Single Strand Conformation Polymorphism Analysis of Ras Oncogene by Capillary Electrophoresis with Laser-Induced Fluorescence Detector

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Single strand conformation polymorphism (SSCP) analysis of the N-ras oncogene was achieved by capillary electrophoresis with a laser-induced fluorescence detector (CE-LIF) using methylcellulose as a molecular sieving agent. The PCR-amplified N-ras oncogene, which is known to have a point mutation at codon 61 in the neuroblastoma, was investigated by CE-LIF combined with SSCP (SSCP-CE-LIF). A mixture of wild- and mutant-type single strand DNA fragments (103 bp) of the N-ras oncogene was separated by buffer solution containing 1.0% methylcellulose and 0.2 μM fluorescent dye (YO-PRO-1) at 25°C.

The SSCP-CE-LIF technique gave good resolution for wild- and mutant-type single strand DNA fragments with separation completed within 7 min. SSCP analysis using a CE system with a LIF detector was successfully applied to the detection of the one point mutation on the N-ras oncogene.

Key words capillary electrophoresis; single strand conformation polymorphism; ras oncogene; PCR

Capillary electrophoresis (CE) is rapidly becoming an important tool for the analysis of the polymerase chain reaction (PCR) amplified products of DNA and for DNA sequencing in place of slab gel electrophoresis, owing to its high resolving power and high speed separation.1—7) We have reported a technique for gene diagnosis of heart disease through human apolipoprotein B genotyping using CE with a laser-induced fluorescence detector (CE-LIF).8) CE-LIF is an important technique for the highly sensitive analysis of DNA fragments of low concentration offering high sensitivity, resolution, accuracy, and speed. We applied the CE-LIF technique to the single strand conformation polymorphism (SSCP) analysis of the N-ras oncogene. Although SSCP analysis by capillary gel electrophoresis (CGE) was reported by Arakawa et al.9) CGE with a UV detector has many disadvantages in sensitivity, reproducibility, and manipulation to fill the capillary with gel compared with CE-LIF using methylcellulose (MC) solution. Several groups have also demonstrated that SSCP analysis can be performed by CE-LIF.10—15) In their systems, however, samples were amplified using primers labeled with one or two dyes,10—14) or using samples post-labeled with two different dyes.15) In the present study, N-ras gene was amplified without primer labeled with fluorescent dye. Furthermore, we used MC as polymer solution instead of linear polyacrylamide (LPA) which is conventionally used in SSCP analysis.10—12,16) MC solution is superior to LPA in terms of handling because of low viscosity, repeatability, and easy preparation.

The N-ras oncogene is mapped to the short arm of chromosome 1 and is known to have a point mutation in codon 12 and 61, which are observed in various tumors such as leukemia and neuroblastoma. As shown in Fig. 1, the wild-type DNA has a codon CAA coding for glutamine (Gln), while the mutant-type DNA has a codon coding for various amino acids due to the difference of one base between the wild- and mutant-types. The mutation of the N-ras oncogene is now considered critical to human leukemogenesis.17,18)

The SSCP method is a superior technique for the detection of mutation of DNA.19) This method is based on the fact that a single strand DNA (ssDNA) molecule has a helix structure, which depends on its size and its sequence. A DNA molecule containing a mutation, even a single base substitution, will have a mobility shift during electrophoresis different to that of the wild type. Because the electrophoretic mobility of the ssDNA molecule in non-denaturing conditions strongly depends on its conformation, mutant- and wild-type ssDNA molecules would be separated by the CE-LIF technique in MC solution. Thus, we attempted the separation of SSCP containing a point mutation using the CE-LIF technique in polymer solution including intercalating dye. To improve the separation of ssDNA molecules, we investigated several parameters, electric field, running temperature, MC concentration, and intercalating dye concentration, which are known to have an influence on the electrophoretic mobility of ssDNA.

We have established a technique for the high performance DNA diagnosis of tumors through point mutation of the N-ras oncogene by the CE-LIF technique using an intercalating dye and a polymer solution. This technique is superior to slab gel electrophoresis and conventional SSCP analysis by the CE technique in sensitivity, reproducibility, speed, and handling.

Experimental

Wild-type and some mutant-type DNA fragments (103 bp) of N-ras oncogene, which have different mutations in codon 61, were amplified by PCR using the N-ras gene primer set (Takara). The genomic DNA of N-ras gene

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Fig. 1. Mutation of N-ras Oncogene
was the contents of the ras mutant set purchased form Takara. The oligonucleotide primers were 20 nucleotides in length. The sequence of the 5' -PCR primer was 5'-GGTGAAACCTGTTTGTTGGA-3'. That of the 3'-PCR primer was 5'-ATACACAGAGGAAGCCTTCG-3'. The PCR mixture (100 μl) contained 20 pmol of each primer, 2.5 mM of the four deoxynucleotide triphosphates (dNTPs), and 2.5 U of Taq DNA polymerase. PCR was performed on a thermal cycler (PHC-3, Techne Inc., Princeton, NJ, U.S.A.) with 35 cycles of amplification (denaturation: 1 min at 94 °C, annealing: 2 min at 55 °C, and extension: 1 min at 72 °C). The length of the generated PCR fragment was checked by CE using a 20bp DNA ladder (Gensura Laboratories Inc., CA).

The PCR products were purified using SUPREC™-02 (Takara) to remove excess primers and dNTPs. The purified DNA samples were diluted 10—100 fold with MilliQ water and stored at −218 °C until use. Prior to CE separation the sample DNA (double strand DNA; dsDNA) was heated at 99 °C for 5 min, quenched, and kept on ice for more than 5 min to give ssDNA. All other chemicals were of analytical reagent or electrophoretic grade from Wako (Osaka, Japan).

A P/ACE 2050 CE system equipped with a LIF (Beckman Instruments, Inc., Fullerton, CA, U.S.A.) was used with negative source polarity. The laser source was operated at a power of 3 mW, excitation was at 488 nm and a 520 nm band pass filter was used as an emission cut off filter. A DB-17 coated capillary (360 μm o.d., 100 μm i.d.; J & W Scientific, Folsom, CA) of 20 cm effective length and 27 cm total length was used. The running buffer solution used in the present study was 50 mM Tris–borate (TB). To 10 ml of the buffer solution were added 0.5—1.0% MC and 1—10 μl of fluorescent dye 1-(4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylidene]-quinolinium)-3-trimethyl-ammonium propane diiodide (YO-PRO-1) which was intercalated into part of the double strand of SSCP. Denatured samples were electrophoretically introduced into the capillary at a negative polarity of 5.4 kV for 10—20 s and were run with running buffer solution containing MC and YO-PRO-1 at a negative polarity of 2.16—6.75 kV (80—250 V/cm). The temperature of the capillary was altered at 15—40 °C. DNA fragments were detected at 520 nm (Ex.; 488 nm).

Results and Discussion

Figure 2 shows the results of SSCP analysis of the wild- and three mutant-type ssDNA fragments of N-ras oncogene by the CE-LIF technique. Two peaks corresponding to ssDNA were observed at around 7 min in each electropherogram. The peaks indicate the sense and anti-sense ssDNA obtained by the denaturation of the N-ras oncogene. The peak at around 7.5 min in Figs. 2B, C, and D corresponded to the residual double strand DNA (dsDNA). The peak of dsDNA is thought to be due to the incomplete thermal denaturation of crude dsDNA at 99 °C and the formation of double strand DNA on cooling. However, the ssDNAs of the wild- and mutant-type N-ras oncogenes were completely separated by CE-LIF. Furthermore, only two peaks were also obtained in the analysis of ssDNA of other mutants, except dsDNA. The discrepancy in the peak height of the two ssDNA in Figs. 2B, C, and D is considered due to the difference in the amount of YO-PRO-1 intercalated into the double strand of ssDNA, since the same peak height was obtained by UV detection without YO-PRO-1. These results indicate that the

![Fig. 2. CE Separation of ssDNA Obtained from N-ras Oncogene](image)

Capillary: 100 μm i.d., 375 μm o.d., total length 27 cm, effective length 20 cm. Running buffer: 50 mM Tris-boric acid, 1.0% MC and 0.2 mM YO-PRO-1. Temperature: 25 °C. Field: 200 V/cm. Injection: 5.4 kV for 15 s. Detection: Ex., 488 nm, Em., 520 nm. Sample: A) wild-type ssDNA, B) mutant (proline)-type ssDNA, C) mutant (arginine)-type ssDNA, D) mutant (leucine)-type ssDNA.

![Fig. 3. Effect of Electric Field on the Separation of the Mixture of Wild- and Mutant-Type ssDNAs](image)

Field: A) 80 V/cm, B) 150 V/cm, C) 200 V/cm, D) 250 V/cm. Sample: wild-type ssDNA + mutant (proline)-type ssDNA. Other conditions are as in Fig. 2.
one point mutation of N-ras oncogene can be detected, if there is a difference in the mobility of wild- and mutant-type ssDNAs. Thus, we examined the separation conditions for SSCP analysis of N-ras oncogene by the CE-LIF technique in detail to achieve high performance gene diagnosis for cancer through the mutation of N-ras oncogene.

The Effect of Electric Field on the Separation of ssDNA

To begin with, we investigated the effect of the electric field on the CE separation. Figure 3 shows the effect of the applied fields (80—250 V/cm) on the separation of the mixture of wild- and mutant-type ssDNAs. Four peaks were assigned by changing the mixing ratio (wild-type ssDNA vs. Pro mutant-type ssDNA). As can be seen, the migration time of each ssDNA decreased with the increase of applied field. If the mixture of wild- and mutant-type ssDNAs can be completely separated, four peaks should be obtained in the electropherogram. The separation of ssDNAs was completed within only 18 min in the range of 80—200 V/cm although the resolution was poor at electric fields of more than 250 V/cm. At 200 V/cm, however, only 7 min was required to separate the four ssDNAs. Consequently, an electric field of 200 V/cm was used in subsequent experiments.

The Effect of Temperature on the Separation of ssDNA

It is known that temperature is a potent influence on the higher-order structure of ssDNA. Thus, it is predicted that the separation of ssDNA of N-ras oncogene varies with the temperature. Figure 4 illustrates the effect of temperature (15—40 °C) on the separation of ssDNAs. Four peaks corresponding to the mixture of wild- and mutant-type ssDNAs were observed at a temperature of 30 °C and below, whereas the mixture was not separated at more than 35 °C. This is because the higher-order structure (conformation) of ssDNA changes with increasing temperature and the viscosity of the buffer solution containing MC decreases with temperature, resulting in poor resolution. As can be seen from Fig. 4, the wild- and mutant-type ssDNAs can be separated at 15—30 °C, and the migration time is reduced by increasing the temperature.

Thus, in the following experiments the temperature was set up at 25 °C in consideration of migration time, resolution, and experimental conditions such as temperature control and buffer handling.

The Effect of MC Concentration on the Separation of ssDNA

We next examined the influence of the MC concentration on the separation of ssDNAs. Figure 5 demonstrates the influence of the concentration (0.5—1.0%) of MC on the separation of ssDNAs. As can be seen from Figs. 5A and B,
the mixture of wild- and mutant-type ssDNAs could not be separated by MC solutions of less than 0.7%. However, four peaks corresponding to wild- and mutant-type ssDNAs were resolved using the 1.0% MC solution. It is conceivable that the polymer network in 0.5—0.7% MC is too large to detect the delicate difference in SSCP of N-ras oncogene. Also, it was difficult to fill the capillary with more than 1.2% MC so-solution because of high viscosity. Thus, the optimal MC concentration was determined to be 1.0% for subsequent experiments.

The Effect of YO-PRO-1 Concentration on the Separation of ssDNA

One of the factors which has a great influence on the higher-order structure of ssDNA is the cation concentration. Figure 6 illustrates the effect of the concentration (0.1—1.0 \( \mu \text{M} \)) of the cationic intercalating dye YO-PRO-1 on the separation of ssDNAs. The intercalation of YO-PRO-1 into dsDNA expands the spacing of successive base pairs, distorts the regular sugar—phosphate backbone, and decreases the pitch of the double helix. As can be seen from Figs. 6A and B, three peaks were present with 1.0 and 0.5 \( \mu \text{M} \) YO-PRO-1 concentrations. However, the separation of the four ssDNA peaks could be achieved within 7 min by using 0.2 \( \mu \text{M} \) YO-PRO-1. As shown in Fig. 6D, poor resolution was obtained below 0.1 \( \mu \text{M} \) YO-PRO-1. From the results, the optimal concentration of YO-PRO-1 was found to be 0.2 \( \mu \text{M} \).

Broad peaks were obtained at higher YO-PRO-1 concentrations, indicating less difference in the higher-order structure of the ssDNAs, therefore, the resolution power was decreased at higher concentration. YO-PRO-1 is intercalated into DNA to delay the migration time, resulting in good separation. On the other hand, we could not achieve a good separation using other intercalating dyes such as 1,1’-(4,4,7,7-tetramethyl-4,7-diazundecamethylene)-bis-4-[3-methyl-2,3-dihydro(benzo-1,3-oxazole)-2-methylidene]-quinolinium tetraiodide (YO-YO-1) and quinolinium 4-[3-methyl-2(3H)-benzothiazolylene)methyl]-1-[3-(trimethylammonio)propyl]-diiodide (TO-PRO-1), which are optimally excited by the argon ion laser (488 nm), although the detection sensitivity of these fluorescent dyes is not very different from YO-PRO-1.

From these results, the mixture of wild- and mutant-type ssDNAs could be separated with high resolution within 7 min (electric field; 200 V/cm) at 25 °C using a 27 cm capillary, 50 mM TB-buffer containing 1.0% MC and 0.2 \( \mu \text{M} \) YO-PRO-1.

Repeatability in SSCP Analysis

We next examined the repeatability of the SSCP analysis by the CE-LIF technique. The analysis of the mixture of wild- and mutant-type ssDNAs was carried out 9 times using the same sample and buffer. The average migration time, mobility, and relative mobility of the peaks W2, M1, and M2 in Fig. 6C for the peak W1 of the wild-type ssDNA are summarized in Table 1. The repeatability of the migration time and the mobility of each ssDNA was about 0.5% relative standard deviation (R.S.D., \( n = 9 \)), but that of the relative mobility of each ssDNA for the peak W1 was in the range of 0.07—0.22% R.S.D., indicating that SSCP analysis by the CE-LIF technique has satisfactory precision for the detection of one point mutation of the N-ras oncogene.

SSCP Analysis of the N-ras Oncogene

Figure 7 shows the CE separation of the mixture of wild- and other mutant-type ssDNAs. In this figure, peaks W1 and W2 show the ssDNA obtained from the wild-type, and peaks M1 and M2 from the mutant-type. Figure 7 indicates that six in seven mutant-type samples could be resolved. However, the mix-

![Fig. 6. Effect of YO-PRO-1 Concentration on the Separation of the Mixture of Wild- and Mutant-Type ssDNAs](image_url)

<table>
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<tr>
<th>Peak</th>
<th>Migration time (min)</th>
<th>R.S.D. (%)</th>
<th>Mobility (cm²V⁻¹s⁻¹)</th>
<th>R.S.D. (%)</th>
<th>Relative mobility (%)</th>
<th>R.S.D. (%)</th>
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</thead>
<tbody>
<tr>
<td>W1</td>
<td>6.78</td>
<td>0.53</td>
<td>2.47 ( \times 10^{-4} )</td>
<td>0.51</td>
<td>1</td>
<td>—</td>
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<tr>
<td>M1</td>
<td>6.83</td>
<td>0.47</td>
<td>2.45 ( \times 10^{-4} )</td>
<td>0.50</td>
<td>0.993</td>
<td>0.07</td>
</tr>
<tr>
<td>M2</td>
<td>6.94</td>
<td>0.48</td>
<td>2.41 ( \times 10^{-4} )</td>
<td>0.54</td>
<td>0.975</td>
<td>0.22</td>
</tr>
<tr>
<td>W2</td>
<td>6.98</td>
<td>0.42</td>
<td>2.39 ( \times 10^{-4} )</td>
<td>0.45</td>
<td>0.969</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Table 1. Repeatability (\( n = 9 \)) of Migration Time, Mobility, and Relative Mobility for ssDNA Obtained from N-ras Oncogene
ture of wild- and mutant (arginine)-type ssDNAs could not be separated using the present experimental conditions. However, from the appearance of three peaks, it is clear that this sample contains the mutant-type DNA, which is different from wild-type DNA.

Conclusion

We reported the effects of electric field, temperature, MC concentration, and YO-PRO-1 concentration on the separation of ssDNAs by the SSCP-CE-LIF technique. N-ras oncogene containing one point mutation could be resolved by the SSCP-CE-LIF technique with high sensitivity.

The SSCP-CE-LIF technique using the intercalating dye, YO-PRO-1, can be used to genetically screen for cancer through the detection of the one point mutation of the N-ras oncogene. This technique offers excellent resolving power, high-speed separation, and good repeatability. In SSCP analysis with the CE-LIF technique using MC polymer solution it is very easy to prepare a buffer solution and refill the capillary with the buffer solution, and it is possible to reuse the capillary any number of times.

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