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ORIGINAL INVESTIGATION

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Molecular characterization of phenylketonuria in Japanese patients

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Abstract We characterized phenylalanine hydroxylase (PAH) genotypes of Japanese patients with phenylketonuria (PKU) and hyperphenylalaninemia (HPA). PKU and HPA mutations in 41 Japanese patients were identified by denaturing gradient gel electrophoresis and direct sequencing, followed by restriction fragment length polymorphism analysis to find a large deletion involving exons 5 and 6. Of 82 mutant alleles, 76 (92%) were genotyped showing 21 mutations. The major mutations were R413P (30.5%), R243Q (7.3%), R241 C (7.3%), IVS4nt-1 (7.3%), T278I (7.3%), E6nt-96A→g (6.1%), Y356X (4.9%), R111X (3.7%), and 442–706delE5/6 (2.4%). Eight new mutations (L52 S, delS70, S70P, Y77X, IVS3nt-1, A132 V, W187 C, and C265Y) and a polymorphism of IVS10nt-14 were detected. In vitro PAH activities of mutant PAH cDNA constructs were determined by a COS cell expression system. Six mutations, viz., R408Q, L52 S, R241 C, S70P, V388 M, and R243Q, had 55%, 27%, 25%, 20%, 16% and 10% of the in vitro PAH activity of normal constructs, respectively. The mean pretreatment phenylalanine concentration (0.83±0.21 mmol/l) of patients carrying the R408Q, R241 C, or L52 S mutation and a null mutation was significantly lower (P<0.0005) than that (1.99±0.65 mmol/l) of patients with both alleles carrying mutations associated with a severe genotype. Simple linear regression analysis showed a correlation between pretreatment phenylalanine concentrations and predicted PAH activity in 29 Japanese PKU patients (y=31.9–1.03x, r=0.59, P<0.0001). Genotype determination is useful in the prediction of biochemical and clinical phenotypes in PKU and can be of particular help in managing patients with this disorder.

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Introduction

Phenylketonuria (PKU) is an autosomal recessive disorder caused by a deficiency of hepatic phenylalanine hydroxylase (PAH). This disease causes severe mental retardation unless an affected child is maintained on a strict low-phenylalanine diet (Scriver et al. 1995). The incidence of PKU as determined by newborn mass screening in Japan is 1/120,000 (Aoki and Wada 1988) and is much lower than in Caucasians (1/10,000; Bickel et al. 1981) and Chinese (1/18,000; Liu and Zuo 1986).

Ninety-nine percent of the mutant alleles in the Danish population have been characterized by denaturing gradient gel electrophoresis (DGGE) and direct sequencing (Guldberg et al. 1993). Characterization of all PKU alleles has been performed in several countries by using this technique. The number of potential mutations identified for the PAH gene has dramatically increased recently; in the latest PAH Mutation Analysis Consortium Database (Nowacki et al. 1997), more than 350 different mutations and polymorphisms had been identified. The various PAH mutations reduce PAH activity to different degrees, explaining the broad phenotypic heterogeneity of this disease from severe PKU to mild hyperphenylalaninemia.

PKU is diagnosed by detecting an elevation of serum phenylalanine concentrations rather than by measuring hepatic PAH activity. Therefore, predicting the clinical severity of PKU from genotypes is an important purpose of genetic analysis in PKU patients. Correlations between mutations in both PAH alleles and clinical phenotypes have been demonstrated, and several mutations have been related to non-classical PKU (mild PKU) phenotypes (Guldberg et al. 1994, 1996; Kayaalp et al. 1997; Svensson et al. 1992; Zschocke et al. 1994). Correlation studies with PAH mutation combinations are limited by the number of combinations and the need for previous

clinical and biochemical data. On the other hand, a predicted PAH activity system can simplify genotype by averaging in vitro PAH activities from both alleles in a COS cell expression system and by taking into account genotype-phenotype correlations. Predicted PAH activity is correlated with pretreatment serum phenylalanine concentrations, phenylalanine tolerance, and serum phenylalanine concentrations after administration of an oral protein load (Eisensmith et al. 1996; Okano et al. 1991b; Svensson et al. 1993; Trefz et al. 1993). The predicted PAH activity system has allowed the prediction of clinical phenotypes in patients with novel or rare mutations without recourse to any previous correlation data.

In previous studies of the Japanese population, 54% of mutant PAH alleles have been genotyped in 36 PKU patients by screening allele-specific oligonucleotides for eight identified mutations (Okano et al. 1992, 1994a). In order to complete the molecular characterization of Japanese PKU alleles, we have found mutations by DGGE analysis and direct sequencing and examined the in vitro PAH activity of mutant PAH cDNA constructs in a COS cell expression system. These studies should enable us to establish the utility of genotype analysis in the prediction of clinical phenotype, in the subsequent treatment of PKU by a suitable protein-restricted diet, and in determining the prognosis of Japanese patients with PKU detected by newborn screeening.

Materials and methods

Patients

Forty-one nonconsanguineous Japanese patients were evaluated biochemically and clinically at various institutes and were determined to have PAH deficiency. Patients with BH4 deficiency were excluded based on the absence of neurologic deterioration on a low phenylalanine diet, analysis of dihydropteridine reductase activity in red blood cells, biopterin loading test, and/or pteridine analysis of urine. The criterion for classical PKU was a plasma phenylalanine concentration of 1.2 mM or more, prior to initiation of a phenylalanine-restricted diet or in the absence of dietary restrictions later in life. The plasma phenylalanine concentration in non-classical PKU is less than 1.2 mM without a phenylalanine-restricted diet. Informed consent for genetic analysis was obtained from all subjects or their parents or guardians.

Denaturing gradient gel electrophresis

Polymerase chain reaction (PCR) amplification of the PAH gene with GC-clamped primers of all 13 exons and flanking intronic regions was performed according to Guldberg et al. (1993). Reaction mixtures contained 0.5–1.0 µg genomic DNA, 2 mM each dNTP, 50 mM KCl, 100 mM TRIS-HCl pH 8.3, 15 mM $MgCl_2$, 0.1% gelatin, 50 ng each primer, and *Taq* DNA polymerase. Thirty-five PCR cycles (94°C for 30 s, 50°C for 60 s, and 72°C for 60 s) were carried out, after which the mixture was incubated at 72°C for 5 min in a Perkin-Elmer Model 9600 Thermocycler. Aliquots of 20 µl GC-clamped PCR product were loaded onto a 6% polyacrylamide gel containing a gradient of urea and formamide ranging from 20% to 80%. Electrophoresis was performed at 160 V for 7 h in TAE buffer (0.04 M TRIS acetate, 1 mM EDTA, pH 8.0) at 61°C on a DGGE apparatus obtained from CBS Scientific.

Genome DNA sequencing

Genomic DNA was isolated from lymphocytes or Epstein-Barr virus transformed lymphoblasts. Exons and their flanking intronic regions with mutations were amplified from genomic DNA by PCR with biotinylated primers. Amplified exonic DNAs were purified by the Dyna-bead system (Dynal). The purified single-stranded DNA was sequenced directly with dimethyl sulfoxide as described elsewhere (Wang et al. 1991a).

Restriction fragment length polymorphism analysis

For Southern hybridization, genomic DNA was digested thoroughly with restriction enzymes *Bam*H1/*Eco*R1, *Eco*RV, and *Xmn*1 in the appropriate buffers and hybridized onto GeneScreen nylon membranes (New England Nuclear). The full PAH cDNA probe was radiolabeled with [α-^{32}P] dCTP (DuPont-NEN) by a Megaprime DNA labeling system (Amersham).

Expression analysis

Mutant human PAH cDNA was synthesized by specific base substitutions by using site-directed mutagenesis in a eukaryotic expression vector (pCDNA3; Invitrogen) containing a full-length human PAH cDNA. Mutant and normal PAH cDNAs were introduced into COS cells in a mixture of 20 mM HEPES pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM dextrose by electroporation with a Gene Pulser apparatus (Bio-Rad) at 200 V with a 960 µF capacitance. The cells were harvested after 72 h culture. To confirm the reproducibility, PAH activity was determined twice as described previously (Okano et al. 1991a, 1994b). The level of PAH activity in cells transfected with various mutant or normal PAH cDNA constructs was normalized for relative variations in the levels of PAH mRNA. PAH mRNA levels in cell extracts were determined by dot-blot hybridization for serially diluted total-RNA samples by using a PAH cDNA probe labeled with [α-^{32}P] dCTP (Du-Point-NEN) by the Megaprime DNA labeling system (Amersham). The level of PAH activity in cells transfected with the mutant PAH cDNA was expressed as a percentage of that in cells transfected with normal PAH cDNA.

Correlation between genotype and phenotype

Twenty-nine Japanese PKU patients with identified genotypes and pretreatment phenylalanine concentrations were analyzed for the correlation between genotype and phenotype. Predicted PAH activity was calculated by averaging in vitro PAH activities of both alleles as determined by COS cell expression analysis and was expressed as a percentage of normal levels, as described elsewhere (Okano et al. 1991b). The nonsense and splicing mutations were given a definition of null PAH activity, including E6 nt-96A→g (Y204 C; Ellingsen et al. 1997). Simple linear regression analysis was used to examine the relationship between pretreatment phenylalanine concentrations and predicted PAH activity, and the correlation coefficient and the probability value were determined according to standard statiscal methods. The differences of pretreatment phenylalanine concentrations among genotype groups were evaluated by Student's *t*-test.

Results

Identification of genotype

DGGE analysis is an effective way of detecting exons with mutations prior to direct sequencing. In our 41 Japanese PKU patients, all mutant exons of 38 alleles in which mu-

tations had previously been identified (R413P, IVS4nt-1, R241 C, R243Q, E6nt-96A→g, Y356X, R111X, R408Q) showed abnormal bands by DGGE analysis. We could distinguish between normal alleles and homozygous mutations. Homozygous R413P mutations showed a slightly faster single band compared with normal controls, and homozygous Y356X mutations showed a slightly slower single band. We confirmed that 7 patients were homozygous for R413P and that 1 patient was homozygous for Y356X by using direct sequencing and demonstrated that the parents were heterozygous carriers for the same mutations as the patients.

We identified 21 different mutations in Japanese patients with PKU, which accounted for 92.7% (76) of 82 independent mutant alleles. The mutations in 74 out of 82 PKU alleles were detected by DGGE analysis and direct sequencing, and the 442–706delE5/6 deletion in 2 out of 8 uncharacterized PKU alleles was detected by Southern hybridization. Since this 442–706delE5/6 showed a large deletion involving exons 5 and 6 in PAH genome DNA, restriction fragment length polymorphism (RFLP) analysis with *Eco*RI/*Bam*HI, *Eco*RV, and *Xmn*I was used to detect this mutation; however, DGGE analysis could not detect the abnormal bands in exons 5 and 6.

The mutational spectrum was 12 missense mutations, 3 nonsense mutations, 4 splicing mutations, and 2 deletions. The defined mutations were distributed from exons 2 through 7, and in exons 11 and 12. The relative frequencies are shown in Table 1. The most prevalent mutation was R413P, which accounted for 30.5% of Japanese PKU alleles. The next four most prevalent mutations were IVS4nt-1, R241 C, R243Q, and T278I; these accounted for 7.3% of Japanese PKU alleles. Nine mutations were found in more than two alleles and represented 76.8% of all mutant alleles. For the other 12 mutations, each mutation was found in only one PKU allele. We found 8 novel mutations (L52 S, S70P, delS70, Y77X, IVS3nt-1, A132 V, W187 C, C265Y) and one novel polymorphism (IVS10nt-14) among the 21 different mutations not recorded in the PAH Mutation Analysis Consortium Database (Nowacki et al. 1997).

Characterization of mutant PAH

Six mutations (L52 S, S70P, W187 C, T278I, C265Y, and V388 M) were examined for PAH activities in a COS cell expression system. The PAH activity of each mutant con-

Table 1 Relative frequency of PAH mutations in Japanese patients with PKU

Mutation	Systemic name	Exon	Number	Frequency (%)
R413P	c.1238 G→C	12	26	30.5
IVS4nt-1	c.442–1 g→a	Intron 4	6	7.3
R241 C	c.721 C→T	7	6	7.3
R243Q	c.728 G→A	7	6	7.3
T278I	c.833 C→T	7	6	7.3
E6nt-96A→g	c.611 A→g	6	5	6.1
Y356X	c.1068 C→G	11	4	4.9
R111X	c.331 C→T	3	3	3.7
442–706del E5/6	c.442–706 del	5&6	2	2.4
L52 S	c.155 T→C	2	1	1.2
S70P	c.208 T→C	3	1	1.2
delS70	c.208–210delTCT	3	1	1.2
Y77X	c.231 T→G	3	1	1.2
IVS3nt-1	c.353–1 g→t	Intron 3	1	1.2
A132 V	c.395 C→T	4	1	1.2
W187 C	c.561 G→C	6	1	1.2
C265Y	c.794 G→A	7	1	1.2
IVS7nt+2	c.842+2 t→a	Intron 7	1	1.2
IVS10nt-14	c.1066–14 c→g	Intron 10	1	1.2
V388 M	c.1162 G→A	11	1	1.2
R408Q	c.1223 G→A	12	1	1.2
Total (21)			76/82	92.7
From other investigations				
P173T[a]	c.517 C→A	6	1	
I224 M[a]	c.672 T→G	6	1	
R261X[b]	c.781 C→T	7	1	
M276V[c]	c.826 A→G	7	10/20	
IVS9nt-6[d]	c.970–6 t→g	Intron 9	1	

[a] Shirahase and Shimada (1992)
[b] Shirahase et al. (1991)
[c] Goebel-Schreiner and Schreier (1993)
[d] Takahashi et al. (1994)

struct was calculated after correcting for the efficiency of transfection into COS cells by determining PAH mRNA levels by dot-blot hybridization of serially diluted RNA from transfected cells. Table 2 shows the results of the PAH activity in expression analysis. PAH activities for the R408Q, R413P, R243Q, and R241 C mutations were obtained from reports previously published (Okano et al. 1994b; Svensson et al. 1992; Wang et al. 1991a, b). Six of the missense mutations had residual enzyme activity in the expression system, whereas four missense mutations had PAH activity below 3% of control levels.

Correlation between genotype and phenotype

The patients were classified into one of the three groups based on predicted PAH activity and clinical phenotype. Patients in the severe genotype group showed a classical PKU phenotype and close to 0% predicted PAH activity, both mutant alleles consisting of missense, nonsense, splicing, or deleted mutations, indicating null PAH activities or less than 3% in vitro PAH activities. Patients in the mild genotype group, comprising compound heterozygote of the severe mutation and the R408Q, L52 S, or R241 C mutation, showed 27.5%, 13.5%, and 12.5% of predicted PAH activity, respectively, and a non-classical PKU phenotype. Patients in the intermediate genotype group, comprising compound heterozygotes of the severe mutation and the S70P, V388 M, or R243Q mutation, showed 10%, 8%, and 5% of predicted PAH activity, respectively, and a classical PKU phenotype. The mean pretreatment phenylalanine concentrations of the severe and intermediate genotype groups were 1.99±0.65 and 1.61±0.49 mmol/l, respectively, and were significantly higher than 0.83±0.21 mmol/l of the mild genotype group.

Simple linear regression analysis was used to define the relationship between pretreatment phenylalanine concentrations and predicted PAH activity in the 29 Japanese PKU patients. Figure 1 shows the correlations between pretreatment phenylalanine concentrations in Japanese patients and PAH activity predicted from genotype, based on in vitro expression analysis of the mutant protein (r=0.59, P<0.001).

Table 2 Results of expression analysis of Japanese PKU missense mutations

Mutation	PAH activity (%)
Wild	100
R408Q	55[a]
L52 S	27
R241 C	25[b]
S70P	20
V388 M	15
R243Q	10[c]
R413P	<3[d]
W187 C	1
T278I	1
C265Y	0

[a] Svensson et al. (1992)
[b] Okano et al. (1994b)
[c] Wang et al. (1991b)
[d] Wang et al. (1991a)

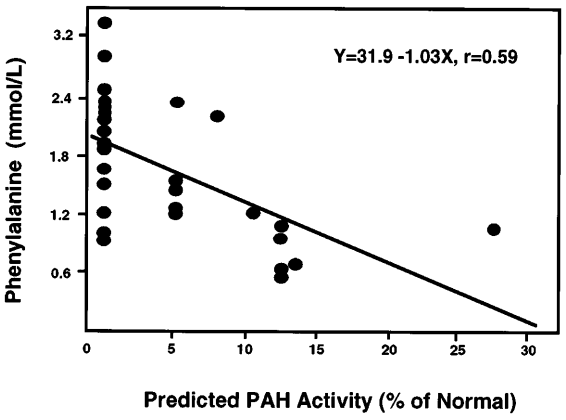

Fig. 1 Predicted PAH activity in relation to the pretreatment phenylalanine concentrations in patients with PKU. Simple linear regression analysis was used to calculate the line equation and the correlation coefficient

Discussion

Our system for identifying mutations in Japanese PKU patients consists of DGGE analysis and direct sequencing, followed by RFLP analysis in order to detect the 442–706delE5/6 mutation, which cannot be detected by DGGE analysis. This mutation was originally found by Trefz et al. (1990) as a unique RFLP pattern in Japanese patients by digesting with *Eco*RI-*Bam*HI, *Xmn*I, *Bgl*II, and *Eco*RV. The 442–706delE5/6 mutation has been characterized by the skipping of exons 5 and 6 in PAH mRNA by means of illegitimate transcription analysis and by a large deletion of 10 kb involving exons 5 and 6 by means of RFLP analysis in genomic DNA (Okano et al. 1994b). We have been able to identify the genotypes of 92.7% of PKU alleles in 41 Japanese PKU patients with 21 different mutations. Twelve mutations were private, only one allele of each being found in Japanese patients. Our 8 novel mutations and one novel polymorphism were all private. Private mutations accounted for only 17% of Japanese PKU alleles. The number of patients in which both mutant alleles have been detected rises from 55% to 86% on inclusion of private mutations, a detection level that is satisfactory for evaluating PKU mutations. Finding private mutations by DGGE analysis is important for providing genetic data on patients with regard to clinical evaluation. Our system of DGGE and RFLP analysis is thus adequate for evaluating PKU patients by genotype.

In the IVS10nt-14 polymorphism, ac is changed to ag upstream of 14 nucleotides of exon 11, i.e., the same sequence as that of a splicing acceptor site. Shapiro and Senapathy (1987) have reported a splicing score system at 3’ splice acceptor sites with nucleotides of CAGG and a poly-pyrimidine tract. The score at the original splice acceptor site in intron 10 is 77.2, whereas the score at the novel cryptic site in intron 10 is 80.9. We suspect that the IVS10nt-14 mutation provides the novel splice acceptor

site and results in the insertion of 13 nucleotides in exon 11 with a frameshift that would cause a premature termination at codon 392 in exon 11. However, we have no direct evidence of the insertion at the PAH mRNA level.

Previously, five other mutations had been reported for various Japanese patients: P173 T, I224 M (Shirahase and Shimada 1992), R261X (Shirahase et al. 1991), and IVS9nt-6 (Takahashi et al. 1994) have been found in only one allele each (Table 1). M276 V has been found in 10 out of 20 PKU alleles on Kyushu Island, located in southwestern Japan (Goebel-Schreiner and Schreiner 1993). The 442–706delE5/6 mutation has also been found in 3 (12%) out of 24 PKU alleles in Kyushu Island (Trefz et al. 1990). In our 41 Japanese patients, we found only 2 alleles (2.4%) carrying the 442–706delE5/6 mutation and no alleles carrying the M276 V mutation, since most of these patients were from the mainland in central Japan. In Hokkaido, an island in northern Japan, R413P mutations have been found in more than 45% of PKU alleles (Nagao 1997), whereas our finding have shown this mutation rate to be 30%. Thus, even though Japan is a small country and the Japanese population is relatively homogeneous, we have found mutations with regional differences.

In our population genetic study, we have reported that PKU mutations must have occurred after the racial divergence between Caucasians and East Asians and that the spectrum of mutations in Japanese PKU patients is similar to that in Chinese patients (Okano et al. 1992). In this study, we have confirmed the origins of the PKU mutations in Japanese in more detail. Nine mutations (IVS4nt-1, R111X, E6nt-96A→g, R241 C, R243Q, IVS7nt+2, Y356X, R408Q, R413P) common between Japanese and Chinese PKU patients have a relatively high frequency in Japanese patients, whereas the private mutations in Japanese patients have not been found in Chinese patients, except for IVS7nt+2 and R408Q (see Nowacki et al 1997 for the PAH Mutation Analysis Consortium Database). The relatively prevalent mutations observed in Japanese PKU patients may have occurred prior to the divergence of the Chinese, Korean, and Japanese peoples, and private mutations may have occurred relatively recently.

In this study, we have classified only two clinical phenotypes of PKU. Our borderline between non-classical PKU and classical PKU in Japanese PKU patients has 10%–15% predicted PAH activity. This borderline for the Japanese population is similar to those previously found in relatively homogeneous European populations (10%–15%; Okano et al. 1991a) and heterogeneous American populations (15%–25%; Eisensmith et al. 1996). Another recent study has defined the category of moderate phenotype in Caucasians as lying between the classical and non-classical PKU (mild PKU) phenotypes; in this category phenylalanine is tolerated in blood at values of 300 mmol/l (Guldberg et al. 1998). The V388 M mutation belongs to the moderate group in that report, and Caucasian patients homozygous for the V388 M mutation have a non-classical (mild PKU) phenotype, with a phenylalanine concentration of 15–20 mg/dl at diagnosis (Desviat et al. 1995). The Japanese patients who were compound heterozygotes of V388 M and severe mutations and in the intermediate genotype group had 8% of the predicted PAH activity and the classical PKU phenotype. Our intermediate genotype group might therefore be similar to this moderate category. Genotype analysis should enable more suitable dietary treatment and control of blood phenylalanine in PKU patients. Japanese PKU patients and Asian PKU patients have different PKU mutations from Caucasians. The genotype of PKU mutations as determined by in vitro PAH activity can thus be used worldwide to evaluate the clinical severity of PKU in different races.

The findings obtained with the predicted PAH activity system should be interpreted with caution. First, predicted PAH activities are higher than their corresponding activities in vivo. However, since the in vitro PAH activity is proportional to in vivo PAH activity in hepatocytes, as demonstrated by the correlation of genotype and biochemical phenotype, the predicted PAH activity system is useful for assessing the relative severity of PKU mutations, the classification of PKU, and the prediction of the clinical course. Secondly, there are cases with discordance between genotype and biochemical phenotype (Kayaalp et al. 1997; Treacy et al. 1997). Treacy et al. (1996) have demonstrated different in vivo metabolic profiles for phenylalanine arising from differences in phenylalanine transamination and renal clearance of transamination metabolites in two siblings with a different PKU phenotype but the same genotype. However, the rate of genotype-phenotype inconsistency is reported to be 4%–23% at seven European centers (Guldberg et al. 1998). A strong correlation has been demonstrated between clinical phenotype and predicted PAH activity in European, American, and our Japanese PKU patients (Eisensmith et al. 1996, Okano et al. 1991a). The genotype at the PAH locus is the major determinant of the metabolic phenotypes of PAH deficiency. The determination of genotypes in patients after newborn mass screening is useful for characterizing clinical and biochemical phenotypes and should permit further optimization of therapy, determination of long-term prognosis, and intensive support of patients and their families.

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