Mutations of the Phenylalanine Hydroxylase (PAH) gene in Filipino Patients with Phenylketonuria

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ABSTRACT

Introduction. Phenylketonuria (PKU), an autosomal recessive metabolic disorder caused by phenylalanine hydroxylase (PAH) deficiency, leads to hyperphenylalaninemia and neurological damage if untreated. This is the first study in the Philippines to identify the disease-causing mutations in the PAH gene of clinically diagnosed Filipino PKU patients.

Methods. The study included four unrelated PKU patients detected by the Philippine Newborn Screening Program from 1996 to 2008. Plasma amino acid analyses for all patients showed increased phenylalanine and low to normal tyrosine levels consistent with the diagnosis of PKU. Mutations in the PAH gene were identified by genomic DNA extraction from dried blood spots of the patients, PAH exon amplification by polymerase chain reaction and subsequent bi-directional DNA sequence analysis.

Results. All patients presented with significantly elevated phenylalanine levels on bacterial inhibition assay and thin layer chromatography. Urinary pterins confirmed the diagnosis of Tetrahydrobiopterin deficiency in two patients while the other 2 patients had the Classical PKU phenotype. Four previously identified mutations in the PAH gene (p.I65T, p.R413P, p.EX6-96A>G, p.R243Q) were identified in those with Classical PKU.

Conclusion. The present results confirm the heterogeneity of mutations at the PAH locus in Filipinos. Neonatal screening and the use of molecular diagnosis significantly aid in the medical management and genetic counseling of patients and their families.

Key Words: Phenylketonuria, PKU, PAH gene, metabolic disorder, phenylalanine hydroxylase

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Introduction

Phenylketonuria (PKU; OMIM 261600), an inborn error of metabolism, is characterized by defective activity of the enzyme phenylalanine hydroxylase (PAH) leading to elevation of plasma phenylalanine. A deficiency of the hepatic PAH or the cofactor tetrahydrobiopterin (BH4) causes the accumulation of phenylalanine which is transaminated to phenylketones. Normally, excess phenylalanine is eliminated from the body by hydroxylation to tyrosine (Figure 1).[1] Deficient enzyme activity, however, causes toxic accumulation of phenylalanine in both the blood and urine, and production of alternate metabolites of phenylalanine. Unless treated early, this metabolic disorder results in growth failure, microcephaly, seizures and mental retardation.[2]

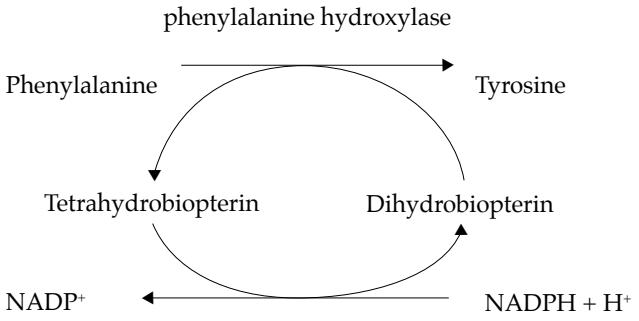

Figure 1. The phenylalanine hydroxylase reaction. Tyrosine is formed by the hydroxylation of phenylalanine. The reaction is catalyzed by phenylalanine hydroxylase, which requires a cofactor tetrahydrobiopterin. The cofactor is oxidized in the reaction to dihydrobiopterin and must be regenerated by dihydrobiopterin reductase with NADPH as the reductant.[1]

This enzyme defect, transmitted as an autosomal recessive trait, is found in most populations. As determined by neonatal screening, the incidence of PKU in Europe is 1/10,000, in China 1/11,000, in Korea 1/41,000 and in Japan 1/120,000.[3] The Philippine Newborn Screening Program, as of August 2008, estimates the crude incidence at 1/116,006. Since 1996, nine (9) patients have so far been confirmed positive by neonatal screening. Three of the 9 patients have

since migrated to other countries, 2 were lost to follow-up and the 4 patients included in this study are presently being followed up at the Metabolic Clinic of the Department of Pediatrics, Philippine General Hospital (PGH).

The study of PKU at the molecular level became possible with the cloning and characterization of the full-length and functional human PAH cDNA (GenBank NM_000277).[4] The human PAH gene (GenBank NC_000012) maps to chromosomal locus 12q23.2, contains 13 exons, spans 90 kilobases and is expressed in the liver and in the kidney.[5-7] Sequencing of all 13 exons provides a mutation detection rate of about 99% with more than 500 different mutations reported to date.[2,3,8]

We herein present the mutation analysis of the PAH gene in clinically diagnosed PKU patients in the Philippines. To our knowledge, this is the first study to define the PKU mutation profile in a Filipino population.

Methods

Subjects

Four of the nine (9) unrelated PKU patients confirmed positive by neonatal screening have been included in the study and are presently being followed up at the Metabolic Clinic of the Department of Pediatrics, Philippine General Hospital. Consanguinity was not reported among the families included. Informed consent was obtained from the parents of the patients.

Plasma amino acid analyses for all four patients showed increased phenylalanine and low to normal tyrosine levels consistent with the diagnosis of PKU (data not shown). All patients also presented with significantly elevated phenylalanine levels on bacterial inhibition assay and thin layer chromatography.

Molecular characterization of the PAH locus

Genomic DNA was extracted from peripheral blood according to standard protocols using the QIAmp Blood DNA Midi Kit (QIAGEN Inc., Valencia, Calif.) with PCR amplification of the 13 exons done using primer sequences as described.[9] Cycling conditions were as follows: 95 ^{o}C denaturation for 10 min; followed by 40 cycles of 95 ^{o}C for 50 sec, 60 ^{o}C for 30 sec, and 72 ^{o}C for 50 sec; and a final elongation step at 72 ^{o}C for 10 min. Bi-directional DNA sequencing was carried out directly on PCR amplified products using the ABI PRISM 3730xl electrophoresis system (Applied Biosystems, USA).

Results and Discussion

Newborn screening results of all four patients showed elevated initial phenylalanine levels on dried blood spot of 240 to 400 umol/L except for patient 2 who did not have a screen done at birth and thus was picked up late. Patients 1 and 2 initially presented with symptomatology ranging from hypotonia, transient tachypnea, decreased sensorium, slow feeding and poor activity, all of which were initially managed as sepsis by the unsuspecting pediatrician. Patient 2, unfortunately, was only referred to our institution at 3 months of age with tachypnea, spasticity, seizures and esotropia. A phenylalanine level of 1500 umol/L confirmed the diagnosis of PKU. Patients 3 and 4 were initially asymptomatic (Table 1).

Two of the 4 patients included in the study have been diagnosed as having Tetrahydrobiopterin (BH4) deficiency based on urinary pterins (data not shown) and the other 2 remaining patients, patients 3 and 4, have the classical PKU phenotype.

All four patients are on a phenylalanine restricted diet with phenylalanine levels monitored regularly. Patients 1 and 2, diagnosed as having BH4 deficiency, have both presented with seizures at 2-4 months of age and are presently on substitution therapy with BH4 tablets, levodopa/carbidopa, and 5-hydroxytryptophan, in addition to dietary treatment.

Direct sequence analyses of the PAH gene was done to determine the molecular background in 4 unrelated clinically diagnosed PKU patients. Three missense mutations [c.194T>C (p.I65T), c.1238G>C (p.R413P) and c.728G>A (p.R243Q)] and one splice mutation [c.611A>G (p.Ex6-96A>G)] were identified to be compound heterozygous in 2 patients included in this present study (Table 2). These previously reported disease-causing mutations were all found to be located in the highly conserved regions spanning exons 3, 6, 7 and 12 of the PAH gene.[3]

Though mutation profiles and their frequencies vary among populations, pathogenic PAH alleles may also reflect geographic origins, genomic diversity and population structures.[10] Interestingly, the mutations identified in this study have been commonly reported in Asians especially among the Chinese, Koreans and the Japanese with c.1238G>C (p.R413P) found to be the most prevalent allele among Japanese PKU patients. On the other hand, the missense mutation c.194T>C (p.I65T) was previously reported to be a prevalent founder mutation in Europeans especially among the Iberians and the Irish.[3,11] Our genotyping studies revealed patient 3 to be compound heterozygous for the missense mutations p.I65T and p.R413P (Figure 2).

The c.611A>G transition in codon 204 of the PAH gene, found to be compound heterozygous in patient 4 (Figure 3), was initially reported to result in a tyrosine to cysteine substitution (p.Y204C).[12] However, this finding has since been refuted with the A>G transition, rather than causing a missense mutation, actually giving rise to aberrant mRNA splicing with a 96-nt. deletion at the 3' end of exon 6 (p.Ex6-96A>G) which occurred through the generation of a fully active and novel 5'donor splice site.[13]

Patient 4 also presented with the c.728G>A transition which changed the amino acid arginine to glutamine at position 243 (p.R243Q). This has been found to be one of the most prevalent alleles in Northern Chinese PKU patients with a relative frequency of 22%.[3,14]

Despite sequencing the entire PAH coding region, no

Table 1. Clinical features of the PKU patients

Patient	Initial Phe peak (umol/L)	BW (kg)	AOG (wk)	Age at onset/ diagnosis	Clinical Course
Patient 1 1yo/M (BH4 Def)	350 1400	2.4	38	2d/19d	At 1 wk: jaundice, sepsis At 19 days: slow feeding, decreased sensorium, spastic, anthropometrics <10th percentile At 3-4 mos: seizures At present: no seizures, developmental delay
Patient 2 2 yo/F (BH4 Def)	1500	1.82	36	2d/3mos	At birth: transient tachypnea, sepsis At 2.5 mos: tachypnea, spasticity, seizures, esotropia At present: developmental delay, truncal hypotonia, spastic extremities
Patient 3 2yo/F (Classic)	Day 5: 400 Day 11: 2000 Day 16: 1700	2.5	38	5d/20d	At birth: good cry/suck/activity At present: slow weight gain, development at par with age
Patient 4 3yo/F (Classic)	240 1600 650	2.24	38	5d/14d	At birth: good cry/suck/activity At 1 mo: rashes At present: asymptomatic, development at par with age

Table 2. List of pathogenic mutations found (Reference GenBank NC_000012)

Patient	Mutation	Protein effect	Location	Type of Mutation	Reference
Pt. 1	?	?	?	?	?
Pt. 2	?	?	?	?	?
Pt. 3	c. 194T>C	p.165T	Exon 3	missense	Waters et al 1999[11] Song et al 2005[3]
	c.1238G>C	p.R413P	Exon 12	missense	Wang et al 1991[14] Song et al 2005[3]
Pt. 4	c.611A>G	p.Ex6-96A>G	Exon 6	splicing	Ellingsen et al 1997[13]
	c.728G>A	p.R243Q	Exon 7	missense	Wang et al 1991[14] Song et al 2005[3]

mutation was identified in the 4 alleles of patients 1 and 2. It is possible that the mutations may be present in other numerous loci affecting the synthesis and regeneration of the cofactor BH4.[15]

At present, PKU serves as a model for the control of genetic disease. It was the first identified neurogenetic disorder, the first treatable inborn error of metabolism, and the first inherited metabolic disease subjected to population screening with its main goal of treatment being the prevention of mental retardation.[2] Early diagnosis by early detection in neonatal screening is deemed essential because this metabolic disorder cannot otherwise be suspected since the clinical signs of developmental delay and mental retardation do not appear until later in infancy or childhood with affected newborns usually appearing normal.[16]

PAH deficiency is diagnosed by newborn screening using the Guthrie bacterial inhibition assay (BIA) test. Other tests currently in use are the fluorometric analysis and tandem mass spectrometry (MS). These have brought excellent prognosis for individuals with PAH deficiency treated early since it is advised that an early and well-maintained treatment by dietary restriction of phenylalanine must commence in the neonatal period or no later than early infancy for normal development and prevention of CNS involvement.[2,17]

The identified PAH genotype in patients allows efficient prediction of the clinical form of PKU and therefore, provides optimal treatment for the affected individual. The established genotype-phenotype correlation in a number of the PAH gene mutations makes molecular genetic testing in PKU valuable in the precise diagnosis of PKU suspected on the basis of mass newborn screening data.[18]

Conclusion

The present results confirm the heterogeneity of mutations at the PAH locus in Filipinos. It further allows understanding of the structure and function of the mutant protein which is useful for genotype/phenotype correlation in patients.[3] Despite the present results not being able to provide genotype-phenotype correlations, it shows the

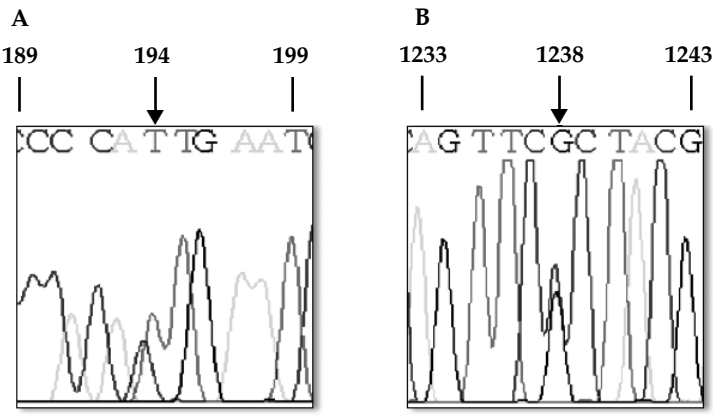

Figure 2. Chromatogram of PKU patient 3 showing the missense mutations A. c.194T>C in exon 3 responsible for p.165T; and B. c.1238G>C in exon 12 resonsible for p.R413P.

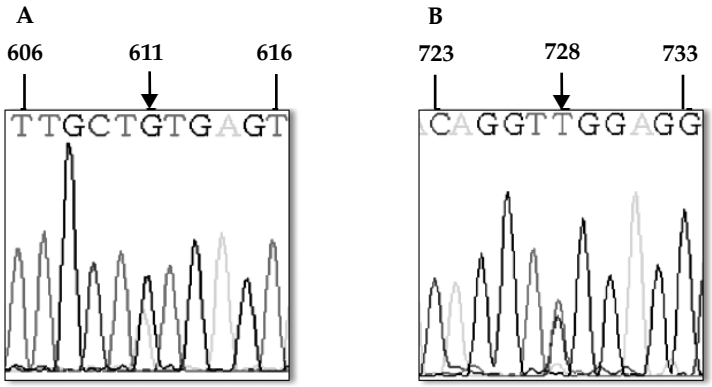

Figure 3. Chromatogram of PKU patient 4 showing A. c.611A>G in codon 204 of exon 6 responsible for p.EX6-96A>G, splicing; and B. the missense mutation c.728G>A responsible for p.R243Q.

heterogeneity of mutations at the PAH locus in Filipinos and implies that our mutation spectrum may be similar to other Asian populations.

In countries abroad, assessing the PAH gene is becoming routine to evaluate patients identified by newborn screening. Direct mutation analysis of the PAH gene may well be considered beneficial for confirmatory diagnostic testing, genetic counseling for carrier detection for at risk family members and if an option, effective prenatal diagnosis.

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