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Molecular Genetics of Phenylketonuria in Orientals: Linkage Disequilibrium between a Termination Mutation and Haplotype 4 of the Phenylalanine Hydroxylase Gene

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Summary

Phenylketonuria (PKU) is a common metabolic disorder among Chinese, with a prevalence of about 1 in 16,500 births. This frequency is very similar to that among Caucasians. Individual exons of the phenylalanine hydroxylase (PAH) gene with flanking introns were amplified by polymerase chain reaction and cloned into M13 for sequence analysis. An *Arg*^{111}-to-*Ter*^{111} mutation has been identified in exon 3 of the PAH gene in a Chinese PKU patient. The mutation is in linkage disequilibrium with the mutant haplotype 4 alleles which are the most prevalent haplotype among the Orientals. The mutation accounts for about 10% of the Chinese PKU alleles and is absent from the Caucasians, demonstrating that independent mutational events have occurred in the PAH locus after racial divergence.

Introduction

Classical phenylketonuria (PKU) is an autosomal recessive genetic disorder caused by a severe deficiency in hepatic phenylalanine hydroxylase (PAH). The disorder is characterized by an accumulation of phenylalanine in the serum, resulting in hyperphenylalaninemia and abnormalities in aromatic amino acid metabolism. Untreated patients will develop severe postnatal brain damage and mental retardation which is irreversible (Folling 1934). PKU is the most common inborn error in amino acid metabolism, with an average prevalence of about 1 in 10,000 Caucasians (for review, see Scriver et al. 1988). Previous biochemical and clinical observations have demonstrated that phenotypically PKU is a very heterogeneous disease (for review, see Guttler 1980).

The study of PKU at the molecular level became possible with the construction of a full-length and functional human PAH cDNA (Kwok et al. 1985; Ledley et al. 1985). It was used to identify extensive RFLPs in the human PAH locus (Woo et al. 1983; Lidsky et al. 1985*b*), which permitted prenatal diagnosis for PKU (Lidsky et al. 1985*a;* Ledley et al. 1988).

RFLP haplotype analysis of the human PAH locus has revealed the presence of at least 43 different haplotypes (Woo 1988), and haplotypes 1–4 compromise approximately 90% of all mutant PKU alleles among Caucasians of northern-European ancestry (Chakraborty et al. 1987). Molecular analysis of genomic DNA isolated from a patient homozygous for mutant haplotype 3 has revealed a G-to-T transition at the splice-donor site of intron 12 of the PAH gene (DiLella et al. 1986*b;* Marvit et al. 1987). In a mutant haplotype 2 allele a second mutation was identified to be a C-to-T transition in exon 12 of the gene, resulting in the substitution of *Arg* to *Trp* at residue 408 of the enzyme (DiLella et al. 1987). These mutations are tightly linked to the respective mutant haplotypes, providing strong evidence for linkage disequilibrium (Woo 1989).

Recently, PKU has also been found to be prevalent in the Chinese population, with an average incidence

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of 1 in 16,500 births (Liu 1985). Extensive RFLP haplotype analysis revealed that haplotype 4 accounts for approximately 80% of both normal and mutant PKU alleles (Chen et al. 1989; Daiger et al. 1989). Since the haplotype distribution in Orientals is very different from that in Caucasians, the mutant PAH alleles in the Oriental population may be distinct from those in the Caucasian population. In the present report, we describe the characterization of a mutant Oriental PAH allele that is in linkage disequilibrium with haplotype 4.

Material and Methods

Patients

Thirty-nine Chinese PKU families with complete haplotype data were evaluated clinically at the Children's Hospital in Shanghai and at the Institute of Basic Medical Sciences in Beijing (Daiger et al. 1989). The presence of classical, severe PKU in affected children was confirmed by Guthrie test and clinical criteria described elsewhere (Guthrie and Susi 1963; Guttler 1980), with plasma phenyalanine level in excess of 20 mg/dl. The proband selected for molecular analysis is a classical PKU child from south China and is homozygous for RFLP haplotype 4.

PCR Amplification of Exonic Regions in the PAH Gene

Phosphorylated primers for polymerase chain reaction (PCR) amplification of exonic regions of the human PAH gene were purchased from Genetic Designs, Inc., Houston. Individual exons with flanking intronic sequence of the PAH gene were amplified and analyzed independently. Target sequences were amplified in a 100-μl reaction volume containing 1 μg genomic DNA, 1.5 mM each of dATP, dGTP, dCTP, and dTTP, 67 mM Tris-HCl (pH 8.8), 16.6 mM ammonium sulfate, 2.5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 6.7 μM EDTA, 170 μg BSA/ml, 10% of DMSO, and 1 μM each of an amplification primer pair. Samples were heated at 97°C for 7 min to denature the DNA and were allowed to cool at 37°C for 2 min. A 2.5-U sample of *Taq* DNA polymerase (Perkin Elmer Cetus) was added to each sample, and the amplification was initiated by incubation at 63°C for 2 min. The following cycles consisted of DNA denaturation at 92°C for 1 min, annealing at 37°C for 2 min, and primer extension at 63°C for 2 min. A total of 40 cycles were performed. A 10-μl sample of PCR mixture was analyzed on 4% NuSieve agarose gels, and the amplified products were purified using Centricon 10 (Amicon).

M13 Cloning and Sequencing of PCR Products

M13mp18 (RF) DNA was cut to completion using *Sma*I (Biolabs) and was treated with alkaline phosphatase (Boehringer Mannheim). A 1-μg sample of PCR-amplified DNA was ligated with 0.6 μg dephosphorylated M13mp18 vector in 50 μl. The ligated DNA was used for transformation of *Escherichia coli* TG-1 cells which were then plated on XGAL/IPTG agar. Seven to 10 independent recombinant clones containing the corresponding exons were sequenced. Dideoxy sequencing was performed using the Sequenase DNA sequencing kit (United States Biochem).

Mutation Analysis of PCR-amplified Products by Oligonucleotide Hybridization

A pair of oligonucleotide probes (19-mer), one specific for the normal allele and the other specific for the mutant allele, were end-labeled with ^{32}P dATP (6,000 Ci/mmol; New England Nuclear). Genomic DNA samples were individually PCR amplified as described above. A 2.5-μl sample of amplified material was denatured in 0.2 M NaOH and was applied directly by dot blot on to zeta-probe membrane (Bio-Rad) in 1 M NH_4OAC. Hybridization was carried out using labeled normal and mutant probe separately according to a method described elsewhere (DiLella et al. 1988).

Results

Amplification of Exon 3 and Flanking Introns of the PAH Gene

Figure 1*A* shows the primers for PCR amplification of exon 3 plus the flanking intronic regions of the human PAH gene. The amplified fragment is expected to be 300 bp in length. Figure 1*B* shows the patient's genomic DNA both without amplification (lane 2) and after amplification for various cycles (lanes 3–6). There is a distinct 300-bp DNA band that was absent in lane 2 but that progressively accumulated during amplification. Southern blotting and hybridization using a labeled PAH cDNA probe confirmed that the amplified DNA fragment contained exonic PAH sequence.

Identification of a Nonsense Mutation in Exon 3 of the PAH Gene

The amplified fragment from this PKU patient was subcloned in M13mp18, and seven independent clones were characterized by sequence analysis. There is a C-to-T transition at the first base of codon 111, result-

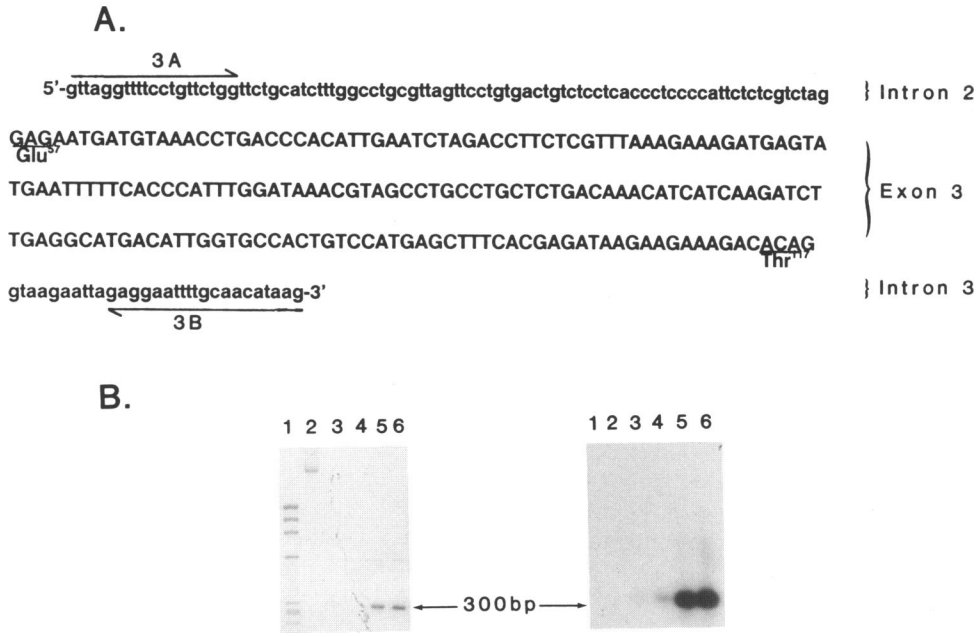


Figure 1 *A*, Sequence of exon 3 plus flanking introns of the human PAH gene. The sequence (capital letters for exon, small letters for introns) was determined from a fragment of exon 3–containing region in a human genomic DNA clone (DiLella et al. 1986*a*). The exon encodes amino acid residues 57–117 in the enzyme. The locations of PCR primers are shown by the arrows. Primer 3A (*5'*-GTTAGGTTTTCCTGTTCTGG-3') is the same strand of intron 2, 65–84 nucleotides *5'* of exon 3. Primer 3B (*5'*-CTTATGTTGCAAAATTCCTC-3') is complementary to the sense DNA strand of intron 3, 11–30 nucleotides *3'* of exon 3. *B*, Agarose-gel electrophoresis of the PCR-amplified product (*left*) and Southern hybridization with PAH cDNA probe (*right*). Lane 1, *Hae*III-digested Ox174 DNA marker; lane 2, 0.1 μg patient's genomic DNA without amplification; lanes 3–6, 0.1 μg patient's genomic DNA after 10, 20, 30, and 40 cycles of PCR amplification, respectively.

ing in the substitution of an *Arg* (CGA) to a stop codon (TGA) in exon 3 of the gene. (fig. 2). This mutation was present in three of the seven independent clones, suggesting that it is an authentic mutation and that the patient may be a compound heterozygote.

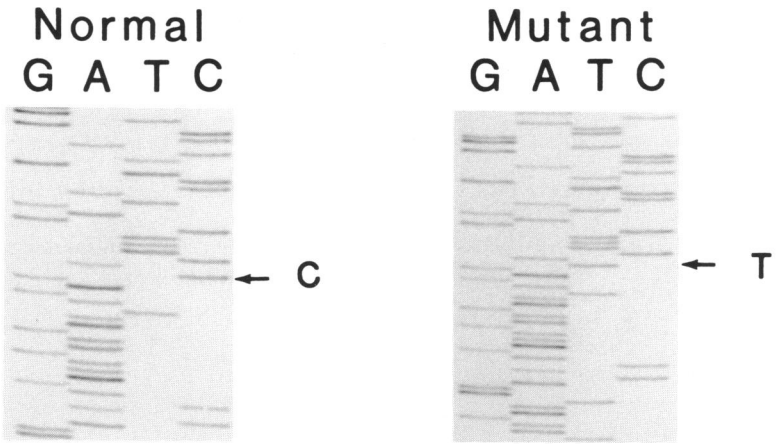
Mutation Verification in Patient's Genomic DNA and Mendelian Transmission

To verify that the nonsense mutation is indeed present in genomic DNA of the patient and one of the parents, genomic DNA samples from the proband and both parents were independently amplified by PCR and were analyzed by dot-blot hybridization using oligonucleotides corresponding to the mutant and normal alleles. The mutant probe hybridized with the proband sample (fig. 3*A*), demonstrating that the termination mutation is indeed present in the patient's genomic DNA and is not an artifact of PCR. The mutant probe also hybridized with the paternal sample but not with the maternal sample, illustrating the source of the mutant allele in this family. The normal probe hybridized with samples from all three members of the nuclear family (fig. 3*A*), indicating successful amplification of the maternal sample, which must bear a different mutant allele. This second mutant allele has been transmitted to the proband, who must therefore be a compound heterozygote.

Additional Oriental nuclear PKU families were similarly analyzed, and the results for two such families bearing the termination mutant allele are also shown (fig. 3, families B and C). In family B, the mutant allele is maternal in origin and the proband is also a compound heterozygote; in family C, both parents are carriers of this mutant allele and the proband is a homozygote.

Linkage Disequilibrium between the Termination Mutation and Haplotype 4

In 39 PKU families from various geographic locations in China, oligonucleotide hybridization analysis was performed with complete haplotyping data, to determine whether there is linkage disequilibrium between

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                                        Arg111
Normal sequence :  5'- GAG CTT TCA CGA GAT AAG AAG -3'

Mutant sequence :  5'- --- --- --- TGA --- --- --- -3'
                                    Ter111
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Figure 2 Identification of a nonsense mutation in exon 3 of the human PAH gene. Sequence analysis of the exon 3–containing regions of a normal and a mutant allele. The C-to-T transition results in the substitution of the Arg^{111} codon to a termination codon.

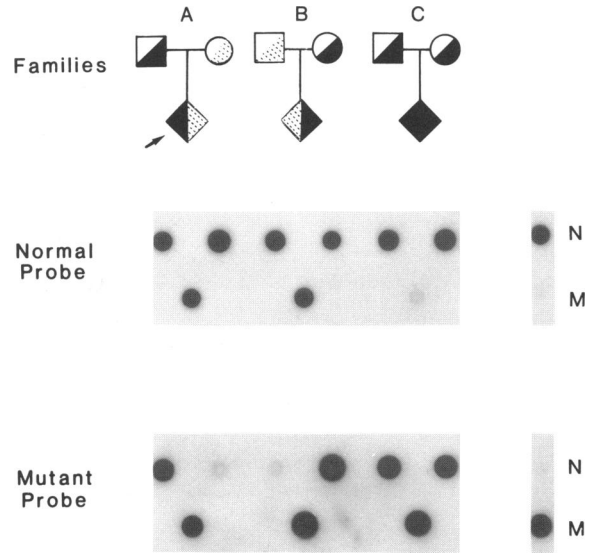

Figure 3 Transmission of the termination mutant alleles in three different Chinese PKU families as analyzed by PCR amplification followed by dot-blot hybridization using specific oligonucleotide probes. The probe used to detect the substitution at amino acid 111 in exon 3 were the following: the normal probe (5′-GAGCTTTCA-CGAGATAAGA-3′), which is the sense strand, and the mutant probe (5′-ACTTATCTCATGAAATGCTC-3′), which is the antisense strand.

the termination mutation and haplotype 4. A total of five such mutant alleles were found among 78 mutant alleles, and none were found among 78 normal alleles. Furthermore, all five mutant alleles were associated with haplotype 4 of the mutant PAH gene (table 1). The data not only suggest the existence of tight linkage between this specific mutation and haplotype 4 but also demonstrate that there are multiple mutations associated with this haplotype. In addition, we have analyzed the presence of this mutant allele in 17 Chinese PKU patient samples without haplotyping data due to the lack of complete parental samples. It was observed that two patients were homozygous for this mutant allele and that three patients were compound heterozygotes. Thus seven of the 34 additional mutant alleles were repre-

Genomic DNA was isolated from leukocytes of different family members. Exon 3–containing regions (300 bp) were amplified by PCR, dot-blotted onto Z-probe membrane, and hybridized with the specific oligonucleotide probes as described in methods. The proband in family A (indicated by arrow) was the one characterized by molecular analysis. Solid symbols in pedigrees represent the Arg^{111}-to-Ter^{111} mutant alleles; hatched symbols represent uncharacterized PAH mutant alleles; and open symbols represent normal PAH alleles.

Table 1

Population Studies of *Arg*^{111}-to-*Ter*^{111} Mutation among Chinese and Caucasians

	Total no. of Signals/No. Analyzed		
	Chinese		Caucasians
Haplotype(s)	Normals	Mutants	Mutants
1	0/1	0/1	0/9
2	0/3	0/3	0/8
3	0/4	0/2	0/8
4	0/49	5/60	0/29
5–23	0/21	0/12	0/2

sented by the termination mutation allele analyzed. This mutant accounts for about 10% (12 of 112 mutant alleles) of PKU genes in the Chinese population.

Discussion

We identified a new molecular lesion in the PAH gene of a Chinese PKU patient. This mutation is characterized by a single nucleotide substitution in exon 3 of the PAH gene encoding amino acid residue number 111, where an arginine codon CGA is changed to a termination codon TGA. This mutation will cause the loss of approximately two-thirds of the PAH polypeptide and is the first PKU mutation identified in the Oriental population.

The mutation occurred only on the haplotype 4 background in the Oriental population, suggesting the existence of linkage disequilibrium between mutation and haplotype, which has previously been observed in Caucasians (DiLella et al. 1986*b*, 1987). In this case, however, the linkage disequilibrium is not inclusive, as there are mutant haplotype 4 alleles that do not bear this particular mutation. Our finding provides strong evidence for the hypothesis that different PKU mutations have occurred on the PAH gene of the same haplotype background, which has been observed previously in β-thalassemia (for review, see Orkin and Kazazian 1984).

Extensive RFLP haplotype analysis of the PAH locus revealed that the haplotype distribution in the Oriental population is quite different from that in the Caucasian population (Daiger et al. 1989). Analysis of 56 Caucasian PKU alleles has shown that the *Arg*^{111}-to-*Ter*^{111} mutation is absent in the Caucasian population (table 1). Likewise, the *Arg*^{408}-to-*Trp*^{408} and the intron 12 splicing mutation were never observed in the Oriental population. This evidence suggests that multiple mutations occurred independently in the two populations after racial divergence.

Since more than 80% of both normal and mutant PKU alleles are represented by haplotype 4 in the Oriental population, haplotyping is not very informative for effective prenatal diagnosis. Direct mutation analysis will therefore be important for this purpose. PCR amplification has greatly enhanced our ability to identify carriers of specific mutant alleles. Further characterization of PKU mutations in Orientals will also eventually provide the necessary information for effective prenatal diagnosis and carrier detection of PKU in that population.

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