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Fluorescence-based polymerase chain reaction–single-strand conformation polymorphism analysis of p53 gene by capillary electrophoresis

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Abstract

Mutation of the p53 gene plays an important role in neoplastic progression in human tumorigenesis. Polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) techniques are now available for the detection of point mutations. The original method using polyacrylamide gel electrophoresis is disadvantageous, particularly for clinical tests and for analysis of large numbers of samples. Therefore, using an automated capillary electrophoresis (CE) technique with a molecular-sieving polymer solution, we have devised a completely automatic fluorescence-based PCR–SSCP system (CE–FSSCP) for the differential detection of point mutations that dose not require SSCP with radioisotopes and polyacrylamide gels. This automatic CE–FSSCP system was developed for reproducible operations in the denaturation of double-stranded DNA and electrophoresis of single-stranded DNA. The detection system consists of a 100 W I_2 lamp and photomultiplier. We performed CE–FSSCP with a 2% linear polyacrylamide polymer solution containing 5% glycerol. Four tissue specimens of lung tumors with mutations in exon 7 of the p53 gene were found to have mutant alleles; a six-base-pair deletion at codons 247–248, a one-base-pair deletion at codon 260, a one-base-pair deletion at codon 244 and a GGC to CGC substitution at codon 244. We expect this technique to prove useful for the clinical DNA diagnosis of human cancers, determination of the therapeutic effect of anticancer agents and for the study of the molecular aspects of the mechanisms involved in the pathogenesis of human cancers.

Keywords: Genes; Polymerase chain reaction; Detection, electrophoresis; Proteins; DNA

1. Introduction

Tumor suppressor genes are thought to actively prevent the development or progression of various types of tumors of diverse tissue origin [1]. An essential feature of this model is the assumption that the functions of tumor suppressors are lost or altered in some way in tumors compared with those in the normal tissue from which the tumor originated [2]. The characteristics of three clear candidate tumor suppressor genes isolated thus far tend to support this assumption. The most well studied of these is the human retinoblastoma susceptibility gene (RB). The RB gene is frequently rearranged or mutated in many different types of tumors, suggesting that its protein product may play a central role in controlling cell growth in most, if not all, tissues [3]. The second gene, DCC, encodes a protein with similarities to cell

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adhesion molecules and may function to maintain normal cell–cell interactions [4]. The oncoprotein p53 is the third protein with some of the characteristics of a tumor suppressor. Evidence has recently been obtained that mutation of the p53 gene plays a major role in the pathogenesis of a variety of human tumors. The p53 gene is frequently mutated in a wide variety of human cancers [5]. The presence of mutations has been detected in exons 5 through 8, which contain 86% of all mutations reported for the p53 gene [6].

Polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) techniques are now available for the detection of point mutations of the p53 gene. PCR–SSCP analysis is a rapid and efficient method for the detection of mutations and polymorphisms in genomic or cDNA sequences [7]. In the original method, the target sequence is amplified and radioisotopically labeled simultaneously and then separated by polyacrylamide gel electrophoresis in a single-stranded state [8]. Mutations are detected as mobility shifts caused by secondary conformational changes of single-stranded amplification products. However, mutation detection with radioisotopes has a disadvantage in that it is complex and time-consuming. It is therefore inadequate for use, particularly for clinical tests or for the analysis of large numbers of samples. Several groups have demonstrated that SSCP analysis can be performed by electrophoresis in small gels with detection of bands by silver-staining [9–11] and by ethidium bromide-staining [12]. These nonradioactive methods are convenient for detecting some mutations, but it is probably impossible to develop a completely automatic SSCP system using them because of the requirement for slab gel electrophoresis.

Recent studies have shown that CE is not only a rapid and convenient method for the analysis of PCR products, but that it can be used in a completely automatic SSCP system. Due to the temperature sensitivity of the secondary structure of single-stranded DNA fragments, accurate temperature control is required for reproducibility of SSCP. Efficient joule heat dissipation can be achieved in CE due to the forced cooling of the outer surface of the capillary. Moreover, operations such as column preconditioning, sample injection, electrophoresis and detection proceed automatically in CE, if an autosampler is used. This means that CE is suitable for automatic and reproducible gene analysis. DNA mutation detection with CE has progressed rapidly in both screening and diagnostics methodologies. PCR–RFLP studies have been reported by several groups [13–15]. In addition, SSCP has been used with both denaturing and nondenaturing gel-filled CE [16] and entangled solution CE [17–20]. In this study, using a fluorescence-based PCR technique, we have devised a completely automatic CE–FSSCP system with molecular-sieving polymer solutions, a simple and rapid procedure for the differential detection of point mutations and which does not require SSCP with radioisotopes and polyacrylamide gels.

2. Experimental

2.1. Human tissue samples and DNA extraction

Sixty-three specimens of lung cancer were obtained from the Respiratory Disease Center, Kyoto Katsura Hospital. Cancer specimens and nornnal tissues were frozen in liquid nitrogen and stored at −80°C. Genomic DNA was extracted from tissues by proteinase K digestion and phenol–chloroform extraction using the method of Sambrook et al. [21], with minor modifications.

2.2. Primers

Oligonucleotides were synthesized as primers for PCR, based on the published p53 gene sequence for exon 7 flanking the intron/exon [22]. The designations and sequences for each primer were as follows: EX-07f, 5′-TCTTGGGCCTGTGTTATCTC-3′; EX-07r, 5′-AGGGTGGCAAGTGGCTCC-3′; EX-F07r, F-5′-TCTTGGGCCTGTGTTATCTC-3′. The number in each designation indicates the region of the exon of the p53 gene subjected to examination by PCR–SSCP or PCR–FSSCP analysis. The letters "f" and "r" indicate forward and reverse primers, respectively, for each region. The reverse primers were labeled at their 5′ ends with fluorescein derivatives by the Fluore method (Pharmacia Biotech, Uppsala, Sweden). "F" indicates carboxyfluorescein linked to the 5′-nucleotide via a linker and phosphate.

2.3. PCR–SSCP analysis

The target sequences were amplified by PCR in 50 μl of solution containing a 1 μM solution of the primers, 100 μM concentrations of dATP, dCTP, dGTP and TTP, 100 ng of human genomic DNA and 0.125 units of Amplitaq (Perkin-Elmer Roche, Branchburg, NJ, USA) in the buffer recommended for the enzyme. All PCR reactions were performed in a Robo-Cycler (Stratagene, La Jolla, CA, USA) over 40 cycles. Each reaction cycle included denaturation at 95°C for 1 min, primer annealing at 56°C for 1.5 min, and primer extension at 72°C for 1.5 min. A 10-μl volume of the PCR products was diluted two-fold with a buffer consisting of 20 mM EDTA, 96% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol, heated at 95°C for 5 min and then placed on ice for 5 min. Then 10 μl of this solution was applied to each lane of a 7.5% neutral small polyacrylamide gel (9×8 cm). Electrophoresis was performed at 5 V/cm for 3 h. The gel was stained with ethidium bromide and visualized under UV light.

2.4. Cloning and DNA sequencing of PCR products

PCR using EX-07f/EX-07r as primer pairs was performed as described in Section 2.3. The amplified fragments were cloned in the TA cloning site of the pCR II cloning vector (Invitrogen, San Diego, CA, USA) using T4 DNA ligase (Takara Shuzo, Kyoto, Japan). Approximately twenty recombinant colonies were selected and cultured in TB (Terrific Broth) medium. Sequence analyses of these PCR-amplified fragments in the cloning vector was performed using the ALF II automated DNA sequence analyzer with M13 primers (Pharmacia Biotech).

2.5. PCR–FSSCP analysis using an automated CE system

The automatic CE–FSSCP system was developed for reproducible operations in the denaturation of double-stranded DNA and electrophoresis of single-stranded DNA. A diagram of the system is shown in Fig. 1. Twenty sample tubes 500 μl in site and commercially available can be placed in a sample carrousel. A motor-driven arm picks up one of the sample tubes and carries it to a heated hole at 95°C. The tube remains there for a period of time to denature the double-stranded DNA fragments and is then moved (by the motor-driven arm) to a 0°C cooling hole, to prevent reanealing of single-stranded DNA, followed by placement in the inlet carrousel for sample injection. All procedures are controlled by parameter input to the software system and are completely automated. The periods of heating and cooling are chosen within the range of 0–10 min. For electrophoresis, a high-voltage electric field in the range of −30 kV to +30 kV, at up to 300 μA, is applied with a high-voltage power supply (HCZ 30PN-OW Matsusada Precision, Shiga, Japan), while the capillary cassette is thermostatted by forced-air controlled between 10 and 45°C, with an accuracy of ±0.1°C. The capillary cassette used was fitted with a 75 μm-I.D. synthetic silica capillary (CE0121, Otsuka Electronics, Osaka, Japan) of 300 mm in length and 225 mm to the detector. The detection system consists of a 100 W I_2 lamp (MHF-D100LR, Moritex, Tokyo, Japan) as a light source, and the exciting light is focused on the capillary cell with a ball lens (Moritex) through an optical fiber (Super Eska SH-4001, Mitsubishi Rayon, Tokyo, Japan) and a bandpass filter of 480 nm center and 11 nm width (Barr Associates, Westford, MA, USA). The emitted light is received by a photomultiplier (Hamamatsu Type H5783 P, Hamamatsu Photonics, Shizuoka, Japan) through a sharp-cut filter of 550 nm (OG550, Schott Glaswerke, Mainz, Germany). The range of detection of this optical system was examined using fluorescein (Wako, Osaka, Japan) in a 1×TBE (90 mM Tris, 90 mM boric acid and 2 mM EDTA, pH 8.5)-filled capillary. The S/N ratio of a $5 \cdot 10^{-7}$ M fluorescein solution was estimated to be 13.5 when the signals were collected at 1 s intervals. Linearity of sensitivity was obtained in the range of $2 \cdot 10^{-8}$ to $2 \cdot 10^{-5}$ M. The inner surface of the capillary was coated with polyacrylamide using the method of Hjertén [23] in order to suppress the electroosmotic flow. PCR using the EX-07f/EX-F07r primer pair, with the reverse primer labeled at the 5′ end with fluorescein, was performed as described above. A 100-ng amount of genomic DNA was amplified in a total volume of 50 μl in the buffer recommended by Perkin-Elmer Roche. The PCR products were diluted

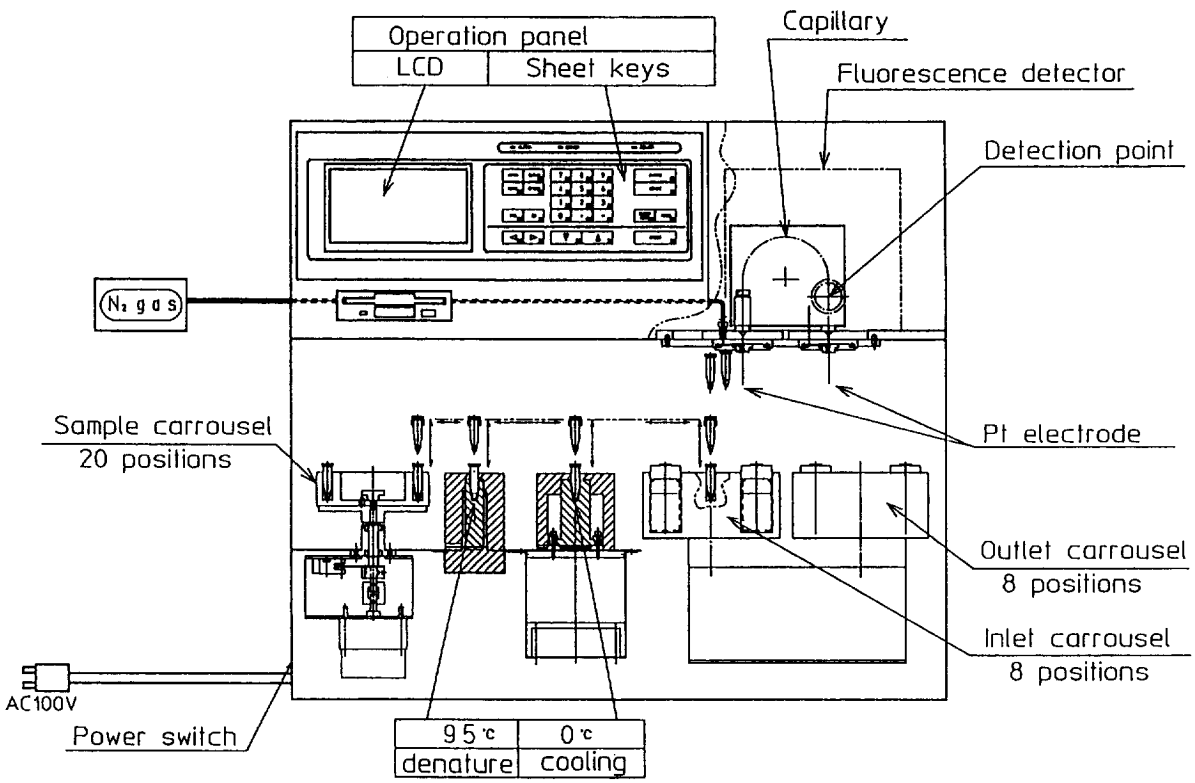

Fig. 1. An automatic CE–FSSCP system.

two-fold with a formamide buffer, heated at 95° for 5 min, placed at 0°C for 5 min and then injected at low pressure (0.5 p.s.i.; 1 p.s.i.=6894.76 Pa) for 30 s. The separation was carried out at 10 kV with a system current of 15 μA and the was capillary maintained at 25°C. The molecular-sieving polymer was 1×TBE containing 2% polyacrylamide polymer or 2% polyacrylamide polymer with 5% glycerol. The polyacrylamide (M_r=700 000–1 000 000) solution (10% in water) was purchased from Tokyo Kasei (Tokyo, Japan).

3. Results and discussion

3.1. PCR–SSCP analysis using polyacrylamide gels

Exon 7 of p53 was amplified from DNA extracted from specimens obtained from 63 patients with lung cancer. The PCR products were subjected to PCR–SSCP analysis using small polyacrylamide gels. The results of PCR–SSCP analysis of exon 7 are summarized in Fig. 2. Mutations were observed in 4 of the 63 cases, in patients 104, 106, 137 and 232. The migration pattern of the normal p53 allele from normal lung tissues is shown in N. In the tumor DNA (T), a new band of allelic single-stranded DNA molecules appeared, corresponding to mutated alleles. Moreover, for patient 137, we were able to detect a band of single-stranded DNA corresponding to a mutated allele and a band of heteroduplex DNA (hetero).

3.2. Cloning and DNA sequencing

The results obtained by PCR–SSCP analysis were confirmed by DNA sequencing. The fragments of the mutated allele of the p53 gene were amplified and cloned, and mutated clones were subsequently selected by PCR–SSCP. Sequences were analyzed for three clones thought to contain mutated alleles, based

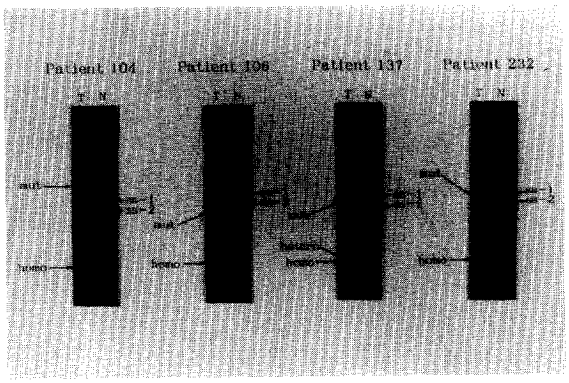

Fig. 2. PCR–SSCP analysis using polyacrylamide gels. PCR products were electrophoresed in a 7.5% polyacrylamide gel and stained with ethidium bromide. Mobilities of DNA fragments amplifed from tumor samples (T) were different from those amplified from normal tissue samples (N) in exon 7. Homo, hetero, ss-1 and ss-2 indicate the positions of homoduplex, heteroduplex and single-stranded DNA molecules. The positions of strands of mutated alleles are indicated by mut.

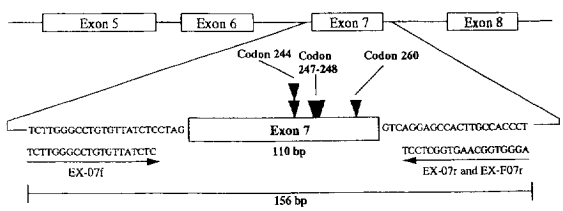

Fig. 3. Schematic representation of mutation sites detected by sequence analysis. EX-07f, EX-07r and EX-F07r were synthesized as a sense primer and antisense primers. The EX-F07r to be used for CE–FSSCP analysis is labeled at the 5′-end with fluorescein. The mutation sites are shown as triangles.

on results of SSCP analysis. The results of sequence analysis are shown in Table 1. Patient 104 exhibited a deletion of six nucleotides at codons 247–248, patient 106 had a deletion of one nucleotide at codon 260, patient 137 had a deletion of one nucleotide at codon 244 and patient 232 had a substitution from glycine (GGC) to arginine (CGC) at codon 244. The presence of these mutations was detected in codons 244 through 260, corresponding to the latter half of exon 7 (Fig. 3).

3.3. Analysis using an automatic CE–FSSCP system

Polyacrylamide gel electrophoresis of DNA is a widely used high-resolution method of analysis. However, in the capillary electrophoresis format, shrinkage of the gels result in formation of bubbles saturated with water vapor, particularly at high field strengths. These drawbacks preclude the repeated use of a gel-filled capillary and thereby prevent automation of runs. Therefore, molecular-sieving polymer solutions have become very popular as substitutes for gels. They are replaceable, i.e., the same capillary can be used in many experiments, and after each run the used polymer solution is sucked or pressed out of that capillary and replaced by a fresh one. We have devised a simple and rapid procedure using an automatic CE–FSSCP system with molecular-sieving polymer solutions. We measured the temperature changes that took place when a 50-μl volume of water in a sample tube was transported automatically from the sample carrousel, to a heated hole, a cooling hole and to the inlet carrousel for electrophoresis with a thermocouple in sample solution. The result shows corresponding temperature transitions to those observed with quick manual operations using a hot water bath and iced water (data not shown).

Table 1
Alterations of exon 7 of the p53 gene

Patient	Codon	Mutation	Amino acid change
232	244	GGC to CGC	Gly to Arg
137	244	one-base pair deletion	Frame shift
104	247–248	six-base pair deletion	Deletion
106	260	one-base pair deletion	Frame shift

Four mutated DNA samples (patients 104, 106, 137 and 232) were analyzed using the automatic CE–FSSCP system. We checked for the presence of mutations in exon 7 using a reverse primer (EX-F07r) with a fluorescein moiety attached to an extra G residue. The PCR products were diluted two-fold with a formamide buffer to prevent re-annealing of single-stranded DNA and then were subjected to CE–FSSCP analysis. Fig. 4 illustrates the CE–FSSCP electropherograms of the PCR-amplified mixture in a 225 mm (effective length)×75 μm capillary equilibrated with 2% polyacrylamide polymer solution. The DNA from normal tissue (normal) had a peak for non-reacted primer (n-primer), a peak

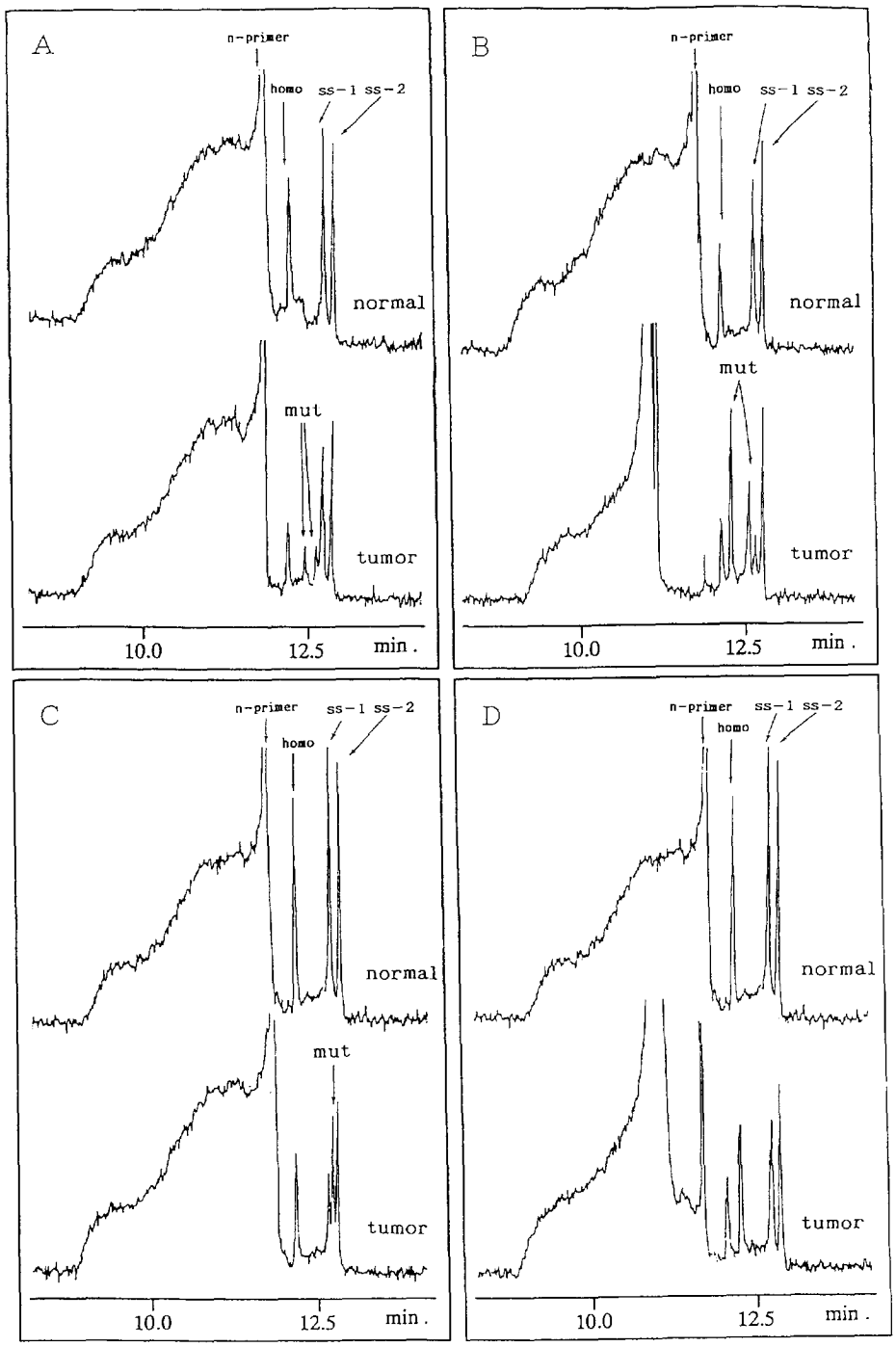

Fig. 4. CE–FSSCP analysis using a 2% polyacrylamide polymer system. DNA samples examined were from cancer tissue (tumor) and normal tissue (normal) from patients 104, 106, 137 and 232. For patients 104 (A), 106 (B) and 232 (C), cancer tissue samples yielded a DNA peak in addition to the peak produced by normal tissue samples, but this new peak was not detected for patient 137 (D). Homo, ss-1 and ss-2 indicate positions of homoduplex and single-stranded DNA molecules. The peaks of mutated alleles are indicated by mut.

for homoduplex DNA molecules (homo) and two peaks for single-stranded DNA molecules (ss-1 and ss-2) for all normal samples from four patients in Fig. 4. Electropherographic patterns are quite similar among the four patients, since only the antisense strand amplified with a reverse primer is fluorescently labeled, one peak of single-stranded DNA is expected in fluorescence detection. However two peaks of single-stranded DNA could be observed. Two possible explanations are considered. First, the second peak of single-stranded DNA (ss-2) may reflect the linear shape of the strand without secondary structure, although the first one (ss-1) has the secondary structure characteristics of the strand. Second, two peaks may corespond to two different stabilized states of secondary structure, a possibility that is less likely than the first prospect. In contrast, in patients 104 and 106, DNA from tumor tissue (tumor) had four peaks for single-stranded DNA molecules. Two of these peaks for the tumor DNA migrated to the same positions as for the normal DNA and the two peaks indicated by "mut" were mutated p53 alleles (Fig. 4A–B). In patient 232 (Fig. 4C), a new peak appeared in the tumor DNA, corresponding to mutated p53 alleles. However, for patient 137 we did not detect peaks for mutated p53 alleles (Fig. 4D). Under the conditions we used, the non-reacted primers were observed as a broader peak with a fronting shoulder. In particular, the patterns on electropherograms of the region of the primers are not similar for tumor samples from patient 106 (Fig. 4B, lower) and patient 137 (Fig. 4D, lower). We cannot interpret the reason for this irregularity of the primer, but the matrix of the samples may make the migration behavior of the primer unstable in the sieving medium of 2% polyacrylamide.

To perform the task shown in Fig. 4, a polymer buffer system was chosen and optimized. On testing of several polymer buffer systems, the glycerol buffer system was found to be ideal for CE–FSSCP. Polyacrylamide gel electrophoresis containing glycerol is widely used for PCR–SSCP analysis. The conformation of single-stranded nucleic acid is presumably determined by the balance between thermal fluctuation and weak local stabilizing forces such as short intra-strand base pairings and base stackings. Therefore, changes in environmental conditions such as temperature and the presence of denaturant are likely to cause changes in conformation, which can be detected in SSCP analysis as alterations in mobility [8]. In the polymer-sieving mode of CE, the addition of glycerol has been demonstrated to enhance resolution, especially in the presence of borate [24]. Fig. 5 illustrates the electropherograms obtained using sieving medium of 2% polyacrylamide containing 5% glycerol. The peak for non-reacted primer (n-primer) was sharper, with no fronting shoulder, both for normal and tumor samples from all four patients. The electropherograms show the same pattern and the peak resolution is improved. The addition of glycerol to the sieving buffer apparently fixes the migration properties of each nucleic acid components. In patients 104, 106 and 232 (Fig. 5A–C), for tumor DNA, new peaks appeared, corresponding to mutated p53 alleles. Moreover, for patient 137 (Fig. 5D), a mutated p53 allele was observed as a peak of single-stranded DNA molecules (mut) and as a peak of heteroduplex DNA molecules (hetero). The results of analysis using the two methods are outlined in Table 2. As can be seen, there was a 100% correlation between the results obtained with PCR–SSCP analysis by polyacrylamide gel electrophoresis and those from CE-FSSCP analysis using 2% polyacrylamide polymer buffer containing 5% glycerol. It is our belief that the improved separation of DNA fragments achieved with this polymer buffer system is due not only to the effect of a change in the conformation but also to an interaction between glycerol and other components in the buffer. Cheng and Mitchelson [24] proposed that when glycerol is introduced into the (hydroxypropyl)methylcellulose (HPMC) buffer, dimeric spirane complexation reactions may occur between glycerol, borate and HPMC. The first such reaction is the attachment of glycerol molecules to the HPMC polymers at borate–monodiol intermediates, by reaction of glycerol with the free hydroxyl groups of the tetraborate. The second is the formation of glycerol–borate multimers that may further cross-link HPMC complexes to reduce the effective pore size of the polymer network. We suspected that the influence of glycerol on polyacrylamide polymer buffer containing borate is essentially the same as that described for the interaction between glycerol, HPMC and the borate buffer system.

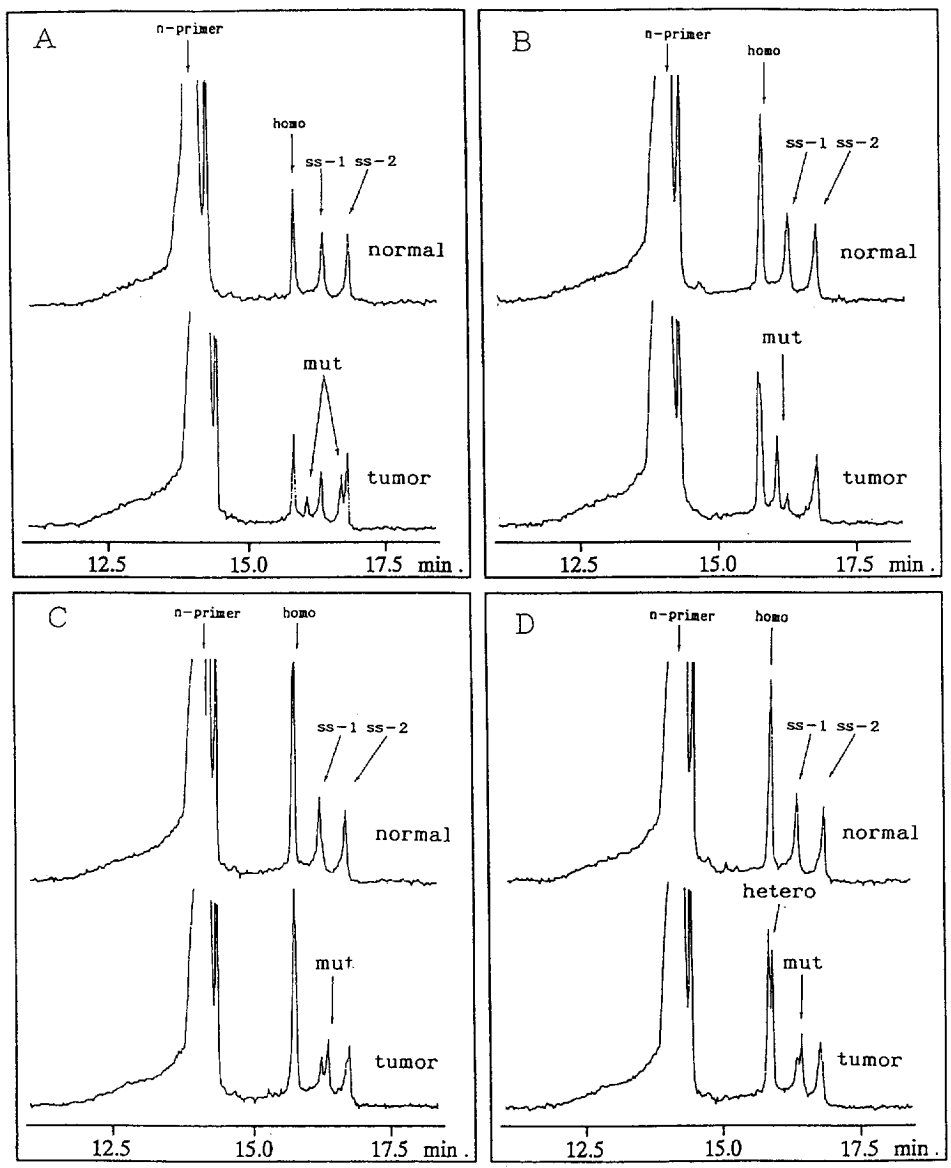

Fig. 5. CE–FSSCP analysis using a 2% polyacrylamide polymer system with 5% glycerol. Mobilities of DNA fragments amplified from cancer tissue samples (tumor) were different from those amplified from normal tissue samples (normal). Moreover, a peak corresponding to heteroduplex DNA molecules (hetero) was detected for patient 137 (D). Homo, ss-1 and ss-2 indicate positions of homoduplex and single-stranded DNA molecules, respectively. The position of strands of mutated alleles are indicated by mut.

Mutation detection by PCR–SSCP with CE has already been reported with the use of UV detection [16–19] and laser-induced fluorescence (LIF) [20]. The disadvantages of UV detection are non-selectivity between sense and antisense strands and relatively low sensitivity. Non-selectivity results in complicated patterns on electropherograms that are unfavorable for clinical examination. Fluorescence detection with labeling of one of the primers with a fluorescent dye simplifies the peak pattern to be identified. Most gene analysis with CE requiring high sensitivity can use LIF for detection. The system we developed uses

Table 2
Comparison of the detection of exon 7 mutations of the p53 gene using CE–FSSCP and PCR–SSCP analysis

Patient	PCR–SSCP result	CE–FSSCP result	
		without glycerol	with 5% glycerol
104	ss DNA	ss DNA	ss DNA
106	ss DNA	ss DNA	ss DNA
137	ss DNA+hetero	ND	ss DNA+hetero
232	ss DNA	ss DNA	ss DNA

ss DNA: The mutated alleles were observed as the peaks of single-stranded DNA.
hetero: The mutated alleles were observed as the peaks of hetero duplex DNA.
ND: not detected.

fluorescence detection with a cheap I_2 lamp, and the excitation wavelength is tunable with an appropriate bandpass filter. The sensitivity is less than that of LIF detection, but is approximately 100 times higher than that of UV detection. Accordingly, our system may be useful for clinical examinations. Moreover, the CE–FSSCP system features an automatic heating–cooling process to melt double-stranded DNA molecules instead of manual handling of samples, as reported in previous studies. CE–FSSCP analysis can be performed automatically and sequentially for twenty samples.

4. Conclusions

The results of the present study show that CE can be used to produce an automated PCR–FSSCP system. CE–FSSCP not only enables rapid, automated analysis, but direct entry of data into a computer is advantageous when a large number of PCR products must be analyzed. Moreover, results can be interpreted quantitatively, since the bands are detected as peaks in fluorograms. In clinical testing, large numbers of tumor and normal tissue samples must be analyzed. This task is currently performed primarily by visual inspection and interpretation of patterns on X-ray film and ethidium bromide- or silver-stained polyacrylamide gels. This entire process will be much easier and less error-prone when data are entered directly into a computer, interpreted there, and processed for calculation. Future development of software for the automated CE–FSSCP analysis should include allele identifcation, an interface for transfer of data to other applications and possibly normalization of mobility.

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