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Original Article

Non-radioactive single-strand conformation polymorphism (SSCP) analysis of relatively long PCR products

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Abstract

The SSCP technique is based on the appearance of new "refolding" conformations during electrophoresis due to mutation. In order to develop a simple, non-radioactive SSCP analysis method so that it can reliably detect single nucleotide changes in PCR products up to 500 bp in length, extensive optimisation trials were performed. The best separation of SSCP bands of PCR products up to 500 bp in length was obtained with 14.5 to 15.5% polyacrylamide gels that had been run in the cold room at 15 V/cm for 65 to 70 hours. After the pre-run, 5 μl of PCR product was mixed with 10 μl of denaturing-loading dye and the mixture was heated to 94 °C for 10 min. Total mixture volume of the 15 μl was loaded into the well without quenching. After electrophoresis, the gels were stained with SYBR® Gold nucleic acid stain for 30-40 minutes and photographed under UV light using a SYBR® gel stain photographic filter. This method alongside with confirmatory sequencing has been utilised to identify three novel mutations in the 5′-regulatory region of the human *CYP3A4* gene. The final results were very satisfactory and the optimised method was able to reliably reveal new mutations in amplified DNA from blood samples. In fact the non-radioactive SSCP method developed here seems to be robust enough to analyse PCR products up to 500 bp long.

Key words: Non-Radioactive SSCP, PCR, SYBR® Gold

INTRODUCTION

SSCP is a widely used method for mutation detection because of its simplicity and versatility. In the original SSCP protocol (1), the region of interest in the genome or cDNA is amplified by PCR, using radio labelled PCR primers or nucleotides, to generate a 200-350 bp radioactive product which is then denatured and electrophoresed in a large-format (40×20 cm) 8-10% non-denaturing polyacrylamide gel. The mobility of single-stranded nucleic acid molecules electrophoresed under non-denaturing conditions is determined by both their fragment length and their secondary structure which is sequence dependent. A DNA strand may adopt several conformations for any given set of electrophoresis conditions and these are visualised as separate bands in the gel. The test sample will take up a different conformation if there is a sequence change and will migrate differently during electrophoresis. Mutations are thus detected as differences of mobility of the DNA bands between control and test. Some or all of the bands may show a shift, a single base change being sufficient to alter secondary structure and hence mobility. Because of the lack of theoretical background to predict conformation, and therefore the mobility of single-stranded DNA in gel electrophoresis, optimal conditions for

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separation of conformers has to be determined by empirical observation. This method has several drawbacks. It requires the use of radioisotopes and electrophoresis on large denaturing gels to affect separation and detection of the single-strand conformers. Also, auto radiography (usually with multiple exposures) requires substantial time to produce a clear image of the resulting SSCP pattern.

There are now several reports describing non-radioactive (or 'cold') protocols for SSCP analysis. However, some require the purchase of expensive additional equipment or use of silver staining techniques. In 'cold' SSCP as used in this project, there is no need to use radioactivity and the gel is made of high percentage polyacrylamide (14-20%) in a min-format which makes this method safer and very easy to perform with simple instrumentation.The bands can also be visualised by staining the gel with conventional DNA stains (3).

In the current experiment, this simple 'cold' SSCP analysis method has been further developed so that it can reliably detect single nucleotide changes in PCR products of up to 500 bp in length. Performing SSCP on long PCR products will make mutation detection experiments quicker, easier, less cumbersome and more reliable as well as cost effective due to a substantial decrease in overlapping regions (especially when large numbers of PCR products are needed to be analysed). Furthermore, using a novel DNA stain, SYBR® Gold, has provided a better condition to visualise the SSCP bands. Application of this dye which has been introduced in 1999 and is 10-fold more sensitive and safer than ethidium bromide (4, 5) has been recently increased (6, 7). This system and confirmatory sequencing have been utilised to identify three novel mutations in the 5′ regulatory region of the human *CYP3A4* gene (8).

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents used in the experimental work were purchased from *Sigma-Aldrich Ltd* (Dorset, England) unless specified below.

Acrylamide 40% and Bis-acrylamide 2% stock solutions were purchased from *Promega Corporation* (Madison, WI, USA). SYBR® Gold nucleic acid stain (10000× concentration in DMSO) and SYBR® gel stain photographic filter were supplied by *Molecular Probes* (Oregon, USA). Advantage®-HF 2 (high fidelity) PCR kit was purchased from *Clontech Laboratories Inc* (Palo Alto, CA, USA). Wizard® genomic DNA purification kit was purchased from *Promega Corporation* (Madison, WI, USA). QIAquick PCR purification was supplied by *QIAGEN* (Hilden, Germany). PCR primers were purchased from *MWG Biotech AG* (Ebersberg, Germany). On arrival, the primer tubes were centrifuged for a few seconds to collect the dry DNA at the bottom of the tube. An appropriate volume of TE buffer or nuclease-free water was added to the tubes and, after rehydration for 2 minutes, the solution was vortexed for 15 seconds and stored at –20 ^{o}C. Polyacrylamide gel electrophoresis was performed using a Bio-Rad mini Protean II™ set, *Bio-Rad Laboratories* (Hercules, USA).

PCR

Specific primers were designed to amplify the 5′ regulatory region of the *CYP3A4* gene as 3 short overlapping segments to aid the SSCP analysis (Table 1). Their relative positions are shown in Figure 1. All PCR amplifications were carried out in a 50 µl reaction volume, using the Advantage-HF 2 (high fidelity) PCR kit (Clontech, Palo alto, California, USA). This kit is an AdvanTaq™-based system designed to amplify cDNA or genomic DNA templates with exceptionally

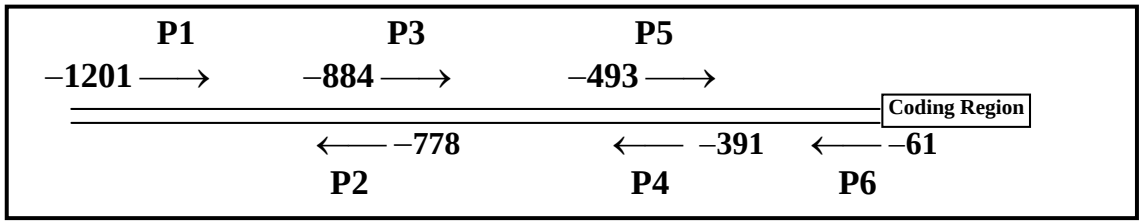

Figure 1. The *CYP3A4* proximal promoter region and position of the PCR primer sets.

Table 1. Primer pairs used for PCR amplification of the *CYP3A4* 5′ regulatory region.

Sequence	Position*	Size (bp)
P1: 5′–GACCACTGCCCCATCATTGC–3′ **P2:** 5′–GCTGGTGGAGTTGACTTAGC–3′	–1201/–778	424
P3: 5′–GCACAGCCAAGAGCTCTGGC–3′ **P4:** 5′–CTTGCCCTTGTCTCTATGGC–3′	–884/–391	494
P5: 5′–GGCACAGGCACACTCCAGGC–3′ **P6:** 5′–TGCTGGGCTATGTGCATGGAGC–3′	–493/–61	433

* Numbering system defines the first coding **A**TG as position +1.

high fidelity. The Advantage-HF 2 Polymerase Mix is a high-performance PCR system that combines AdvanTaq DNA polymerase (a 5′-exonuclease-deficient genetically modified variant of *Taq* polymerase) with a minor amount of proof-reading polymerase (such as *Pfu* DNA polymerase) and TaqStart™ Antibody The dual-polymerase system provides both high sensitivity and flexibility in amplifying a wide range of DNA templates, and TaqStart provides automatic hot-start PCR. Each reaction contained 5 µl of 10× HF 2 PCR buffer, 5 µl of 10× HF 2 dNTP mixture, 6 µl of relevant primer mixture (15 pmol each), 1 µl of 50× Advantage-HF 2 polymerase mixture, 100-200 ng (1 µl) of genomic DNA template and nuclease-free water up to 50 µl. The reaction conditions were: 90 seconds at 94 ºC for 1 cycle; 30 seconds at 94 ºC, 30 seconds at 65 ºC and 1 minute at 72 ºC for 30 cycles; 7 minute at 72 ºC for 1 cycle.

To perform SSCP a stock solution of 20% acrylamide mixture (39:1 acrylamide to bis-acrylamide) was prepared and stored at cold room. 100 ml of this solution contained 48.75 ml of 40% acrylamide solution (from Promega), 25 ml of 2% bis-acrylamide solution (from Promega), 10 ml of 5× TBE buffer, 10 ml of 50% Glycerol and water up to 100 ml. After extensive optimisation trials (e.g. using gels of different strength, running the gels in the cold room or at room temperature and for different times) the best separation of SSCP bands for *CYP3A4* promoter PCR products up to 500 bp in length was obtained with 14.5 to 15.5% gels that had been run in the cold room at 15 V/cm for 65 to 70 hours. To prepare a 10 ml 15% acrylamide gel the following components were mixed; 7.5 ml of 20% acrylamide mixture (see above), 10 µl of TEMED (tetramethylethylenediamine), 10 µl of 10% ammonium persulphate (freshly prepared) and water up to 10 ml. Each gel (8 cm × 7.3 cm × 1 mm) was cast using the Bio-Rad mini Protean II™ set. The gels

were ready after about 1.5 hours. Before sample loading, the gels were pre-run in 1× TBE for about 1 hour. After the pre-run, 5 µl of PCR product was mixed with 10 µl of denaturing-loading dye (95% formamide, 4 M urea, 0.1% bromophenol blue, 0.1% Xylene cyanol FF and 0.5 µl 15% Ficoll) and the mixture was heated to 94 °C for 10 min (9). The total mixture volume of 15 µl was loaded into the well without quenching (2). A 1 kb DNA ladder (from Promega) was also added to one of the wells. The electrophoresis was carried out as indicated above. After electrophoresis the gels were stained with SYBR® Gold nucleic acid stain (Molecular probes, Oregon, USA) for 30-40 minutes and photographed under UV light using a SYBR® gel stain photographic filter (4). The stock SYBR® Gold stain was diluted 10000-fold to make a 1× staining solution e.g. in TE (10 mM Tris-Cl, 1 mM EDTA, pH 7.5-8.0). After adding enough staining solution to completely cover the gel, it was incubated in 1× staining solution for 10 to 40 minutes. During staining, the gel was protected from light by covering it with aluminium foil or by placing it in the dark. The gel was agitated gently at room temperature. No de-staining was required.

DNA sequencing of the variant PCR products

PCR products producing altered SSCP patterns were re-amplified from the corresponding genomic DNA samples and purified with the Ultra Clean™ 15 DNA purification kit (MO BIO, Solana beach, California, USA). The purified samples were then used directly for DNA sequencing of both strands. Where a variation in DNA sequence compared to wild type was found, the initial PCR product was also sequenced for confirmation. In the case of novel mutations, additional repeat amplification and sequencing was performed. Numbering of nucleotides was carried out by assigning the figure +1 to the base A in the ATG translation initiation codon and –1 to the base before this A, (http://www.imm.ki.se/CYPalleles).

RESULTS

SSCP analysis for the –493/–61 (P5/P6) segment of the *CYP3A4* promoter showed altered SSCP patterns for ten out of the 101 PCR products indicating likely mutations. The photograph in Figure 2 is an example showing the SSCP patterns produced. In this example, all samples have either two or three bands except one (lane 6) which has 4 bands indicating the possible presence of mutations in the DNA. The occasional presence of the uppermost band does not seem to be related to changes in DNA sequence in the PCR product but is due to either the concentration of the PCR product or the extent of single strand formation during denaturation.

In segment –884/–391 (P3/P4), eighteen out of the 101 PCR products showed an altered SSCP band pattern indicating possible mutations (Figure 3). In this example, all samples have three bands except one (lane 3) which has 4-5 bands indicating the possible presence of mutation. SSCP analysis of the PCR products from –1201/–778 (P1/P4) segment showed only one sample with an altered band pattern (Figure 4). All samples have three bands except one (lane 2) which has a split pattern in the uppermost band indicating possible mutation.

In sequencing the –493/–61 section, the previously described *CYP3A4*1B* allele containing –392 A→G (10) was found to be heterozygous in 9 out of the 10 variant samples. A new allele (designated *CYP3A4*1E*) (8) containing a –369 T→A transversion was found in one sample. It should be noted that the SSCP pattern of the sample with the new mutation was not distinguishable from that of the other 9 variant samples. As it has previously been

shown (8), all detected mutations were heterozygous. It should be mentioned here that in order to confirm the new T→A mutation, 4 different PCR products were prepared from the relevant genomic DNA and sequenced (both sense and antisense strands) using 2 different DNA sequencing machines to confirm robustness of the procedure. The results were the same in all cases. The –884/–391 sequencing revealed 17 samples with a novel allele (designated *CYP3A4*1F*) (8) containing a –747 C→ G transversion. The remaining sample showed a 9-nucleotide heterozy-gous insertion (ATGGAGTGA) after –845 G. The insertion was also detected in the SSCP and sequencing analysis of the overlapping –1201/–778 segment of the promoter and was the only sample from this segment with an altered SSCP pattern. It should be noted that in addition to the insertion at –845 this sample also contained heterozygously a –392 A→G transition. All DNA samples containing novel mutations were subjected to confirmatory PCR and sequencing to eliminate the possibility of PCR artefacts.

DISCUSSION

Single-strand conformation polymorphism (SSCP) facilitates rapid identification of genetic polymorphisms within segments of DNA up to 300 bp and is still widely used for mutation detection experiments in different genes (3, 11, 12). However, the need for relatively short PCR products in this method was an intensive labour and time-consuming. Therefore, optimisation of the method to reach the ability of analysing PCR products up to 500 bp clearly makes it very easy and applicable for further genetic studies.

The SSCP results obtained clearly showed that the "Cold SSCP" method for mutation detection was reliable and repro-ducible (as described above by detection of 28 genetic variations among 101 samples) to detect changes in mobility due to alteration

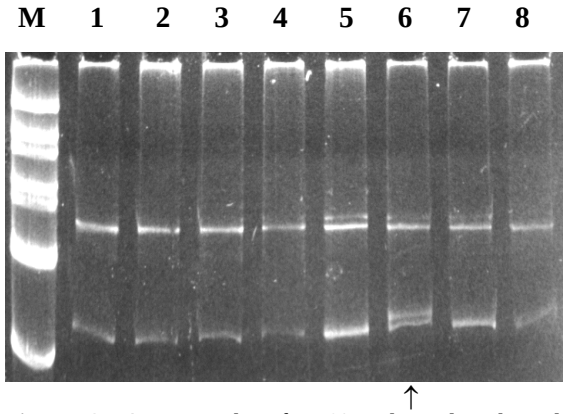

Figure 2. An example of 15% polyacrylamide gel electrophoresis and SSCP analysis of PCR products from the –493/–61 segment of the *CYP3A4* promoter.**M:** DNA marker Lanes **1-8:** SSCP analysis of PCR products from different genomic DNA samples. Arrow indicates a PCR product producing an extra lower SSCP band.

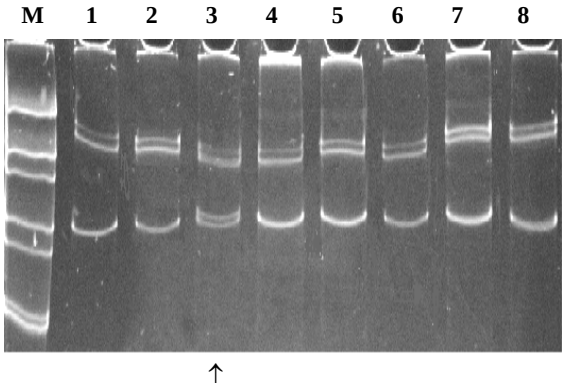

Figure 3. An example of 15% polyacrylamide gel electrophoresis and SSCP analysis of PCR products from the –884/–391 segment of the *CYP3A4* promoter.**M:** DNA marker **Lanes 1-8:** SSCP analysis of PCR products from different genomic DNA samples. Arrow indicates a PCR product producing an extra lower SSCP band.

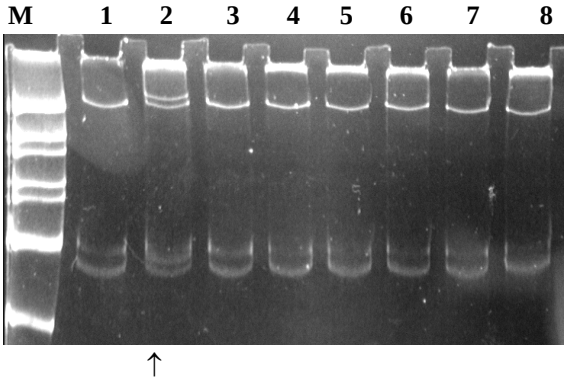

Figure 4. An example of 15% polyacrylamide gel electrophoresis and SSCP analysis of PCR products from the –1201/–778 segment of the *CYP3A4* promoter. **M:** DNA marker Lanes **1-8:** SSCP analysis of PCR products from different genomic DNA samples. Arrow indicates a PCR product producing an extra upper SSCP band.

in single strand conformation. All detected extra SSCP bands showed nucleotide changes in further sequencing reactions [8]. In sequencing of randomly selected samples no changes were observed. Also, the staining dye "SYBR Gold" was found to be sufficiently sensitive in comparison to conventional dye ethidium bromide (data are not been shown due to very poor quality of the images produced).

Optimisation of 'Cold' SSCP also took a relatively long time due to the larger than usual (> 300 bp) PCR products being examined. There is in fact only one publication suggesting the possibility of performing SSCP on PCR products of around 500 bp (13). In this study, different techniques from the literature (2, 3, 4, 9, 14) were combined to develop a reliable method, particularly different polyacrylamide gel concentrations and loading dye with high concentrations of denaturants. Moreover, it was found to be difficult to perform the high percentage (e.g. 15%) polyacrylamide gel electro-phoresis of PCR products of more than 400 bp in a reasonable time scale. PCR products of this size, as single strands, move very slowly in high percentage gels. As a consequence, the application of high voltages was necessary to perform efficient separation, however such voltages could cause the gel to melt and jeopardise the electrophoresis process. Due to the lack of suitable instrumentation (e.g. refrigerated buffer circulation) to perform 'cold' SSCP at high voltages and reduce the electrophoresis time to a matter of a few hours, SSCP electrophoresis had to be performed in the cold room at lower voltage and took almost three days to complete. However, the final results were very satisfactory and the optimised method was able to reliably reveal new mutations in amplified DNA from blood samples. In fact the 'Cold' SSCP method developed here seems to be robust enough to analyse PCR products up to 500 bp long.

In fact, the strategy designed to screen for novel mutations in the *CYP3A4* 5'-regulatory region worked very well and is easily applicable to further large-scale population screening studies. Indeed the relatively high number (28 in total) of the mutations found clearly demonstrated the robustness and reliability of the cold SSCP protocol that was developed. The only modification which seems to be necessary for large scale applications is to provide suitable conditions to reduce the electrophoresis time by applying higher voltages. This could be achieved by using a thermostatically controlled refrigerated circulator to maintain a constant preset temperature of the buffer during electrophoresis in order to prevent melting of the gel due to high voltages.

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