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Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 1537–1544

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Analysis of single-strand conformation polymorphisms by capillary electrophoresis with laser induced fluorescence detection[1]

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Received 3 September 1996; accepted 11 October 1996

Abstract

Detection of point mutations in genomic DNA is important for diagnosis of inherited characteristics and genetic diseases. A point mutation in a specific region of DNA amplified by polymerase chain reaction (PCR) can be detected with single-strand conformation polymorphism (SSCP) analysis. Analysis of SSCP by laser-induced fluorescence capillary electrophoresis in entangled polymer solution (CE-LIF) has been developed in the present paper. K-*ras* genes including seven mutations were amplified with primer labeled with Texas Red at its 5′ end. The labeled PCR products were dissociated to single strands by heating and separated with capillary gel electrophoresis and He-Ne laser-excited fluorescence detection. Our results suggest that all fragments having normal (Gly) and mutated (Ala, Arg, Cys, Ser, Val, Asp) sequences at codon 12 can be distinguished. Analysis of SSCPs with CE-LIF is well suited for clinical analysis of SSCPs because of its high sensitivity, resolution, reproduciblity and speed. © 1997 Elsevier Science B.V.

Keywords: Single strand conformation polymorphism; Capillary electrophoresis; Laser induced fluorescence detection; Multiple sheath-flow capillary-array electrophoresis; K-*ras* gene; Polymerase chain reaction

1. Introduction

Point mutations are nucleotide changes in genomic DNA, which may result in heritable genetic diseases. Their analysis has become more important in the diagnosis of genetic and infectious diseases and cancers. Therefore, rapid and easily used analytical techniques must be developed to detect point mutations. Point mutations can be detected with such techniques as denaturing gradient gel electrophoresis [1], RNase A cleavage [2], allele-specific polymerase chain reaction (allele specific PCR) [3], PCR-restriction fragment length

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[1] Presented at the Seventh International Symposium on Pharmaceutical and Biomedical Analysis, August, 1996, Osaka, Japan.

PII S0731-7085(96)01988-7

polymorphism [4], heteroduplex polymorphism analysis [5], and SSCP [6]. Of these techniques, SSCP analysis is the simplest and most sensitive. The basis of SSCP analysis is that the conformational change of single-stranded DNA caused by mutation, results in a mobility shift on non-denaturing gel electrophoresis The detection of a slight conformation difference with conventional electrophoresis requires strict running conditions, such as low temperature, and takes several hours. Analysis of SSCP with capillary electrophoresis (CE) has been developed by Kuypers [7]. The advantages of this method are high resolution, reproducibility, speed, and ease of analysis. In the present paper, we studied the analysis of SSCPs by capillary electrophoresis in entangled polymer solution (CE) with laser induced fluorescence (LIF) detection (CE-LIF). A k-*ras* gene including seven mutations was amplified with a primer labeled with Texas Red at its 5′ end. The labeled PCR products were dissociated to single strands by heating and separated by CE with He-Ne laser-excited fluorescence detection. The results show that all the fragments having normal (Gly) or mutated (Ala, Arg, Cys, Ser, Val, Asp) sequences at codon 12 can be distinguished. Further more, SSCP analysis was carried out with multiple sheath-flow gel capillary-array electrophoresis to analyze many samples simultaneously. CE-LIF is well suited for clinical analysis of SSCPs because of its high sensitivity, resolution, reproducibility and speed.

2. Experimental

2.1. Apparatus

A Model 270A capillary electrophoresis (CE) system (ABI, Foster City, CA, USA) was used. The separations were monitored on-capillary tube at 260 nm. The CE system with LIF detector was an apparatus made at the Hitachi Central Research Laboratory, and the fluorescent labeled-DNA fragments were detected with LIF detection on capillary tube. A high-voltage direct current power supply (HCZE-30 PN 0.25-LDS, Matsusada Precision Device, Japan) and a He-Ne laser (PMS Electro-optics) were used. A multiple sheath-flow gel capillary-array electrophoresis system, as reported by Kambara et al. [8], was used.

2.2. Materials

γ-Methacryloxypropyltrimethoxysilane was purchased from Sigma (St Louis, MO, USA). Acrylamide, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Silica capillary tubing (100 μm inner diameter, 375 μm outer diameter) was obtained from GL Sciences (Tokyo, Japan). Gene Amp PCR reagent kit with Amplitaq DNA polymerase was from Perkin-Elmer Cetus (Norwalk, CT, USA). K-*ras* genes including seven mutations at codon 12 were from Takarashuzo (Kyoto, Japan). Texas Red labeled primers and non fluorescent labeled primers used for PCR were obtained from Toagosei Chemical Industries (Tokyo, Japan). Other chemicals were of reagent grade.

2.3. Procedures

2.3.1. Preparation of capillary gel

The fused-silica capillary tubing was used for CE. Acrylamide polymerization was accomplished in the capillary according to the methods of Paulus and Ohms [9]. The polymer was covalently attached to the walls of the fused silica capillary with γ-methacryloxypropyltrimethoxysillane. Solutions consisting of 9% acrylamide dissolved in 100 mmol l^{-1} Tris-250 mmol l^{-1} borate buffer (pH 7.8) were polymerized by adding both *N*,*N*,*N*′,*N*′-tetramethylethylene diamine (TEMED) solution and ammonium persulfate solution. The polymerizing solution was quickly introduced into

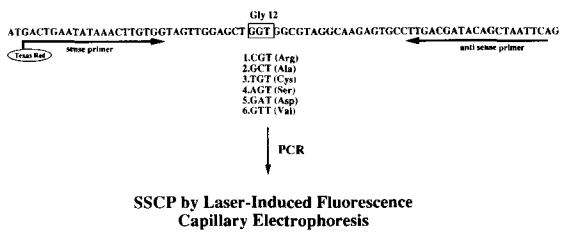

Fig. 1. The schema of PCR of k-*ras* gene.

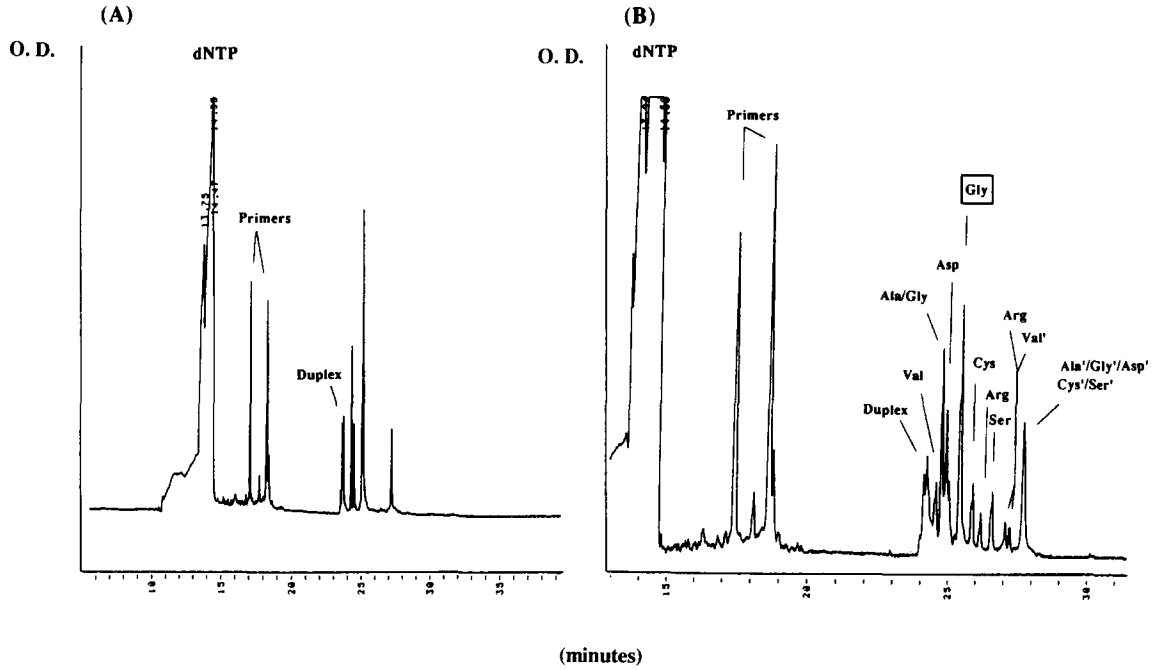

Fig. 2. Electropherograms of SSCP obtained from (A) normal (Gly) and (B) a mixture of seven kinds of k-*ras* codon 12 [normal (Gly) and muted (Ala, Arg, Cys, Ser, Val, Asp)] by CE with UV detection.

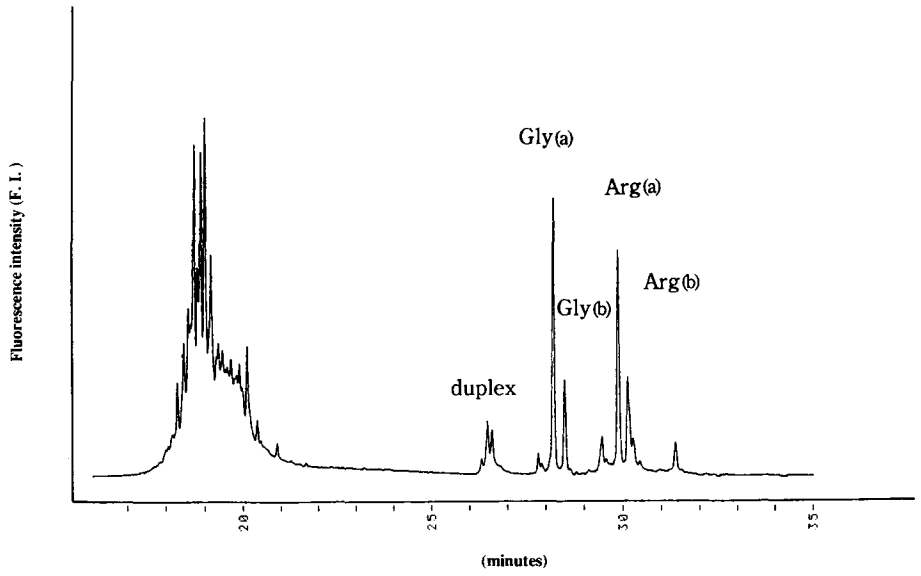

Fig. 3. Electropherogram of SSCP obtained from a mixture of normal (Gly) and mutant (Arg) by CE with LIF detection.

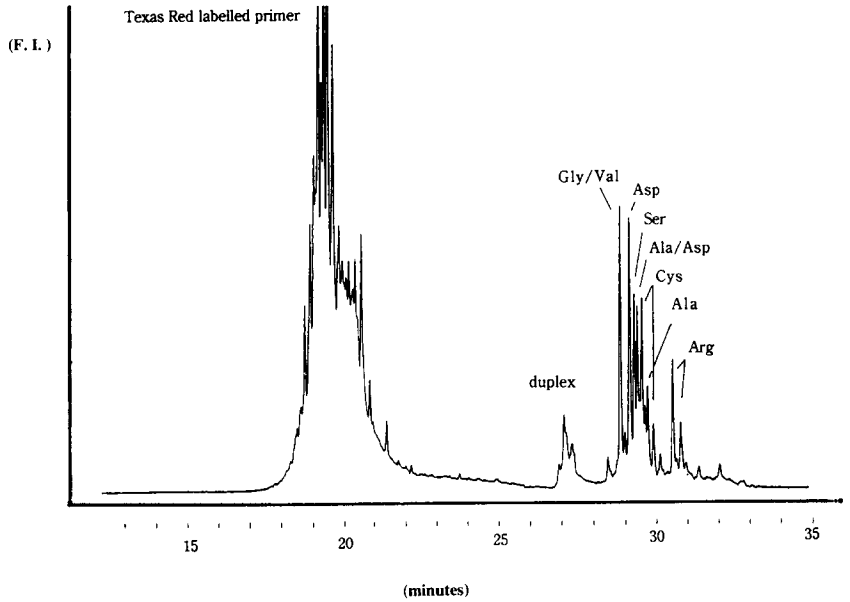

Fig. 4. Electropherogram of SSCP obtained from a mixture of seven kinds of k-*ras* codon 12 [normal (Gly) and muted (Ala, Arg, Cys, Ser, Val, Asp)] by CE with LIF detection.

the treated capillary by a vacuum injection system equipped with an ABI model 270 A CE system for 10 min or by a syringe and left overnight at room temperature. In a CE experiment, the electrode of the injection side was attached to the negative side of a power supply. Sample injections were performed electrophoretically for, typically, 10 s at 100 V cm^{-1} for UV detection and 3 s at 100 V cm^{-1} for LIF detection. Electrophoresis was performed with 25 mmol l^{-1} Tris–glycine buffer (pH 8.5) at 200 V cm^{-1}, 20°C. The DNA fragments were detected at 260 nm or by a LIF detection using the 594 nm line of a He-Ne laser (5 mW) for excitation, and 615 nm for emission.

2.3.2. *SSCP of k-ras gene*

Normal (wild) and mutant DNA of k-*ras* (231 bp) were subjected to PCR with Texas-Red labeled sense primer (5′GACTGAATATAAACTTGTGG) and antisense primer (5′GAATTAGCTGTATCGTCAAG) to generate fluorescent-labeled 71 bp fragments for LIF detection and to PCR with non fluorescent labeled sense primer and antisense primer to generate non fluorescent-labeled 71 bp fragments for UV detection. These PCR products of the k-*ras* gene contain a point mutation at codon 21. The schema of PCR of the k-*ras* gene is illustrated in Fig. 1. The PCR reaction mixture consisted of 50 mmol l^{-1} KCl, 10 mmol l^{-1} Tris–HCl (pH 8.3) 1.5 mmol l^{-1} $MgCl_2$, 0.001% gelatin, 200 μmol l^{-1} dNTPs, 1.6 μmol l^{-1} each of Texas Red labeled sense primer (or non-fluorescent labeled sense primer) and antisense primer, 10 ng of k-*ras* DNA, and 1.5 units of Taq DNA polymerase in a total volume of 50 μl. PCR was carried out for 30 cycles as follows: 0.5 min at 94°C, 2 min at 55°C, and 1 min at 72°C. PCR products, generated from normal and mutant genes, were separately precipitated with ethanol. The precipitated PCR products were dissolved in 20 μl of solution containing 10 mmol l^{-1} Tris–HCl (pH 7.5) containing 1 mmol l^{-1} ethylenediamine tetraacetic acid (TE buffer). Sample solutions were heated to 99°C for 6 min and cooled in ice for 6 min. The samples were analyzed by CE with UV detection. For CE with the LIF detector, sample solutions were further diluted 40 to 500 fold with TE buffer before heating. The diluted sample was denatured as described above and analyzed by CE with LIF detection.

Table 1
Migration times of SSCP obtained from the combinations of normal and various mutants

No.	Sample	T (°C)	ds-DNA (duplex)		ss-DNA (a)	ss-DNA (b)
1	Gly (Ref)	20.0	27.34	Gly	29.12	29.44
2	Gly+Arg	20.0	26.46	Gly	28.18	28.48
				Arg	29.86	30.11
3	Gly+Ala	20.0	26.23	Gly	27.96	28.26
				Ala	28.50	28.83
4	Gly+Val	20.0	26.25	Gly	28.02	28.34
				Arg	28.02	28.34
5	Gly+Asp	20.0	26.66	Gly	28.42	28.70
				Asp	28.70	29.03
6	Gly+Cys	20.0	6.73	Gly	28.49	28.79
				Cys	29.19	29.55
7	Gly+Ser	20.0	26.74	Gly	28.51	28.80
				Ser	28.96	29.30
8	All Mixture	20.0	27.08	Gly	28.84	
				Val	28.84	
				Asp	29.14	
				Ser	29.29	
				Ala	29.39	
				Cys	29.54	
				Arg	30.52	

Single strand DNA (ssDNA) (a) and (b) is corresponding to major and minor SSCP, respectively.

3. Results and discussion

Analysis of SSCPs is widely used to detect single point mutations because of its simplicity and more sensitivity. Recently, some groups reported SSCP analysis using CE with UV detection [7,10,11]. We also performed a detailed study of running temperature, gel concentration, and PCR product size for separating SSCPs with CE-UV detection [12]. These results suggest that CE is well suited for SSCP analysis. However, CE is not sufficiently sensitive to detect SSCPs in small amounts of human DNA, even after 30 cycles of PCR. Hebenbrock et al. reported SSCP using CE with two-dye laser-induced fluorescence detection [13]. The method made possible a specific detection of sense or antisense SSCP and a highly sensitive detection of SSCP. In the present paper, we tried to develop a highly sensitive method of SSCP analysis with CE-LIF detection and SSCP analysis by multiple sheath-flow capillary-array electrophoresis to analyze many samples simultaneously. PCR products labeled with florescence dye were prepared as follows. Oncogene, k-*ras* including seven mutations was PCR-amplified with sense primer labeled with Texas Red at the 5′ end and non fluorescent labeled antisense primer. The PCRs were performed for 5 to 30 cycles on a DNA thermal cycler to evaluate the sensitivity of CE-LIF. For SSCP analysis with UV detection, non florescent labeled sense primer was used instead of Texas-Red labeled sense primer.

3.1. SSCP with CE-UV

Fig. 2 shows results of PCR-SSCP for a k-*ras* mutant with CE-UV detection. Fig. 2(A) and (B) show SSCP of k-*ras* codon 12 normal (glycine) and SSCP of a mixture of seven kinds of k-*ras* codon 12 mutant by UV detection, respectively. The SSCP analysis obtained by UV detection shows a complex electropherogram because of deoxynucleotide triphosphate (dNTP), two primers (sense and antisense), and single strand DNAs (SSCP) having UV absorbance at 260 nm. In Fig. 2(A), two small peaks corresponding to minor SSCPs were observed at 24 and 27 min in addition to large peaks corresponding to major

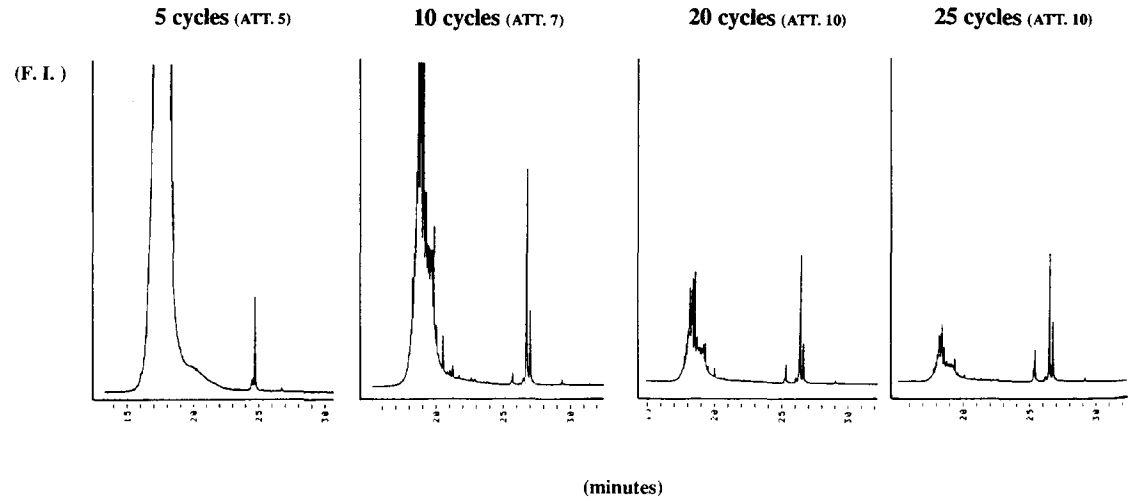

Fig. 5. Effect of PCR-cycle number on the sensitivity for SSCP detection.

SSCPs at 24 and 25 min. Thus, the SSCP pattern of k-*ras* codon 12 normal (glycine) showed four peaks. It is essentially expected to show two large peaks corresponding to sense and antisense SSCPs. The minor peaks observed on the electropherogram (Fig. 2(A)) are thought to be adducts of the major PCR product resulting from the addition of an adenine owing to nonspecific PCR. The identity of the adduct was confirmed by gel filled capillary electrophoresis for DNA sequence (data not shown).

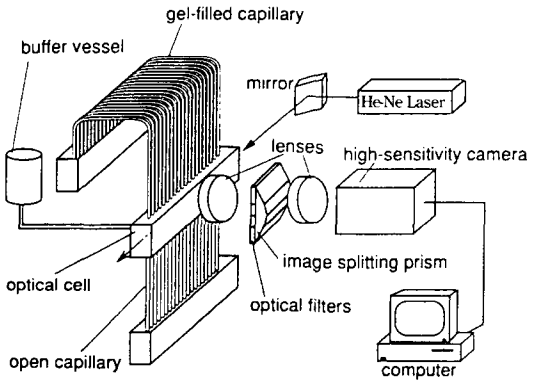

Fig. 6. Schematic view of the multiple sheath-flow capillary-array electrophoresis system.

3.2. SSCP with CE-LIF

Fig. 3 shows a typical electropherogram of SSCPs obtained with CE-LIF. The sample was a mixture of k-*ras* codon 12 normal (glycine: -GGT-) and mutant (arginine: -CGT-). As shown in Fig. 3, SSCPs of normal (Gly) and mutant (Arg) k-*ras* DNA were observed at approximately 28 (Gly (a) and (b)) and 30 min (Arg (a) and (b)), respectively. The peaks of Gly (b) and Arg (b) are adenine adducts of each peak (a) (major SSCP) as observed with UV detection. The peaks observed at approximately 19 min were from Texas Red labeled primer. Fig. 4 shows electropherogram for SSCP analysis of seven kinds of k-*ras* codon 12 (normal (Gly) and mutated (Ala, Arg, Cys, Val, Asp)). Using the CE-LIF, we could separate all seven SSCP variations within 31 min. The result show that the electropherogram obtained with LIF detection was clearer than that obtained with UV detection. Thus, distinct SSCP patterns could easily be differentiated from other SSCP patterns. Migration times of SSCPs obtained from combinations of normal k-*ras* and various mutants are listed in Table 1. The results show that normal k-*ras* (Gly) and mutants other than valine could easily be distinguished. Although SSCPs of normal (Gly) and mutant valine k-*ras* were indistinguishable, the SSCPs could be separated when

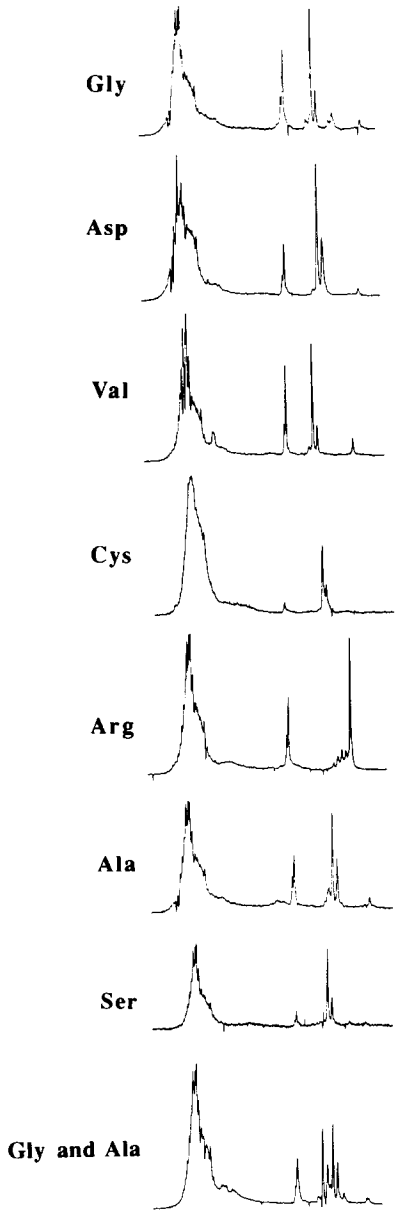

Fig. 7. Electropherograms of SSCPs obtained from seven kinds of k-*ras* mutant at codon 12 and a mixture of normal (Gly) and mutant (Ala) with multiple sheath-flow capillary-array electrophoresis.

Texas Red labeled antisense primer was used instead of sense primer (data not shown).

3.3. Precision

The precision of migration times obtained with CE-LIF for SSCPs of glycine at k-*ras* codon 12 was examined. The relative standard deviation ($n = 5$) was 2%.

3.4. Sensitivity and PCR cycle number

CE-LIF is highly sensitive. It has been reported that the minimal concentration of Texas Red labeled DNA detectable with CE-LIF is 10^{-13} mol l^{-1} [8]. Approximately 1000 molecules of fluorescent labeled DNA fragments can be detected so that SSCPs can be detected from small amounts of human DNA and number of PCR cycle can be reduced to obtain more specific PCR product. Fig. 5 shows the results of SSCP analysis obtained by reducing the number of PCR cycle from 30 to 5. Valine mutant of k-*ras* gene was used for this experiment. The results obtained with valine mutant show that SSCPs produced by only five cycles of PCR can be detected satisfactory with CE-LIF and that minor products (adenine adducts produced by nonspecific PCR) observed after 10 to 30 cycles are not observed on electropherograms with five cycles. In contrast, CE with UV detection could not detect SSCPs produced with PCR of less than 10 cycles.

3.5. SSCP analysis with multiple sheath-flow gel capillary-array electrophoresis

With multiple capillary electrophoresis, many samples can be examined simultaneously [8]. The multiple sheath-flow assembly for capillary array is shown in Fig. 6.

In this paper, we performed preliminary experiments with a multiple capillary electrophoresis system for SSCP analysis. PCR products obtained from normal and mutant k-*ras* were precipitated with ethanol and denatured. Individual samples of k-*ras* mutant at codon 12 and mixtures of normal (Gly) and mutant (Ala) k-*ras* DNA were examined with a multiple capillary array. The results are shown in Fig. 7. Eight samples could be

analyzed simultaneously within 30 min. With this system, SSCP analysis of 100 to 200 samples can be performed simultaneously. Further studies on the separation of SSCP with a multiple capillary electrophoresis system are in progress in our group.

3.6. Conclusion

In conclusion, the proposed CE-LIF system is extremely sensitive and can be used to specifically detect SSCPs labeled with fluorescent dye. Analysis of SSCPs with the proposed CE-LIF system clearly distinguished normal and mutant alleles, thus facilitating rapid diagnosis of cancers and genetic diseases. CE-LIF is well suited for clinical analysis of SSCP.

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