Diverse PAH transcripts in lymphocytes of PKU patients with putative nonsense (G272X, Y356X) and missense (P281L, R408Q) mutations

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Abstract The majority of mutations in the human phenylalanine hydroxylase (PAH) gene that lead to the recessive disease phenylketonuria (PKU) are believed to affect the activity or stability of the PAH enzyme. In this study we have performed in vivo analyses of lymphocyte PAH mRNA from PKU patients homozygous for the PKU missense mutations P281L and R408Q as well as the nonsense mutations G272X and Y356X. The mutations G272X, P281L and R408Q, which are located outside the consensus splice site sequence, result in transcripts with one or more exons skipped in addition to full-length transcripts. The mutation Y356X results in transcripts with one or more exons skipped, but no full-length transcripts. Our findings question the value of functional and structural predictions of mutations at the protein level without analyses of the corresponding transcript.

Key words: Phenylketonuria mutation; Phenylalanine hydroxylase; RNA splicing

1. Introduction

Phenylketonuria (PKU) is an autosomal recessive human genetic disorder caused by the absence or severe reduction of phenylalanine hydroxylation. At present, more than 290 mutations associated with PKU have been reported in the hepatically expressed human phenylalanine hydroxylase gene (hPAH) [1]. Among these PKU mutations more than 60% are classified as missense mutations within the protein coding part of the gene, while 11% are classified as splice mutations based on changes in the 5′ or 3′ consensus splice site. However, at least for a few percent of the missense mutations, a splice defect may be predicted based on the creation of a new 5′ or 3′ consensus splice site [2].

In vitro protein expression analyses have shown that PKU mutations may result in complete loss of enzyme activity, increased protein turnover or abnormal kinetics of the enzyme (for a review see [1]).

However, some mutant PAH proteins show a residual enzymatic activity too high to explain the PKU phenotype of patients with these mutations. Also, the effect of some mutations in the PAH gene has been predicted from 3D computer modelling of the structure of the PAH enzyme [3]. However, all these analyses are based on predictions of amino acid substitutions in the PAH protein without any in vivo evidence for the existence of single base substituted transcripts encoding these proteins.

Previously, we have shown that an A→G mutation at cDNA position 611 in exon 6 of the hPAH gene, believed to cause the amino acid substitution Y204C, leads to the creation of a new, functionally active 5′ donor splice site [2].

In this work we expanded the study on missense and stop mutations by in vivo mRNA analyses of four exonic point mutations in samples from homozygous patients. The experiments had to be carried out on transcripts of the hPAH gene from cultured lymphocytes of PKU patients, as hepatic mRNA from PKU patients is not accessible. We found that the four mutations, which lie outside normally considered splice site consensus sequences, all lead to abnormal splicing of the hPAH mRNA.

2. Materials and methods

2.1. Patients

The genotypes and phenotypes of PKU patients (Table 1) have previously been described [4]. The patient homozygous for the R408Q mutation had a mild PKU phenotype (823 μM pre-treatment serum phenylalanine), while the patients homozygous for the P281L, G272X and Y356X mutations, respectively, had the classical metabolic phenotype of PKU (>1200 μM phenylalanine).

2.2. RT-PCR

Isolation of illegitimately transcribed hPAH mRNA from cultured lymphocytes (ectopic transcripts) of the PKU patients, cDNA synthesis and nested PCR were performed as previously described [2]. In addition, PCR primers used for a second round of PCR were 5′-GATCCTGTGTACCGTGCAAG-3′ (forward, cDNA positions 451–470) and 5′-CTCCATCAACAGATTCACAGC-3′ (reverse, cDNA positions 1386–1406)). RT-PCR on a normally spliced hPAH mRNA using these conditions amplified a full-length PCR product of 957 bp. RT-PCR products were visualized in UV light after electrophoresis in 1.5% or 2% agarose gels and ethidium bromide (EtBr) staining. RT-PCR products were measured by the intensity of EtBr fluorescence in UV light (GDS 8000 System, UVP Ltd., Cambridge, UK). cDNA sequencing was performed on both strands as previously described [2] applying the same primers as for the second round of PCR.

3. Results

Blood samples from patients homozygous for the mutations P281L, R408Q, G272X and Y356X were cultured. mRNA was subsequently extracted and analyzed by RT-PCR as described.

RT-PCR amplification of mRNA isolated from normal blood donors produced exclusively a 957 bp product from the 3′ end of the hPAH coding region, corresponding to cDNA positions 451–1406 (exon 5–5′ UTR), representing the normally spliced 3′ end of hPAH mRNA (confirmed by DNA sequencing) (Fig. 1A–C).

Two fragments were amplified from lymphocyte mRNA isolated from the patient homozygous for the P281L mutation

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(Fig. 1A). DNA sequencing showed that the largest fragment represented a full-length transcript while the smaller represented a transcript with exon 8 deleted. Both mutant fragments contained the 842C→T mutation (P281L) of exon 7 found in the genomic DNA from this patient. The shortest transcript represents a double mutant transcript from a single allele (P281L/Δexon 8).

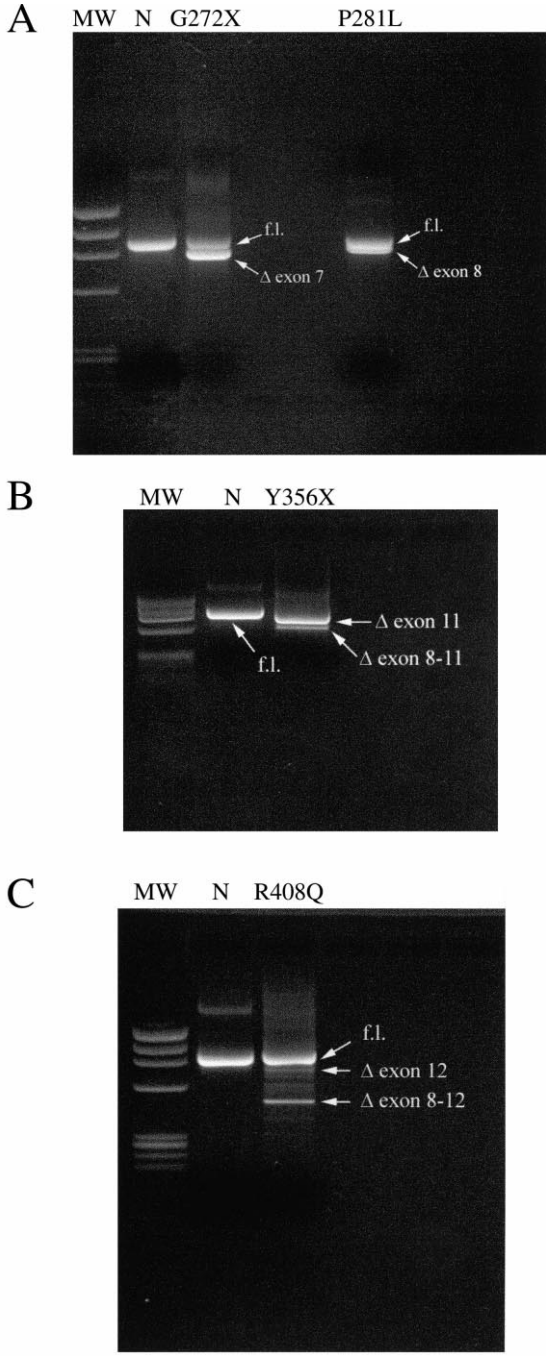

Fig. 1. A–C: RT-PCR analysis of lymphocyte mRNA from homozygous PKU patients. PCR amplification using mRNA samples isolated from cultured lymphocytes from normal blood donors (N) and PKU patients homozygous for the G272X and P281L mutations (A), Y356X mutation (B) and R408Q mutation (C). PCR products were separated on 2% agarose gels and stained with EtBr. f.l., full-length PCR product.

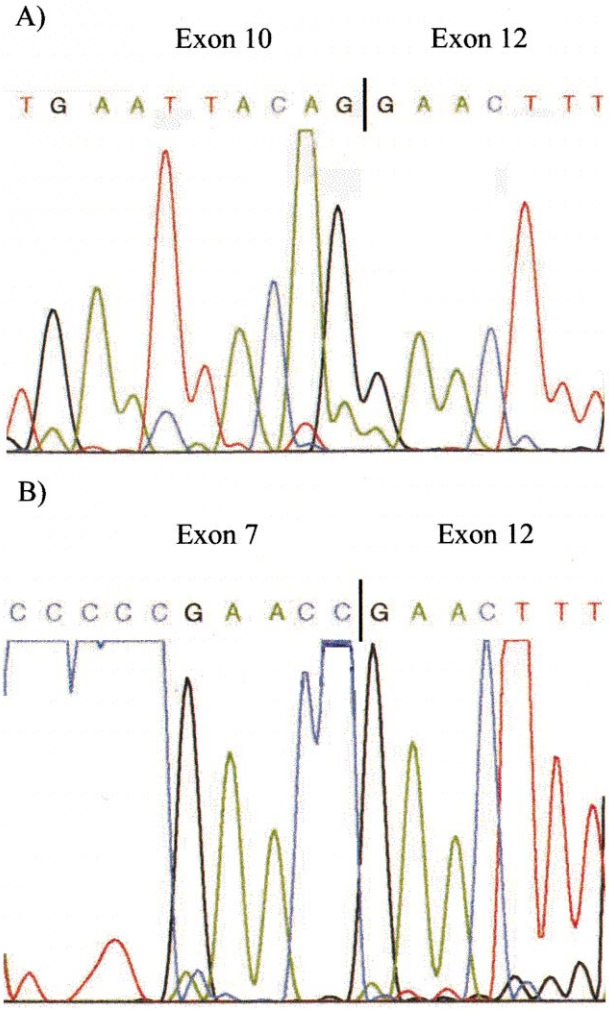

Fig. 2. DNA sequence of the two transcripts found in lymphocytes isolated from a patient homozygous for the Y356X mutation. A: Transcript with exon 11 deleted. B: Transcript with exons 8–11 deleted.

The RT-PCR analysis of the patient homozygous for the R408Q mutation revealed three differently sized fragments (Fig. 1C). In addition to the full-length transcript (normally spliced transcript) with the 1223G→A mutation, the two shorter fragments had deletions of exon 12 and exons 8–12 in the transcripts, respectively.

The RT-PCR products amplified from mRNA from the patient homozygous for the G272X mutation were the full-length transcript with the 814G→T mutation, as well as a Δexon 7 transcript (Fig. 1A). Analysis of the patient homozygous for the Y356X mutation revealed two differently spliced transcripts lacking exon 11 and exons 8–11, respectively (Figs. 1B and 2). No full-length transcript was amplified from lymphocyte mRNA from this patient.

All the exon skipping observed predicts frame-shifts and premature stop codons in translation, except for the Δexon 8–11 transcript in the patient homozygous for Y356X. This transcript predicts a protein with an internal deletion. The skipping of exons 8–11 preserves the original reading frame, but results in a new codon made up of the last two nucleotides in exon 7 and the first nucleotide in exon 12 (CCG, Pro).

All results were confirmed by duplicate experiments.

Table 1
PAH transcripts in lymphocytes of PKU patients

Patient genotype (homozygous)	Phenotype	Nucleotide change[a]	RT-PCR products[b]	%	Predicted translation of mRNA	Mutant enzyme activity (%)
R408Q	mild PKU	1223G→A	full-length	75	R408Q	55
			Δexon 12	10	frameshift, premature stop codon	n.a.
			Δexons 8–12	15	frameshift, premature stop codon	n.a.
P281L	classical PKU	842C→T	full-length	50	P281L	<1
			Δexon 8	50	frameshift, premature stop codon	n.a.
G272X	classical PKU	814G→T	full-length	25	G272X	n.d.
			Δexon 7	75	frameshift, premature stop codon	n.a.
Y356X	classical PKU	1068C→G	n.d.	n.d.	Y356X	n.a.
			Δexon 11	70	frameshift, premature stop codon	n.a.
			Δexons 8–11	30	deletion of amino acids 282–400	n.a.

n.d.: not detectable; n.a.: mutant protein not analyzed.
[a]Nucleotide positions refer to revised cDNA numbering from the PAH Gene Mutation Analysis Consortium, 1994.
[b]RT-PCR products were quantitated on the GDS 8000 system (UVP, Cambridge, UK) by fluorescence after gel electrophoresis and EtBr staining.

4. Discussion

The predicted mutant PAH proteins previously reported for mutations G272X, P281L and R408Q have been functionally analyzed in several in vitro protein expression systems [1]. These analyses have focused on the effect of the amino acid substitution predicted from the mutation detected in genomic DNA, without looking for the existence of a mutant transcript encoding these mutant proteins.

R408Q is associated with a mild PKU phenotype, but the hPAH residual activity of $>50\%$ [5] is in contradiction with a PKU diagnosis. However, the level of residual PAH activity needed to correct a PKU phenotype is still unclear. The partial exon skipping described here, resulting in transcripts encoding non-functional PAH proteins, might be the additional mechanism necessary to explain that the R408Q mutation is associated with a PKU phenotype.

Repeated amplifications of transcripts from non-PKU individuals (normal alleles) did not reveal any aberrantly spliced RT-PCR products. This suggests that the abnormally spliced transcripts observed are a consequence of the mutations studied in this work.

Liver biopsies can normally not be collected from PKU patients, and our studies are restricted to illegitimately transcribed mRNA isolated from cultured lymphocytes. However, the transcripts from the splice mutation IVS 12 nt1g/a mutation was studied both in liver [6] and in cultured lymphocytes [2] and the mutation caused aberrant splicing in both types of cells.

The relative amounts of different transcripts within a sample were determined (Table 1) based on the principles of allele-specific quantification [7]. The data represent a rough quantification and documents that there is a difference in the expression level or mRNA stability between the differently spliced forms of PAH caused by a single mutation (Fig. 1, Table 1). However, mRNA from human liver should be used for a more precise quantification of the expression level/stability of the differently spliced forms of PAH.

Several hypotheses have been proposed to explain exon skipping. A change in the secondary mRNA structure, with consequent aberrations in splicing, has been suggested in several studies [8–10]. Recognition of a nonsense codon prior to splicing, with the subsequent skipping of the mutant exon in order to preserve an open reading frame, has also been proposed [11]. In addition, preservation of the open reading frame by exon skipping has been shown for other types of exonic mutations [12].

Exon enhancers and purine-rich exon splicing elements are strong sequence signals within exons that promote splicing, and several studies that identified such sequence motifs have shown that exon skipping can be caused by mutating these sequences [13,14]. Recently, several reports speculate that the observed exon skipping associated with nonsense mutations may very well be the result of disrupting such exonic sequences relevant for the splicing process [15,16]. In fact, this has been shown for a nonsense mutation in the dystrophin gene, where a G to T transversion resulting in partial exon skipping was shown by in vitro studies to disrupt the function of a splicing enhancer sequence [17].

For all of the studied mutations, except for the Y356X mutation, both the full-length transcripts containing the mutation and transcripts with skipped exons were found. This suggests that these mutations create new splice sites/information to a variable degree and that the molecular basis of PKU caused by these mutations is complex.

Recently Erlandsen et al. [3] reported the 3D structure of the human PAH enzyme. By 3D computer modelling, they explained the molecular mechanisms of the enzymatic phenotypes in PKU/HPA patients. However, in this study we have demonstrated that the molecular basis of the PKU disease is complex, and that exonic mutations generally believed to only affect the PAH protein also cause aberrant RNA processing.

If aberrant transcripts are a frequent consequence of exonic point mutations, a re-examination of a large number of disease-related mutations, with mechanisms we thought to be known, is required. This may explain some of the missing correlation between genotype and phenotype observed for many mutations in genetic diseases.

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