Identification and functional analysis of mutations of the phenylalanine hydroxylase gene in patients with phenylketonuria

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Sang-Wun Kim

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ABSTRACT

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Phenylketonuria (PKU) is an inborn error of metabolism that results from a deficiency of phenylalanine hydroxylase (PAH). Determining the relationship between genotype, phenotype, and tetrahydrobiopterin (BH_4)-responsiveness should provide very useful information on dietary and therapeutic strategies. Therefore, we characterized the *PAH* mutations of 78 independent Korean patients with PKU or hyperphenylalaninemia. Eleven *PAH* missense mutations, including six novel mutations found in this study, have been functionally and structurally analyzed. To analyze the effects of *PAH* missense mutations on the PAH protein structure, model structures of PAH protein and the composite tetramer were constructed using the software program, SHEBA.

PAH nucleotide sequence analysis revealed 39 different mutations, including ten novel mutations. The novel mutations consisted of nine missense mutations and a

novel splice site variant. We also observed three BH_4-responsive mutants. No PAH activity was detected for some mutants. However, the residual activities associated with other mutants ranged over a wide spectrum. In some severe *PAH* mutations detected in classical PKU, PAH enzyme activity was increased when treated with BH_4. The composite model structure of PAH revealed that dimer stability was reduced in the BH_4-responsive mutants, whereas tetramer stability remained normal. This study suggests that even patients with classical PKU can be treated with BH_4 supplementation. Further studies to validate these structural and functional analyses of BH_4 responsiveness should facilitate the development of new therapeutic strategies for PKU patients.

Key words: phenylketonuria, hyperphenylalaninemia, phenylalanine hydroxylase, mutation, tetrahydrobiopterin, expression, structure, modeling

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I. INTRODUCTION

Phenylketonuria (PKU; MIM 261600) is an autosomal recessive metabolic disease caused by a deficiency of phenylalanine hydroxylase (PAH; EC 1.14.16.1). PAH is a hepatic enzyme that catalyses the hydroxylation of L-phenylalanine (L-Phe) to L-tyrosine (L-Tyr) using tetrahydrobiopterin (BH_4) as a cofactor.[1] This reaction is the rate-limiting step in the catabolic pathway of phenylalanine, which results in the complete degradation of the amino acid.

The *PAH* spans about 90 kb on chromosome 12q and comprises 13 exons. It has three structural domains consisting of an N-terminal regulatory domain (residues

1–142), a central catalytic domain (residues 143–410), and a C-terminal tetramerization domain (residues 411–452). [2] The active PAH enzyme is composed of four monomeric proteins. Recent studies of PAH crystal structure have provided information on the active site and the binding sites of its substrate and cofactor.[3-5]

More than 490 different mutations of the *PAH* have been reported (*PAH* Mutation Analysis Consortium Database, http://www.mcgill.ca/pahdb). The mutation profile of the *PAH* is not restricted to any one region, but is spread throughout the structural domains. The mutations lead to a variety of clinical and biochemical phenotypes with different degrees of severity, from mild hyperphenylalaninemia to classical PKU.[6] Several studies have investigated the relationship between genotype and phenotypic expression.[7,8]

Synthetic BH_4 is routinely used in replacement therapy to treat patients with one of the rare genetic defects of cofactor biosynthesis or regeneration.[9] Since Kure *et al*. described four BH_4-responsive patients with known mutations in the *PAH* gene in 1999, many BH_4-responsive *PAH* mutations have been reported[10–17] and a new treatment modality for PKU has been proposed. BH_4 treatment of responsive patients has resulted in successful control of blood phenylalanine levels with progressive relief or withdrawal from the phenylalanine-restricted diet.[18–20] Determining the relationship between genotype, phenotype, and BH_4-responsiveness should provide very useful information on dietary and therapeutic strategies.

Therefore, we analyzed the *PAH* in 78 patients with PKU and their families to study genotype–phenotype relationships and to help with genetic counseling. And then we analyzed the mutation spectra of the *PAH* in Korean patients and compared them with those of other ethnic groups, including Japanese and Chinese. Furthermore, we investigated the enzyme activities of 11 *PAH* missense mutations identified in this study, including six novel mutations (P69S, G103S, L293M, G332V, S391I, A447P), two mutations common in east Asian patients with PKU (R243Q, R413P), and three BH_4-responsive mutations (R53H, R241C, R408Q), using transient protein overexpression in mammalian cells. Novel mutations were defined by exclusion from the PAHdb (http://www.pahdb.mcgill.ca) and previously reported mutations on PubMed (http://www.ncbi.nlm.nih.gov/PubMed/). To investigate the effects of BH_4 on mutant PAH enzyme expression and activity in mammalian cells, we analyzed BH_4 responsiveness in vitro using the natural cofactor BH_4 ([6R]-5,6,7,8-tetrahydro-L-biopterin dihydrochloride). The effects of *PAH* missense mutations on the protein structure were also analyzed.

II. MATERIALS AND METHODS

1. Subjects

This study was approved by the institutional review board of the National Institute of Health, Korea. The study included 78 unrelated families with PAH deficiency. Participants were recruited from the Korean PKU family support group. Most of them were identified in neonatal screening, and PAH deficiency was diagnosed by conventional biochemical methods. Patient severity was assigned to classical PKU, moderate PKU, or MHP, according to the plasma phenylalanine concentration prior to phenylalanine restriction diet. The level for classical PKU was 1,200 μM or more; the level for moderate PKU 600–1,200 μM; the level for MHP less than 600 μM. Informed consent for DNA analysis was obtained from the patients and their families.

2. BH_4 loading test

For the BH_4 loading test, patients without a phenylalanine restriction diet were administered orally at a dose of 20 mg/kg (for the patients under 36 months old) or 7.5 mg/kg (for the patients over 36 months old). Blood phenylalanine levels were measured before, 1, 2, 4, 6, 8, 12, and 24 h after administration. The BH_4 loading test was considered positive when initial plasma phenylalanine concentration decreased

by at least 40% after 12 h. Urinary pterin analysis and dihydropteridine reductase (DHPR) assay were performed to exclude 6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiencies.

3. Mutation analysis

Genomic DNA was isolated from peripheral blood leukocytes using the QIAamp DNA blood kit following the manufacturer's instruction (Qiagen, Hilden, Germany). All 13 exons including exon–intron boundaries and 2 kb of the 5'-upstream region of the *PAH* were amplified by PCR. PCR amplicons were extracted from an agarose gel using a gel extraction kit (Qiagen, Hilden, Germany). Direct sequencing was performed using a BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 2.0 (Applied Biosystems, Foster City, CA, U.S.A) and analyzed with an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA, U.S.A) according to the standard methods. When available, parental DNA samples were sequenced to confirm trans configurations in compound heterozygotes and to distinguish homozygosity from hemizygosity. In addition, *PAH* in 50 normal individuals were analyzed to confirm that the novel sequence variations were not polymorphisms but real pathogenic mutations. Novel mutations were defined by exclusion from the PAHdb (http://www.pahdb.mcgill.ca) and previously reported mutations on PubMed (http://www.ncbi.nlm.nih.gov/PubMed/).

4. Construction of PAH expression plasmids and mutagenesis

The full-length human *PAH* cDNA, phPAH247 (American Type Culture Collection, Manassas, VA, U.S.A) was digested with the restriction endonucleases *EcoR*I and *Nhe*I and ligated into pcDNA3.1(+) vector digested with *EcoR*I and *Xba*I. For mutagenesis, *PAH* cDNA was subcloned into the *EcoR*I and *Apa*I sites of the pBlueScript II SK(–) phagemid vector and mutagenized with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A) (Table 1). After DNA sequence verification of the desired mutations, all mutant *PAH* cDNAs were subcloned from pBlueScript II SK(–) into pcDNA3.1(+).

Table 1. Oligonucleotides used for the mutagenesis.

Mutations	Primer Sequence(5'-3')
R53H	GCATTGGCCAAAGTATTGC**A**CTTATTTGAGGAGAATGATG
P69S	TGACCCACATTGAATCTAGA**T**CTTCTCGTTTAAAGAAAGATGAG
G103S	CTTGAGGCATGACATT**A**GTGCCACTGTCCATGAG
R241C	CTTGCACTGGTTTC**T**GCCTCCGACCTGTGG
R243Q	GGTTTCCGCCTCC**A**ACCTGTGGCTGG
L293M	GTTGGGACATGTGCCC**A**TGTTTTCAGATCGCAGC
G332V	GGTTTACTGTGGAGTTTG**T**GCTCTGCAAACAAGGAG
S391I	CCTGTATTACGTGGCAGAGA**T**TTTTAATGATGCCAAGGAG
R408Q	CTGCCACAATACCTC**A**GCCCTTCTCAGTTCGC
R413P	CCCTTCTCAGTTC**C**CTACGACCCATACACC
A447P	AATTGGAATCCTTTGCAGT**C**CCCTCCAGAAAATAAAGTAAAG

5. Transient eukaryotic expression of PAH

To achieve 60%–80% confluence at the time of transfection, COS7 monkey kidney cells were plated one day before transfection in Dulbecco's Modified Eagle's Medium (Invitrogen, San Diego, CA, U.S.A) supplemented with 10% heat-inactivated fetal bovine serum. Cells were transfected in 60 mm plates with 4 μg of wild-type or mutant pcDNA3.1(+)–*PAH*, using Lipofectamine Plus Reagent (Invitrogen, San Diego, CA, U.S.A). For the negative control, COS7 cells were transfected with the pcDNA3.1(+) vector, the parent plasmid lacking the *PAH* cDNA

insert. All transfections included 1 μg of the pcDNA3.1/His/LacZ vector (Invitrogen, San Diego, CA, U.S.A) as a control for transfection efficiency. Cells were harvested for RNA and protein extraction 48 h after transfection. A portion of the cells was lysed in Reporter Gene Assay Lysis Buffer as described in the β-Gal Reporter Gene Assay (Roche Applied Science, Mannheim, Germany), and the total protein concentration was determined with the Bradford assay (Bio-Rad, Munchen, Germany). β-Galactosidase activity was measured using the β-Gal Assay Kit (Invitrogen, San Diego, CA, U.S.A).

6. PAH activity assays

Enzyme activity was assayed by measuring the conversion of L-[^{14}C]phenylalanine to L-[^{14}C] tyrosine at 37 °C in a volume of 50 μL. The crude extract (100 μg) was incubated for 5 min in the presence of 0.2 μCi of L-[^{14}C]Phe (460 μCi/mmol; Amersham, Buckinghamshire, UK), 0.25 mM L-Phe, 40 units of beef liver catalase, and 250 mM Tris-HCl (pH 7.8). All products were purchased from Sigma (Sigma–Aldrich, Milano, Italy). After 5 min, the reaction was initiated by the addition of BH_4 (Schircks, Jona, Switzerland) to a final concentration of 0.8 mM. The reaction was stopped after 60 min by the addition of 5 μL of carrier L-Phe/L-Tyr (0.1 M each) and incubation in boiling water for 5 min. Samples were then centrifuged at 12,000 × g for 5 min and 15 μL of the supernatant was spotted onto a thin-layer chromatography (TLC) plate (TLC Silica Gel 60 plates, Merck, Darmstadt, Germany).

TLC was performed using a mixture of chloroform:methanol:ammonia (55:35:10) for 60 min and the plate was dried for 10 min. The radioactivity of L-[^{14}C]phenylalanine and converted L-[^{14}C]tyrosine was quantified using ImageQuant after visualization with a PhosphoImager (Molecular Dynamics, Sunnyvale, CA, U.S.A). The residual activities of mutant PAH enzymes were expressed as a percentage of the wild-type PAH enzyme activity and normalized to transfection efficiencies based on β-galactosidase activities. All the *PAH* mutants were transfected three times, enzyme activities were also measured three times, and the mean enzyme activities were calculated.

7. In vitro BH_4-responsiveness assay

To test the in vitro BH_4 responsiveness of the wild-type and mutant *PAH*, we simultaneously transfected COS7 cells with 4 μg of wild-type or mutant pcDNA3.1(+)–*PAH* in two 60 mm dishes. All transfections included 1 μg of pcDNA3.1/His/LacZ vector as a control for transfection efficiency. Five hours after transfection, the culture media were replaced with fresh DMEM with or without 1 mM BH_4. The cells were harvested 48 h after transfection and PAH enzyme activities were measured as described above.

8. Western blot

Fifty micrograms of the same crude extracts analyzed for both PAH and

β-galactosidase activities were resolved electrophoretically on 10% polyacrylamide gel and blotted onto polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA, U.S.A). The membrane was hybridized with a 1:200 dilution of murine anti-PAH antibody (PH8; Immunocor, San Diego, CA, U.S.A), and subsequently with a horseradish-peroxidase-conjugated anti-mouse-IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A). The signals were then visualized using ECL Plus enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A).

9. RT–PCR analysis

Total RNAs were isolated using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). RT reactions were conducted using 100 pmol/μL random primer (Takara Shuzo, Kyoto, Japan). The PCR amplification of cDNAs was performed on each RT sample and a no-template control using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, U.S.A). To specifically detect *PAH* transcripts, the following primers were used at 10 pmol: forward primer, 5´-CTTGTATAAAACCCATGCTTGCT-3´; reverse primer, 5´-GTAATTCACCAAAGGATGACAGG-3´. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified as an internal control: forward primer, 5´-AGACAAGATGGTGAAGGTCG-3´; reverse primer, 5´-TCATGAGCCCTTCCACGATG-3´. After PCR amplification, samples were

resolved electrophoretically on 1.5% agarose gels and visualized with ethidium bromide.

10. Composite model structure

No crystal structure of PAH with three key domains (regulatory, catalytic, and tetramerization) had been deposited in the PDB database as of January 2005. Therefore, initially, a model structure of the PAH protein was derived from the three-dimensional structures of a rat tyrosine hydroxylase (2toh) and a rat phenylalanine hydroxylase (2phm); 2toh contains catalytic and tetramerization domains and 2phm contains catalytic and regulatory domains. The 2toh protein was structurally aligned to 2phm and the coordinates of 2toh were transformed with the structural alignment program, SHEBA.[21] The sequence identity and C-α-atom root-mean-square deviation (cRMS value) after their superposition were 59% and 0.9 Å, respectively. A PAH composite model structure with three key domains was then constructed, and the mutation-affected residues were refined using the modeling package GEMM.[22] The composite model structure of the tetramer was then constructed to estimate the functional consequences of the mutations.

III. RESULTS AND DISCUSSION

1. Mutation analysis

PAH nucleotide sequence analysis of 78 unrelated PKU probands revealed 39 different mutations (Tables 2, 3). Among 78 patients, two mutation alleles were detected in 59 patients (75%), either compound heterozygous or homozygous (52 and seven, respectively). Only one mutation allele was revealed in 19 patients. Ten novel mutations were identified in this study. These novel mutations included nine missense substitutions: P69S, G103S, N207D, T278S, P281A, L293M, G332V, S391I, and A447P. From database comparison, the glycine[103] is found to be conserved among human, mouse, and rat. The remaining mutated amino acid residues were even more strictly conserved among human, mouse, rat, and zebrafish. A novel splice-site variant, IVS10–3C>G, was also detected. The -3 sequence of the splicing acceptor site is a strictly conserved sequence, and this substitution might result in aberrant splicing products. No novel frameshift mutations or nonsense mutations were detected.

Table 2. Genotypes for mutations of the *PAH* in 78 Korean phenylketonuria (PKU) patients.

Patient number	*PAH* allele 1	*PAH* allele 2	Class[b]
1	IVS4–1G>A	A259T	Classical
2	P407S	R413P	Moderate
3[a]	Y356X	R408Q	Moderate
4	S70[del]	L255S	MHP
5	N207D	Y325X	Classical
6	Y204C	?	Classical
7	Y204C	?	NA
8	Y356X	?	Classical
9	IVS4–1G>A	R243Q	Classical
10[a]	R241C	T278I	Moderate
11	R243Q	?	NA
12	R243Q	?	NA
13	A259T	T278I	NA
14	A345T	G332V	Classical
15	G103S	R413P	NA
16	IVS4–1G>A	V388M	NA
17	R243Q	R252Q	NA
18	A259T	?	NA
19	R413P	Y325X	NA
20	IVS2nt–2T>C	?	Moderate
21	Y204C	Y204C	NA
22	Y325X	V388M	Classical
23	Y204C	?	Classical
24	IVS4–1G>A	V388M	NA
25	R241C	R241C	MHP
26[a]	R53H	R243Q	Moderate

Table 2 (Continued)

Patient number	*PAH* allele 1	*PAH* allele 2	Class[b]
27	N207D	?	NA
28	Y204C	Y204C	Classical
29	R111X	R243Q	NA
30	A259T	?	NA
31	R176X	A259T	NA
32	IVS4–1G>A	?	Classical
33	Y204C	Y356X	Classical
34	R158Q	R243Q	NA
35	IVS4–1G>A	L293M	Classical
36	IVS4–1G>A	Y356X	NA
37	R243Q	P281A	Classical
38	D84Y	Y356X	NA
39[a]	R241C	R243Q	Moderate
40	Y204C	?	Classical
41	R243Q	V388M	Classical
42	P69S	R261Q	NA
43	S70[del]	IVS4–1G>A	Classical
44	IVS4–1G>A	?	Classical
45	R413P	?	Classical
46	Y204C	?	NA
47	W187X	Y356X	Classical
48	IVS4–1G>A	R243Q	NA
49	R243Q	?	Moderate
50[a]	R241C	A259T	Moderate
51	IVS4–1G>A	S310F	Moderate
52	Y204C	Y325X	MHP
53	R243Q	A345T	NA
54	IVS4–1G>A	T278S	Classical

Table 2 (Continued)

Patient number	*PAH* allele 1	*PAH* allele 2	Class[b]
55	T278I	R413P	NA
56	G332E	?	NA
57	A259T	?	NA
58	T278I	Y356X	Classical
59	Y204C	R243Q	NA
60[a]	R241C	A259T	Moderate
61	IVS10–3C>G	IVS10–3C>G	NA
62	Y204C	P281L	NA
63	R53H	V388M	MHP
64	A447P	?	NA
65	R243Q	A345T	Classical
66	R176X	S391I	Classical
67	IVS4–1G>A	R261X	NA
68[a]	R241C	R243Q	Moderate
69	IVS4–1G>A	IVS4–1G>A	Classical
70	R243Q	Y325X	NA
71	Y356X	?	NA
72	IVS4–1G>A	P281L	Moderate
73	G239S	P281L	Classical
74	R243Q	Y356X	NA
75	Y204C	Y204C	Classical
76	R241C	R241C	MHP
77	A259T	T278I	NA
78	Y204C	R243Q	NA

[a]BH_4 responsive type

[b]NA not available

Table 3. Spectrum of *PAH* mutations detected in this study.

Mutation name	Normal	Mutation	Location	Allele frequency	Relative frequency (%)	Refer-ences[a]
R53H	CGC	CAC	Exon 2	2	1.3	1
IVS2–2T>C			Intron 2	1	0.6	2
P69S	CCT	TCT	Exon 3	1	0.6	Novel
S70[del]	TCT	c.208-210 delTCT	Exon 3	2	1.3	1
D84Y	GAT	TAT	Exon 3	1	0.6	1
G103S	GGT	AGT	Exon 3	1	0.6	Novel
R111X	CGA	TGA	Exon 3	1	0.6	1
IVS4–1G>A	GT	AT	Intron 4	16	10.1	1
R158Q	CGG	CAG	Exon 5	1	0.6	1
R176X	CGA	TGA	Exon 6	2	1.3	1
W187X	TGG	TAG	Exon 6	1	0.6	1
E6–96A>G			Exon 6	16	10.1	1
N207D	AAT	GAT	Exon 6	2	1.3	Novel
G239S	GGT	AGT	Exon 7	1	0.6	1
R241C	CGC	TGC	Exon 7	9	5.7	1
R243Q	CGA	CAA	Exon 7	19	12	1

Table 3 (Continued)

Mutation name	Normal	Mutation	Location	Allele frequency	Relative frequency (%)	Refer-ences[a]
R252Q	CGG	CAG	Exon 7	1	0.6	3
L255S	TTG	TCG	Exon 7	1	0.6	1
A259T	GCC	ACC	Exon 7	9	5.7	1
R261Q	CGA	CAA	Exon 7	1	0.6	1
R261X	CGA	TGA	Exon 7	1	0.6	1
T278I	ACC	ATC	Exon 7	5	3.2	1
T278S	ACC	AGC	Exon 7	1	0.6	Novel
P281L	CCT	CTT	Exon 7	3	1.9	1
P281A	CCT	GCT	Exon 7	1	0.6	Novel
L293M	TTG	ATG	Exon 8	1	0.6	Novel
S310F	TCT	TTT	Exon 9	1	0.6	1
Y325X	TAC	TAG	Exon 10	5	3.2	4
G332E	GGG	GAG	Exon 10	1	0.6	1
G332V	GGG	GTG	Exon 10	1	0.6	Novel
A345T	GCT	ACT	Exon 10	3	1.9	1
IVS10–3C>G			Intron 10	2	1.3	Novel

Table 3 (Continued)

Mutation name	Normal	Mutation	Location	Allele frequency	Relative frequency (%)	Refer-ences[a]
Y356X	TAC	TAA	Exon 11	9	5.7	1
V388M	GTG	ATG	Exon 11	5	3.2	1
S391I	AGT	ATT	Exon 11	1	0.6	Novel
P407S	CCT	TCT	Exon 12	1	0.6	1
R408Q	CGG	CAG	Exon 12	1	0.6	1
R413P	CGC	CCC	Exon 12	5	3.2	1
A447P	GCC	CCC	Exon 13	1	0.6	Novel
Total				136	86	

[a](1) Mutations reported in the PAHdb, (2) mutation reported by Song *et al*.[23], (3) mutation reported by Chien *et al*.[24], (4) mutation reported by Park *et al*.[25].

R243Q, IVS4–1G>A, and E6–96A>G were the most prevalent mutations in Korean patients with PKU. They have been reported to be some of the most frequent mutations in Asian populations (Table 4) and accounted for 51 of the 156 total chromosomes (32.7%) in this study.

Table 4. Relative frequencies of common *PAH* mutations found in oriental populations.

Mutation	Relative frequencies (%, allele frequency/total subject chromosome)			
	78 Korean	41 Japanese[26]	52 Chinese[a,27]	25 Taiwanese[24]
R243Q	12	7.3	18.3	6
IVS4–1G/A	10.1	7.3	7.7	2
E6–96A>G	10.1	6.1	11.5	4
R241C	5.7	7.3	NA	32
A259T	5.7	0	NA	0
Y356X	5.7	4.9	6.7	0
T278I	3.2	7.3	NA	0
Y325X	3.2	0	NA	0
V388M	3.2	1.2	NA	0
R413P	3.2	30.5	8.7	4
R111X	0.6	3.7	10.7	4
R408Q	0.6	0	NA	14
Total detected	86	92.7	66.5	90

[a]NA not available

It is well known that different ethnic groups have their own distinctive and diverse *PAH* mutant allele series that include one or a few prevalent founder alleles.[28] In comparison of *PAH* mutation data among ethnic groups, there are the correlations between mutation and genetic history of investigated populations. For example, in Europe, there are several prevalent founder alleles, including R408W, IVS12+1G>A, IVS10–11G>A, and Y414C, that represent the expansion, migration, and genetic drift of European populations.[28] In particular, the R408W mutation has a frequency of 20%–84% in patients with PKU in Eastern Europe and Germany. However, these mutations are rarely detected in oriental populations. In a previous study, Okano *et al*.[27] reported the frequency and distribution of *PAH* mutations among Japanese, Korean, and Chinese patients. Because the study was undertaken in the early 1990s, it was restricted to screening for previously isolated mutations. Unidentified but relatively frequent alleles, such as R241C, were not investigated, and only ten Korean patients were included, which is a relatively small number to represent Korean allelic distribution. The present study, with 78 participants, extends these previous results to give a more comprehensive understanding of *PAH* allele distribution and frequency in Koreans. Although some overlaps of mutant allele distribution are observed among Japanese, Chinese, and Korean populations, there are several significant differences (Table 4).

R243Q, E6–96A>G, and IVS4–1G>A, the most frequent mutations in our study, are also frequently detected in Japanese, Chinese, and Taiwanese. However, R111X, a frequent mutation in Japanese and Chinese patients, is very rare in Korean patients.

The mutant R413P is the most prevalent allele in Japanese, but a very small proportion of probands have the R413P allele in Korean and Taiwanese. The mutant IVS4–1G>A occupied a relatively larger proportion in Korean mutant allele profiles than in Japanese or Chinese. Although the mutant A259T was not detected in any other oriental population studies, it was identified in nine different families in this study.

2. BH_4 loading test

Interestingly, the two R241C homozygous patients (patient 25 and patient 76) showed MHP, and all compound heterozygous individuals with R241C (2 with R241C/R243Q, another 2 with R241C/A259T and 1 with R241C/T278I) showed BH_4 responsiveness (Table 2, Fig. 1).

In a previous study, PAH with R241C substitution showed to have 25% of residual activity in the COS cell expression system.[29] Guldberg *et al*.[6] assigned the patient with a R241C genotype to the MHP category. It was also reported that the blood phenylalanine levels of R241C/R413P patients was decreased by oral administration of BH_4.[10] R241 is located near the cofactor binding region and does not directly interact with the cofactor, so the mutation may lead to relatively mild structural deformities.[30] Our data are consistent with these previous reports.

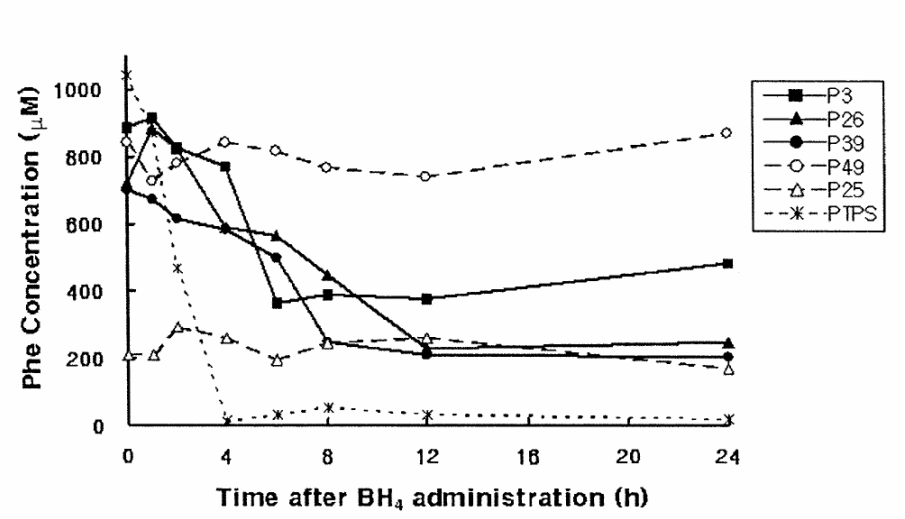

Figure 1. Profile of blood phenylalanine concentration changes during the BH_4 loading test. Filled square, patient 3 with Y356X/R408Q genotype; filled triangle, patient 26 with R53H/R243Q; and filled circle, patient 39 with R241C/R243Q showed the BH_4 responsive pattern; and open triangle, patient 25 with R241C/R241C; and open circle, patient 49 with R243Q/? showed the nonresponsive pattern.

Patient 3 (genotyped with Y356X/R408Q) also represented BH_4 responsiveness. Y356X is a null mutation and may not be the BH_4-responsive allele. R408Q was reported to be associated with near-normal levels of residual activity in eukaryotic and prokaryotic expression system.[8] The residual activity of R408Q and the BH_4

responsiveness of patient 3 indicate that R408Q is one of the BH_4-responsive alleles. Our data added R53H to the list of BH_4-responsive *PAH* alleles. Patient 26 (genotyped with R53H/R243Q) represented BH_4 responsiveness. The facts that R243Q was associated with classical PKU in our study and another R53H heterozygous patient was MHP suggested that R53H had some residual enzyme activity and brought out the responsiveness in patient 26.

The BH_4 response pattern between the PTPS-deficient patient and the PKU patient are somewhat different (Fig. 1). Phenylalanine levels of the PTPS patient was dramatically and completely decreased to the normal level after administration of BH_4; in the PKU patient, the decrease was relatively retarded, and the blood phenylalanine concentration remained at the higher-than-normal level. The basal phenylalanine level of patient 25 (R241C homozygote) was too low to represent BH_4 responsiveness.

In the BH_4-non-responsive patients, the phenylalanine level remained at the same level as the starting point (Fig. 1). Some moderate PKU patients (patient 49 and 72) did not respond to the BH_4. This result suggests that BH_4 responsiveness requires some residual enzyme activity, but all the cases with mild phenotype are not associated with the BH_4 responsiveness.

3. PAH activity assays

PAH enzyme activities encoded by the 11 *PAH* mutants ranged from 0% to 118% (Fig. 2, Table 5). The expression levels of the mutant enzymes R53H, P69S, G103S, R408Q, and R413P, detected by Western blot, were similar to the wild-type expression and enzyme activities were 79%, 52%, 39%, 118%, and 66%, respectively.

In the mutants R241C, R243Q, L293M, and A447P, the expression of mutant enzymes was reduced relative to that of the wild-type, with enzyme activities of 28%, 32%, 41%, and 8%, respectively. The mutant enzymes G332V and S391I were not detected by Western blot, and consequently no residual enzyme activities could be measured. The enzyme activities of the BH_4-responsive *PAH* mutants were 28%–118% (79% in R53H, 28% in R241C, 118% in R408Q).

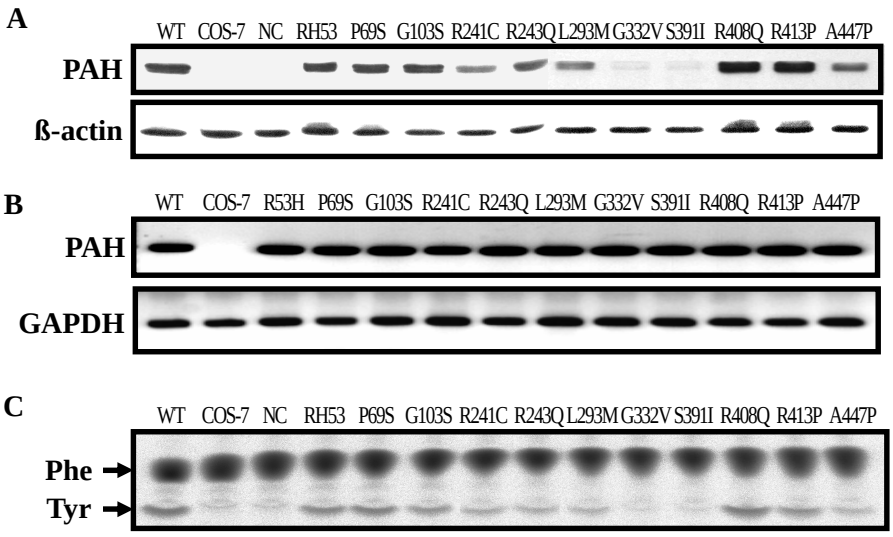

Figure 2. (A) Western blot, (B) RT–PCR, and (C) relative PAH enzyme activities of wild-type and mutant forms of PAH in COS7 cells assayed by measuring the conversion of L-[^{14}C]Phe to L-[^{14}C]Tyr using a natural cofactor (BH_4). WT, wild-type; NC, negative control.

Table 5. PAH enzyme activities and in vitro BH_4 responsiveness test of PAH enzyme activities.

Mutations	PAH enzyme activities[*,†]		PAH enzyme activities[**,††]	
	Mean	Range	BH_4 (–)	BH_4 (+)
WT			100	161
NC			0	0
R53H	79	75–82	63	139
P69S	52	38–68	45	60
G103S	39	14–63	16	24
R241C	28	14–40	26	38
R243Q	32	22–38	17	36
L293M	41	15–81	55	57
G332V	0	0	0	0
S391I	0	0	0	2
R408Q	118	109–129	129	306
R413P	66	62–80	51	95
A447P	8	7–12	6	24

[*] expressed as a percentage of wild-type PAH activity; [**] expressed as a percentage of BH_4-untreated wild-type PAH activity; WT, wild-type *PAH* control; NC, negative control; BH_4(–), No BH_4 in culture medium; BH_4(+), 1 mM BH_4 in culture medium 5h after transfection. [†] All the *PAH* mutants were transfected three times, enzyme activities were also measured three times, and mean enzyme activities were calculated. [††] See Materials and methods.

Compared with cells expressing the wild-type PAH protein, enzyme assay revealed decreased PAH activities in the lysates of cells in which various missense mutant proteins were expressed. The activity of some mutants was undetectable, meaning that they were effectively less than 1% of wild-type activity. In contrast, the residual activities associated with other mutants ranged over a wide spectrum. Measurement of immunoreactive PAH protein almost always indicated a corresponding decrease in the protein level (Fig. 2A). Therefore, the specific activity of most mutant PAH proteins did not differ from that of the wild-type. Quantitation of *PAH* mRNA levels in the cell lysates revealed no differences between the wild-type and mutant forms (Fig. 2B). Therefore, mutation effects at the RNA level do not explain the decreased levels of PAH protein. This triad of characteristics has typified increasing numbers of missense mutations for over a decade and it has usually been ascribed to the instability of the mutant protein. However, ‘‘instability’’ is a loose term that begs several questions.

4. In vitro BH_4-responsiveness assay

Wild-type PAH treated with 1 mM BH_4 showed increased enzyme activity (161%) with no increase in PAH protein (Table 5, Fig. 3). COS7 cells showed neither endogenous PAH protein expression nor enzyme activity. When treated with 1 mM BH_4, clinically proven BH_4-responsive *PAH* mutants showed increased PAH enzyme activity (R53H, 63% → 161%; R241C, 26% → 38%; R408Q, 129% → 306%). Mutants P69S (45% → 60%), G103S (16% → 24%), R243Q (17% → 36%), L293M (55% → 57%), R413P (51% → 95%), and A447P (6% → 24%) also showed increased PAH enzyme activity. Mutant G332V treated with 1 mM BH_4 showed neither PAH protein expression nor enzyme activity.

However, mutant S391I showed increased PAH protein expression and enzyme activity when treated with 1 mM BH_4. For mutant R413P, Shintaku *et al.*[31] described that it is one of severe *PAH* mutations detected in classical PKU, result in nonfunctional *PAH* allele, however PAH enzyme activity was increased when treated with BH_4 in our study. It suggests that even patients with classical PKU can be treated with BH_4 supplementation.

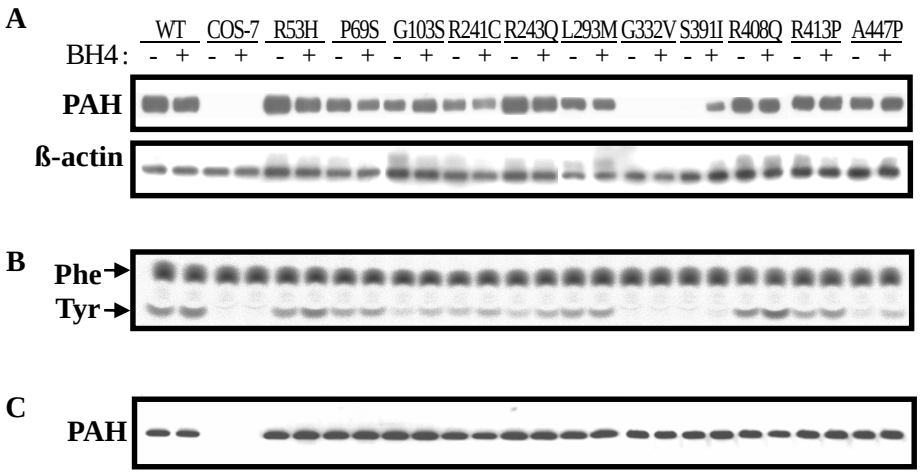

Figure 3. In vitro BH_4-responsiveness test of PAH enzyme activities. (A) Western blot, (B) relative PAH enzyme activities of wild-type and mutant forms of PAH, and (C) RT–PCR. BH_4(–), cultured without BH_4; BH_4(+), cultured with 1 mM BH_4.

Several studies have investigated the molecular mechanisms underlying BH_4 responsiveness and several mechanisms have been proposed: (i) decreased affinity for BH_4 binding, (ii) stabilization of the active tetrameric or dimeric forms of the mutant proteins and protection from proteolytic cleavage, (iii) BH_4-induced changes in BH_4 biosynthesis, and (iv) *PAH* mRNA stabilization [11]. Thöny *et al*.[32] reported that BH_4 has a chaperon-like effect on PAH synthesis and/or is a protecting cofactor against enzyme auto-inactivation and degradation without affecting gene expression or *PAH* mRNA stability. Particular amino acid residues may have key roles in the correct folding of transient intermediates en route.

5. Composite model structure

The composite model structure of the tetramer was constructed to estimate the functional consequences of the mutations (Fig. 4). Of the 11 missense mutations analyzed in this study, three BH_4-responsive mutations (R53H, R241C, R408Q) were located at opposite sides of the BH_4-binding pocket. Specifically, the composite model structure of the PAH tetramer revealed that these three mutations were in close proximity to the dimer interface (Fig. 4).

Each of the four tetramerization domains (residues 428–425) forms a coiled-coil helical complex, and once these complexes are formed, the four monomers adopt a two-fold symmetry whereby two regulatory domains face each other in a dimer and the two dimers form a tetramer. The R53H, R241C, and R408Q mutations occurred along the interface region of the regulatory domain (Fig. 4). This observation suggests that upon mutations of R53H, R241C, or R408Q, dimer stability is reduced. Increased BH_4 levels rescue dimer stability and, consequently, enzyme activity also recovers to normal.

Other mutations, P69S, R243Q, S391I, R413P, and A447P, are also located at the interface region of the two dimers (Fig. 4).

A

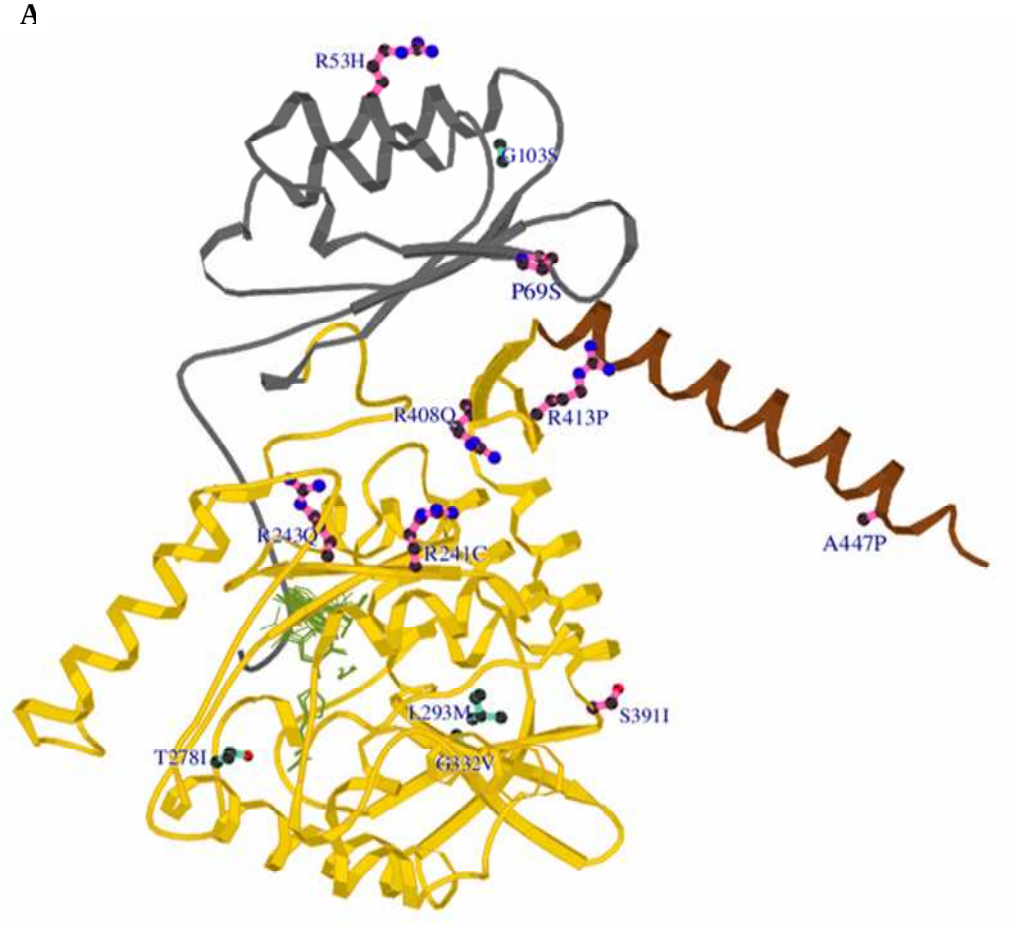

Figure 4. MolScript representation of phenylalanine hydroxylase.

(A) MolScript representation for polymerization-related mutations, side-chain carbon atoms are pink, nitrogen atoms are blue, and oxygen atoms are red. The remaining side-chain carbon atoms are light green. BH_4 and substrate analogues transferred from the structures of the PAH superfamily are shown as a dark green stick representation. Regulatory, catalytic, and tetramerization domains are colored gray, dark yellow, and brown, respectively.

B

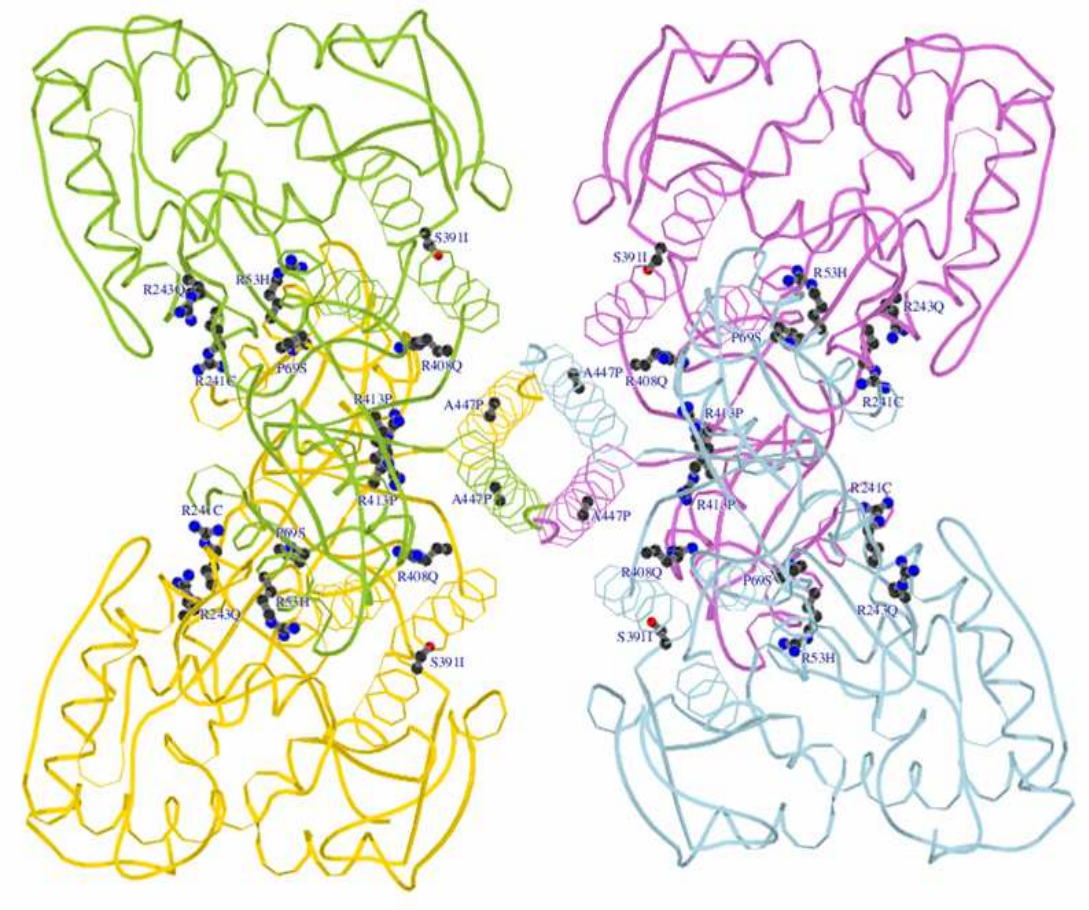

Figure 4. (continued)

(B) A composite model structure of PAH tetramer is displayed with polymerization-related mutations, R53H, P69S, R243Q, S391I, R413P, R241C, R408Q, and A447P. Each chain in PAH tetramer is shown in different color.

IV. CONCLUSION

In summary, we screened the *PAH* in 78 Korean PKU-affected families and identified 39 mutations, including ten novel mutations. Although the Korean mutation profile of *PAH* is similar to those of the nearest oriental populations, there are several different characteristic features. We investigated the enzyme activities and BH_4-responsiveness of 11 *PAH* missense mutants. Although the activities of some mutants were undetectable, the residual activities of other mutants ranged over a wide spectrum. In some severe *PAH* mutations detected in classical PKU, PAH enzyme activity was increased when treated with BH_4. This study suggests that even patients with classical PKU can be treated with BH_4 supplementation. The composite model structure of PAH revealed that dimer stability of the BH_4-responsive mutants is reduced, whereas tetramer stability remains normal. This result suggests that increased BH_4 levels rescue dimer in stability and, consequently, enzyme activity is restored to normal. Further studies to validate these structural and functional analysis of BH_4 responsiveness in *PAH* mutants should facilitate the development of new therapeutic strategies for patients with hyperphenylalaninemia.

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Abstract (in Korean)

한국인 페닐케톤뇨증 환자의 페닐알라닌 수산화효소 유전자의 돌연변이 동정과 기능적 분석

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김상운

페닐케톤뇨증은 페닐알라닌을 타이로신으로 전환시키는 페닐알라닌 수산화효소 (phenylalanine hydroxylase, PAH)의 결핍에 의해서 생기는 상염색체 열성 유전질환이다. 본 연구에서는 한국인 페닐케톤뇨증 환우회 가족 중 연구참여에 동의한 78 가족의 말초 혈액 임파구로부터 DNA를 추출하고 *PAH* 유전자 염기서열을 분석하여 돌연변이 양상을 알아보고 발견된 *PAH* 유전자 돌연변이를 바탕으로 페닐케톤뇨증 환자의 임상양상과 *PAH* 유전형과의 연관성을 알아보았다. 총 39개의 돌연변이를 발견하였으며 이 중 10개 (P69S, G103S, N207D, T278S, P281A, L293M, G332V, S391I, A447P, and IVS10-3C>G)는 처음 발견된 것이며 3개 (R53H, R241C, R408Q)는 tetrahydrobiopterin (BH_4)-반응형이었다. 이 중에 총 11개의 돌연변이 (P69S, G103S, L293M, G332V, S391I, A447P, R243Q, R413P, R53H, R241C,

R408Q) 에 대하여 유전자의 발현 정도와 효소 활성도를 측정하였으며 그 결과 다양한 정도의 활성도를 보였다. 페닐케톤뇨증을 유발하는 일부 돌연변이도 BH_4 투여 후 페닐알라닌 수산화효소의 발현과 활성도가 증가하였는데 이것은 일부 전형적인 페닐케톤뇨증 환자에서도 BH_4 투여를 하면 혈중 페닐알라닌이 감소 할 수도 있음을 시사한다. 페닐알라닌 수산화효소 모형 분석 결과 BH_4-반응형 돌연변이에서는 이합체 (dimer)의 안정성은 감소되어 있었으나 사합체 (tetramer)의 안정성은 정상이었다.

본 연구에서는 돌연변이 *PAH* 유전자의 발현 정도와 효소의 활성도를 측정하고 구조적 분석을 통하여 페닐케톤뇨증 환자의 치료와 유전상담에 도움이 되고자 하였다.

핵심 되는 말: 페닐케톤뇨증, 고페닐알라닌혈증, 페닐알라닌 수산화효소, 돌연변이, 발현, 구조, 모형