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BRIEF COMMUNICATION

A Phenylalanine Hydroxylase Amino Acid Polymorphism with Implications for Molecular Diagnostics

Mutations in the gene encoding phenylalanine hydroxylase (PAH, EC 1.14.16.1) are associated with various degrees of hyperphenylalaninemia, including classical phenylketonuria (PKU). We examined the *PAH* gene in a Brazilian PKU family of African origin and identified three missense variants, R252W (c.754C → T), K274E (c.820A → G), and I318T (c.953T → C), the two latter of which were transmitted *in cis*. Expression analyses in two different *in vitro* systems showed that I318T is associated with profoundly decreased enzyme activity, whereas the enzyme activity of K274E is indistinguishable from that of the wild-type protein. Detailed kinetic analyses of PAH expressed in *E. coli* showed that the K274E mutant protein has kinetic properties similar to that of the wild-type protein. Population studies have suggested that the K274E variant occurs on approximately 4% of African-American *PAH* alleles, whereas the neonatal screening incidence of PKU among African Americans is only 1:100,000. This is to our knowledge the first demonstration of a PAH missense variant with no apparent association to PAH deficiency. Awareness of this common variant may be helpful to laboratories that perform molecular diagnosis of PAH deficiency in populations of African origin.

Key Words: **phenylalanine hydroxylase; phenylketonuria; amino acid polymorphism; *in vitro* expression.**

Human phenylalanine hydroxylase (PAH, EC 1.14.16.1) is a hepatic enzyme that catalyzes the irreversible conversion of phenylalanine to tyrosine. Mutations in the *PAH* gene segregate in an autosomal recessive pattern in families with phenylketonuria (PKU), a disorder characterized by hyperphenylalaninemia and development of mental retardation in patients exposed to normal amounts of dietary phenylalanine. The frequency of PAH deficiency varies considerably among different ethnic groups, from 1 in 4000 in Ireland to 1 in 119,000 in Japan (1).

More than 400 different sequence variants at the *PAH* locus have been identified worldwide, the vast majority being missense mutations (2). The multitude of mutations is reflected in the phenotypic variability of PAH deficiency, spanning from classical PKU requiring rigorous phenylalanine restriction to mild hyperphenylalaninemia with no need for dietary correction (3). The systematic compilation of mutation data has made mutational analysis of the *PAH* gene in hyperphenylalaninemic newborns an important diagnostic tool, not only for establishing PAH deficiency as the cause of hyperphenylalaninemia and for genetic counseling, but also for predicting the phenotype. In many cases, there is a correlation between the *PAH* mutation genotype and the metabolic phenotype, and large-scale studies have established the phenotype associations for a number of *PAH* mutations (4,5). However, there are also several reports of cases where patients with identical *PAH* mutation genotypes exhibit different degrees of PAH deficiency, suggesting that the *PAH* genotype is not always the only determinant of the metabolic phenotype, as is seen for a number of genetic disorders (6–8).

Another potential pitfall inherent in the use of genetic data in the diagnosis and treatment of PAH deficiency is that only a minority of known mutations have unambiguously been shown to cause enzyme impairment. Due to the relatively large size of the *PAH* gene (13 exons and a 1356-bp open-reading frame), mutations have usually been identified by partial gene analysis, implying that the search for mutations is stopped once two putative disease-causing sequence variants have been identified. Furthermore, as reported recently (9), *in vitro* expression analysis of PAH mutations performed on a

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large-scale provides only some indications as to their pathogenic nature. In this report we describe a missense variant in the *PAH* gene, which was initially considered disease causing on the basis of partial gene analysis and family studies, but on the basis of complete gene analysis, expression studies, and population studies appears to be a silent polymorphism.

MATERIALS AND METHODS

Mutation Analysis

Chromosomal DNA extraction, mutation screening of the *PAH* gene by denaturing gradient gel electrophoresis (DGGE), and direct sequencing were carried out according to previously described procedures (10). Blood for gene analysis was collected from each individual after informed consent, and for children, after consent from the parents. The research protocol was approved by the Ethics Committee of the University Hospital.

In Vitro Expression Analyses

Expression in *Escherichia coli* and by *in vitro* translation was performed as recently described (9). Briefly, wild-type and mutant (K274E, I318T, or R252W) pET-hPAH constructs were expressed in the *E. coli* strain, HMS174 DE3 (Novagen Inc., Madison, WI). SDS-PAGE analysis of *E. coli* extracts followed by immunoblotting was done using the monoclonal anti-PAH antibody PH8 (BD Biosciences-PharMingen, San Diego, CA). PAH enzyme activity was determined by measuring the conversion of [^{14}C]phenylalanine to [^{14}C]tyrosine in extracts from *E. coli.* Wild-type and mutant pcDNA3-PAH constructs (11) were expressed using the T7-based coupled transcription-translation system (Promega Corp., Madison, WI), according to the manufacturer's instructions.

Purification and Characterization of Recombinant PAH

Recombinant wild-type and K274E-mutant PAH were obtained by expression in *E. coli* as maltose-binding fusion proteins (using the pMAL vector system) and subsequent removal of the fusion partner (12). For determination of PAH activity, the purified enzymes (30 μg/ml) were incubated for 5 min at 30°C in the presence of 0.2 mM tetrahydrobiopterin, 1 mg/ml catalase, 0.1 mM Fe(II)SO_4, and different concentrations of phenylalanine in NaHepes (100 mM, pH 7.0). Tyrosine was quantitated by high-performance liquid chromatography, as previously described (13). Equilibrium denaturation, size-exclusion chromatography, and fluorescence measurements were performed as described (14).

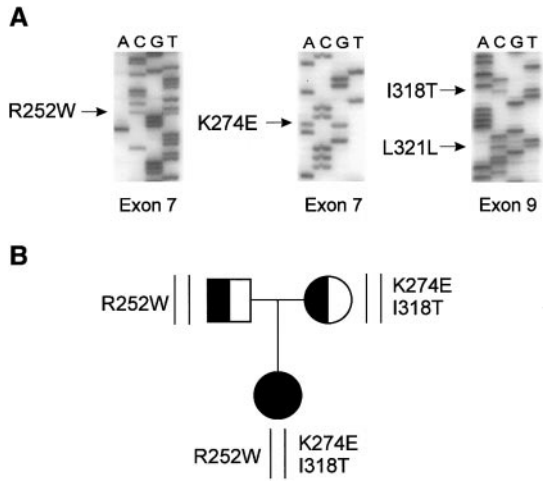

FIG. 1. Segregation *in cis* of *PAH* mutations K274E and I318T in a Brazilian PKU family. (A) Partial sequence ladders of *PAH* exons 7 and 9, showing the R252W (c.754C → T), K274E (c.820A → G), and I318T (c.953T → C) variants in the proband. (B) Pedigree showing the segregation of the mutant K274E-I318T and R252W *PAH* alleles.

RESULTS

During screening for neonatal hyperphenylalaninemia in Brazil, we identified a patient of African origin with an elevated blood phenylalanine concentration of 37.5 mg/dl (2.3 mmol/L), corresponding to a classical PKU phenotype. Initially, PCR amplification and direct sequence analysis of exon 7 of the *PAH* gene had revealed two mutations: the known classical-PKU mutation, R252W (c.754C → T), and a novel mutation, K274E (c.820A → G) (15). Examination of the parents' *PAH* alleles showed that R252W was inherited from the father and K274E from the mother. However, by exhaustively scanning the *PAH* gene by PCR in combination with DGGE, we later identified an additional mutation in exon 9: a T-to-C transition at the second base of codon 318, resulting in the substitution of isoleucine with threonine (Fig. 1A). The I318T (c.953T → C) variant was present in DNA from both the patient and her mother, and thus was inherited in *cis* with the K274E mutation (Fig. 1B).

To further investigate the implications of the three mutations, K274E, R252W, and I318T, on PAH activity, mutant and wild-type forms of the

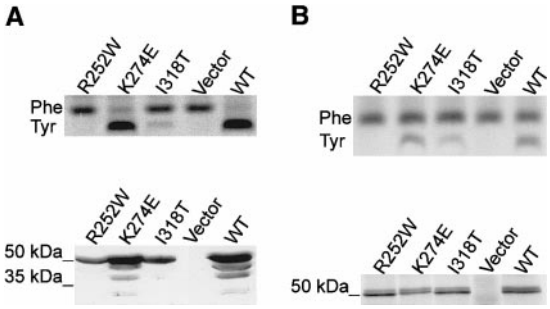

FIG. 2. *In vitro* expression analysis of wild-type PAH and the mutant variants, R252W, K274E, and I318T. (A) Expression in *E. coli.* Upper panel, PAH enzyme assay measuring the conversion of [^{14}C]phenylalanine to [^{14}C]tyrosine in extracts. Lower panel, immunoblotting of PAH. (B) Expression in rabbit reticulocyte lysates. Upper panel, PAH enzyme assay. Lower panel, SDS-PAGE analysis of *in vitro* translation reactions incorporating ^{35}S-labeled methionine.

human PAH cDNA (bases 1–1556, equivalent to the PAH reading frame) were inserted into a bacterial expression vector (pET-11) and expressed in *E. coli.* Western blot analysis of bacterial extracts showed a major band corresponding to the full-length protein of 52 kDa, and additional bands migrating 5–7 kDa faster, corresponding to truncated proteolytic PAH polypeptides made by *E. coli* (16). Because the pathogenic effect of *PAH* missense mutations is often due to misfolding and increased degradation of PAH (9,17–19), it usually is not reasonable to accurately calculate residual activities associated with mutant PAH enzymes. Nevertheless, incubation of *E. coli* extracts in the presence of the substrate L-phenylalanine and the natural cofactor, tetrahydrobiopterin (BH_4), clearly showed that PAH-252W is completely devoid of enzyme activity and that PAH-318T has significantly reduced enzyme activity. In contrast, PAH-274E has a level of activity that is similar to that of the wild-type protein (Fig. 2A). Similar results were obtained when the mutant and wild-type forms of PAH cDNA were examined by *in vitro* translation in rabbit reticulocyte lysates (Fig. 2B).

In order to obtain pure enzyme for detailed kinetic analyses, the K274E mutant enzyme was expressed as a fusion protein in *E. coli* (pMAL-system), cleaved from the fusion partner, and purified to homogeneity. The purified protein showed a specific activity and kinetic properties comparable to those of the wild-type enzyme (Table 1). When the mutant and wild-type enzymes were analyzed in parallel in identical experiments, both showed a positive cooperativity (Hill coefficient ~2.4) and they were both inhibited at substrate concentrations >0.5 mM (Fig. 3) (13).

TABLE 1
Kinetic Parameters

	$[S]_{0.5}$ (L-Phe) (μM)	K_m (BH_4) (μM)	Hill coefficient (L-Phe)
PAH-WT	121	24	2.4
PAH-274E	125	22	2.4

Note. Kinetic parameters for the wild-type (WT) enzyme are from Knappskog and Haavik (13). Data represent average values from triplicate experiments with less than 10% difference between measurements.

Most of the known PKU-associated PAH mutants appear to suffer from a decreased stability *in vivo* and *in vitro* and an increased intrinsic tendency to aggregate (9,17–19). Thus, although the K274E mutant was expressed at similar levels and had kinetic properties similar to the wild-type protein, a decreased stability *in situ* could affect its catalytic efficiency under *in vivo* conditions. We have recently determined the thermodynamic stability and oligomeric structure of wild-type human PAH and found that in the presence of increasing concentrations of urea, a stepwise dissociation occurs of the native tetrameric protein to increasingly denatured dimeric and monomeric forms (14). In the present

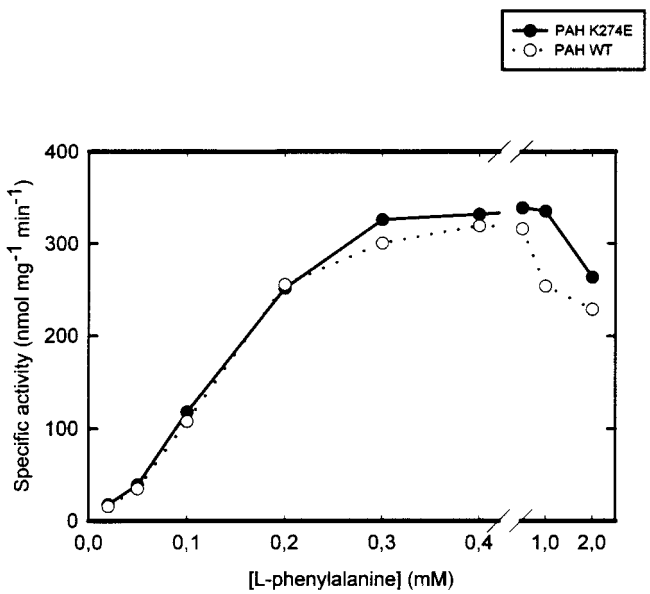

FIG. 3. The effect of L-phenylalanine on the activity of wild-type hPAH (○) and the K274E mutant (●).

study, we determined the molecular dimensions of either the wild-type PAH or the K274E mutant, which previously had been exposed to 0–8 M urea. In these experiments, no significant differences between the wild-type and the mutant forms were observed, indicating that the two enzymes had similar oligomeric structures and thermodynamic stabilities (data not shown).

DISCUSSION

In a recent study of the *PAH* gene in African Americans, we identified the K274E variant in 8% of individuals ($n = 13$) with no family history of PAH deficiency (unpublished data). Although we have not identified a K274E homozygote among normal controls and therefore cannot formally exclude that K274E is associated with PAH deficiency, several lines of evidence strongly suggest that it is a silent variant that should be classified as an amino acid polymorphism. First, a carrier frequency of 8% for a disease-causing mutation would not be compatible with the low incidence of PAH deficiency (1:100,000) among African Americans. Second, K274E is associated with normal enzyme activity in two different *in vitro* expression systems and has kinetic properties similar to that of the wild-type protein. Third, in a patient with PAH deficiency, K274E was inherited in *cis* with a second putative disease-causing mutation, I318T, that was not present on normal K274E alleles and caused reduced enzyme activity *in vitro*. Fourth, lysine at residue 274 of human PAH is not highly conserved during evolution and is substituted with various other hydrophilic amino acids in other species, most notably glutamic acid in *Pseudomonas aeruginosa* (20). Fifth, analysis of the crystallographic structure of PAH indicates that residue 274 is positioned on the molecule surface, supporting the notion of a residue that can be changed to a glutamic acid without disturbing inter- or intramolecular interactions.

Double mutant alleles have been reported in a wide range of inherited disorders and represent a significant problem in molecular diagnostics. At least three double mutant *PAH* alleles have been identified in patients with PAH deficiency, including R413P-Y414C (21), R252W-R408W (22), and E183Q-R408W (23). These alleles are all rare and probably arose by the coincidental introduction of a second mutation into one of the common Y414C or R408W mutant *PAH* alleles. The K274E mutation described in this report is to our knowledge the first example of a missense variant that occurs on normal *PAH* alleles at a relatively high frequency. Our findings underscore the importance of examining the entire disease-associated gene in diagnostic settings and suggest that the disease-causing status of some mutations should be revisited, for example, by *in vitro* expression analysis or sequence alignment of related genes of the same or other species (24).

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