

UK), 0.25 μ M control primers, 0.5 U DNA polymerase (Advanced Biotechnologies, Epsom, UK), and 50 ng genomic DNA. One duplicate reaction set was specific for the normal sequence (845G) and a second reaction set was specific for the mutated sequence (845A); both reaction sets used a common forward primer (5'-AAGGTGACACATCATGTGACC-3'), with a reverse primer specific for either 845G (5'-CTGGGTGCTCCACCTGGC-3'), or 845A (5'-CTGGGTGCTCCACCTGGT-3'), resulting in a PCR product of 232 base pairs. Control primers, which amplified an 842 base pair fragment of the human growth hormone gene⁸ were included in all reactions. Amplification conditions involved an initial denaturation step for one minute at 96°C, followed by five cycles consisting of denaturation for 25 seconds at 96°C, annealing for 45 seconds at 70°C, and extension for 30 seconds at 72°C, then a further 21 cycles where the annealing temperature was lowered to 65°C, plus four cycles where annealing was for one minute at 55°C, and extension was for two minutes at 72°C. A final extension step for five minutes at 72°C completed the amplification.

Results and discussion

The duplicate reaction sets typing for either 845G or 845A were analysed by electrophoresis on a 1.5% agarose gel; PCR products were

visualised by staining with ethidium bromide and photographed under ultraviolet illumination. Results from three representative individuals are shown in fig 1. The results demonstrate that the PCR-SSP method is highly specific for the haemochromatosis associated mutation 845G→A in the HFE gene and that the method could be used as an aid to diagnosis, or for screening purposes.

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High resolution single strand conformation polymorphism analysis using formamide and ethidium bromide staining

T Xie, S L Ho, O C K Ma

Abstract

Single strand conformation polymorphism (SSCP) analysis using ethidium bromide can be improved by adding formamide as the denaturant. This gives higher resolution than previous SSCP methods; it had 100% sensitivity in the discrimination of 14 PCR samples from two different genes, even for a long fragment close to the upper limit of 250 base pairs. This modified procedure is a rapid, simple, safe, and yet highly sensitive method for detecting structural differences in DNA fragments.

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Keywords: single strand conformation polymorphism; formamide; ethidium bromide

Polymerase chain reaction (PCR) amplification of DNA fragments followed by single strand conformation polymorphism (SSCP) analysis is a sensitive method for detecting genetic polymorphisms or mutations. Detection by autoradiography¹ or silver staining² is time consuming, costly, and inconvenient. Non-isotopic SSCP (cold SSCP) using ethidium bromide staining^{3,4} facilitates the rapid identification of structural changes in PCR products. Currently, the denaturant used most frequently in cold SSCP is either sodium hydroxide⁴ or methylmercury hydroxide.³ The former has been reported in some cases to yield inconsistent results³; the latter is an extremely toxic and volatile compound⁵ and great care has to be taken to avoid skin contact with methylmercury and inhalation of its aerosol.

University
Department of
Medicine, University
of Hong Kong, Hong
Kong
T Xie
S L Ho

Department of Clinical
Biochemistry
O C K Ma

Correspondence to:
Dr Ho, University
Department of Medicine,
University of Hong Kong,
Queen Mary Hospital, Hong
Kong.

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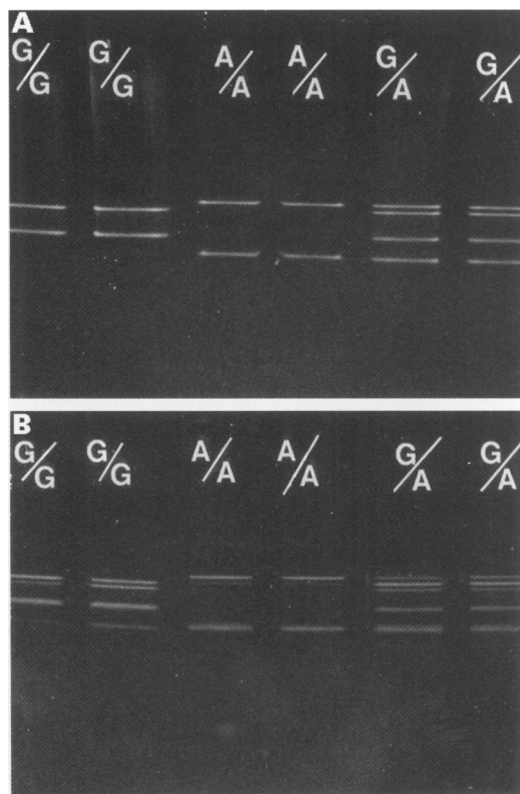


Figure 1 SSCP analysis of the human catechol O-methyltransferase gene; (A) with formamide and (B) without formamide. Three pairs of PCR products (from left to right) from individuals homozygous for G/G and A/A (each with two bands of ssDNA) and heterozygous for G/A (four bands of ssDNA) with different mobility shifts. The various bands showed a high resolution in (A) and a lower resolution with additional artefactual bands in (B).

Therefore, it is desirable to find an alternative denaturant that gives a high resolution but is less toxic.

Formamide has been used previously as the denaturant in the silver staining method for SSCP⁶ but not in ethidium bromide staining. Silver stained SSCP analysis has high resolution but there is a brown or yellow background caused by non-specific deposition of insoluble silver salts.⁷ Hence, we hypothesised that formamide might maintain the high resolution of bands without the background stain, if it were used with ethidium bromide instead of

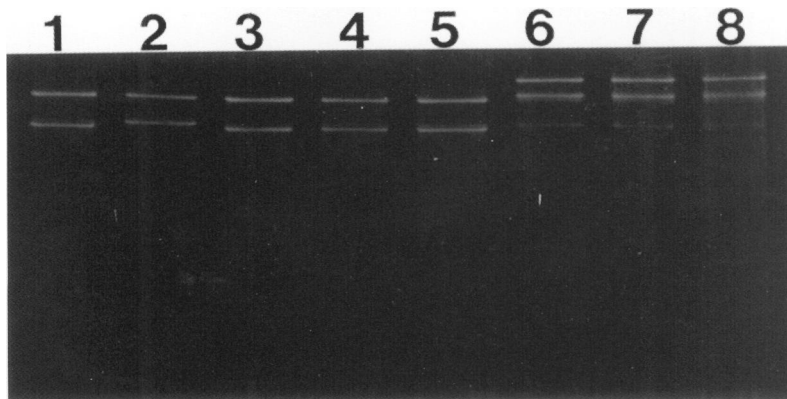


Figure 2 SSCP analysis of the p53 gene using formamide as the denaturant. Eight PCR products comprising 184 base pairs were derived from different cell lines with known single base mutations. All of them were detected successfully by different mobility shifts as shown. Lane 1: cell line SW480 (G→A mutation at codon 273, positive control); lane 2: cell line 915 (no mutation, negative control); lanes 3, 4, and 5: cell lines 666, 1915, and Met1, respectively (G→A mutation at codon 273); lanes 6, 7, and 8: cell lines CNE1, CNE2, and Sune-1, respectively (G→C mutation at codon 280).

silver. In this paper, we compared the sensitivity of SSCP analysis using formamide and sodium hydroxide as a denaturant in ethidium bromide stained polyacrylamide gels.

Methods

We tested 14 PCR products amplified from two different genes: six from the human catechol O-methyltransferase gene and eight from human p53, a tumour suppressor gene.

The catechol O-methyltransferase gene has a polymorphism at codon 1947 (G/G, or A/A, or G/A) that is associated with high or low activity of the enzyme in erythrocytes. The forward and reverse primers used were: 5'-CTGCACAGGCAAGATCGTGGA-3' and 5'-TCCAGGTCTGACAACGGGTCA-3', respectively. The optimal PCR conditions were: primers (25 μM), genomic DNA (0.1–0.4 μg), dNTPs (0.2 μM), MgCl₂ (2 μM), and use of the "hot start" PCR at 94°C for five minutes before adding Taq polymerase (1 unit; Advanced Biotechnologies, Epsom, Surrey, UK); then 94°C for one minute, 65°C for 30 seconds, 72°C for 30 seconds, for 26 cycles, and a final extension time of five minutes at 72°C. The amplified fragment length was 234 base pairs.

The PCR product (volume 10–15 μl; 20–100 ng of DNA) was mixed with an equal volume of sequencing stop buffer containing 98% formamide. The other constituents were 10 mM NaOH, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. A control experiment was carried out simultaneously with 10 mM sodium hydroxide and no formamide. No purification of the PCR product was necessary before SSCP analysis when only one desired major band was present. The mixture was denatured at 100°C for seven minutes then plunged into ice immediately for five minutes. Next, the mixture was loaded on to a 20% Tris borate EDTA (TBE) polyacrylamide gel (8.0 × 8.0 × 0.1 cm; 39:1 acrylamide:bisacrylamide; Novex, San Diego, California, USA). Electrophoresis was carried out in 1.5× TBE buffer, initially at 400 V for two minutes, followed by 300 V for 2.5 hours, using the thermoflow system (Novex) and refrigerated circulator (Pharmacia Biotech, Uppsala, Sweden) to maintain consistent temperature. Different running temperatures ranging from 10°C to 25°C were tried, and the optimal temperature was found to be 15°C for this particular fragment. After electrophoresis, the gel was stained with a 0.5 μg/ml solution of ethidium bromide for 20 minutes, then washed with water for five minutes. Ethidium bromide stained bands were visualised using a 340 nm ultraviolet viewing box and photographed with an exposure time of one second.

We also tested detection of a single base mutation (G→A and G→C mutations) in exon 8 of the human p53 tumour suppressor gene, using a further eight PCR products and similar conditions (except using a running temperature of 20°C).

Results

We found that formamide denatured gels showed better resolution than those denatured using sodium hydroxide alone (fig 1); better even than any other published pictures. Although sodium hydroxide also showed some different mobility shifts in SSCP, gel lanes often showed additional artefactual bands comprising incomplete denatured single strands, which made it difficult to detect the polymorphic bands (fig 1B). In contrast, the use of formamide as a denaturant did not result in any ambiguity and gave high resolution in distinguishing the various alleles (fig 1A) of the polymorphic region tested.

Others^{6,7} have used different volume ratios of formamide to PCR product (3:1 or 1:1) in silver stained SSCP. We did not find any difference in the resolution of the ethidium bromide stained SSCP using volume ratios of 1:1, 2:1, or 3:1. We found that a 1:1 volume ratio was the most practical because of the limited well volume of the gel. We also compared various concentrations of sodium hydroxide (ranging from a final concentration of 10–50 mM) as denaturants in our cold SSCP analyses but did not find any significant difference in the resolution (data not shown). Different gel concentrations of 6% and 20% homogeneous polyacrylamide TBE gels, and 4–20% gradient polyacrylamide gels (Novex) were used: we found that the 20% homogeneous polyacrylamide TBE gel demonstrated the best resolution of this polymorphism.

In addition, this method detected and distinguished all the other known single base mutations in exon 8 of the human p53 gene within the eight PCR products tested (fig 2).

Discussion

To our knowledge, this ethidium bromide stained cold SSCP procedure using formamide

as the denaturant has not been reported before. It gave a higher resolution than previous SSCP methods and had 100% sensitivity in the discrimination of 14 PCR samples from two different genes, even for a long fragment close to the upper limit of 250 base pairs. We believe that this modified procedure is a rapid, simple, safe, and yet highly sensitive method for detecting structural differences in DNA fragments.

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