Identification of exonic deletions in the PAH gene causing phenylketonuria by MLPA analysis

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Abstract

Background: Multiplex ligation probe amplification (MLPA) is a sensitive and efficient technique for molecular diagnosis of diseases involving deletions or duplications of large genomic regions. In phenylketonuria (PKU), most of the mutant alleles correspond to missense mutations and large deletions have been scarcely identified. In this study, we report for the first time the use of MLPA analysis on PKU patients to detect exonic deletions.

Method: DNA from 22 unrelated PKU patients with an incomplete genetic diagnosis after standard mutation detection analysis were subjected to MLPA analysis. Deletions were confirmed by long-range PCR and sequence analysis.

Results: The technique identified two large genomic deletions in the phenylalanine hydroxylase (PAH) gene, of 6.6 kb and 1.8 kb, including exons 3 and 5, respectively. The chromosomal breakpoints were established by long-range PCR and chromosomal walking, confirming the involvement of repetitive sequences in the deletions.

Conclusion: MLPA may complement routine mutation screening in PKU patients, although, in the sample studied, exonic deletions in the PAH gene do not appear to be a frequent cause of PKU.

Keywords: Multiplex ligation probe amplification (MLPA); PKU; Exonic deletions; PAH gene; Mutations

1. Introduction

Over 490 mutations causing phenylketonuria (PKU) have been reported in the phenylalanine hydroxylase (PAH) gene (OMIM 261600) (http://www.pahdb.mcgill.ca), most of them corresponding to point mutations causing missense changes. Given the high genetic heterogeneity most diagnostic laboratories use different mutation scanning procedures such as denaturing gradient gel electrophoresis (DGGE) plus sequencing to identify mutations in affected patients, with a mutation detection rate of 95–99%. It is generally assumed that the uncharacterized PKU alleles may correspond to mutations in regulatory or deep intronic regions or to heterozygous exonic deletions, which are overlooked by standard mutation detection methods.

Large genomic deletions involving one or more exons in the PAH gene have been scarcely described [1–6], suggesting they are rare. However, the proportion of deletions may be underestimated, given that mutation scans do not systematically include searches for them.

Among the recently developed techniques to detect deletions and duplications, multiplex ligation probe amplification (MLPA) has been described as a sensitive, fast and effective gene dosage method [7,8]. Recently, an MLPA kit specific for the PAH gene has become available and we have applied this technique for the analysis of Spanish PKU patients with an incomplete genetic diagnosis, candidates for harbouring an exonic deletion.

2. Patients and methods

Twenty-two unrelated PKU patients were included in the analysis. In 18 of them, DGGE analysis identified only one
mutant allele, while in two patients, a discordant inheritance was observed after analyzing the parents (patient homozygous, one parent does not carry the mutation). In the remaining two patients, PCR repeatedly failed to amplify one PAH exon (exon 3 and exon 5, respectively), while DGGE analysis of the remaining exons was normal.

For MLPA analysis, 125 ng of DNA purified from whole blood was used as starting material with the SALSA PO55 PAH MLPA kit available from MRC Holland (www.mrc-holland.com). After hybridization, ligation and amplification according to the instructions of the manufacturer, 2 μl of the PCR products were mixed with 0.2 μl of ROX-labeled internal size standard (250–500 Genescan), separated on an ABI Prism 3700 Genetic Analyzer (Applied Biosystems) and analyzed using the Genescan software. For data analysis, the peak sizes and areas were transferred to an Excel file. For normalization, relative probe signals were calculated by dividing each measured peak area by the sum of all peak areas of that sample. The ratio of each relative probe signal from patients compared to control samples was then calculated. An exon deletion was considered when the ratio was lower than 0.7 and the corresponding samples were subjected to a second MLPA analysis.

Long-range PCR was performed using AccuTaq DNA polymerase (Sigma) following the manufacturers’ recommendations. Briefly, 500 ng of genomic DNA was subjected to PCR amplification in 50 μl reaction volume containing the enzyme buffer 1×, 500 μM of dNTPs, 400 nM of each primer, 2% DMSO and 2.5 units of polymerase. The primers were designed to have $T_m \geq 70$ °C to allow a two-step cycling in the PCR reaction as follows: initial denaturation of 98 °C for 30 s, 30 cycles of 94 °C for 15 s, 68 °C for 8–10 min, final extension of 68 °C for 10 min, soak at 4 °C. Primer sequences, which are available upon request, were based on the intronic sequences of the PAH gene obtained from the Ensembl Genome browser at http://www.ensembl.org.

For analysis of intronic sequences to identify repetitive elements, the RepeatMasker software was used (http://woody.embl-heidelberg.de/repeatmask/).

3. Results

At our center, we routinely screen by DGGE analysis and sequencing Spanish and Latinamerican PKU patients, populations with a higher genetic heterogeneity compared to northern Europe [9,10]. In the past few years, the mutation detection rate in this sample has been ∼96%, in line with previous studies.

In the present study, 22 unrelated PKU patients referred to our laboratory and with an incomplete genetic diagnosis were subjected to MLPA analysis. In one patient (20780), no peak corresponding to exon 3 was observed and, in another (1095), the peak corresponding to exon 5 was missing. This correlated with the lack of amplification of the respective exons during previous DGGE analysis which already suggested the presence of an homozygous exonic deletion. This was also observed in one affected sibling of each patient. In another patient (1492) with a missense mutation in one allele, MLPA analysis revealed a significant reduction (∼40%) in the peak area corresponding to exon 5, suggestive of an heterozygous deletion. In the remaining patients, no differences in peak areas of the PAH exonic probes were observed.

The MLPA results were confirmed by long-range PCR using intronic primers hybridizing at both sites of the presumed deletions of exons 3 and 5. Exon 3 of the PAH gene is flanked by large introns of 17.8 kb (intron 2) and 17.2 kb (intron 3). In intron 3, an RFLP corresponding to a $Pvu$ II site and an STR microsatellite have been described [11,12]. Examination of the updated genomic sequence at the Ensembl genome browser (http://www.ensembl.org) covering the PAH gene (contig AC069227) reveals that the STR region is located 5.3 kb downstream of exon 3 and the $Pvu$ II RFLP site is 1.5 kb 3′ to exon 3. In patient 20780, PCR successfully amplified the STR

![Fig. 1. Breakpoint analysis and schematic figure of PAH gene exon 3 deletion. Long-range PCR encompassing exon 3 using intronic primers would result in an amplicon of ∼15 kb, which could not be successfully amplified in a control sample (C) while a shorter ∼8 kb product was detected in patient 20780 with a homozygous deletion in exon 3 recognised by MLPA analysis. Sequence analysis showed a deletion of 6604 bp spanning from intron 2 to intron 3, and an insertion of 8 nucleotides, denoted as c.169-4951del6604ins8.](http://www.ensembl.org)
region but failed to amplify the PvuII RFLP region, denoting that it is included in the deletion. An antisense primer hybridizing to the STR region was used for long range PCR in combination with different primers hybridizing in intron 2, revealing a deletion spanning approximately 7 kb, estimated by size comparison (Fig. 1). Chromosomal walking identified the deletion breakpoint and revealed a small insertion of unknown origin at the site of the deletion (Fig. 1). The mutation deletes 6604 nucleotides and can be named as AF404777.1:g.45041_51645delinsGGCACCTG or c.169-4951del6604ins8, according to the recommended nomenclature of genomic deletions [13] and (http://www.genomic.unimelb.edu.au/mdi/mutnomen/).

Introns 4 and 5 flanking exon 5 are also large, 10.8 kb and 11.3 kb, respectively. Using different intronic primers and long-range PCR with DNA samples from patient 1095, a deletion of approximately 2 kb was detected (Fig. 2). Amplification from DNA samples of patient 1492, heterozygous for the exon 5 deletion by MLPA analysis and parents from patient 1095, showed two products, of normal size and ∼2 kb smaller, confirming they are heterozygous carriers of the deletion allele (Fig. 2). Chromosomal walking allowed us to establish the deletion breakpoints between intron 4 and intron 5 (Fig. 2), corresponding to a deletion of 1881 bp, which can be named as: AF404777.1:g.76772_78653del1881 or c.442-1556del11881.

Large genomic deletions are often caused by recombination events between repetitive elements scattered throughout the genome, most abundantly, Alu repeats [14]. The PAH intronic sequences involved in the identified deletions were analyzed using the Repeatmasker software (http://woody.embl-heidelberg.de/repeatmask/). Analysis of the intronic sequences involving exon 3 deletion revealed high similarity of two Alu sequences, an AluYa5 element in intron 2 and an AluSp element in intron 3. On the other hand, the exon 5 deletion starts in the middle of a simple (TA) repeat sequence in intron 4 and the 3′ deletion breakpoint lies near a (TG) repeat in intron 5.

4. Discussion

In this study, MLPA analysis performed on samples from 22 PKU patients highlighted two different PAH gene deletions, involving exon 3 and exon 5, respectively, which were confirmed by long-range PCR and sequence analysis.

Two patients were homozygous for a deletion allele and one patient was heterozygous with a missense mutation on the other allele. None of the remaining 19 patients showed any indication of carrying an exonic deletion, as judged by MLPA analysis, indicating that large PAH deletions are unfrequent, at least in the population studied. It remains to be seen whether uncharacterized alleles correspond to deep intronic mutations causing splicing aberrations, or to mutations in promoter or regulatory regions. For the patients included in this study with discordant inheritance of the mutant alleles, a false negative MLPA result or other causes (non-paternity, uniparental disomy, “de novo” mutation, etc.) could explain the results obtained.

The deletion involving exon 3 characterized in this work (c.169-4951del6604ins8) spans 6.6 kb. Two different PAH exon 3 deletions have been reported in the literature, although the exact deletion breakpoints were not identified. A 2.5 kb deletion has been reported in a Sicilian family [3] and another estimated in 6.7 kb identified in all PKU cases among Yemenite Jews, with a presumed origin in the capital of Yemen before the beginning of the 18th century [2]. It seems plausible that the exon 3 deletion detected in this study in one patient born to consanguineous parents of Arab descent is identical by descent to the Yemenite deletion. However, we cannot discard that the deletion may not be exactly the same in both populations.

A deletion in exon 5 has been previously reported in three patients [6]. However, the size of the deletion could not be determined so we cannot compare with the one identified in this study in two unrelated Spanish families.

In both cases, repetitive sequence elements, Alu elements or simple repeats, are potentially involved in the generation of the

Fig. 2. Breakpoint analysis and schematic figure of PAH gene exon 5 deletion. Long-range PCR using intronic primers encompassing exon 5 results in an amplicon of ∼6 kb, detected in control samples (C) while a shorter 4.3 kb product is detected in patient 1095 with a homozygous deletion in exon 5 recognised by MLPA analysis. Both bands are visible in patient 1492, heterozygous for exon 5 deletion by MLPA analysis, and in the father (F) of patient 1095. Sequence analysis of the smaller band showed a deletion of 1881 bp spanning from intron 4 to intron 5, denoted as c.442-1556del11881.
deletion, as previously shown for human inherited diseases and cancer [15].

This study is the first report regarding the use of MLPA analysis on PKU patients. Although this technique does not effectively increase the PKU mutation detection rate, it may be used to complement standard mutation analysis, in cases when the genetic diagnosis is incomplete.

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