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ORIGINAL ARTICLE

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The molecular basis of phenylketonuria in Koreans

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Abstract Phenylketonuria (PKU) is an inborn error of metabolism that results from a deficiency of phenylalanine hydroxylase (PAH). We characterized the *PAH* mutations of 79 independent Korean patients with PKU or hyperphenylalaninemia. *PAH* nucleotide sequence analysis revealed 39 different mutations, including ten novel mutations. The novel mutations consisted of nine missense mutations (P69S, G103S, N207D, T278S, P281A, L293M, G332V, S391I, and A447P) and a novel splice site variant (IVS10−3C > G). R243Q, IVS4−1G > A, and E6−96A > G were the most prevalent mutations, as they accounted for 32% of the total mutant alleles in this study. Although some common characteristics of allele frequency and distribution were identified among oriental populations, several distinctive characteristics were revealed in Korean patients. Although the R413P allele is the most prevalent form (30.5%) in Japanese, we detected it in only five chromosomes from 158 independent chromosomes (3.2%). The A259T allele, which has not yet been found in oriental populations, was frequently found in this study. We also observed that tetrahydrobiopterin (BH_4) responsiveness was associated with specific genotypes (R53H, R241C, and R408Q), suggesting there are some correlations between phenotype and genotype.

Keywords Phenylketonuria · Phenylalanine hydroxylase · Mutation · Allele · Frequency · Korean

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Introduction

Phenylketonuria (PKU; MIM 261600) is an inborn error of metabolism inherited as an autosomal recessive trait. The incidences of PKU vary among ethnic populations: 1/10,000 in Caucasian (Bickel et al. 1981), 1/120,000 in Japanese (Aoki and Wada 1988), and 1/18,000 in Chinese (Liu and Zuo 1986). In Korea, PKU incidence is estimated, from the neonatal screening that was introduced in 1998, to be about 1/41,000.

PKU results from a deficiency of phenylalanine hydroxylase (PAH). The *PAH* gene spans about 90 kb on chromosome 12q and comprises 13 exons. PAH is a hepatic enzyme that catalyzes hydroxylation of phenylalanine to tyrosine using tetrahydrobiopterin (BH_4) as a cofactor. It has three structural domains consisting of an N-terminal regulatory domain, a catalytic domain, and a C-terminal tetramerization domain. The active PAH enzyme is comprised 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.

The mutation profile of the *PAH* gene is not restricted to any one region but spreads throughout the entire structural domains and shows enormous diversity. More than 460 different mutations of the *PAH* gene have been identified and recorded in the PAH Mutation Analysis Consortium Database (PAHdb; Scriver et al. 2003). The severity of the disease is also diverse from mild hyperphenylalaninemia (MHP) to classical PKU, which is characterized by pretreatment blood phenylalanine levels or dietary tolerance (Guldberg et al. 1998). Another phenotypic characteristic is BH_4 responsive-

ness. The serum phenylalanine levels of BH_4-responsive patients are controlled by BH_4 oral administration without phenylalanine restriction diet. Several studies have investigated the relationship between genotype and this diverse phenotypic expression. Determining the relationship between genotype and phenotype would provide very useful information for planning dietary and therapeutic strategies.

Therefore, we analyzed the *PAH* gene in 79 patients with PKU and their families to study genotype–phenotype relationships and to help with genetic counseling. Furthermore, we analyzed the mutation spectra of the *PAH* gene in Korean patients and compared them with those of other ethnic groups, including Japanese and Chinese.

Subjects and methods

Subjects

This study was approved by the institutional review board of the National Institute of Health, Korea. The study included 79 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.

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-pyruvoyltetrahydropterin synthase (PTPS) deficiencies.

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* gene were amplified by PCR. PCR amplicons were extracted from an agarose gel using a gel extraction kit (Qiagen). Direct sequencing was performed using a BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 2.0 (PE Applied Biosystems, Foster City, CA, USA) and analyzed with an ABI 3100 automated sequencer (PE Applied Biosystems) 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* genes 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/).

Results and discussion

PAH nucleotide sequence analysis of 79 unrelated PKU probands revealed 39 different mutations (Tables 1, 2). Among 79 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, and no mutations were detected in two 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.

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 3) and accounted for 51 of the 158 total chromosomes (32.2%) in this study.

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 (Zschocke 2003). 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 (Zschocke 2003). In particular, the R408W mutation has a frequency of 20–84% in PKU patients in Eastern Europe and Germany. However, these mutations are rarely detected in oriental populations. In a previous study, Okano et al. (1992)

Table 1 Genotypes for mutations of the *PAH* gene in 79 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
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
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
79	?	?	NA

[a] BH_4 responsive type
[b] *NA* not available

reported the frequency and distribution of *PAH* gene 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 79 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 3). 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. R413P is the most prevalent allele in Japanese, but a very small proportion of probands have the R413P allele in Korean and Taiwanese. IVS4−1G > A occupied a relatively larger proportion in Korean mutant allele profiles than in Japanese or Chinese. Although A259T was not detected in any other oriental population studies, it was identified in nine different families in this study.

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 1, Fig. 1). In a previous study, PAH with R241C substitution showed to have 25% of residual activity in the COS cell expression system (Okano et al. 1994). Guldberg et al. (1998) 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 (Kure et al. 1999). R241 is located near the cofactor binding region and does not directly interact with the cofactor, so the

Table 2 Spectrum of *PAH* mutations detected in this study

Mutation name	Normal	Mutation	Location	Allele frequency	Relative frequency (%)	References[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	12.0	1
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
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. (2003), (3) mutation reported by Chien et al. (2004), (4) mutation reported by Park et al. (1998)

Table 3 Relative frequencies of common *PAH* gene mutation found in oriental populations

Mutation	Relative frequencies (%, allele frequency/total subject chromosome)			
	79 Korean	41 Japanese (Okano et al. 1998)	52 Chinese (Okano et al. 1992)[a]	25 Taiwanese (Chien et al. 2004)
R243Q	12.0	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	86.0	92.7	66.5	90.0

[a]*NA* not available

mutation may lead to relatively mild structural deformities (Erlandsen and Stevens 2001). Our data are consistent with these previous reports.

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

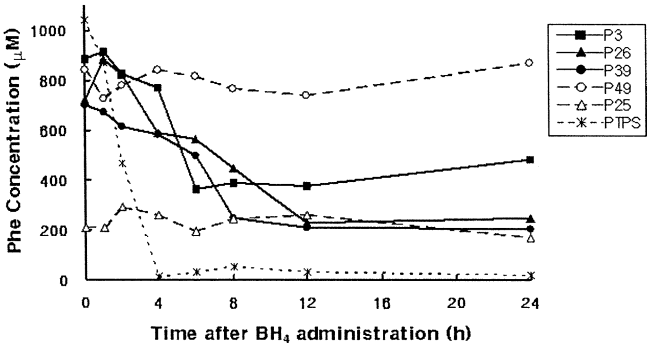

Fig. 1 Profile of blood phenylalanine concentration changes during the BH_4 loading test. *Filled square*, patient 3 with Y356X/R408Q genotype; *filled triangle*, patient 26 with R53H/R243Q; and *filled circle*, patient 39 with R241C/R243Q showed the BH_4 responsive pattern; and *open triangle*, patient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

reported to be associated with near-normal levels of residual activity in eukaryotic and prokaryotic expression system (Pey et al. 2003). 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.

In summary, we screened the *PAH* gene in 79 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. The relationship of genotype and phenotype, especially BH_4 responsiveness of some patients, was also described. This study would contribute to the diagnosis, genetic counseling, and planning of the dietary and therapeutic strategy in PKU patients.

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