



Variations in genotype-phenotype correlations in phenylketonuria patients

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ABSTRACT. Phenylalanine hydroxylase deficiency is a trait inherited in an autosomal recessive pattern; the associated phenotype varies considerably. This variation is mainly due to the considerable allelic heterogeneity in the phenylalanine hydroxylase enzyme locus. We examined the genotype-phenotype correlation in 54 phenylketonuria (PKU) patients from Minas Gerais, Brazil. Two systems were used. The first was a phenotype prediction system based on arbitrary values (AV) attributed to each mutation and the second was a correlation analysis. An AV was assigned to each mutation: AV = 1 for classical PKU mutation; AV = 2 for moderate PKU mutation; AV = 4 for mild PKU mutation, and AV = 8 for non-PKU hyperphenylalaninemia mutation. The observed phenotype for AV analysis was the clinical diagnosis established by the overloading phenylalanine test. Among the 51 PKU patients that we analyzed based on this trait, in 51% the predicted phenotype did not match the observed phenotype; the highest degree of concordance was found in patients with null/null genotypes. The genotype was observed to be a good predictor of the clinical course of the patients and significant correlations were

found between phenylalanine values at first interview and predicted residual activity, genotype and arbitrary value sum.

Key words: Phenylketonuria; Minas Gerais, Brazil; Genotype; Phenotype; Correlation

INTRODUCTION

Phenylketonuria (PKU, OMIM 261600) and other hyperphenylalaninemias (HPA) are caused by mutations in the gene encoding phenylalanine hydroxylase enzyme (PAH, EC 1.1.4.16.1). More than 500 mutations in PAH gene have been described. Most PKU patients have two different mutations (compound heterozygotes). Interactions between two different mutant polypeptides in the tetrameric enzyme molecule increase disease complexity. The large number of possible allelic combinations makes prediction of the phenotype difficult (Dipple and McCabe, 2000; Scriver and Kaufman, 2001; Kasnauskiene et al., 2003; Kim et al., 2006; Bercovich et al., 2008; Danielle et al., 2009). *In vitro* expression analysis studies have demonstrated a large range of residual activities among different mutations (null to 75%) (Waters et al., 1998; Jennings et al., 2000; Waters, 2003).

It has been demonstrated that PAH genotype does not completely explain phenotypic manifestations and that other factors influence phenylalanine homeostasis (Kayaalp et al., 1997; Mallolas et al., 1999; Scriver and Waters, 1999; Benit et al., 1999). Correlation studies are difficult because of high allelic heterogeneity and broad phenotypic variability. Recently, a system was developed for estimating genotype-phenotype correlations based on the prediction of the phenotypic impact of each mutation (Guldberg et al., 1998; Guttler et al., 1999).

In Minas Gerais State, in southeastern Brazil, nine mutations account for 80% of the PKU alleles (Santos et al., 2006, 2008), which facilitates investigation of their phenotypic effect. Some of the mutations that are common in Minas Gerais, such as V388M, IVS2+5G>A, and IVS2+5G>C, are little known elsewhere. We examined genotype-phenotype correlations for 28 different genotypes in 54 PKU patients.

MATERIAL AND METHODS

Subjects

Fifty-four PKU patients were analyzed; they had been detected by the Minas Gerais State Neonatal Screening Program. Four different types of information were available concerning these patients: phenylalanine levels in neonatal screening tests, established by fluorimetry (Clague and Thomas, 2002); phenylalanine levels at the first consultation (pretreatment phenylalanine values), phenylalanine values in the overloading test (O'Flynn et al., 1980), which was done at six months of age, and dietary phenylalanine tolerance estimated at three, six, and nine months of age.

Based on the phenylalanine overloading test, patients were assigned to one of the four phenotypic PKU subtypes (classical PKU, moderate PKU, mild PKU, or HPA). Patients with serum phenylalanine values higher than 1200 μ M were classified as classic PKU, those with values between 900 and 1200 μ M, as moderate PKU, and those between 600 and 900 μ M as

mild PKU. Patients with values lower than 600 μM were classified as HPA and were not included in our sample (O'Flynn et al., 1980; Guttler, 1980).

Information about the genotype and phenylalanine overloading test was available for all individuals. Phenylalanine values in the neonatal screening tests were available for 53 patients and dietary phenylalanine tolerance data was available for 47 patients. Serum phenylalanine values at the first interview were available for 50 patients.

Genotyping

Genomic DNA isolation and mutation analysis was done previously by PCR and RFLP or based on single-strand conformation polymorphism and sequencing (Santos et al., 2006, 2008).

Mutations in the PKU patients included six missense mutations (I65T, R261Q, R270K, V388M, L348V, and R158W) and nine null mutations, corresponding to five splice-site mutations (IVS2nt5g>c, IVS2nt5g>a, IVS7nt1g>a, IVS10-11G>A, and IVS12nt1g>a), one stop codon (R261X), and three missense mutations (R252W, P281L, R408W) with null effect demonstrated by *in vitro* expression analysis studies. Predicted residual enzymatic activity (PRA) for each of these mutations has been previously reported (Waters et al., 1998; Erlandsen and Stevens, 1999; Rivera et al., 2000; Pey et al., 2003; Anonymous, 2003), except for R158W. Mean PRA values were calculated for each genotype.

Phenotypic prediction system

Mutations were assigned to one of the four phenotype categories (classic, moderate, mild, and HPA non-PKU), according to Guldborg et al. (1998). An arbitrary value (AV) was assigned to each mutation: AV = 1 for classical PKU mutation; AV = 2 for moderate PKU mutation; AV = 4 for mild PKU mutation, and AV = 8 for non-PKU HPA mutation. Phenotypes resulting from a combination of the two mutant alleles were expressed as the sum of the two mutations' AVs (Guldborg et al., 1998). For two mutations (R270K and R158W) present in three individuals (5.5%) there were no AV estimates in the literature, and therefore, these patients were excluded from those analyses that depended on AV information.

Statistical analysis

Statistical analysis was implemented with SAS/STAT® (SAS, 2003). Spearman correlation was estimated among pretreatment phenylalanine levels (values from neonatal screening test and at the first interview), dietary phenylalanine tolerance at three, six and nine months of life, serum phenylalanine in the overloading phenylalanine test, diagnosis, established on the basis of the phenylalanine overloading test, mean PRA and AV sum. Genotypes were coded in two different ways. Initially, a simple sequential order was used. Thereafter, codes based on the residual activity of each allele were constructed. Alleles classified as null or with some residual activity were distributed among three genotype classes: null/null; null/residual; residual/residual. Linear regression was calculated between each of the above variables and genotype. A significance level of $P < 0.05$ was considered for all the analyses.

RESULTS

Based on the phenylalanine overloading test, 31 patients were classified as classic PKU, 17 individuals were classified as moderate PKU and only one patient was classified as mild PKU.

We analyzed 28 different genotypes formed by combinations of 15 PAH mutations (Table 1). Among the 54 individuals, 23 were homoallelic and 31 were heteroallelic. In the latter group, 13 individuals were functionally hemizygous (one null mutation and one mutation with some residual activity).

Table 1. Predicted versus observed phenotypes in 54 phenylketonuria (PKU) individuals from Minas Gerais, Brazil.

Genotypes	N	AV sum	Predicted phenotype	Observed phenotype	Inconsistencies
V388M/V388M	6	4	Mo/Mi	5 C/1 Mo	5
R261Q/R261Q	5	4	Mo/Mi	3 C/2 Mo	3
R261Q/V388M	4	4	Mo/Mi	1 C/2 Mo/1 Mi	1
V388M/I65T	4	4	Mo/Mi	3 C/1 Mo	3
V388M/IVS10-11G>A	4	3	Mo	2 C/2 Mo	2
IVS2+5G>A/IVS2+5G>A	3	2	C	C	
R261Q/R252W	2	3	Mo	2C	2
R261Q/L348V	2	4	Mo/Mi	1 C/1 Mo	1
V388M/R252W	2	3	Mo	2 C	2
I65T/I65T	2	4	Mo/Mi	1 C/1 Mo	1
IVS2+5G>C/IVS10-11G>A	2	2	C	C	
IVS10-11G>A/IVS10-11G>A	2	2	C	1 C/1 Mo	1
R261Q/IVS10-11G>A	1	3	Mo	C	1
R261Q/R408W	1	3	Mo	Mo	
R261Q/R270K	1	?	?	Mo	?
R261Q/I65T	1	4	Mo/Mi	Mo	
IVS2+5G>C/IVS2+5G>C	1	2	C	C	
IVS10-11G>A/IVS12+1G>A	1	2	C	C	
P281L/R261X	1	2	C	Mo	1
P281L/R252W	1	2	C	Mo	1
P281L/P281L	1	2	C	C	
R252W/IVS2+5G>C	1	2	C	C	
R252W/R252W	1	2	C	C	
IVS2+5G>A/L348V	1	3	Mo	C	1
IVS10-11G>A/L348V	1	3	Mo	C	1
IVS7+1G>A/L348V	1	3	Mo	Mo	
R158W/R158W	1	?	?	C	?
R270K/R270K	1	?	?	C	?
Total	54				

AV = arbitrary value; C = classic PKU; Mo = moderate PKU; Mi = mild PKU.

Phenotypic prediction system

The results of the phenotype prediction based on AVs are summarized in Table 1. In our sample, 14 patients had null/null genotypes, 11 had functionally hemizygous genotypes and 25 individuals had genotypes with two mutations and some residual enzymatic activity. Among the 51 patients for whom AV information was available, 25 (49%) showed a phenotype (clinical classification based on phenylalanine overloading tests) in accordance with the AV predicted for their genotype. In this group, 11 patients had genotypes with two null mutations (involving

mutations IVS2+5G>A, IVS2+5G>C, IVS10-11G>A, IVS10-11G>A, IVS12+1G>A, P281L, R252W). The rest of the group was composed of four patients with functionally hemizygous genotypes and 10 individuals had two missense mutations with residual enzymatic activity.

However, in 26 patients (51%) the observed phenotypes were different from the expected phenotypes (Table 1). There were three genotype-phenotype inconsistencies among the individuals bearing two null mutations (R252W/P281L, R261X/P281L and IVS10-11G>A/IVS10-11G>A) that were not associated with classic PKU, as would have been expected. Seven individuals had functionally hemizygous genotypes and 15 had genotypes composed of two mutations with some residual enzymatic activity. All genotypes composed of two missense mutations (N = 23) conferred more than one phenotype, except for R261Q/I65T, which was observed in only one individual.

Statistical analysis

Spearman correlation analysis revealed a highly significant negative correlation ($r = -0.55554$; $P < 0.0001$) between PRA values and phenylalanine values at the first consultation (pre-treatment values). The AV sums significantly correlated with PRA, phenylalanine values at the first consultation and genotype ($P < 0.0001$). However, no significant correlation was observed between PRA and phenylalanine values in the neonatal screening test. In the regression analysis, phenylalanine values at the first interview, dietary phenylalanine tolerance at nine months, PRA and AV sum (dependent variables) were associated with the genotype (independent variable), $P = 0.0016$, 0.002 , 0.0012 , and 0.0003 , respectively. Dietary phenylalanine tolerance measured at three, six and nine months of age correlated with each other (tolerance at three months x tolerance at six months, $r = 0.38895$ and $P = 0.0069$; tolerance at six months x tolerance at nine months, $r = 0.48951$ and $P = 0.0005$; tolerance at three months x tolerance at nine months, $r = 0.32258$ and $P = 0.0270$). The diagnosis was correlated with the overloading test value, as expected ($r = 0.82022$; $P < 0.0001$).

DISCUSSION

It has been proposed that PAH genotype is not a rigorous predictor for clinical progression in phenylketonuria patients. Many factors can influence phenotypic variation in PKU, such as interindividual variations in intestinal absorption, hepatic uptake of dietary phenylalanine, rate of incorporation of phenylalanine into proteins, rates of influx of phenylalanine across the blood brain barrier, mutations affecting tetrahydrobiopterin (which works as a co-factor, but also protects PAH enzyme against proteolytic degradation), as well as interactions of the PAH gene with other loci (Kayaalp et al., 1997; Scriver and Waters, 1999; Dipple and McCabe, 2000; Dipple et al., 2001; Scriver, 2002, 2007).

Two different approaches for phenotype-genotype correlation studies can be used; one is based on an arbitrary value phenotypic prediction system, which usually does not involve a formal correlation test and another based on pairwise correlations/regressions.

Arbitrary value-based phenotypic prediction system

The AV-based method for phenotypic prediction has been used in some studies (Gütler et al., 1999; Acosta et al., 2001; Kasnauskienė et al., 2003). In each study, metabolic phenotypes were predicted by observing functionally hemizygous individuals in each population.

We adopted the same AV categorization system (Guldberg et al., 1998). This system was also adopted in some other studies (Zschocke, 2003; Aulehla-Scholz and Heilbronner, 2003).

We investigated 15 mutations distributed among 28 different genotypes. The most frequent homozygous genotypes were R261Q/R261Q (N = 5) and V388M/V388M (N = 6). The R261Q mutation was classified as moderate (Guldberg et al., 1998). However, in our sample, three of five individuals homozygous for this mutation had classic PKU. Also, three of four patients with genotypes composed of R261Q and any known null allele were also associated with classic PKU. The same occurred with the homozygous V388M/V388M genotype. This mutation had been classified as moderate based on only one functional hemizygous patient (Guldberg et al., 1998). In our sample, five of six patients with a homozygous genotype had classic PKU and only one had moderate PKU. Moreover, four of six patients bearing V388M and any null allele also presented classic PKU, suggesting that the effect of this mutation is more severe than previously reported.

The L348V mutation is another example of the same situation. Although reported as moderate, classic PKU was observed in association with two L348V/null genotypes and in one of two R261Q/L348V genotypes. Therefore, this mutation was also more frequently associated with classic PKU in our sample.

We also observed that heterozygote genotypes constituted by two mutations with similar severities lead to a less severe phenotype than that observed if they were in homozygosis, as previously described by Guldberg et al. (1998). In our sample, patients with R261Q/V388M genotype had a milder phenotype than R261Q/R261Q or V388M/V388M homozygous individuals. However, the suggestion that severity of the illness is generally determined by the less severe allele (Guldberg et al., 1998; Kasnauskienė et al., 2003) was not confirmed in our study. Ten of 13 genotypes of this type (R261Q/null, V388M/null, L348V/null) were associated with classic PKU, which does not support the above-cited generalization.

Better agreement with previous reports was observed for those genotypes composed of null/null mutations, but even among these, some genotypes (R252W/P281L, R261X/P281L and IVS10-11G>A/IVS10-11G>A) were not associated with classic PKU, as would have been expected. On the other hand, all genotypes constituted of two missense mutations were associated with both classic and moderate PKU (Table 1).

In vitro expression data for the R158W mutation are not available; however, this mutation distorts the enzyme active-site structure and probably results in classic PKU, as does the R158Q mutation at the same site (Erlandsen et al., 2003). The R270K mutation gives low residual enzyme activity (2.1%; Leandro et al., 2006). Patients that were homozygotic for either of these two mutations had classic PKU in our study.

Taken together, these results point to an efficiency for the AV system of 49% in our sample. This system was particularly useful as a predictor of clinical course for patients bearing null/null genotypes. The efficiency of the method based on AV estimates will vary depending on the set of mutations in a specific population. For some populations, the system proved to be highly useful. For example, this system gave 96% concordance in a sample in which the most common mutations were null mutations (Kasnauskienė et al., 2003).

Statistical analysis

Good predictors for the clinical course of the patient are important for directing treatment; using statistical analysis, we were able to identify these predictors. Phenylalanine values

from the neonatal screening test did not significantly correlate with any other parameters. This could be because at the time the test is applied, up to the fifth day of life, some children may not have stabilized feeding. This result suggests that this value would be a weak predictor.

Phenylalanine pretreatment values (at first interview) were significantly correlated with PRA and AV sum. We found highly significant regression analysis values between AV sum and genotype, and between genotype and phenylalanine values at first interview. The genotype explained 20% of the phenylalanine value variation at first interview, contrasting with absence of correlation with phenylalanine values in the overloading test. However, phenylalanine pretreatment values (at first interview) did not correlate with tolerance and phenylalanine overloading test values.

The correlations found among the tolerances at ages of three, six and nine months suggest good consistency of these values. These results suggest that genotype is a good predictor of clinical progress in phenylketonuria patients. The predictor is the genotype itself, and not functional values attributed to it.

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