Two distinct mutations at a single *Bam*HI site in phenylketonuria

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Abstract

Classical phenylketonuria is an autosomal recessive disease caused by a deficiency of hepatic phenylalanine hydroxylase (PAH). The abolition of an invariant *Bam*HI site located in the coding sequence of the PAH gene (exon 7) led to the recognition of two new point mutations at codon 272 and 273 (272^{Gly→stop} and 273^{Ser→Phe}, respectively). Both mutations were detected in north eastern France or Belgium and occurred on the background of RFLP haplotype 7 alleles. The present study supports the view that the clinical heterogeneity in PKU is accounted for by the large variety of mutant genotypes associated with PAH deficiencies.

Phenylketonuria (PKU) is an autosomal recessive disease resulting from deficiency of a liver specific enzyme, phenylalanine hydroxylase (PAH, E.C. 1.14.16.1). The extensive use of restriction fragment length polymorphisms (RFLPs) has defined RFLP haplotypes at the PAH locus¹ and shown linkage disequilibrium between particular mutant genotypes and RFLP haplotypes in PKU.² Occasionally, the observation of abnormal restriction fragments has helped in identifying the mutant genotype(s) in this disease.³ In this study we show that the alteration of an invariant *Bam*HI site in the PAH gene has led to the discovery of two different mutations. Both occurred on the background of haplotype 7 PKU alleles originating from northern Europe. Since these two new mutations are located in exon 7 of the PAH gene, it appears that this particular exon carries the highest number of mutant genotypes identified in

PKU so far (seven mutations). This observation may have practical implications for future strategies of genetic screening in PAH deficiency.

Material and methods

PATIENTS

The five patients reported here were born either in Belgium (four patients) or in north eastern France (one patient). All five children were diagnosed through systematic newborn screening programmes and met the criteria for typical PKU (low tolerance for dietary phenylalanine, averaging 250 mg/day).⁴

METHODS

Genomic DNA was isolated from peripheral blood leucocytes and digested for determination of RFLP haplotypes by Southern blotting using seven restriction enzymes: *Pvu*II (two separate polymorphisms), *Bgl*II, *Xmn*I, *Msp*I, *Hind*III, *Eco*RV, and *Eco*RI. The latter enzyme generates polymorphic bands of 11 or 17 kb but this pattern is complicated by the presence of an additional invariant band which comigrates with the polymorphic 17 kb fragment. For this reason, the *Eco*RI polymorphism is better identified with a combined *Eco*RI + *Bam*HI digestion which cleaves the 17 and 11 kb fragments into 8.3 and 6.5 kb fragments, respectively.⁵

For screening mutations at codons 272 to 273, genomic DNA was submitted to 30 cycles of polymerase chain reaction (PCR) amplification of 192 bp spanning exon 7 of the PAH gene (primer 5': 5' ATCCCAGCTTGCACTGGTTTCCGCC 3', primer 3': 5' AGATGACGCTCAGTGTGCCT 3'). Amplified exons were recovered from 1% agarose gels and either digested using *Bam*HI or cloned into M13 mp18 phages for sequencing both strands using the Sanger dideoxy method.⁶

Results

Southern blot analysis of the first two patients was consistent with heterozygosity for mutant RFLP haplotype 7 at the PAH locus. This haplotype is defined by eight RFLP genotypes, including an 11 kb

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Figure 1 (Top) Southern blot analysis of a nuclear family (father (F), mother (M), and proband affected with PKU (P)) using restriction enzymes *Eco*RI (E), *Bam*HI (B), or both (E+B). The polymorphic bands are indicated by arrows. The abnormal 13 kb restriction fragment resulting from the absence of an invariant *Bam*HI site is indicated by an asterisk. (Bottom) Schematic representation of the PAH gene in the region of exons 5 to 9 (boxes). Both polymorphic (dotted lines) and constant restriction sites (arrows) for *Eco*RI (E) and *Bam*HI (B) are presented. The mutant *Bam*HI site in exon 7 is indicated by an asterisk.

polymorphic *Eco*RI fragment (fig 1; father 11/11*, mother 17/17*, proband 11*/17*). However, double digestion of genomic DNA with *Eco*RI + *Bam*HI did not produce the 6.5 kb band expected from the digestion of the 11 kb *Eco*RI fragment of the mutant allele (fig 1: father 6.5/11*, mother 8.3/8.3*, proband 11*/8.3*). Further evidence for the modification of the invariant *Bam*HI site in exon 7 was provided by digestion of genomic DNA with *Bam*HI only, which normally gives rise to invariant 8.3 and 4.7 kb fragments (fig 1: father 8.3/13*, mother 8.3/8.3*, proband 13*/8.3*).

In an attempt to identify the mutation(s) responsible for this particular restriction pattern, exon 7 of the PAH gene, which contains the modified *Bam*HI site, was amplified and sequenced in both patients. Surprisingly, two distinct mutations were identified at this *Bam*HI site (GGA TCC). In one patient, a C to T transition at codon 273 (TCC→TT*C) led to substitution of a serine by a phenylalanine in the protein (273^{ser→phe}, fig 2). In the other, a G to T transition at codon 272 (GGA→T*GA) transformed a glycine residue into a stop codon (272^{gly→stop}, fig 2).

In order to estimate the frequency of the mutations at codons 272 to 273 in PAH deficient children, 270 mutant genes were screened for the suppression of the *Bam*HI site by either Southern blotting (252 genes) or digestion of amplified exon 7 (18 genes). Fig 3 shows that the restriction enzyme *Bam*HI normally digests the 192 bp amplified fragment into two smaller fragments of 117 and 75 bp, respectively (fig 3, control). A mutation at this site prevents this fragment from digestion and produces one single 192 bp band (fig 3, P3*). This procedure resulted in the detection of three additional mutant genes which were further sequenced and identified as mutations at codon 272

Figure 2 (Right) Normal sequence of exon 7 of the PAH gene (codons 265 to 277) with reference to the constant *Bam*HI site (nt 1036 to 1041). (Left) Sequences of mutant exon 7 in two patients: 273 TCC→TT*C (*ser→phe*), and 272 GGA→T*GA (*gly→stop*).

Figure 3 (Bottom) Schematic representation of PCR amplification of 192 bp of the PAH gene spanning exon 7 (nt 929 to 1064 of the coding sequence). Oligonucleotide primers 5' and 3' are shown by arrows. The constant BamHI site is indicated. (Top) Agarose gel electrophoresis of amplified exon 7 either digested with BamHI or non-digested (nd). C: control DNA; P1, 2, 3: three PKU patients; F, M: father and mother of patient P3; P3nd: cloned exon 7 of patient P3.

(272^{gly→stop}). No mutant homozygote was found, but a consistent segregation of the mutant genotype with the mutant haplotype was observed in nuclear families (fig 3, F, M, and P3).

Finally, the suppression of the invariant BamHI site was observed on the background of haplotype 7 mutant genes only, and was not found among 252 normal alleles and 119 non-haplotype 7 mutant alleles ($p < 0.001$).

Discussion

The identification of two distinct mutations at a single restriction site in exon 7 of the PAH gene deserves attention for several reasons. First, the two mutations are likely to account for the PKU phenotype in our patients since they resulted in a translation termination, or in a basic to neutral amino acid substitution in the protein, for mutations at codons 272 and 273 respectively.

Second, both mutations occurred on the background of mutant haplotype 7 genes only. This rare mutant haplotype (4% in our series) is usually associated with typical PKU in France⁴ and is linked to a mutation at codon 252 in Mediterranean countries (252^{arg→trp}, $p < 0.001$).⁶ In the present study, which concerns

PKU children from north eastern France and Belgium, the mutant haplotype 7 genes were found to carry different genotypes, namely mutations at codons 272 and 273. Recently, Eiken *et al* have also observed the mutation at codon 272 (272^{gly→stop}) on the background of mutant haplotype 7 genes in Norway (J Apold, personal communication). It appears, therefore, that at least three mutant genotypes are associated with mutant haplotype 7 alleles, thus confirming the view that a single mutant haplotype may well carry several genotypes at the PAH locus. The close proximity of the two mutations is surprising and no simple explanation relates these molecular events to each other. Their location at a single restriction site is therefore probably fortuitous, as is their occurrence on the background of mutant haplotype 7 alleles. The actual cause of their detection is the extensive use of BamHI for determination of RFLP haplotypes introducing an obvious bias in the recognition of these mutant genotypes among all PKU alleles.

Finally, the present report might have practical implications for population genetics of PKU. Indeed, screening for both mutations is now feasible by either Southern blotting of genomic DNA or more easily by BamHI digestion of amplified exon 7. However, if one considers the total number of mutations in this particular exon (mutations at codons 243, 252, 261, 272, 273, 280, and 281),⁶⁻⁸ it appears that the systematic screening for these various mutations on the same amplified exon is now feasible rapidly and cheaply by denaturing gradient gel electrophoresis, conformation polymorphism studies, or allele specific oligonucleotide hybridisation.

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