Missense Mutations Prevalent in Orientals with Phenylketonuria: Molecular Characterization and Clinical Implications

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Two missense mutations in the phenylalanine hydroxylase (PAH) genes of Orientals with phenylketonuria (PKU) have been identified. A G-to-A transition in exon 7 of the gene results in the substitution of Gln199 for Arg243 (R243Q) and accounts for 18% of all PKU chromosomes among Chinese. An A-to-G transition in exon 6 of the gene results in the substitution of Cys204 for Tyr204 (Y204C) and identifies about 13 and 5% of all PKU chromosomes in the Chinese and Japanese populations, respectively. The R243Q construct produced less than 10% of normal PAH activity in in vitro expression analysis in a eukaryotic cell system, and patients homozygous for this substitution exhibit a severe clinical phenotype. These results are consistent with previous findings in this expression system. The Y204C construct, however, produced near normal levels of PAH enzyme activity and immunoreactivity in this in vitro expression system. Because this substitution is present only on PKU chromosomes, it is a valuable marker for identifying the corresponding mutant allele for carrier screening of PKU. With the characterization of these two substitutions, about 60% of PKU alleles in China can now be identified. The continuing search for additional PKU mutations will permit effective carrier screening and prenatal gene diagnosis of PKU in East Asia. © 1991 Academic Press, Inc.

INTRODUCTION

As one of the more prevalent inherited metabolic disorders in man, classical phenylketonuria (PKU) is a direct consequence of liver phenylalanine hydroxylase (PAH) deficiency. This deficit results in an accumulation of phenylalanine in the serum and is accompanied by irreversible mental retardation in untreated children (Fölling, 1934; Jervis, 1953; Scrivener et al., 1988). As PKU exhibits a heterogeneous clinical phenotype (Güttler, 1980), it was predicted that different molecular lesions in the PAH gene may be responsible for different enzymatic defects, each of which confers a relatively unique phenotype (Scrivener et al., 1988).

Initially, PKU was considered a disease that primarily affected Caucasians, as 1 in 50 are carriers, and the molecular genetics of PKU have been studied extensively in Caucasians (Scrivener et al., 1988). With the isolation of full-length human PAH cDNA as a probe, eight polymorphic sites in or near the PAH locus that can discriminate over 43 different RFLP haplotypes were identified (Woo, 1988). Four of these RFLP haplotypes, haplotypes 1-4, account for approximately 90% of all PKU chromosomes in Caucasians (Chakraborty et al., 1987; Daiger et al., 1989a). Furthermore, many molecular defects have been characterized in Caucasian populations, most found to be linked tightly to the respective mutant haplotype (DiLella et al., 1986b, 1987; Lichter-Konecki et al., 1988; Abadie et al., 1989; Dworniczak et al., 1989; Lyonnet et al., 1989; John et al., 1989, 1990; Apold et al., 1990; Avigad et al., 1990; Okano et al., 1990a–c, 1991; Svensson et al., 1990; Wang et al., 1990).

The molecular basis of PKU is much less clear in the Oriental population, where the disease is also prevalent with a carrier frequency of 1 in 65 (Liu and Zu, 1986). Preliminary RFLP analysis revealed that haplotype 4 dominated in that population, accounting for more than 80% of both normal and mutant chromosomes (Daiger et al., 1989b). These results implied a quite different and probably a much less heterogeneous genetic background of PKU in Asia. Recently, three PKU mutations that are in linkage disequilibrium with RFLP haplotype 4 have been identified in Chinese (Wang et al., 1989, 1991a,b). These mutations together account for about 27% of all PKU alleles among the Chinese. In addition, the R408W mutation, previously observed to be prevalent on haplo-
type 2 PKU chromosomes in Caucasians, has been observed on a haplotype 44 background in Chinese patients with classical PKU (Tsai et al., 1990).

Some of these newly identified PKU mutations are unevenly distributed among different Asian populations. For example, the R413P mutation is maintained at a high frequency in northern Chinese and Japanese populations, but is relatively rare among southern Chinese (Wang et al., 1991a). In contrast, the mutation in the intron 4 splice acceptor site (IVS-4) is more prevalent among southern Chinese than among northern Chinese or Japanese (Wang et al., 1991b). These findings are compatible with the previous observations that polymorphic IgG markers of southern Orientals are distinct from those of northern Orientals (Matsumoto, 1988; Zhao and Lee, 1989) and support the hypothesis of multiple origins for PKU in Asia. The continuing identification of PKU mutations in Orientals will not only further delineate the molecular basis of PKU but also help to establish an effective carrier screening program for PKU in Asia. This study reports two additional mutations in the PAH gene that are prevalent among Chinese and documents the first case of successful prenatal gene diagnosis of PKU by direct genotype analysis.

MATERIALS AND METHODS

Patients

Fifty-two Chinese, 11 Japanese, and 25 Caucasian PKU families with full haplotype data were selected for this study (Daiger et al., 1989a). Genomic DNAs were isolated from peripheral blood samples of both parents and affected children. Eight RFLP sites in or near the PAH gene locus were analyzed, and haplotypes of each member were then determined according to the classification of Woo (1988). The clinical and biochemical phenotypes of the PKU patients in these families were evaluated separately in their home institutes according to standard criteria (Guthrie and Susi, 1963; Gütüttler, 1980). The serum phenylalanine levels of fasting, untreated patients who were between 10 months and 3 years of age were determined by fluorescence spectroscopy (Holton and West, 1970). The family chosen for prenatal gene diagnosis has one classical PKU child with no previous RFLP or mutation information available and the mother was in her second pregnancy. Fetal genomic DNA was prepared from amniotic fluid cells.

PCR Amplification and Direct Sequencing

The human PAH gene has previously been identified as having 13 exonic regions (DiLella et al., 1986a). Oligonucleotide primers were designed for the amplification of each exon based on flanking intronic sequence (unpublished data). Exonic regions were amplified using the polymerase chain reaction (PCR) and the amplified products were purified using Centricor-30 microconcentrators (Amicon) and Nusieve low-melting agarose gel as previously described (Wang et al., 1990). Direct sequencing of PCR products was performed as previously described (Wang et al., 1990).

Dot-Blot Hybridization Analysis

The 13 exon-containing regions of the PAH gene from each member of these PKU families were amplified and the amplified material was blotted onto Zeta probe membrane (Bio-Rad). ASO probes (end-labeled with [γ-32P]ATP) for both the normal and the mutant sequence were then applied separately on the filters and stringent washing was performed as previously described (DiLella et al., 1988).

In Vitro Expression Analysis

The two single-base substitutions were introduced separately into a full-length human PAH cDNA (PAH 247) by site-directed mutagenesis performed in M13 (Zoller and Smith, 1983). The mutant PAH cDNAs were then subcloned into the eukaryotic expression vector pCDNA I and transfected into COS cells by electroporation (Bio-Rad). After 48 to 72 h of transient expression, transfected cells were harvested and crude cell extracts prepared. PAH enzyme activity, transcribed PAH RNA, and PAH immunoreactive protein were then examined by PAH enzyme assay, Northern blot analysis (Ledley et al., 1985, 1987), and Western blot analysis (Robson et al., 1982), respectively.

RESULTS

Direct Sequencing of Exons 6 and 7 of the PAH Gene following PCR Amplification

The PCR primers designed for the amplification of exon 6 are shown in the legend to Fig. 1, and the PCR primers designed for the amplification of exon 7 have been previously described (Okano et al., 199Oc). The top portion of the left panel in Fig. 1 shows part of the normal exon 6 sequence (Kwok et al., 1985), while the corresponding sequence in the PKU patient is shown on the right. A and G bands are both present at the same position in the sequence from the PKU patient, indicating that this substitution occurred on only one of the two alleles. This individual is therefore heterozygous for this substitution. This A to G transition at
FIG. 1. Characterization of two single-base substitutions in exons 7 and 17 of the PAH gene by PCR-mediated direct sequencing. Primers for the PCR-mediated amplification and sequencing of exon 7 have been described previously (30). Primers for the PCR-mediated amplification of exon 6: 6A (5'-CACAGTTTCTG-GTCCCCGAC 3') is the sense strand of intron 5, and 6B (5'-CTCTCCCTTCTCAATCCTC-3') is the antisense strand of intron 6. Sequence primer for exon 6: 5'-AACCTCCGTTCTG--C3'. The DNA sequences on the left show part of the normal exon 6 (upper left) or 7 sequence (lower left), while the sequences to the immediate right are the corresponding regions of exons 6 and 7 that contain the substitutions. An extra G band appears at the same position as the normal A band in mutant exon 6, clearly demonstrating that a single-base substitution has occurred on one of the two alleles in this patient (upper sequences). This A-to-G transition results in the substitution of cysteine for tyrosine at amino acid 204 (Y204C) (top right panel). In exon 7 (bottom left), an A band is present instead of the normal G band, indicating that both alleles of this patient bear this substitution. This G-to-A transition results in the substitution of glutamine for arginine at amino acid 243 (R243Q) (bottom right panel).

the second base of codon 204 results in the substitution of cysteine for tyrosine at amino acid 204 of the PAH protein (Y204C) (Fig. 1, top right).

The bottom portion of the left panel shows a comparison of normal exon 7 sequence (left) with that of an individual bearing a single-base substitution of A for G at the second base of codon 243 (right). The absence of any normal sequence suggests that this individual is homozygous for this substitution, which causes the substitution of glutamine for arginine at amino acid 243 of the PAH protein (R243Q) (Fig. 1, bottom right).

Kindred Analysis Using Allele-Specific Oligonucleotide (ASO) Probes

The results of dot-blot hybridization for the R243Q and Y204C substitutions in three PKU families are shown in Fig. 2. Genomic DNAs from all members of families A and C were amplified for exon 7 and those from families B and C were amplified for exon 6. Amplified products were dot-blotted onto Zeta-probe membrane and hybridized separately with ASO probes specific for the normal or mutant sequence. Probes for the R243Q substitution in exon 7 (left): 5'-TTCGCCCTGGACCTG-GT-3' (normal) and 5'-CACAGTTTGGAGCCGGA-A3' (mutant). Probes for the Y204C substitution in exon 6 (right): 5'-ATGTGTTGATGACCATGTC-3' (normal) and 5'-TGTTGACT-CACACCAAGCAT-3' (mutant). RFLP haplotypes are listed at the bottom of the symbols representing each family member (center). The solid symbol represents the presence of the R243Q substitution, the hatched symbol represents the Y204C substitution, the dotted symbol represents unknown PKU mutations, and the hollow symbol represents normal PAH alleles.

Site-Directed Mutagenesis and Expression Analysis

Both substitutions were introduced separately into a full-length human PAH cDNA by site-directed mutagenesis followed by enzyme analysis in crude extracts of transfected COS cells. With the full-length human PAH cDNA as a probe, comparable amounts of exon 6-containing regions were successfully amplified, as shown by the hybridization of the normal probes to all three members of families A, B, and C. In family A, only the maternal and patient samples hybridized to the R243Q probe, indicating that both the mother and the patient bear this substitution. In family B, the Y204C probe hybridized to paternal and patient samples, demonstrating that both father and patient are heterozygous for this substitution. In family C, the paternal and patient samples hybridized to the R243Q probe and the maternal and patient samples hybridized to the Y204C probe. Thus, in this family, the patient must be a compound heterozygote for these two substitutions. In a comparison of the presence of these two substitutions with the specific RFLP haplotype of each family member, it is also clear that both are linked to mutant RFLP haplotype 4.

FIG. 2. Dot-blot analysis of the Y204C and R243Q substitutions in three PKU families using allele-specific oligonucleotide probes. Families A, B, and C are three Chinese families, each with one PKU child. Genomic DNAs from all members of families A and C were amplified for exon 7 and those from families B and C were amplified for exon 6. Amplified products were dot-blotted onto Zeta-probe membrane and hybridized separately with ASO probes specific for the normal or mutant sequence. Probes for the R243Q substitution in exon 7 (left): 5'-TTCGCCCTGGACCT-GT-3' (normal) and 5'-CACAGTTTGGAGCCGGA-A3' (mutant). Probes for the Y204C substitution in exon 6 (right): 5'-ATGTGTTGATGACCATGTC-3' (normal) and 5'-TGTTGACT-CACACCAAGCAT-3' (mutant). RFLP haplotypes are listed at the bottom of the symbols representing each family member (center). The solid symbol represents the presence of the R243Q substitution, the hatched symbol represents the Y204C substitution, the dotted symbol represents unknown PKU mutations, and the hollow symbol represents normal PAH alleles.
FIG. 3. PAH enzyme assay for Y204C and R243Q constructs. 0, 25, 50, 100, and 200 µg of protein extract from COS cells transfected with the normal (upper panel), the Y204C mutant (middle panel), or the R243Q mutant (lower panel) PAH cDNA-pCDNA I constructs were assayed for PAH activity. Both the substrate [\(^{3}C\)phenylalanine (upper band of each panel) and the product [\(^{3}C\)]tyrosine (lower band of each panel) are shown by autoradiography after their separation by thin-layer chromatography.

of PAH mRNA were detected by Northern blot from extracts of COS cells transfected with pCDNA I containing the normal PAH cDNA, the Y204C-PAH cDNA, or the R243Q-PAH cDNA. No PAH mRNA was detected from extracts of COS cell without transfection (data not shown).

PAH enzyme activity was determined in 0, 25, 50, 100, and 200 µg of crude extracts from COS cells transfected with pCDNA I containing the normal PAH cDNA (Fig. 3, upper panel), the Y204C-PAH cDNA (Fig. 3, middle panel), or the R243Q-PAH cDNA (Fig. 3, lower panel). The results of this experiment demonstrated that the R243Q-PAH cDNA construct produced less than 10% of the PAH activity associated with the normal control, while the PAH activity produced by the Y204C construct was not significantly different from that produced by the normal control. The level of PAH enzyme activity observed in these experiments was directly proportional to the amount of PAH immunoreactivity, as determined by Western blot analysis (data not shown).

**Correlation between Genotype and Clinical Phenotype**

The biochemical and clinical phenotypes of 11 Chinese PKU patients either homozygous or compound heterozygous for the R243Q and/or Y204C substitutions are summarized in Table 1. The patient homozygous for the R243Q substitution (A) has a severe biochemical phenotype, with serum phenylalanine of 36.5 mg/dl. This finding is consistent with the severe enzyme deficiency suggested by *in vitro* expression analysis. A second patient (B) is a compound heterozygote for the R243Q and R413P substitutions. The R413P substitution is a missense mutation previously shown to produce less than 10% of normal PAH activity. This patient also exhibits a severe PKU phenotype, with serum phenylalanine levels exceeding 20 mg/dl. This result is again consistent with previous findings of PKU mutations expressed in the COS cell system (Okano et al., 1990c; Wang et al., 1991a). A third patient, who is compound heterozygous for the R243Q and Y204C substitutions (patient G), exhibits a mild PKU phenotype, with a serum phenylalanine level of 17.5 mg/dl. Interestingly, patients who are compound heterozygous for the Y204C substitution and other PKU alleles manifest diverse biochemical phenotypes (G–K).

**Population Screening among Orientals and Caucasians**

Genomic DNAs from 52 Chinese, 11 Japanese, and 25 Caucasian PKU families were amplified separately for exons 6 and 7. The PCR products were dot-blotted and probed using oligonucleotide probes specific for the Y204C or R243Q substitution. Nineteen chromosomes bearing the R243Q substitution were detected, representing 18% of all PKU chromosomes in this Chinese sample population. Twelve chromosomes from this group, along with two additional alleles lacking in haplotype data, were found to contain the Y204C substitution, accounting for about 13% of all PKU alleles in this population. Only 1 of 22 chromosomes in the Japanese population bore the Y204C substitution, representing about 5% of all PKU chromosomes in that population. Linkage disequilibrium between these two substitutions and mutant RFLP haplotype 4 was further established (Table 2). Nei-

<table>
<thead>
<tr>
<th>Patient</th>
<th>Haplotype</th>
<th>Substitution</th>
<th>Phenylalanine in serum (mg/dl)*</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>4/4</td>
<td>243/243</td>
<td>36.5</td>
</tr>
<tr>
<td>B</td>
<td>4/4</td>
<td>243/413</td>
<td>20.0</td>
</tr>
<tr>
<td>C</td>
<td>4/3</td>
<td>243/ND</td>
<td>30.0</td>
</tr>
<tr>
<td>D</td>
<td>4/4</td>
<td>243/ND</td>
<td>26.2</td>
</tr>
<tr>
<td>E</td>
<td>4/9</td>
<td>243/ND</td>
<td>26.9</td>
</tr>
<tr>
<td>F</td>
<td>4/7</td>
<td>243/ND</td>
<td>30.0</td>
</tr>
<tr>
<td>G</td>
<td>4/4</td>
<td>204/243</td>
<td>17.5</td>
</tr>
<tr>
<td>H</td>
<td>4/4</td>
<td>204/111</td>
<td>20.0</td>
</tr>
<tr>
<td>I</td>
<td>4/4</td>
<td>204/ND</td>
<td>16.5</td>
</tr>
<tr>
<td>J</td>
<td>4/4</td>
<td>204/ND</td>
<td>15.6</td>
</tr>
<tr>
<td>K</td>
<td>4/4</td>
<td>204/ND</td>
<td>31.4</td>
</tr>
</tbody>
</table>

* Determined fluorometrically during pretreatment period.

b Not determined.
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Table 2
Population Genetics of the R243Q and Y204C Substitutions in the Human PAH Gene

<table>
<thead>
<tr>
<th>Substitution</th>
<th>Population</th>
<th>Haplotype 4 Alleles</th>
<th>Non-haplotype-4 alleles</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
<td>Mutant</td>
<td>Normal</td>
</tr>
<tr>
<td>R243Q</td>
<td>Chinese</td>
<td>0/72</td>
<td>19/81a</td>
<td>0/32</td>
</tr>
<tr>
<td></td>
<td>Japanese</td>
<td>0/17</td>
<td>0/21</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Caucasians</td>
<td>0/16</td>
<td>0/26</td>
<td>0/34</td>
</tr>
<tr>
<td>Y204C</td>
<td>Chinese</td>
<td>0/72</td>
<td>12/81c</td>
<td>0/32</td>
</tr>
<tr>
<td></td>
<td>Japanese</td>
<td>0/17</td>
<td>1/21</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Caucasians</td>
<td>0/16</td>
<td>0/20</td>
<td>0/34</td>
</tr>
</tbody>
</table>

* The ratios represent the number of samples that hybridized to the mutant ASO probe/total number of alleles analyzed.
* $\chi^2 = 6.61, P < 0.01.$
* $\chi^2 = 3.94, P < 0.05.$

There of these substitutions has been detected in Cauca- sian PKU families.

Prenatal Diagnosis for an Oriental PKU Family with ASO Probes

A Chinese family with one classical PKU child requested prenatal diagnosis during the course of pregnancy. There were no RFLP or mutation data available for this family. Genomic DNAs from both parents and the affected child were isolated from peripheral leukocytes, and fetal genomic DNA was obtained from amniotic fluid cells. Exonic regions of the PAH gene were amplified for each member of this family, and ASO probes for the four most prevalent PKU mutations were selected for the initial examination (Wang et al., 1989, 1991a, this report). As shown in Fig. 4, oligonucleotide probes specific for the normal or the R243Q substitution both hybridized to the parental and fetal samples in this family, indicating that these three individuals are heterozygous for this substitution. However, only the R243Q probe hybridized to the sample obtained from the affected child in this family, indicating that this individual is homozygous for this substitution. Since the fetus bears one normal and one PKU chromosome, it should be phenotypically normal, a diagnosis confirmed shortly after birth.

Discussion

Two new molecular lesions present in the human PAH gene have been characterized in Chinese PKU patients. The first, identified in exon 7 of the gene, is a G-to-A transition at the second base of codon 243, resulting in the substitution of Gln for Arg in the mutant protein (R243Q). Previous studies on PKU mutations and PAH enzyme activity have suggested that exon 7, which encodes a segment of the PAH protein located within the enzymatic core region (Iwaki et al., 1986), is particularly susceptible to missense mutation (Abadie et al., 1989). The current report provides further evidence that the region of the PAH protein encoded by exon 7 clearly plays a critical role in enzyme function.

Clinically, patients homozygous for this substitution exhibit a severe biochemical phenotype, with serum phenylalanine levels exceeding 20 mg/dl. In vitro expression analysis in eukaryotic cells indicates that the R243Q mutation produces less than 10% of normal PAH activity. Additional experiments demonstrated that the reduction in PAH enzyme activity was not due to a decrease of PAH mRNA but is most probably the result of instability of the mutant PAH protein, since only trace steady-state levels of this protein were detected by Western analysis. The association between this level of in vitro PAH activity and a severe PKU phenotype is consistent with previous
results obtained using this expression system (Okano et al., 1990c; Wang et al., 1991a). Although this level of in vitro activity is higher than those observed in liver biopsy samples obtained in patients with classical PKU (Trefz et al., 1981), this discrepancy may merely reflect differences in the conditions of gene expression in these two systems. For example, COS cells may differ significantly from hepatocytes at many levels, including regulation of the PAH gene or post-translational modification of the PAH protein. In addition, the concentrations of substrates, cofactor, and other important mediators of PAH activity may vary significantly between the two PAH assay systems. Nevertheless, within this in vitro expression system, a strong correlation between the level of PAH activity and the biochemical or clinical PKU phenotype can be demonstrated (Okano, Eisensmith, and Wang, unpublished observations).

The second substitution, in exon 6 of the PAH gene, produces the substitution of tyrosine for cysteine at amino acid 204 (Y204C). This change could interfere with the function of the naturally occurring cysteine at residue 203, which is also located within the enzymatic core region of the molecule. Furthermore, this change could permit the formation of a new disulfide bond. However, when expressed in the COS cell system, the Y204C construct produces PAH enzyme activity similar to that of the normal control and thus appears to be silent in this in vitro system. Nevertheless, this substitution is tightly linked to mutant RFLP haplotype 4 in Oriental populations and has not been found on normal chromosomes. Thus, two possible explanations arise. First, this substitution may in fact be a mutation responsible for PKU, as supported by population genetic analyses and genotype/phenotype comparisons. If this is the case, one explanation may be that our in vitro expression system might not perfectly reflect the in vivo conditions in this specific circumstance as previously discussed. The second possibility is that this substitution is merely a polymorphism linked to mutant haplotype 4, as suggested by in vitro expression analysis. If this is true, there should be other molecular defects responsible for the PKU phenotype present on these mutant chromosomes. However, if there are other defects present on these chromosomes, they must lie outside the exonic regions and the intron/exon junctions, as these regions have all been carefully examined by DNA sequencing. In either event, this substitution can serve as a valuable marker for identifying 13% of PKU chromosomes in the Chinese population.

Newborn screening and restricted dietary therapy have been accepted as routine procedures for controlling PKU in many developed countries. However, life-long dietary restriction, as recommended, not only represents a significant burden to these families and society as a whole but also involves uncertainties for prognosis. Despite treatment, many PKU patients may still show significant declines in IQ scores, learning disabilities, and psychological problems (Williamson et al., 1981; Koch et al., 1984). Furthermore, this therapy will increase the incidence of maternal PKU, which may then affect PKU clinics in the future (Scrivner, 1985). The limitations of treatment, then, increase the importance of implementing an effective carrier screening program that can reliably identify couples at risk and make prenatal diagnosis in these cases critical. Although the isolation of the human PAH cDNA previously allowed direct detection of the PAH gene in the developing fetus by RFLP haplotype analysis, the application of RFLP haplotype analysis to PKU chromosomes in Orientals is limited by the relative homogeneity of this population. Thus, direct mutation analysis is much more important in this population than in relatively heterogeneous Caucasian populations. With the substitutions reported in the present study, over 60% of all PKU chromosomes can be identified in Oriental populations, which will aid in the future screening of PKU carriers among Orientals.

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