RESEARCH NOTE

Improvement of the sensitivity and resolution of PCR-SSCP analysis with optimized primer concentrations in PCR products

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Introduction

Polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) is a widely utilized screening method for detection of mutations. In this method, the target sequence of interest is amplified by PCR and separated as single-stranded molecules by electrophoresis in a nondenaturing polyacrylamide gel (Orita *et al*. 1989). However, the sensitivity and resolution of PCR-SSCP can be influenced by many parameters, including size and GC content of the PCR product (Li *et al*. 2003); gel temperature during electrophoresis (Chen *et al*. 1995); buffer composition (e.g. ionic strength, pH; Kukita *et al*. 1997); buffer additives, mainly glycerol, formamide, polyethylene glycol (Markoff *et al*. 1997; Paccoud *et al*. 1998); gel matrix composition and concentration (Savov *et al*. 1992), as well as primer concentration in PCR product (Cai and Touitou 1993).

It is generally considered that specific PCR amplification is important in detection of mutations by using PCR-SSCP (Orita *et al*. 1989; Li *et al*. 2003). Moreover, it has been noted that addition of either forward or reverse primer in PCR product may apparently alter SSCP banding patterns, which is presumably caused by the interaction between primer(s) and single-strand DNAs (ssDNA) (Almeida *et al*. 1998; Hennessy *et al*. 1998). Previously, Cai and Touitou (1993) demonstrated that the lowest primer concentration in PCR product affecting SSCP banding patterns is around 6 nM, and that primer-concentration-dependent intermediate SSCP patterns appear in unpurified PCR product amplified with regular amounts of primers (30–150 nM). In the present study, we used two identified PCR fragments of the promoter region in the gene for HLA class II histocompatibility antigen DR-1 beta chain (*HLA-DRB1*) and *Fok*I polymorphism in exon 2 of the gene for vitamin D (1,25-dihydroxyvitamin D3) receptor (*VDR*) to evaluate the effect of specific primers with different concentrations on the sensitivity and resolution of PCR-SSCP analysis. Our results provide useful information for optimization and improvement of this method for the detection of mutations.

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Material and methods

PCR

The sequences of forward and reverse primers used for PCR amplification of the promoter region in *HLA-DRB1* and exon 2 in *VDR* loci were 5′-AGA TCT GTT TCA GAA GAG GAC CTT CA-3′, 5′-CAG GGA GCT TCA GAC ACA CCA T-3′ (Singal and Qiu 1996); 5′-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3′, 5′-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3′ (Harris *et al*. 1997), respectively (Boya, Shanghai, China). PCR mixture in a final volume of 25 μl contained 10 mM KCl, 8 mM $(NH_4)_2SO_4$, 10 mM Tris-HCl (pH 9.0), 0.05% NP-40, 1.5 mM $MgCl_2$, 80 μM of each dNTP, 2.1 pmol of each primer and 1U *Taq* DNA polymerase. For amplification of specific fragment, 2 ng plasmid DNA [pGEM®-T Easy vector *DRB1*] (Promega), or 50 ng genomic DNA (*VDR*) was used as template. All PCR experiments were performed on a Perkin-Elmer 2700 thermal cycler (Applied Biosystems), and cycling conditions included predenaturation at 94°C for 2 min, and 30 cycles of 94°C for 30 s, 62°C (*DRB1*) or 68°C (*VDR*) for 40 s, and 72°C for 40 s, and final extension at 72°C for 5 min. After amplification, PCR product was purified with low-melting agarose

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(Gibco BRL, Invitrogen) and quantified with Ultrospec 2100 *pro* (Amersham Biosciences).

SSCP

Nondenaturing polyacrylamide gel preparation

The 20% (w/v) stock solution of acrylamide (Fluka Chemika, Buchs, Switzerland) and bis-acrylamide (USB, Cleveland, USA) was prepared with the weight ratio of 49:1. Twenty μl of N, N, N', N'-tetramethylethylenediamine (TEMED; Promega), and 280 μl 10% (w/v) ammonium persulphate (Gibco BRL, Invitrogen) were added as polymerization catalysts to 40-ml gel mix. Both gel buffer and electrophoresis buffer solution were 1× Tris–borate–EDTA (1× TBE, 89 mM Trisaminomethane, 89 mM boric acid and 2 mM EDTA, pH 8.3).

Sample preparation

Two μl of purified *HLA-DRB1* promoter or *VDR* exon 2 PCR product (0.15 pmol) and 1 μl specific primers with various concentrations were mixed in the molar ratios 1:3, 1:6, 1:12, 1:25, 1:50, 1:100 and 1:200. Seven μl of gel loading buffer (95% deionized formamide v/v, 20 mM EDTA (pH 8.0), 0.05% bromophenol blue, 0.05% xylene cyanol) was then added to the PCR product. The samples were denatured at 99°C for 10 min, and then chilled immediately on ice for 10 min.

Electrophoresis

Vertical slab gel units with gels of 160 mm × 160 mm ×1 mm were used. The cooled denatured mixture was loaded onto 10% polyacrylamide gel, and run in 1× TBE buffer solution on constant voltage of 110 V at room temperature for 15 h. After electrophoresis, the gel was silver-stained according to the following conditions: gel was fixed in solution of 10% (v/v) ethanol and 10% (v/v) glacial acetic acid for 10 min, stained in 0.1% (w/v) silver nitrate solution for 40 min, developed in 3% (w/v) sodium hydroxide solution containing 0.15% (v/v) formalin, and finally stopped with distilled water.

Results and discussion

The PCR fragment of 250 bp in *HLA-DRB1* promoter was amplified with pGEM®-T Easy vector - *DRB1* plasmid DNA as template. Figure 1 shows that SSCP banding patterns were shifted when PCR product was mixed with either each primer or both primers in the molar ratios 1:3, 1:6, 1:12, 1:25, 1:50, 1:100 and 1:200. In the various mixtures, the corresponding primer concentrations were 45 nM, 90 nM, 180 nM, 375 nM, 750 nM, 1500 nM and 3000 nM respectively. We found that adding either forward or reverse primer caused only one specific band to be shifted (figure 1, lanes 3 and 4), while two bands were shifted with addition of both primers (figure 1, lanes 2 and 12), and that the banding pattern of PCR product mixed with specific primers in the molar ratio 200:1 (figure 1, lane 12) was significantly clearer than that without primer addition (figure 1, lane 5). These results confirmed the idea that optimal primer addition in SSCP analysis led to hybridization of primer to ssDNA and reduction of DNA reannealing, and subsequently the banding mobility shifted and the sensitivity was greatly improved (Almeida *et al*. 1998; Hennessy *et al*. 1998).

In addition, with increase in primer concentration (45 nM, 90 nM, 180 nM, 375 nM, 750 nM, 1500 nM, 3000 nM) the SSCP banding patterns showed that the two shifted bands got stronger gradually (figure 1, lanes 6–12) and the two original bands tended to weaken by degree (figure 1, lanes 6–11), and even disappeared completely (figure 1, lane 12). Intermediate patterns appeared at primer concentrations between 45 nM and 1500 nM. In a previous study it has been proved that the lowest primer concentration in PCR product able to influence the SSCP banding patterns is around 6 nM (Cai and Touitou 1993). It suggested that when concentration of unincorporated primers in PCR product increased from 6 nM to 1500 nM the intermediate patterns of unpurified PCR product would interfere with mutation screening in PCR-SSCP analysis. Therefore the exact result of mutation screening could be obtained by purification or increasing primer concentration to 3000 nM. However, optimal PCR primer addition is relatively simple, rapid, sensitive and cost-effective compared to purification of PCR product.

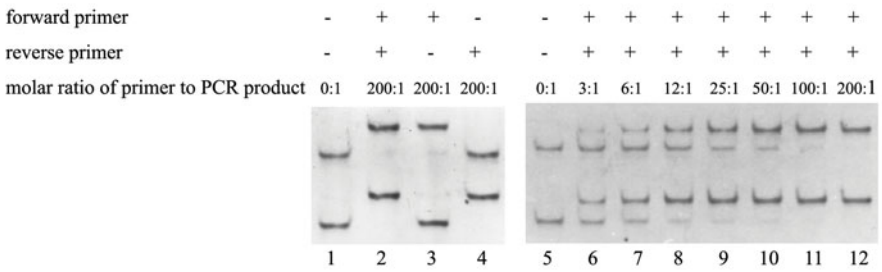

Figure 1. SSCP banding patterns of PCR fragment from *HLA-DRB1* promoter with plasmid DNA as template, influenced by different concentrations of specific primers.

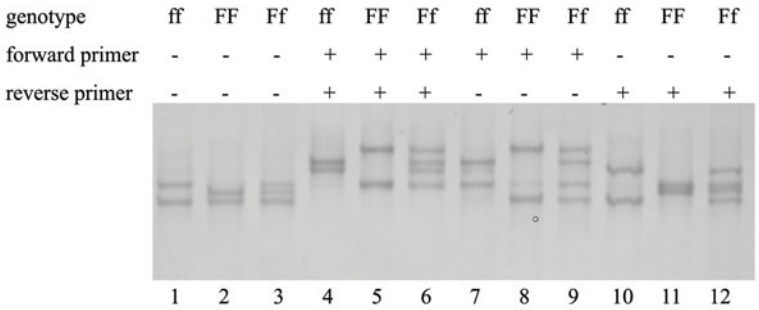

Figure 2. SSCP banding patterns of PCR fragment of *Fok*I polymorphism in exon 2 of *VDR* with genomic DNA as template, affected by specific primers. The primers were mixed with PCR products in the molar ratio 200:1.

Further, to evaluate whether sensitivity and resolution of PCR-SSCP in mutation detection can be improved by primer concentrations, we analysed the banding pattern and electrophoretic mobility of the identified 266-bp PCR product fragments from *VDR*, which was amplified with genomic DNA as template. Figure 2 indicates that the specific primers had similar effect on genomic DNA of homozygote (*FF* and *ff*) and heterozygote (*Ff*) as plasmid DNA (*DRB1*). Three fragments without primers showed relatively low resolution of the bands (figure 2, lanes 1–3). By adding either each primer or both primers to the PCR products in the molar ratio 200:1, the banding patterns of all three samples were changed (figure 2, lanes 4–12). In particular, the resolution of the bands was greatly improved by adding both primers (figure 2, lanes 4–6).

In conclusion, our present study provides additional evidence that electrophoretic mobility in SSCP is influenced by primer concentrations, and also suggests that adding both primers to the PCR product, in the optimal ratio of 200:1, not only improves the sensitivity of SSCP by reducing DNA reannealing, but also excludes interference from intermediate banding patterns caused by excessive unincorporated primers in PCR product and enhances the reproducibility of mutation screening through PCR-SSCP.

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