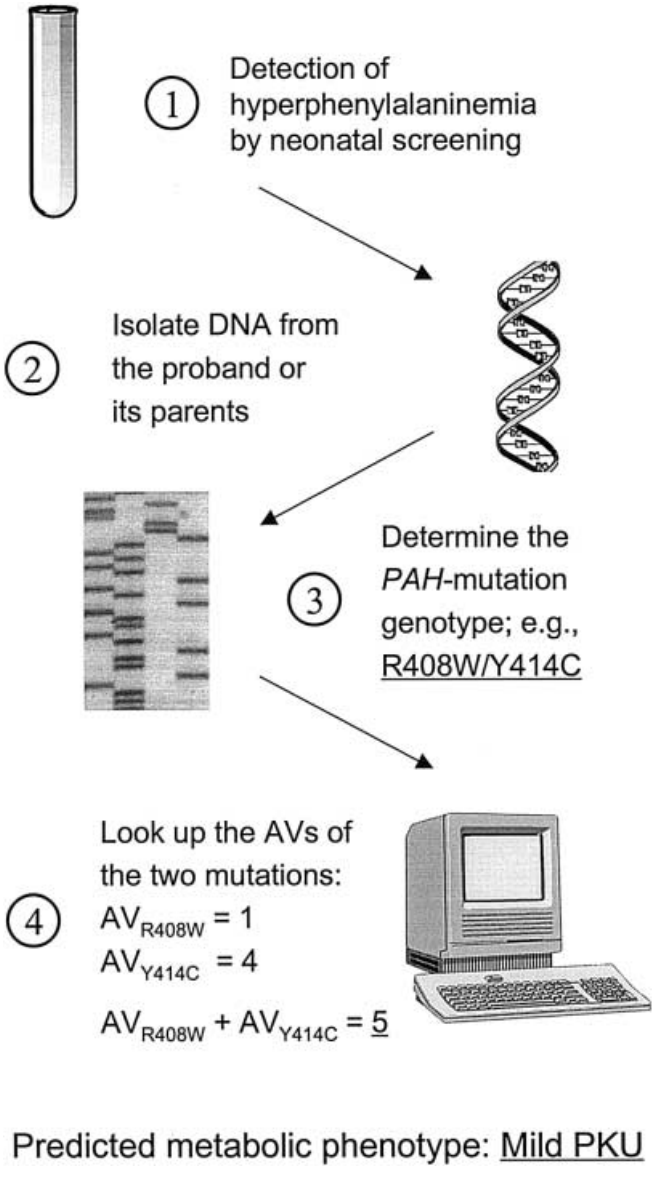

Fig. 1 Diagnosis and classification of PAH deficiency on the basis of PAH mutation genotype analysis. Following detection of hyperphenylalaninaemia by neonatal screening, the genotype is ascertained by mutation analysis of the PAH gene in DNA isolated from the proband or both its parents. The predicted phenotype is calculated on the basis of the AVs of the two mutations according to Table 1. The AVs of 105 PAH mutations are listed in [3]

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