Identification of Disease-Causing Mutations in Phenylketonuria by Denaturing Gradient Gel Electrophoresis Using the DCode™ System

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Introduction

Phenylketonuria (PKU) is a hereditary disease that gives rise to elevated blood levels of phenylalanine. The disease most frequently is caused by mutations in the phenylalanine hydroxylase *(PAH)* gene. This gene is situated at the human chromosomal locus 12q22-q24.1 and encodes a hepatic enzyme (phenylalanine 4-monooxygenase, EC 1.14.16.1) that hydroxylates phenylalanine. The large variety of mutations described in this gene results in a broad range of plasma phenylalanine concentrations associated with phenotypic differences ranging from the severest form of PKU to the mildest hyperphenylalaninaemia. PKU is autosomal recessively inherited; carriers therefore are phenotypically normal, with no pronounced elevated phenylalanine or tyrosine blood levels. The need for a reliable method to identify persons carrying *PAH* mutations is evident. To identify mutations in each of the 13 exons of the *PAH* gene, Guldberg et al. (1993) reported a method based on denaturing gradient gel electrophoresis (DGGE). For the rapid screening of PKU patients and their relatives for mutations in the *PAH* exons and intron/exon boundaries, we further refined this method by using multiplex (MP) polymerase chain reaction (PCR) amplifications combined with DGGE for MP-DGGE analysis (Michiels et al. 1996).

Methods

Multiplex PCR Amplification of *PAH* DNA

Genomic DNA of PKU patients was prepared from dried blood spots on Guthrie cards using the Chelex® 100 extraction procedure (Walsh et al. 1991). Alternatively, 0.5 ml fresh EDTA-treated blood sample was treated with 1 ml haemolysis buffer (HB) at 4°C for 10 min. White blood cells were pelleted (5 min at 2,500 rpm in a microfuge) and washed with HB until no red cell debris was left. After the removal of the supernatant, 500 µl of 5% Chelex 100 (Bio-Rad Laboratories, Inc.) suspension in water was added and mixed thoroughly. This step was followed by incubations at 56°C for 30 min and subsequently at 100°C for 5 min. After centrifuging (3 min at 2,500 rpm), the supernatant was stored at –20°C. Five µl of these DNA preparations was used in a 20 µl multiplex amplification reaction of the *PAH* exons and their intron-exon boundaries. GC-clamped primers for DGGE analysis of the *PAH* gene have been described by Guldberg et al. (1993). To achieve comparable PCR amplification efficiencies and nonoverlapping but high mutation resolution DGGE patterns, combinations of these primer sets were evaluated (Michiels et al. 1996). Amplifications were carried out in 20 µl reaction mixtures, containing PCR reaction buffer (PerkinElmer, Inc.) with 1.5 mM $MgCl_2$, 200 nM each dNTP, 800 nM each primer (MP1 (exon 1 + exon 4 + exon 13), MP2 (exon 2 + exon 6 + exon 8), MP3 (exon 3 + exon 5 + exon 9), MP7 (exon 7 + exon 11), MP10 (exon 10 + exon 12)), and 1.5 U of Taq DNA polymerase (PerkinElmer). The PerkinElmer 9600 thermal cycler was set at 5 min denaturation at 95°C, followed by 40 cycles of 10 sec denaturation at 95°C, 20 sec annealing at 56°C, and 40 sec elongation at 72°C. After cycling, final elongation was done at 72°C for 5 min. Finally, to generate heteroduplexes between heterozygous *PAH* gene fragments, the PCR fragments were incubated for 5 min at 95°C, 60 min at 65°C, and 60 min at 37°C. Complete amplification mixes were used for DGGE analysis.

MP-DGGE

MP-DGGE was performed under two different electrophoresis conditions. A 6% polyacrylamide gel containing a 20–70% (for MP2, MP3, and MP10) or a 30–80% (for MP1 and MP7) gradient (Bio-Rad Model 475 gradient delivery system) of urea and formamide was run at 60°C in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Electrophoresis conditions were 130 V for 6 hr on the DCode universal mutation detection system (Bio-Rad). Gels were stained in 1 µg/ml ethidium bromide and visualized by ultraviolet (UV) transillumination.

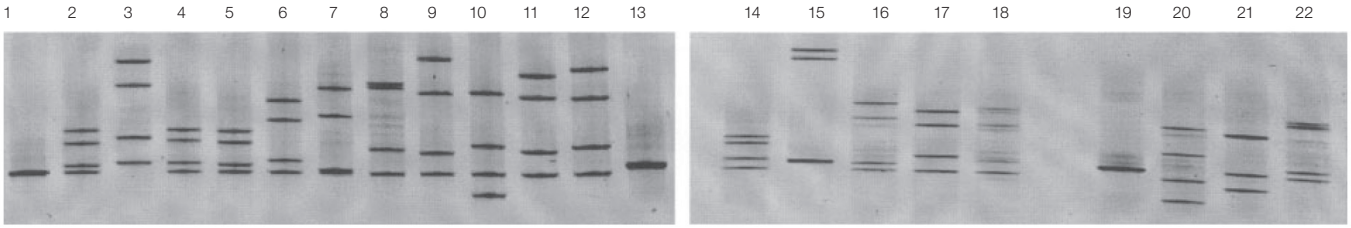

Fig. 1. Mutations detected in exon 7 of the *PAH* gene using DGGE analysis. Lane 1, WT/WT; lane 2, R243Q/WT; lane 3, R243X/WT; lane 4, V245V/WT; lane 5, R252W/WT; lane 6, R261Q/WT; lane 7, R261P/WT; lane 8, R261X/WT; lane 9, G272X/WT; lane 10, Y277D/WT; lane 11, E280K/WT; lane 12, P281L/WT; lane 13, V245V/V245V; lane 14, V245V/V245V + R243X/WT; lane 15, G272X/WT + E280K/WT; lane 16, V245V/WT + G272X/WT; lane 17, V245V/V245V + IVS7nt1/WT; lane 18, V245V/WT + P281L/WT; lane 19, V245V/WT + Y277D/WT; lane 20, V245V/WT + R243X/WT; lane 21, V245V/WT + IVS7nt1/WT + R261Q/WT; lane 22, V245V/WT + R243Q/WT.

The point mutations were confirmed by sequencing the PCR-amplified fragments with the non-GC-clamped primer of each exon, using the Applied Biosystems Model 373A DNA sequencing system and the PRISM Ready Reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems).

Results

DGGE allows detection of more than 95% of the PKU-causing mutations. In the Belgian population, we observed about 100 different mutations using DGGE technology. Among these, seven new mutations were identified (Michiels et al. 1998). The high mutation-resolving power of DGGE is illustrated in Figure 1. This figure shows all the mutations or mutation combinations we located in exon 7 of Belgian PKU patients. The screening of PKU patients and their relatives is rather laborious when exon-specific amplification and DGGE conditions are used. Therefore, to obtain compatible coamplifications with comparable efficiencies, different exon PCR amplifications were combined. Moreover, to make sure that the resolving power to identify the exon-specific mutations remains intact and that the DGGE patterns of the combined exons do not interfere with each other, the multiplex PCR amplifications have to be resolved subsequently on denaturing gradient gels. This resulted in a MP-DGGE of the *PAH* gene (Michiels et al., 1996). Examples of such an MP-DGGE are shown in Figure 2. The 13 different *PAH* exons are analyzed in 5 distinct multiplex PCR and DGGE patterns. Each exon shows distinct mutations, demonstrating that the resolving power of the DGGE gel remains intact. In addition, the DGGE patterns for the different exons do not overlap. Cycle sequencing of the DGGE DNA bands excised from the gel, as described in Methods, allows the identification of new mutations.

Several DNA preparation protocols (homemade (see Methods) and commercially available kits such as Split Second (Boehringer Mannheim) and DNA Easy-Prep (Lifecodes Corporation)) have been tested and can all be used in the MP-DGGE assay. All of them gave comparable results in the multiplex PCR and DGGE experiments.

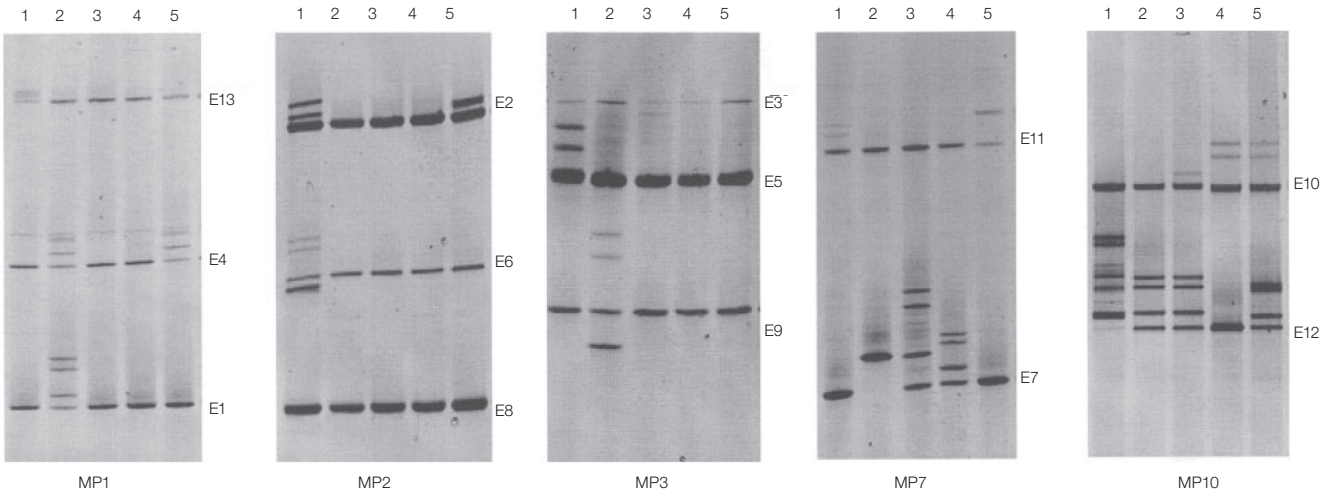

Fig. 2. Examples of MP-DGGE analysis. Heterozygous mutations detected are Q20X in exon 1 (MP1, E1, lane 2); IVS3nt-22c/t in intron 3 (MP1, E4, lanes 2, 5); IVS12nt-35c/t in intron 12 (MP1, E13, lane 1); Q232Q in exon 6 (MP2, E6, lane 1); L48S in exon 2 (MP2, E2, lane 1); IVS12nt19t/c in intron 2 (MP2, E2, lane 5); R158Q in exon 5 (MP3, E5, lane 1); L311P in exon 9 (MP3, E9, lane 2); I65T in exon 3 (MP3, E3, lane 3); L385L in exon 11 (MP7, E11, lane 1); combination of V245V and R261Q in exon 7 (MP7, E7, both homozygous in lane 2 and both heterozygous in lane 3); V245V in exon 7 (MP7, E7, lane 4); IVS10nt-11g/a in intron 10 (MP7, E11, lane 5); combination of D415N and IVS12nt1g/a in exon 12 and at the splice junction of intron 12 (MP10, E12, lane 1); R408W in exon 12 (MP10, E12, lanes 2, 3); K341T in exon 10 (MP10, E10, lane 3); S349P in exon 10 (MP10, E10, lanes 4, 5) and IVS12nt1g/a in intron 12 (MP10, E12, lane 5).

Conclusion

In this report, we demonstrate the use of powerful multiplex PCR amplification combined with DGGE analysis to rapidly identify mutations causing PKU. This MP-DGGE allows analysis of the complete *PAH* gene for three different individuals on one gel, whereas previously such analysis would take three gel runs. Moreover, the MP-DGGE analysis is compatible with all the tested rapid DNA isolation methods commercially available. With this procedure, DNA extracted from dried blood spots on Guthrie cards can be successfully analyzed.

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