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Single-strand conformation polymorphism analysis to detect the p53 mutation in colon tumor samples by capillary electrophoresis

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Abstract

The polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) technique is developed for the detection of point mutations in DNA samples, and is very useful in the research of tumors. The traditional SSCP was carried out with slab gel electrophoresis (SGE), but this is time-consuming and labor-intensive, particularly for clinical diagnoses. We have developed a capillary electrophoresis (CE) method for SSCP detection with a linear polyacrylamide gel solution as the sieving matrix. Twenty colon tumor samples were detected with SSCP–CE and the point mutation in exon 7 of the p53 gene was found in six of the samples. Based on the sequencing results, the accuracy of SSCP–CE was better than that of SSCP–SGE. We hope this rapid and convenient method could be applied in the clinical diagnosis of tumors soon.

Keywords: Single-strand conformation polymorphism analysis; DNA

1. Introduction

The detection of point mutations in DNA fragments has attracted much attention because small alterations in the DNA sequence of a gene can lead to many human diseases, such as tumors, diabetes and heart disease [1–3]. Among these techniques, single-strand conformation polymorphism (SSCP) can detect a point mutation without sequencing, because the point mutation gives rise to a conformational change of single-stranded DNA and results in a mobility shift on neutral polyacrylamide gels [4]. The traditional method used to detect a slight conformational difference is slab gel electrophoresis (SGE), but it required strict running conditions and is labor-intensive.

Recently, the separation of DNA fragments with capillary electrophoresis (CE) has become one of the hottest topics in the field of biochemical analysis [5–7]. CE has even been used in the detection of SSCP because of its high sensitivity and good resolution. Mensink et al. [8] first developed the SSCP–CE method and used it to detect the point mutation in the p53 gene extracted from heterozygous cell lines CEM. The early research focused on the study of the effects of temperature or gel concentration on the resolution and sensitivity of SSCP–CE [9–12], and almost all the capillaries used then were home-made. Therefore, the reproducibility of SSCP–CE is sometimes not ideal.

We have developed a SSCP-CE method recently with the application of commercial coated capillary.

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Both the reproductibility and the detection length of SSCP were improved. Twenty colon tumor samples were detected and the result was compared with that of SSCP–SGE and sequencing. It reveals that the SSCP–CE method is convenient and sufficiently accurate. Further development will focus on the detection of multi-allele mutations by SSCP–CE at the same time.

2. Experimental

2.1. Extraction of DNA fragments from colon tumor samples

Twenty colon tumor samples were obtained from the First Affiliated Hospital of West China University of Medical Sciences. Samples were frozen in liquid nitrogen and stored at −80°C. DNA fragments were extracted from samples by proteinase K digestion and phenol–chloroform according to the method of Sambrook et al. [13] with minor modification.

2.2. Primers and PCR

Exon-specific primers were synthesized on a Beckman Oligo 1000M DNA Synthesizer (Fullerton, CA, USA), and their sequences are shown in Table 1. The target polymerase chain reaction (PCR) products amplified with these primers were 196-base pair (bp) DNA fragments (Condons 1797–1993). The PCR system consisted of 500 mmol/l KCl, 10 mmol/l Tris–HCl (pH 8.4), 1.0 mmol/l $MgCl_2$, 200 μmol/l each of dNTPs (A, G, C, T), 1 μmol/l primer, 100 ng of extracted genomic DNA and two units of Taq DNA polymerase (B.M.) in a total volume of 50 μl. PCR was performed on a Perkin-Elmer Model 9600 PCR system (Foster City, CA, USA) for 35 cycles of the following reaction:

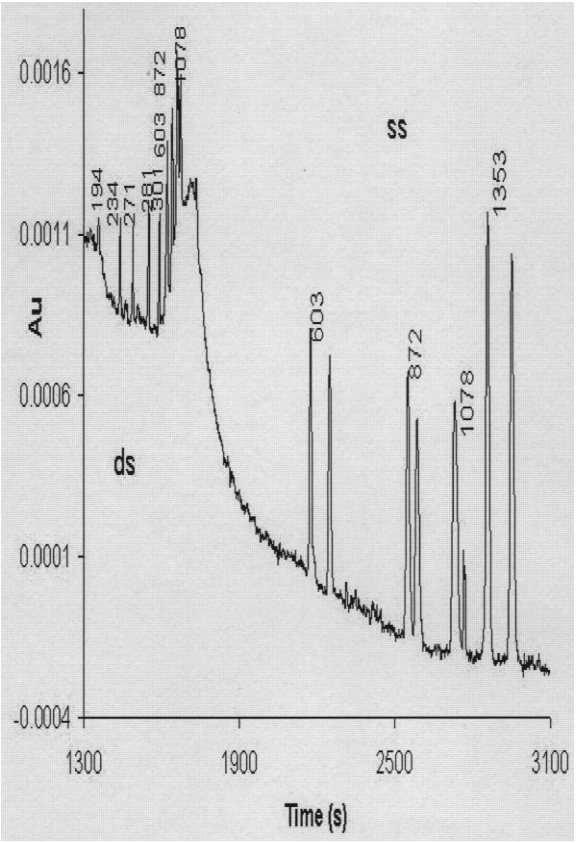

Fig. 1. Electropherogram of denatured ΦX174 RF DNA *Hae*III digest: ds refers to the peak region of double-stranded (ds) DNA fragments, and ss refers to the peak region of single-stranded (ss) DNA fragments. SSCP–CE conditions were reported in Section 2.4.

Table 1
Primer sequence designed for PCR of exon 7 of the human p53 gene

Sample	Type	Primer sequence	Mutation type
A	Forward	5′-GCCACAGGTCTCCCCAAGGC-3′	–
B	Forward	5′-GCCACAGGTCTCCCCAAGAC-3′	G→A
C	Forward	5′-GCCACAGGTCTCCCCAAGTC-3′	G→T
D	Forward	5′-GCCACAGGTCTCCCCAAGCC-3′	G→C
TS	Forward	5′-GCCACAGGTCTCCCCAAGGC-3′	–
N	Forward	5′-GCCACAGGTCTCCCCAAGGC-3′	–
All	Reverse	5′-AGTGTGCAGGGTGGCAAGT-3′	

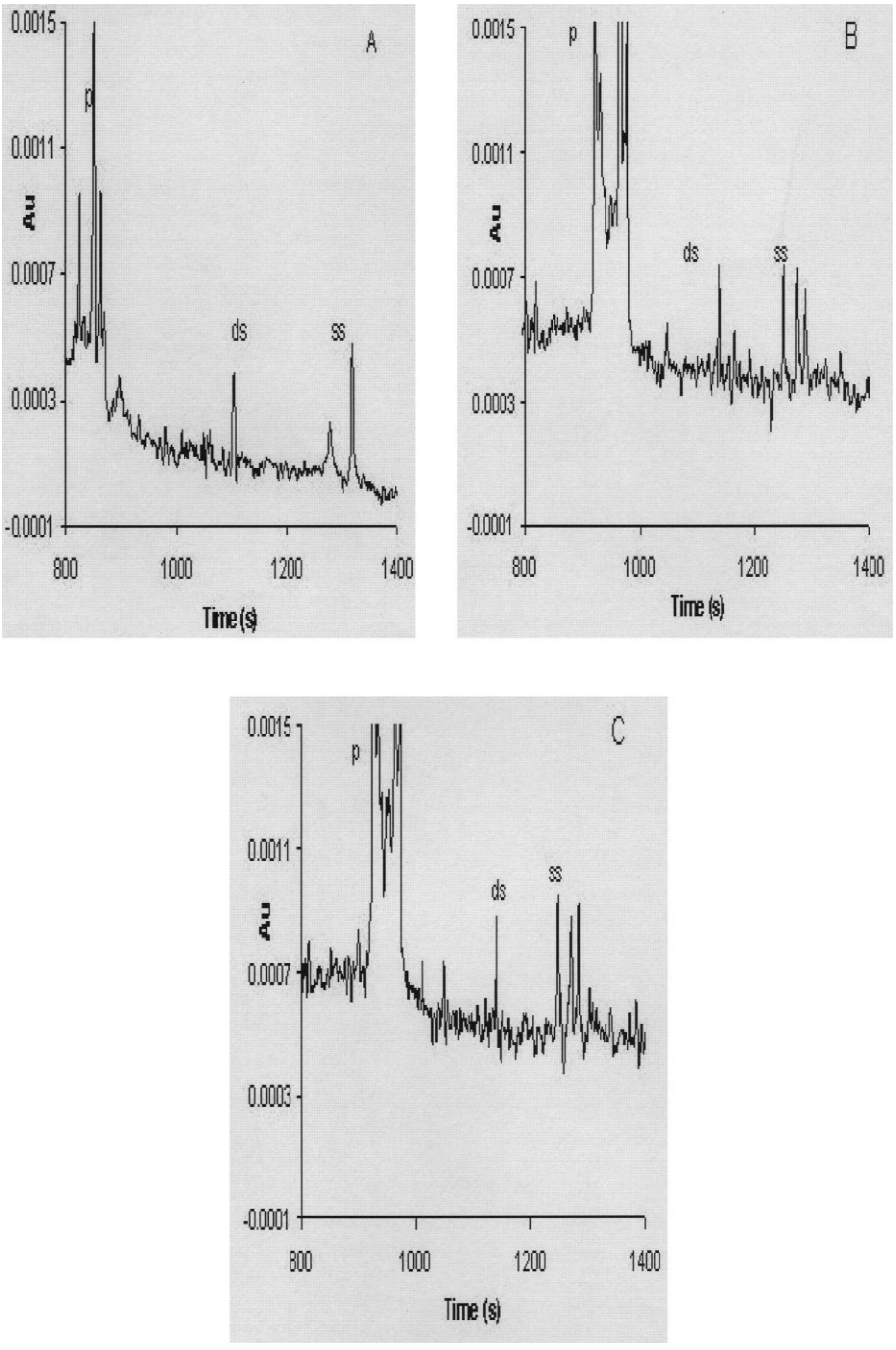

Fig. 2. Electropherogram of denatured PCR products detected by SSCP–CE: (A) normal PCR product A; (B) mixture of A and B (mutation designed in primer of B was G→A); (C) mixture of A and C (mutation designed in primer of C was G→T); (D) mixture of A and D (mutation designed in primer of D was G→C); (E) mixture of A and D separated again after a few days. SSCP–CE conditions were reported in Section 2.4.

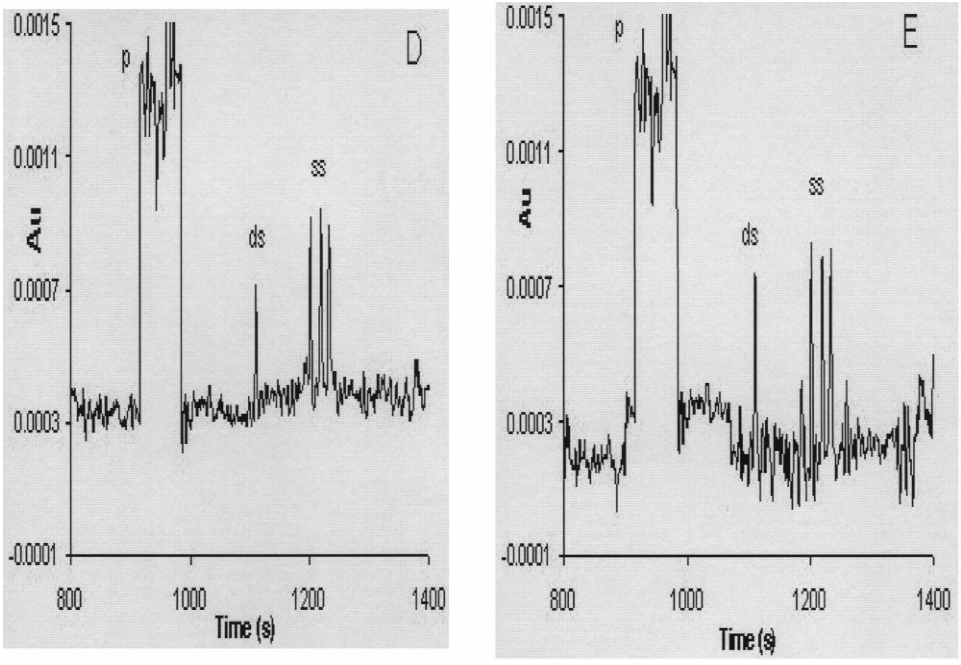

Fig. 2. (*continued*).

denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 1 min.

2.3. SSCP–SGE

PCR products of colon tumor samples (5 μl) were diluted in the loading buffer (95% formamide, 20 mmol/l EDTA pH 8.0, 0.05% xylene cyanol, 0.05% bromphenol blue) at a ratio of 1:1, then heated at 99°C for 10 min and cooled immediately in an ice-bath. Denatured solution (10 μl) was loaded in 10% neutral polyacrylamide gel (acrylamide–bisacrylamide, 49:1) and separated in 1× TBE (100 mmol/l Tris, 90 mmol/l boric acid, 1.0 mmol/l EDTA pH 8.0). All reagents were purchased from Bio-Rad (Hercules, CA, USA). Electrophoresis was performed on a Bio-Rad electrophoresis system (20 cm×20 cm×1 mm) at 100–300 V for 7 h at 25°C. The gel was then stained by $AgNO_3$ solution.

2.4. SSCP–CE

PCR products were first precipitated by isopropanol, and the precipitation was rinsed with 70% ethanol. The supernatant was filtered through a 0.22 μm filter, heated at 99°C for 10 min, and then cooled immediately in an ice-bath. Capillary electrophoresis was performed on a Beckman P/ACE MDQ (Fullerton, CA, USA). Neutral coated capillary (No. 477477, 40 cm×100 μm I.D.) and dehydrated Gel Buffer (No. 477411) were purchased from Beckman. ΦX174 RF DNA *Hae*III marker was purchased from Promega (Madison, WI, USA). The dehydrated Gel Buffer was diluted with distilled water to prepare a 4% T linear gel solution [T=(g acrylamide+g *N,N′*-methylenebisacrylamide)/100 ml solution]. The capillary was rinsed with gel solution for 180 s at 137 895 Pa. The sample was injected at 3447 Pa pressure for 10 s. The electric field strength during separation was 200 V/cm. The wavelength of the UV detector was 254 nm. The temperature of the capillary was 20°C, and that of the sample vial was 4°C.

2.5. DNA sequencing

PCR was performed as described above, and the PCR product was purified by a Promega PCR Preps DNA Purification System Kit. The sequencing

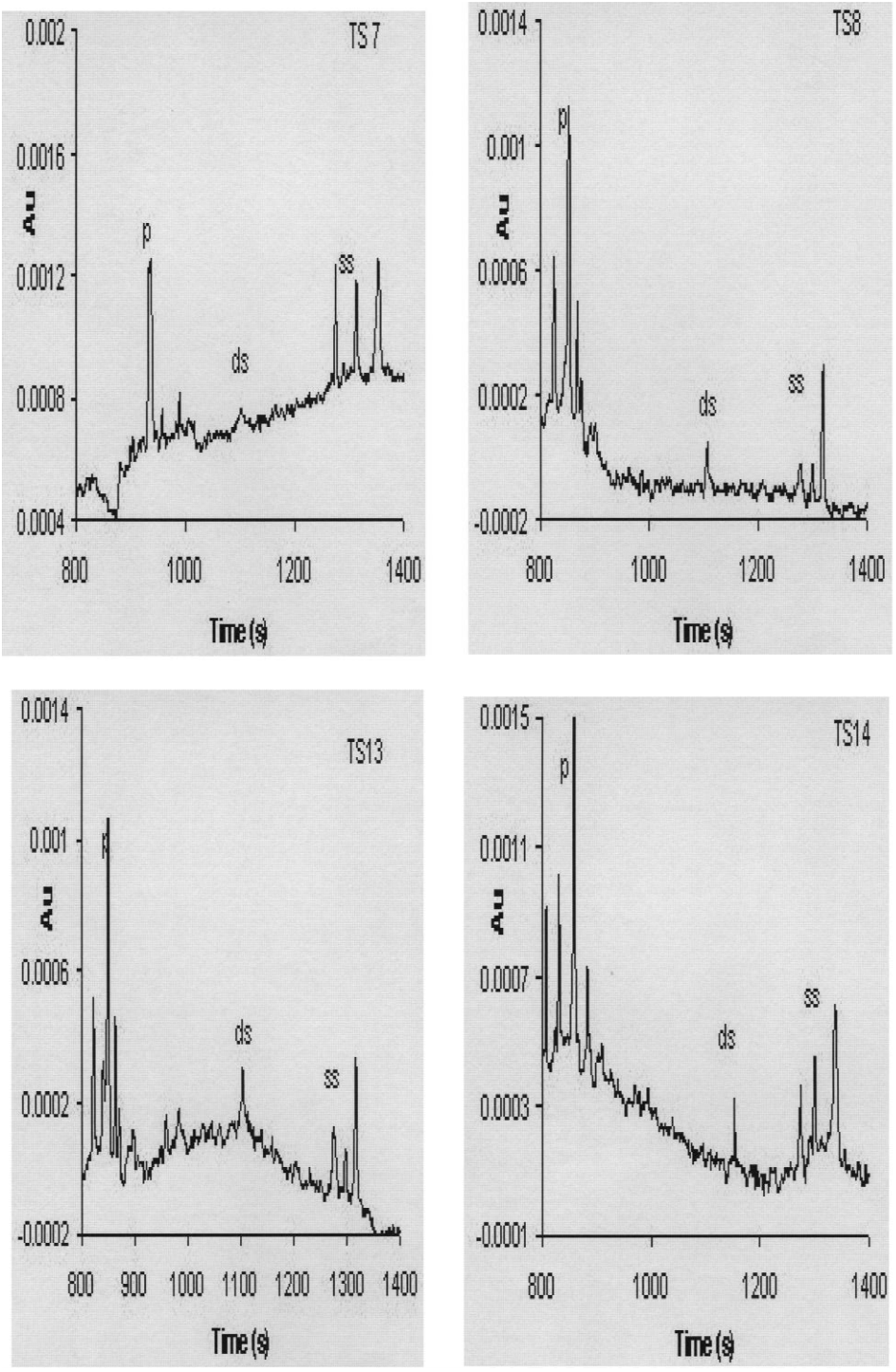

Fig. 3. Electropherogram of denatured colon tumor samples by SSCP–CE: TS7, TS8, TS13 and TS14 refer to colon tumor samples. SSCP–CE conditions were reported in Section 2.4.

reaction was performed with a Thermos Sequence II Dye Termination Cycle Sequencing Kit (Amersham Life Science, Cleveland, OH, USA). Sequencing was performed on a Perkin-Elmer Model 373A ABI.

3. Results and discussion

3.1. Establishment of SSCP–CE method

Fig. 1 shows the electropherogram of the denatured ΦX174 RF DNA *Hae*III marker separated by SSCP–CE under the optimum conditions. The result shows that the method can detect the single-strand conformation polymorphism of DNA fragments, and the detection length was longer than 1000 bases.

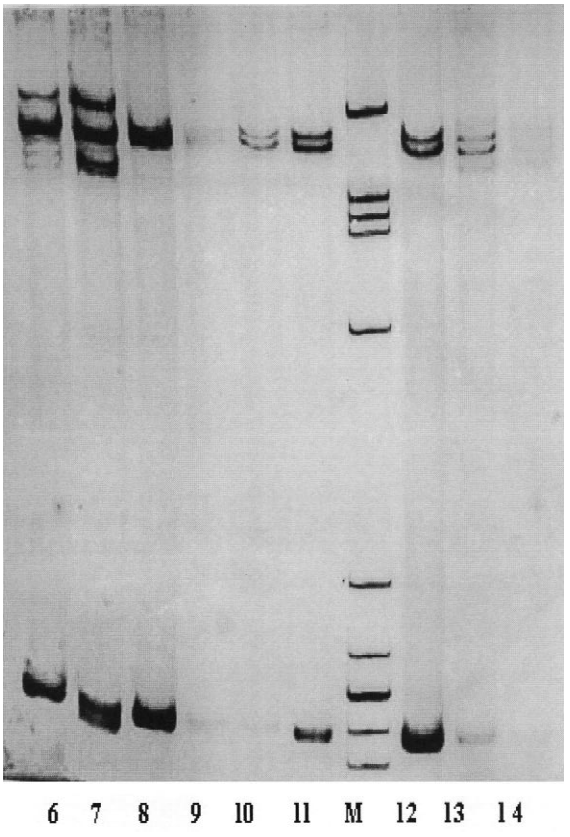

Fig. 4. Electropherogram of denatured colon tumor samples by SSCP–SGE: 6 to 14 refer to colon tumor samples and M refers to the molecular mass marker ΦX174 RF DNA *Hae*III digest. SSCP–SGE conditions as reported in Section 2.3.

Three kinds of point mutation were designed in the forward primer (Table 1), and the mutated PCR products were mixed with the normal one. Fig. 2 shows the electropherogram of these mixtures. The appearance of three peaks in Fig. 2B, C and D demonstrates that SSCP-CE could detect the point mutations (G→A, G→T, G→C), and the reproducibility of the method was rather good (Fig. 2E).

3.2. Detection of point mutations in colon tumor samples by SSCP–CE

Twenty colon tumor samples were detected by SSCP–CE and the point mutation was found in six of the samples (Fig. 3). These samples were also detected by SSCP–SGE (Fig. 4). Based on the sequencing result, it was found that the accuracy of SSCP–CE was better than that of SSCP–SGE (Table

Table 2
Statistics of point mutation in PCR products of colon tumor samples detected by SSCP–CE, SSCP–SGE and sequencing

Colon tumor sample	SSCP–CE	SSCP–SGE	Sequencing
1	−	−	−
2	−	+	−
3	−	−	−
4	+	−	+
5	+	−	+
6	−	+	−
7	+	+	+
8	+	−	+
9	−	−	−
10	−	−	−
11	−	−	−
12	−	−	+
13	+	+	+
14	+	+	+
15	−	−	−
16	−	−	−
17	−	−	−
18	−	−	−
19	−	−	−
20	−	−	−
Mutation frequency (%)	30	25	35
False-positive percentage	0	40	−
False-negative percentage	7.2	26.7	−

Human P53 Gene : 5'-CCACTACAACTACATGTGTAACAGTTCCTGCATGGGCGGCATGA
Tumor patient 7: 5'-CCACTACANGTGNATGTGTTNCNGTTNNNGCGGGGGGNGCNNGA

ACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGGTCAGGAGCCACT
AGCGNANGCTCATCNTCANCATNCTGGNAGTGGACGGGTCNGGGTCNGGNNGCNNN

TGCCACCCTGC-3'
TGTGNNCATGG-3'

Fig. 5. Sequence of the normal p53 gene from site 1880 to 1990 and colon tumor sample 7; sequencing conditions were reported in Section 2.5.

2). The false-positive percentage of SSCP–CE was 0% while that of SSCP–SGE was 40%, and the false-negative percentage of SSCP–SGE was almost four times as large as that of SSCP–CE. The result of sequencing also revealed the reason why the peak in the tumor sample 7 was so high. There were 46 point mutations in 110 bases (Fig. 5), and the mutation frequency was almost 50%.

4. Conclusions

The SSCP–CE method reported here was shown to be convenient and accurate. With this method, clinical diagnosis may soon be realized. The further development of SSCP–CE will focus on increasing the detection sensitivity and the detection of the mutation in multi-alleles at the same time.

Acknowledgements

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