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

Catherine Lynn T. Silao1,2, Daffodil M. Canson1, Karen N. Hernandez1, Mary Anne D. Chiong1,2, Sylvia Capistrano-Estrada1,2, Carmencita David-Padilla1,2

1Institute of Human Genetics, National Institutes of Health, University of the Philippines Manila
2Department of Pediatrics, College of Medicine and Philippine General Hospital, University of the Philippines Manila

ABSTRACT

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). 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.

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

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. 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. 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). 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. 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 °C
denaturation for 10 min; followed by 40 cycles of 95 °C for
50 sec, 60 °C for 30 sec, and 72 °C for 50 sec; and a final
elongation step at 72 °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
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.

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. 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.

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 advisable 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.

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.

### Table 1. Clinical features of the PKU patients

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

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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutation</th>
<th>Protein effect</th>
<th>Location</th>
<th>Type of Mutation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt. 2</td>
<td>c. 194T&gt;C</td>
<td>p.165T</td>
<td>Exon 3</td>
<td>missense</td>
<td>Waters et al 1999, Song et al 2005</td>
</tr>
<tr>
<td>Pt. 4</td>
<td>c.611A&gt;G</td>
<td>Exn6-96A&gt;G</td>
<td>Exon 6</td>
<td>splicing</td>
<td>Ellingsen et al 1997</td>
</tr>
</tbody>
</table>

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.

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. 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.

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. 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. Despite the present results not being able to provide genotype-phenotype correlations, it shows the

---

38 ACTA MEDICA PHILIPPINA
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.

Acknowledgments

Acknowledgments go to the patients and their families for participating in this study. The authors also sincerely thank Kahlil dela Cruz-Rama, Michelle Demata-Rana, Aster Lynn Sur, Drs. Conchita Abarquez and Ambrosio Jumangit III for their technical assistance and the reviewers for their valuable help. This study was supported by the Institute of Human Genetics, National Institutes of Health Philippines.

References