HAPLOTYPE ANALYSIS OF PHENYLALANINE HYDROXYLASE ALLELES IN POLISH FAMILIES WITH PHENYLKETONURIA*

*Department of Human Genetics, Polish Academy of Sciences, Strzeszyńska 32, 60-479 Poznań, Poland, 
 Institute of Pediatrics, Academy of Medicine, Poznań, Poland. 
Department of Medical Genetics, Humboldt University, Berlin, 
German Democratic Republic, and 
Department of Human Genetics, University of Saar, Homburg, Federal Republic of Germany

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Eight polymorphic restriction enzyme sites at phenylalanine hydroxylase locus from the parental chromosomes in Polish families with phenylketonuria were analyzed. Among 28 chromosomes studied, we identified haplotypes found within the Danish population. Haplotype 2 has been found in 25% of affected alleles. One of the patients studied is homozygous for this haplotype.

Classical phenylketonuria (PKU)1 is an autosomal recessive human genetic disorder caused by a deficiency of hepatic phenylalanine hydroxylase (PAH, phenylalanine 4-monooxygenase, EC 1.14.16.1). In the normal human liver, PAH catalyses hydroxylation of phenylalanine to tyrosine. Severe PAH deficiency causes depletion of serum tyrosine and accumulation of phenylalanine and its metabolites, resulting in permanent mental retardation in children if left untreated [1, 2]. PKU is the most common inborn error in amino-acid metabolism. Its incidence among Caucasians is about 1:10 000 and the mutant gene frequency predicts that 1 in 50 individuals is a carrier.

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1 Abbreviations: PHU, phenylketonuria; PAH, phenylalanine hydroxylase; RFLP, restriction fragment length polymorphism; kb, kilobase pair; SSC, (20×), 3 M NaCl, 0.3 M sodium citrate; SDS, sodium dodecyl sulphate.
** Requests for reprints should be addressed to R. Słomski.
of the disease trait [3]. Since there is no reliable biochemical test for identification in vitro of gene carriers [4], molecular techniques were applied to examine genetic variation of the PAH gene associated with PKU [5 - 7]. A full-length cDNA clone of the human PAH gene that contains full information for the enzymatic activity has been isolated [8, 9].

Extensive analysis of restriction fragment length polymorphism (RFLP) of the PAH locus provides the theoretical basis for selection of mutant alleles for further molecular characterization.

Eight restriction sites showing length polymorphism were detected at the structural locus of PAH on human chromosome 12q22 - 24 [10]. The RFLP haplotypes determined by eight restriction enzymes are tightly linked with PKU (maximum lod score of 9.96 at the recombination of 0.00) [11]. The amino acid sequence deduced from the full-length cDNA clone predicts a protein of 451 amino acids with a molecular mass of 51,960. DNA mediated gene transfer experiments demonstrated that phPAH247 contains all the genetic information necessary to code for a functional enzyme [12].

Southern hybridization analysis of DNA isolated from a large number of PKU individuals demonstrated that the disorder is not caused by any obvious deletions in the PAH gene [5]. The genetic lesions causing the loss of enzyme activity in PKU in Polish population are not known. In the present studies we have examined the distribution of PAH hypototypes in Polish families with PKU.

MATERIALS AND METHODS

Plasmid phPAH247 containing a full-length human cDNA for PAH was obtained from S.L.C. Woo (Howard Hughes Medical Institute, Baylor College of Medicine, Houston, U.S.A.). Its sequence characterization has been reported [9]. Plasmid DNA was nick-translated to a specific activity of $2 \times 10^8$ c.p.m./μg.

Total human DNA was isolated from peripheral blood as described by Kunkel et al. [13]. Blood was collected on EDTA, mixed for 5 min with 10 vol. of lysis solution containing 0.32 M sucrose, 5 mM MgCl$_2$, 1% Triton X-100, 0.01 M Tris/HC1, pH 7.6 and after lysis cell nuclei were collected and suspended in solution containing 75 mM NaCl, 24 mM EDTA, pH 8.0, 1% SDS. The nuclear lysate was then incubated with 100 μg/ml Proteinase K (Serva) at 37°C for 16 h. DNA was extracted twice with phenol, once with chloroform and precipitated with ethanol. Ten micrograms of genomic DNA were digested with one of the restriction enzymes: EcoRI, EcoRV, HindIII, MdpI, PvuII, BglII or XmnI (New England Biolabs) in appropriate buffer. Restriction fragments were separated by agarose gel electrophoresis and blotted onto nitrocellulose [14]. The probe was labelled by nick translation.
with $^{32}$P-dATP (Amersham). Prehybridization was performed in a solution containing 50% formamide, 10×Denhardt’s solution, 4×SSC, 0.1% SDS, 0.1% sodium pyrophosphate, 10% dextran sulphate. 50 μg/ml denatured herring sperm DNA at 42°C for 90 min. Hybridization was performed in the same solution in the presence of probe at 42°C for 16 h. Filters were then washed in 2×SSC, 0.1% SDS, at 65°C, for 15 min and in 0.1×SSC, 0.1% SDS at 65°C for 10 min, followed by autoradiography for 1-7 days at −80°C.

RESULTS

High molecular weight genomic DNA was isolated from blood samples of 30 Caucasian individuals from 7 families with the occurrence of phenylketonuria. Each sample of DNA was digested separately with the following restriction enzymes EcoRI, EcoRV, HindIII,MspI, PvuII, BglII and XmnI.

After digestion of genomic DNA with EcoRI and hybridization with phPAH247, a polymorphic 17 kb fragment was observed. The presence of an additional site in each of the 17 kb fragments resulted in its cleavage into a 11 kb fragment. Individuals homozygous for the 17 or 11 kb fragment display an EcoRI restriction pattern as shown in Fig. 1A. The pattern is complicated by the presence of the additional bands.

The phPAH247 hybridized to three EcoRV fragments common to all individuals and a fourth fragment either of 30 kb or 25 kb. Individuals homozygous for the 25 kb fragment have the polymorphic EcoRV site in each of their PAH genes. The 5 kb fragment which is cleaved off from the 30 kb fragment is not detected by the full length cDNA and is comprised of a non-coding sequence (Fig. 1B).

Application of HindIII restriction enzyme in Southern blot analysis of genomic DNA permits detection of the allelic system associated with bands of 4.2 and 4.0 kb (Fig. 1C). An allelic system can be also detected with XmnI enzyme. Restriction site polymorphism results in either 9.4 kb or 6.5 kb fragment (Fig. 1D). A BglII site is polymorphic in respect to phenylalanine hydroxylase and results in fragments of either 3.6 or 1.7 kb in length (Fig. 1E).

The hybridization of full length cDNA with genomic DNA digested with the restriction enzyme Mspl yields fragments, polymorphic of 23 kb and 19 kb (Fig. 1F). Southern analysis of DNA digested with PvuII showed four variant fragments of 19.0, 11.5, 9.1 and 6.0 kb in length created by two polymorphic PvuII restriction sites (Fig. 1G).

In six out of seven families selected for analysis, digestion of genomic DNA with only one restriction enzyme was sufficient to detect linkage of polymorphic bands with normal or mutant alleles. The most informative
Fig. 1. Identification of restriction fragment length polymorphism in human phenylalanine hydroxylase gene. DNA samples were isolated from peripheral blood of members of families with phenylketonuria. DNA samples were digested with one of the restriction enzymes, fractionated in 1% agarose gel, blotted onto nitrocellulose and hybridized according to Southern [14] with the molecular probe phPAH247 consisting of full length of human phenylalanine hydroxylase cDNA. Numbers at the right margin show the size of polymorphic bands. With the exception of Fig. 1H the numbers indicate 1, father; 2, mother; 3, child with phenylketonuria, 4, 5, unaffected siblings.

A. EcoRI polymorphism. Both parents are heterozygous for the size of polymorphic fragments (lanes 1 and 2). The affected child has inherited the fragments of 17 kb (lane 3). The second child is heterozygous for the size of fragments and is a carrier of phenylketonuria.

B. EcoRV polymorphism. Parents are heterozygous for the fragments of 30 and 5 kb (lanes 1 and 2). The affected child has inherited the smaller fragment from both parents (lane 3). One of the phenotypically unaffected siblings has the same genotype as the parents and is a carrier of PKU (lane 4). The other unaffected sibling has a genotype of 30/30 kb and is free of the PKU trait (lane 5).

C. HindIII polymorphism. Parents are heterozygous for the size of fragments (lanes 1 and 2). The affected child is homozygous for the 4.0 kb fragment. This individual has inherited the mutant alleles from father and mother (lane 3).

D. XmnI polymorphism. Father is heterozygous for two fragments of 9.4 and 6.5 kb (lane 1). Mother, who is an obligatory carrier of the PKU is homozygous for the 9.4 kb fragment (lane 2). The affected child has inherited fragments of the same size (9.4 kb) from both parents (lane 3). The phenotypically unaffected sibling has also inherited two alleles of the same size (9.4 kb), a mutant allele from father and a normal allele from mother. This individual is homozygous for the fragments size and is a carrier of the disease (lane 4).

E. BglII polymorphism. Father is an obligatory carrier of PKU but is homozygous for the 3.6 kb fragment (lane 1). Mother shows polymorphism in PAH gene which results in restriction fragments of 3.6 and 1.7 kb (lane 2). The affected child has inherited larger fragments from both parents what indicates that they are mutant alleles (lane 3). The other sibling has the same genotype as the father and first sibling (3.6/3.6 kb). This individual has inherited a normal allele from father and a mutant allele from mother, and is a carrier of the PKU trait.

F. MspI polymorphism. Parents are obligatory heterozygotes for the PKU and are heterozygous for the size of fragments (lane 1 and 2). The affected child has inherited both fragments of the size 19 kb. Future pregnancies within this family can be easily diagnosed by MspI polymorphism.

G. PvuII polymorphism. Four polymorphic fragments are the result of the presence of two independent sets of PvuII RFLP alleles. Father is heterozygous for both fragments sets (a and b) and the pattern shows four bands of 19.0, 11.5, 9.1 and 6.0 kb (lane 1). Mother is heterozygous for the fragments set “a”, homozygous for the set “b” and the pattern shows three bands 19.0, 11.5 and 6.0 kb (lane 2). Two affected siblings are homozygous for both fragments sets and the pattern shows two bands 11.5 and 6.0 kb. They both have inherited fragments alleles of set “a” of 6.0 kb and fragments of set “b” of 11.5 kb (lane 3 and 4).

H. Additional polymorphic HindIII band observed in an phenotypically unaffected child of a PKU family (lane 1). Mother shows typical HindIII polymorphism and is heterozygous for 4.0/4.2 kb bands.
results were obtained after digestion with enzymes EcoRI and MspI. In the case of one family, separate digestions with two enzymes were needed to complete analysis.

In analysis of one DNA sample from an affected family the additional DNA band was observed after digestion of DNA with the restriction enzyme HindIII (Fig. 1H).

The haplotypes defined by RFLP for parents of children with phenylketonuria are shown in Table 1.

**DISCUSSION**

Using a full length PAH cDNA clone as the hybridization probe, extensive restriction fragment length polymorphism can be detected at the human phenylalanine hydroxylase locus. One of the main reasons of polymorphism lies probably in the size of the gene exceeding 90 kb [5]. The potential application of RFLP studies to the prenatal diagnosis of phenylketonuria and the detection of PKU carriers has been already discussed [5]. Normal and mutant phenylalanine hydroxylase genes can be distinguished, in families who have already one previously affected child, by identifying the RFLP haplotypes in each family.

On the basis of large population studies eight restriction enzymes were selected for DNA analysis in PKU: EcoRI, EcoRV, MspI, PvuII, XmnI, HindIII, BglII and SphI. In the present studies seven restriction enzymes were used. In all the cases examined the pattern of RFLP bands characteristic for phenylalanine hydroxylase has been observed. In one family with PKU, in the DNA sample obtained from the unaffected child, an additional hybridization band was observed. Elucidation of this observation will require more extensive studies on a larger number of families. In all the families analysed identification of normal and mutant alleles was possible after digestion of DNA with specific restriction enzymes, but informative results could be obtained only when both parents were heterozygous for polymorphic allele. The mutant allele must be linked in both parental DNAs with the same polymorphic band. The most informative were the analyse obtained with enzymes EcoRI and MspI. In six cases digestion of DNA with one restriction enzyme was sufficient for diagnosis.

Restriction enzymes EcoRI, MspI and XmnI have been found to be the most informative in the RFLP analysis. In one family it was necessary to perform a more complex study using two restriction enzymes, HindIII and MspI, to attain a fully informative result. The data obtained enable us a complete diagnosis using RFLP analysis in future pregnancies in all 7 families we have studied. Furthermore, we discriminated the carrier status in unaffected offsprings. In the Danish population 12 most common RFLP
haplotypes of phenylalanine hydroxylase alleles have been identified [10]. In our studies among 28 parental hyplotypes in Polish families the most common for PKU was haplotype 2. Further analysis of other PKU Polish families will enable full characterization of RFLP haplotypes of phenylalanine hydroxylase alleles. It would be very important to correlate individual RFLP haplotypes of phenylalanine hydroxylase alleles with different clinical stages of phenylketonuria, because the degree of phenylalanine hydroxylase inactivation in patients is variable. It has been shown that in some cases the phenylalanine hydroxylase mRNA was completely absent in liver cells. In Danish population there are 3 RFLP hyplotypes which are the most common for the affected alleles (haplotypes 2, 3 and 4) [15, 16]. Patients with genotypes 2/2, 2/3 and 3/3 have the most severe phenylalanine hydroxylase deficiency. Mutations which have been identified in these two haplotypes are single nucleotide substitutions [15, 16]. As the next step of our studies we propose to compare particular RFLP haplotypes of phenylalanine hydroxylase alleles with the clinical stage of PKU patients.

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Table 1

| Restriction fragment length polymorphism haplotypes of normal and affected parental phenylalanine hydroxylase alleles in Polish families with phenylketonuria |
| DNA samples were isolated from peripheral blood of parents of families affected by PKU. Both parents are obligatory heterozygotes. For each DNA sample separate digestion with indicated restriction enzyme followed by separation in agarose and hybridization with probe phPAH247 were performed. Presence of restriction fragments listed at the bottom of Table within mutant (M) and normal (N) alleles is indicated by "+" or "-", respectively. N.D., not determined, +--, non informative, all members of family are heterozygotes. |
REFERENCES