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Effect of polymer matrix and glycerol on rapid single-strand conformation polymorphism analysis by capillary and microchip electrophoresis for detection of mutations in *K-ras* gene

We present the rapid single-strand conformation polymorphism (SSCP) analysis by capillary and microchip electrophoresis to detect the mutations in *K-ras* gene. Parameters that might affect the analysis of mutation in *K-ras* gene, such as the polymer and the additive in the sieving matrix, have been studied systematically. Under the optimal conditions, the analysis of seven mutants of *K-ras* gene could be finished within 10 min by capillary electrophoresis (CE). Furthermore, with the wild-type gene as the inner standard, the analysis accuracy of mutations could be improved. In addition, by studying the properties of polymer solutions, the matrix suitable for microchip electrophoresis was found, and the detection of mutations in *K-ras* gene could be further shortened to 1 min.

Keywords: Capillary electrophoresis / Microchip electrophoresis / Mutation detection / Single-strand conformation polymorphism DOI 10.1002/elps.200500142

1 Introduction

The sequence of the human genome has been almost completed in early 2001 [1, 2], and the Human Genome Project (HGP) already enters the post-genome-sequencing era [3–6]. During this period, the analysis of gene mutations is of great significance since they are informative markers for clinical diagnostics [7, 8]. Up to now, many methods have been proposed for mutation analysis, such as allele-specific amplification, polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis, DNA sequencing, denaturing gradient gel electrophoresis (DGGE), chemical or enzymatic cleavage of mismatches, heteroduplex assay, and single-strand conformation polymorphism (SSCP) analysis.

Among the above-mentioned methods, SSCP is preferred because of its simplicity and versatility. This method, first proposed by Orita *et al.*, involves polymerase chain reaction (PCR) amplification of the fragment of interest, denaturation of amplified DNA to form single strands, and subsequently the analysis of denatured fragments by electrophoresis [9, 10], during which ssDNA (single-stranded DNA) fragments fold into a three-dimensional (3-D) shape according to their primary sequence so that the wild type and mutant could be distinguished according to the different electrophoretic mobilities.

Traditionally, gel electrophoresis was employed for SSCP analysis [11]. However, the time it consumed was rather long. In the past 10 years, capillary electrophoresis (CE) has been widely accepted as a technique of high efficiency, high resolution, and short analysis time. Besides the analysis of PCR products, the separation of restriction digest fragments, and the sequencing of DNA [12–16], CE has also been successfully applied into SSCP analysis [8, 17–23].

In the post-human-genome-sequencing era, further development of analytical technology for DNA is highly required for high-throughput screening of disease-caus-

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Abbreviations: DEA/DMA, *N*,*N*-diethylacrylamide/*N*,*N*-dimethylacrylamide; **FAM**, carboxyfluorescein; **MC**, methylcellulose

ing genes. Accordingly, microchip electrophoresis, which can offer even faster analysis speed and less sample consumption than CE, has become more and more important [24–28]. Although a lot of papers have been published on the DNA analysis by microchip, the papers on SSCP analysis are still very few [29].

In this paper, with mutations in the *K-ras* gene as the target, which has proven to be related to several kinds of cancers, rapid SSCP analysis by CE and microchip electrophoresis has been achieved under the optimal condition, and such methods have shown great prospect in the clinical examination of *K-ras* gene.

2 Materials and methods

2.1 Apparatus

PCR was carried out on a GeneAmp PCR system 2400 (Perkin-Elmer Applied Biosystems Division, Foster City, CA, USA). CE experiments were performed on a Beckman P/ACE 5010 system equipped with a laser-induced fluorescence detector with the excitation wavelength at 488 nm (Beckman Coulter, Fullerton, CA, USA). Hitachi SV 1100 (Hitachi Electronics Engineering, Tokyo, Japan) and Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) were employed for the microchip electrophoresis-based analyses. For the former one, a light-emitting diode (LED) detector was used with the median excitation wavelength at 470 nm, and the emission wavelength was over 530 nm. For the latter one, a diode laser detector was taken with the excitation wavelength at 635 nm and the emission wavelength in the range of 670–700 nm.

2.2 Reagents and materials

In our experiments, all chemicals were either of reagent or electrophoretic grade. Methylcellulose (MC) (viscosity of 2% aqueous solution at 20°C, 4000 cP) was purchased from Sigma Chemical (St. Louis, MO, USA). *N*,*N*-Diethylacrylamide/*N*,*N*-dimethylacrylamide (DEA/DMA) copolymer was synthesized by the previously described method [30]. Boric acid, tris(hydroxylmethyl)aminomethane (Tris) and glycerol were ordered from Kanto Chemicals (Tokyo, Japan). DNase- and RNase-free water used for sample preparation was obtained from ICN Biomedicals (Aurora, OH, USA). FAM-labeled forward and reverse primers were ordered from Kurabou Industries (Osaka, Japan) with the sequence shown in the caption of Table 1. QIA quick PCR Purification kit (Qiagen, Tokyo, Japan) was used to purify the amplification products. A DB-17-coated capillary (360 μm od, 100 μm id, a coating of diphenyl dimethyl polysiloxane with a thickness of 0.1 μm) (J&W Scientific, Folsom, CA, USA) with a total length of 27 cm and an effective length of 20.2 cm was used for CE-SSCP analysis. Polymethylmethacrylate (PMMA) microchips with a simple cross-channel of 100 μm width, 30 μm depth, and an effective length of 30 mm were obtained from Hitachi Electronics Engineering. Soda lime glass microchips with channels of 50 μm width, 10 μm depth, and an effective length of 15 mm, suitable for 12-sample consecutive analysis, as well as DNA 500 kits, were obtained from Agilent Technologies, Waldbronn, Germany.

Table 1. Mutations in *K-ras* gene (upper) and the sequence of PCR product (lower)

Cell line	Mutated codon	Mutation
A549	12	GGT (Gly) → AGT (Ser)
Lu65	12	GGT (Gly) → TGT (Cys)
MAD-MB231	13	GGC (Gly) → GAC (Asp)
PANC1	12	GGT (Gly) → GAT (Asp)
PSN1	12	GGT (Gly) → CGT (Arg)
SW480	12	GGT (Gly) → GGT (Val)
SW1116	12	GGT (Gly) → GCT (Ala)

5'-GGCCTGCTGAAAATGACTGAATATAAACTTGTGGTA
GTTGGAGCTGGTGGCGTAGGCAAGAGTGCCTTGAC
GATACAGCTAATTCAGAATCATTTTTGTGGACGAATAT
GATCCAACAATAGAGGTAAATCTTGTTTTAATATGCATAT
TACTGGTGCAGGAC-3'.
N shows nested primers; N shows the two mutation codons.

2.3 Procedure

Amplification of each sample listed in Table 1 was carried out with carboxyfluorescein (FAM)-labeled forward and reverse primers. PCR was carried out with 1 cycle at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, and a final extension at 72°C for 10 min. Then the amplification products were kept at 0°C. After purification by a QIA quick PCR Purification kit, the products were denatured at 90°C for 5 min and cooled rapidly on ice for 3 min. Finally, the samples were diluted (20–100)-fold with water before usage. For CE procedure, the sample was introduced by electrokinetic injection at 1 kV for 30–60 s and separated at 20°C. For microchip electrophoresis-based analysis, with the sieving matrix introduced into the crossing channel and wells, the sample was analyzed under an electric field.

3 Results and discussion

For the analysis of mutations by SSCP method, there are many parameters that might affect the resolution of denatured single strands of DNA, such as polymer, additives in the buffer, electric field strength, and the separation temperature. In this paper, a systematic study on the effects of these parameters has been carried out in both CE and microchip electrophoresis systems so that detailed information of mutations in *K-ras* gene could be obtained in the shortest time.

3.1 CE-SSCP analysis

It is well known that the electric field strength has a great effect on the separation of DNA fragments by CE. In our experiments, 300 V/cm was selected as the optimum condition. In addition, although the high-order structure of ssDNA is stable at low temperature, the viscosities of polymeric sieving matrices are high, which could not only prolong the analysis time of samples but also increase the difficulty in filling the capillary. Subsequently, a compromised temperature at 20°C was taken in the following experiments.

Although linear polyacrylamide (LPA) is often employed in DNA analysis by CE because of the relatively high resolution, the preparation of such a solution is not only complex but also toxic, which might prevent it from wide application. In our work, MC, which can provide a wide range of mesh sizes originated by different concentrations in the buffer, was chosen as the sieving matrix. Since the size of the amplified DNA fragments in our work was 162 bp, high-concentration MC was employed as the sieving matrix.

Figure 1A shows the separation of ssDNA of the wild type and PSN1 with 1.5% MC in the buffer. Although we could distinguish them by the electropherograms, the resolution was poor, and the differences on the electrophoretic mobility were not obvious. Since additives with hydroxyl groups have been previously used in CE to improve the separation of DNA [31], in our work the effect of glycerol concentration in the buffer on distinguishing seven mutants of *K-ras* gene was studied systematically. From Fig. 1B it can be seen that better resolution of ssDNAs could be obtained with 5% glycerol added in the buffer. Furthermore, the differences between the electrophoretic migration of the wild type and the mutants became evident. However, with the further increase of the glycerol concentration, the analysis time was relatively long, as shown in Fig. 1C. Accordingly, for the detection of PSN1, 5% glycerol was selected as the buffer additive, the same as that for the analyses of A549, Lu65, SW480,

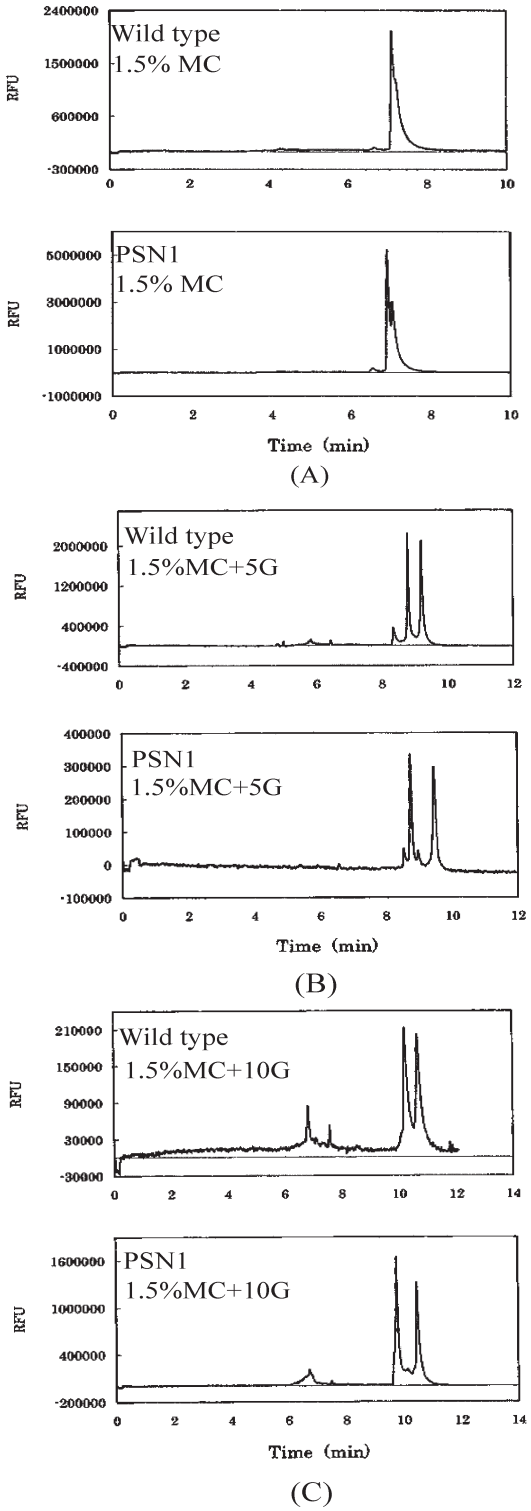

Figure 1. Effects of glycerol concentration in the buffer on CE-SSCP analysis of PSN1 and the wild type of *K-ras* gene. Experimental conditions: 1.5% MC in 50 mM Tris-borate buffer; (A) without glycerol; (B) with 5% glycerol; (C) with 10% glycerol; electric field: 300 V/cm; samples were labeled with FAM; other conditions as shown in Section 2. RFU represents relative fluorescence unit.

Table 2. Effects of buffer on SSCP-CE analysis of mutations in *K-ras* gene

Polymer	pH	Wild type	A549	Lu65	MDA-MB231	PANC1	PSN1	SW480	SW1116
2.0% MC	8.40	–					+	–	
+5G	7.95	+					++		
+10G 1.5% MC	8.40	–	–	–	–	–	+	–	–
+5G	7.85	+	++	+	–	–	++	++	+
+10G	7.50	+	++	+	+	+	++	++	+
1.0% MC	8.40	+					+		
+5G	7.89	+	+	+	+	+	++	++	+
+10G	7.62	–					+	–	
94–4 copolymer	8.50	+	++	–	++		++	–	+

+ indicates the resolution of ssDNA fragments.
++ indicates that ssDNA fragments of wild-type-mutant mixtures were separated and identified.
+5G and +10G indicate that 5% and 10% glycerol were added into the CE buffer.

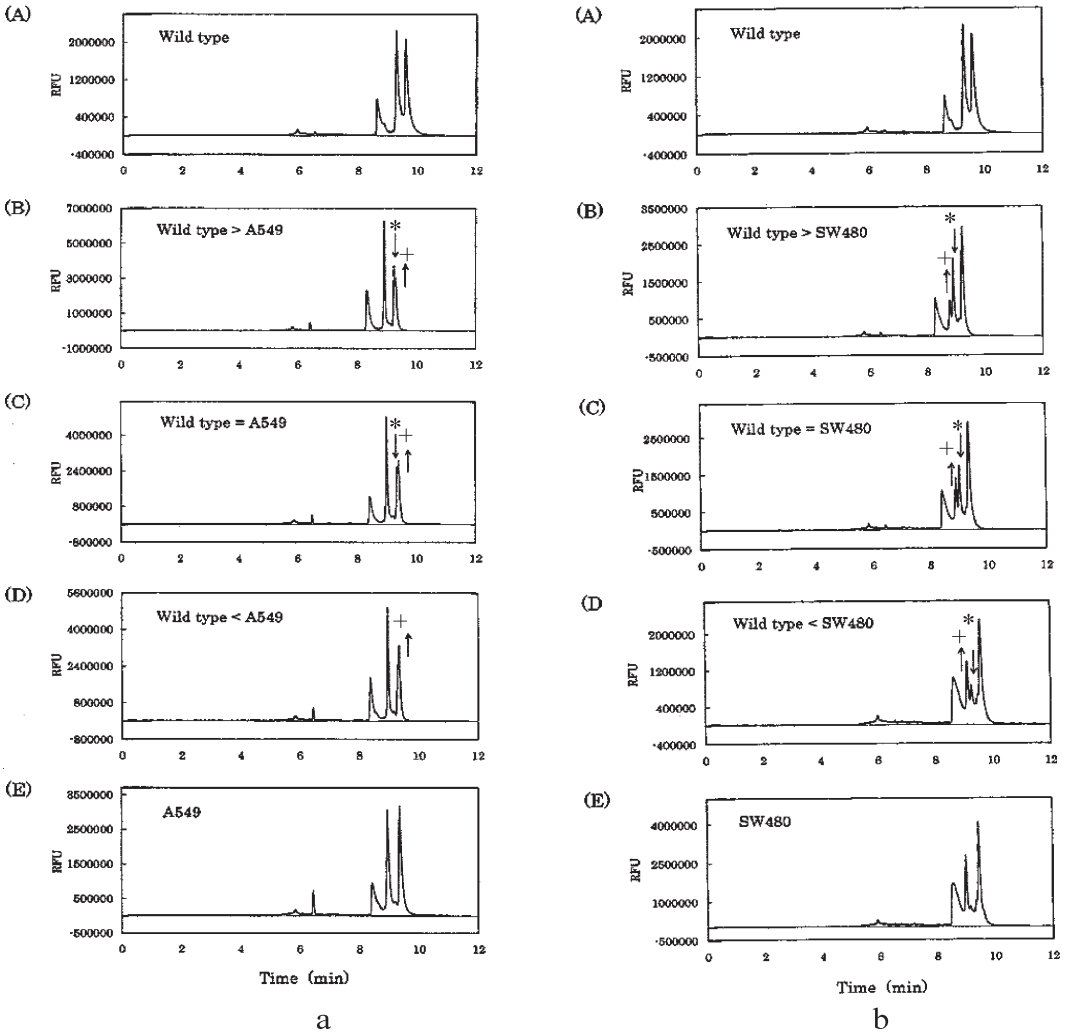

Figure 2. CE-SSCP analysis by mixing mutants and the wild type of *K-ras* gene. Experimental conditions: 1.5% MC, 5% glycerol in 50 mM Tris-borate buffer; other conditions as shown in Fig. 1. Samples: (a) A549; (b) SW480. * Represents the ssDNA from the wild type; + represents the ssDNA from the mutants.

and SW1116; while for MDA-MB231 and PANC1, 10% glycerol could offer better discrimination, as shown in Table 2.

Besides the amount of glycerol, the concentration of MC in the buffer also played an important role in detecting the mutations in *K-ras* genes, as shown in Table 2. Taking PSN1 as an example, although it could be identified from the wide type when MC concentration was over 1.0%, the resolution and the analysis time were different. In our experiment, 1.5% MC with 5% glycerol in the buffer was chosen as the optimal condition. For other mutants, different MC concentrations might be selected according to their electrophoretic behaviors. Under the optimal conditions, the analyses of all mutants in *K-ras* gene could be finished in *ca.* 10 min.

For the SSCP method, the discrimination of single-strand peaks generated from the wild type and the mutant is generally based on their migration times in the electric field. However, the system error might also lead to minor difference on electrophoretic velocity. In order to improve the analysis accuracy, in our experiments, an inner standard method has been employed by mixing the mutant with the wild-type gene. Figure 2 shows the changes of electrophoretic peaks with gradual addition of the ssDNA of A549 and SW480 into the wild-type gene. From this figure, we can see that with more mutant added, the peaks marked by + appeared, and the heights kept on increasing, while those marked by * decreased until they completely disappeared, which meant that the peaks marked by + and * generated from the mutants and the wild type of *K-ras* gene, respectively. Such a method could offer higher reliability for SSCP analysis than the judgment only by the migration times of samples.

Besides MC, DEA/DMA copolymer was also used for detecting the mutations in *K-ras* gene. From Fig. 3A it can be seen that with such a polymer as the sieving matrix, the resolution of ssDNA generated from the wild type and PSN1 was improved, and the difference in their electrophoretic mobility became more obvious. Similar results could also be seen for the analyses of MDA-MB231 (Fig. 3B) and other mutants (Table 2).

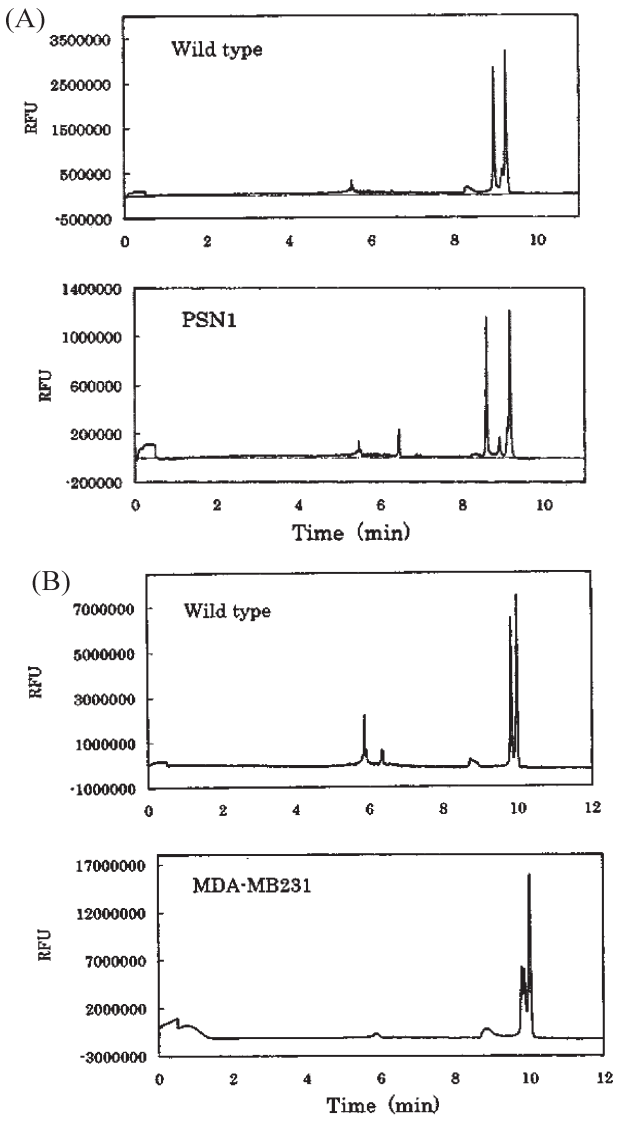

Figure 3. CE-SSCP analysis of the mixture of the wild type of *K-ras* gene with PSN1 and MDA-MB231 in DEA/DMA copolymer solution. Experimental conditions: DEA/DMA in 50 mM Tris-borate buffer; other conditions as shown in Fig. 1. Samples: (A) wild type and PSN1; (B) wild type and MDA-MB231.

3.2 Microchip electrophoresis-SSCP analysis

To develop microchip electrophoresis-based analysis methods for detecting mutations in *K-ras* gene, the properties of the above-mentioned polymer solutions were studied. From Fig. 4 it can be seen that with the addition of glycerol into the buffer, no obvious change of the viscosity was observed. However, the current under the electric field of 300 V/cm in CE was nearly doubled when 5% glycerol was added, and a further increase could be seen on adding more glycerol. Since no cooling system was installed in our microchip electrophoresis systems, a polymer solution that might generate low current is favorable. In addition, the viscosities of 2.0% MC and DEA/DMA copolymer were found to be more than 300 mPa × s, making them difficult to fill into the microchip channel. With the consideration on the resolution, viscosity, and Joule heat, 1.5% MC without glycerol was selected for the microchip electrophoresis-SSCP analysis to detect mutations in *K-ras* gene.

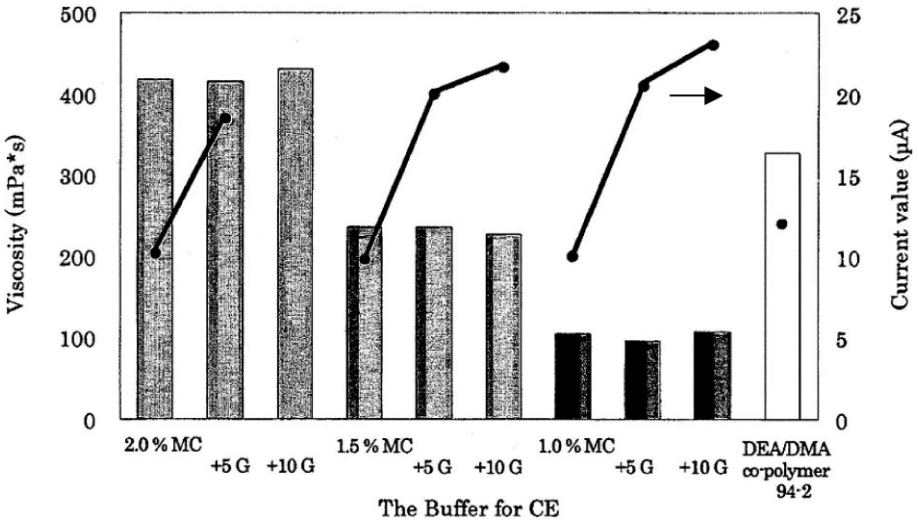

Figure 4. Properties of the sieving matrices used in CE-SSCP for analyzing mutations in *K-ras* gene.

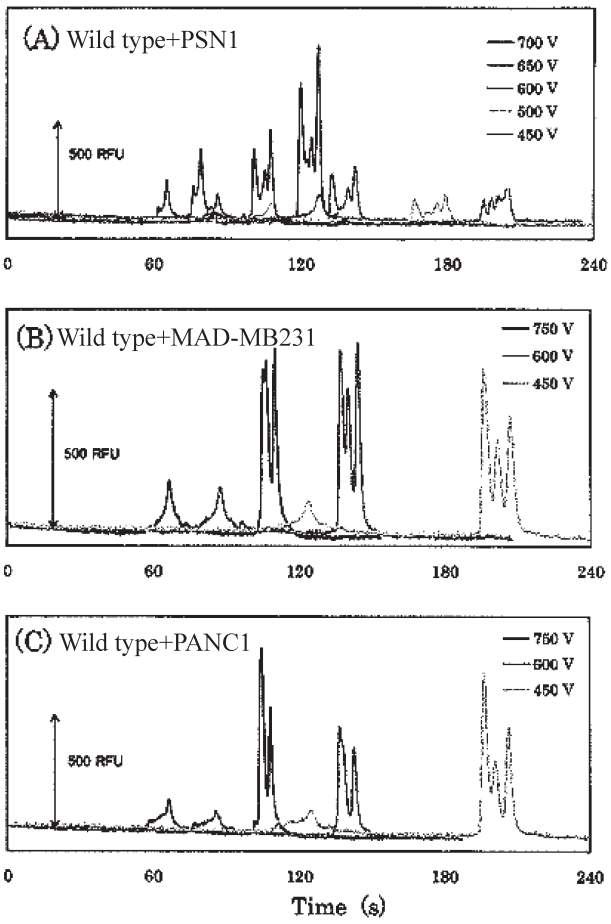

Figure 5. Effects of electric fields on SSCP profiles in microchip electrophoresis. Experimental conditions: 1.5% MC in 50 mM Tris-borate buffer; other conditions are the same as the defaulted conditions of Hitachi SV1100. Samples: mixture of the wild type of *K-ras* gene with (A) PSN1, (B) MAD-MB231, and (C) PANC1.

Figure 5 shows the analyses of PSN1, MAD-MB231, and PANC1 with the wild-type *K-ras* gene added as the inner marker by the Hitachi microchip electrophoresis system. In each electropherogram, over three main peaks generated from the ssDNA generated from the wild type and the mutants of *K-ras* gene could be found within 4 min. In addition, it could be seen that the separation was greatly dependent on the applied voltage. Taking the mixture of the wild type and PANC1 as examples, as shown in Fig. 5C, when the separation voltage was increased to 750 V, although the analysis time was shortened to less than 2 min, we could not distinguish PANC1 from the wild type because of the overlapping of the ssDNA while with low separation voltage, such as 450 V, the differences became evident. This shows that the low separation voltage was favorable to improve the resolution of ssDNA at the cost of a little increase of the analysis time.

In addition, with a DNA 500 kit, the Agilent Bioanalyzer 2100 was also applied for the microchip electrophoresis-SSCP method to detect mutations in *K-ras* gene. From Fig. 6 it can be seen that mutants of *K-ras* gene could be distinguished from the wild type within 1 min by inner standard method, which is of great significance in the clinical examination of mutations in *K-ras* gene.

4 Concluding remarks

In this work, effects of polymer matrix and glycerol on rapid SSCP analysis by CE and microchip electrophoresis for the detection of mutations in *K-ras* gene have been studied. The addition of glycerol into the buffer has proven effective to improve the resolution of ssDNA generated from the wild type and mutants of *K-ras* gene. The

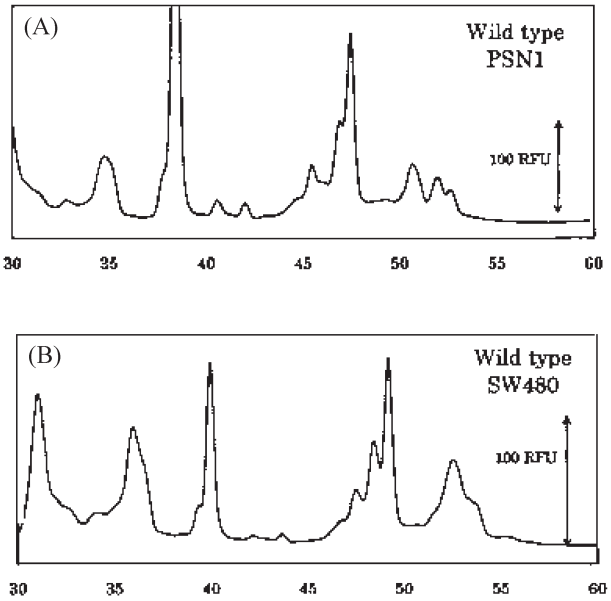

Figure 6. Electropherograms of SSCP profiles of the mixture of the wild type and mutants in *K-ras* gene. Experimental conditions were the same as defaulted by Agilent 2100 Bioanalyzer with DNA 500 kit. Samples: (A) wild type and PSN1; (B) wild type and SW480; labeled by cy5.

optimization of the various types and concentrations of polymers, as well as the electric field strength, has also been shown to be indispensable in obtaining a good separation for SSCP analysis. Under the optimal conditions, the CE-SSCP and microchip electrophoresis-SSCP analyses for detecting the mutants of *K-ras* gene could be finished within 10 and 1 min, respectively, which demonstrates the potential for their further application in the clinical examination of *K-ras* gene.

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