Single-Strand Conformation Polymorphism Analysis by Microchip Electrophoresis for the Rapid Detection of Point Mutation in Human Obesity Gene

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We describe an effective method of microchip electrophoresis (ME) based on single strand conformation polymorphism (SSCP) analysis to rapidly detect the point mutation, Leu72Met, in a human obesity gene. The 207-bp dsDNA in the Leu72Met region, an estimate of the child obesity DNA mutant, was amplified by polymerase chain reaction (PCR) and submitted to a conventional glass microchip analysis with a sieving matrix of 1.75% poly(vinylpyrrolidone) (M_r 1 300 000), 1.0% poly(ethyleneoxide) (M_r 600 000) and 5.0% w/w glycerol. When combined with base stacking (BS) with hydroxide ions, the SSCP-ME provided rapid analysis as well as sensitive detection. The detection sensitivity was effectively enhanced in the OH^- concentration range of 0.01-0.025 M NaOH. The sensitivity and speed of this ME-based SSCP methodology for the rapid detection of Leu72Met point mutations makes this an attractive method for diagnosing childhood obesity in a clinical diagnostic laboratory.

Key Words : Microchip electrophoresis, Human obesity diagnosis, Single-strand conformation polymorphism, Point mutation

Introduction

Ghrelin is a physiological peptide hormone that induces the secretion of somatotrophin (growth hormone, GH), prolactin and adrenocorticotrophin.

It enhances appetite and influences the energy metabolism by decreasing the oxidation of fat.[1,2] The ghrelin gene resides in the third chromosome long arm (3q25-26), consists of four exons, and encodes 117 amino acids of preproghrelin. The active form of ghrelin is formed at amino acid 28 by octanoylation from preproghrelin.[3] A mutation in the ghrelin gene was recently reported to be associated with metabolic disorders such as diabetes and obesity.[4-6] This mutation has three single nucleotide polymorphisms, Arg51Gln, Leu72Met and Gly274Ala. Among them, the Leu72Met mutation is most often detected and might be used to predict the coherence transmutation of childhood obesity, because it is found in the early childhood.[4]

Various methods such as anthropometry,[7] bioelectrical impedance analysis,[8-10] dual energy x-ray absorptiometry,[8,11] ultrasound[11] and skin fold thickness[12] are employed to diagnose obesity. Although these methods are beneficial to diagnose postnatal diseases, they do not provide the genetic information required to analyze hereditary diseases. The methods used to diagnose postnatal diseases are also unsuitable for diagnosing obesity from the viewpoint of preventive medicine. Among the methods used for the direct detection of gene mutations in preventive medicine, SSCP-slab gel electrophoresis is becoming popular on account of its simplicity. Generally, the detection of mutations via conventional SSCP analysis requires PCR amplification of the DNA fragment of interest and denaturation of the double stranded PCR products using heat and formamide, followed by gel electrophoretic separation. The specific DNA fragments stained with silver or ethidium bromide are analyzed using a polyacrylamide TBE gel or an MDE® gel.[13,14] ^{32}P-labeled dNTPs are also incorporated into the PCR products using slab gel electrophoresis in order to increase the detection sensitivity. However, in an attempt to improve the detection efficiency, as well as for greater convenience and safety, many studies have used fluorescent dye-labeled primers or post-fluorescence labeled PCR products instead of the radioactive chemicals in slab gel-based SSCP analysis,[15-17] capillary electrophoresis (CE)-based SSCP analysis[18-25] and, more recently, in ME-based SSCP analysis.[23]

Since the first demonstrations of this method by Manz *et al.*[26] and Harrison *et al.*,[27] ME has rapidly become an important technique for identifying DNA fragments on account of its analytical throughput, speed, small reagent volume, automation, miniaturization, high resolution, etc.[27-31] One of the most important advantages of ME in analyzing DNA fragments is its high speed compared with traditional slab gel electrophoresis and CE. The rapid detection of human obesity DNA was recently demonstrated using a CE-SSCP method.[32] In this study, we demonstrate the advantages provided by translating the CE assay[32] to the microchip format. The optimization of the ME-based SSCP analysis conditions for the ultra-fast, simple and highly efficiency detection of the Leu72Met point mutation, in order to identify its frequency in children with obesity, is also described. The 207-bp DNA of the Leu72Met region was amplified by PCR. The PCR product was denatured at 95 °C for 5 min and snap-cooled on ice. The SSCP profiles for the mutation were obtained in less than 85 s using ME with BS

based on gel electrophoretic separation in a polymer network.

Experimental Section

Materials. Formamide, urea, ethidium bromide (EtBr), nuclease free water, 5× PCR buffer, GoTaq™ DNA polymerase and 5 mM dNTP were purchased from Promega (Madison, WI, USA). The 0.5× TBE buffer (0.089 M Tris, 0.089 M borate and 0.002 M EDTA, pH 8.36) was prepared by dissolving a pre-mixed powder (Amerosco, Solon, OH, USA) in deionized water. The forward primer (5'-AGC AGA GAA AGG AGT CG-3') and the reverse primer (5'-TGT TCA CTG CCA CCT CT-3') were obtained from GenoTech (Daejeon, Korea). The microchip sieving matrixes, 1 300 000 M_r poly(vinyl pyrrolidone) (PVP) and 600 000 M_r poly(ethyleneoxide) (PEO) were purchased from Sigma (St. Louis, MO, USA) and glycerol was acquired from Showa (Japan). The 100-bp DNA ladder was purchased from Seegene (Seoul, Korea).

Genomic DNA Isolation. The genomic DNA and Leu72Me mutant samples used to identify the child obesity DNA mutant were acquired from the Chonbuk National University Medical School.[33]

Polymerase Chain Reaction. Using the method described in a previous report,[32] the 207-bp of the Leu72Met region was amplified in a thermal cycler (Perkin-Elmer model 2400, USA) using the following temperature protocol: 1 min incubation at 94 °C; 30 cycles of denaturing at 94 °C for 60 s, annealing at 56 °C for 60 s, and extension at 72 °C for 60 s; followed by 7 min holding at 72 °C. The 50-μL PCR reaction mixture contained the following: 10 mL each of 10× PCR buffers 1 and 2, 0.5 mM of dNTP, 0.2 pM each of the forward and reverse primers, 1.25 U GoTaq™ DNA polymerase, 59.5% nuclease free water and 3 μL of purified DNA. The amplified DNA fragments showed only 207-bp.

Microchip Electrophoresis-Based SSCP Analysis. One microliter of the PCR product (without purification) was combined with 9 μL of nuclease free water in a 200 µL PCR tube. Unless otherwise specified, the mixture was heated for 5 min at 95 °C and snap-cooled on ice for 3 min before the injection. The SSCP profiles were obtained by introducing the denatured PCR products into the ME system (sample reservoir (2) shown in Figure 1).

ME was performed on a DBCE-100 Microchip CE system (Digital Bio Technology Co., Korea) equipped with a diode-pumped solid-state laser (exciting at 532 nm and collecting fluorescence at 605 nm) and a high-voltage device (DBHV-100, Digital Bio Technology Co., Korea). The microchip was a standard microfluidic chip (MC-BF4-TT100, Micralyne Inc, USA). The chip channel was 50 μm wide and 20 μm deep. The reservoirs were 2.0 mm in diameter and 1 mm deep. A double-T injector with a 100 μm offset was selected as the conventional microfluidic chip. The injection channel length (from reservoir (2) to reservoir (4) in Figure 1) was 8.0 mm, and the separation channel (from reservoir (1) to reservoir (3) in Figure 1) was 85 mm long. Detection was performed at a distance of 65 mm from the injection-T. The ME sieving matrix was made by dissolving 1.75% (w/v) of 1 300 000 M_r PVP and 1% (w/v) 600 000 M_r PEO into the 0.5× TBE buffer (pH 8.36) with 0.5 µg/mL EtBr and 5% w/w glycerol. Between each run, the microchip channel was re-flushed with water for 4 min and a sieving matrix for 3 min. The sieving matrix was filled hydrodynamically by applying a vacuum (EYELA A-3S vacuum aspirator, TOKYO RIKAKIKAI Co., Japan) to the ME reservoir (3). The sample was pipetted into the sample inlet reservoir (2) of the microchip. A normal sample was introduced via a conventional electrokinetic injection into the injection-T region by applying 480 V at the sample outlet reservoir (4) followed by grounding the sample inlet reservoir (2) for 60 s. Separation was achieved by applying 0 V at the buffer inlet (1) and +4.25 kV at the buffer waste (3). The peak areas of the DNA fragments were calculated using OriginPro7.5 software (OriginLab Co., Northampton, MA, USA).

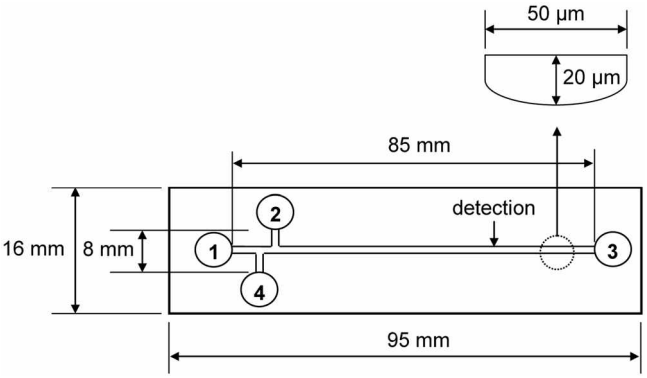

Figure 1. (A) Schematic diagram of the microfluidic chip. Indications: 1, 3: buffer reservoir; 2: sample reservoir; 4: NaOH reservoir.

Enhancement of the Detection Sensitivity Using Base Stacking. The experimental ME setup and the BS method that was applied were similar to those described elsewhere.[34] All of the amplified-PCR products were diluted 1/10 for the BS and the conventional electrokinetic injection method was used. The BS procedure was carried out using the following three steps: (1) OH^- was injected into the channel (0.5× TBE buffer) using the electrokinetic injection method by applying 480 V at the 0.01-0.1 M NaOH inlet reservoir (4) and the buffer inlet reservoir (1) was grounded for 20 s, (2) The PCR products were injected into the channel by applying +480 V at the sample inlet reservoir (2) and the NaOH inlet reservoir (4) was grounded for 60 s (3) The BS and the separation were started by applying +4.25 kV to the buffer inlet reservoir (1) and the buffer outlet reservoir (3) was grounded. The migration time was measured from this moment for all experiments.

Results and Discussion

PCR Amplification. The PCR amplification conditions were optimized using the mutant and wild type DNA templates as well as the primers flanking each of the

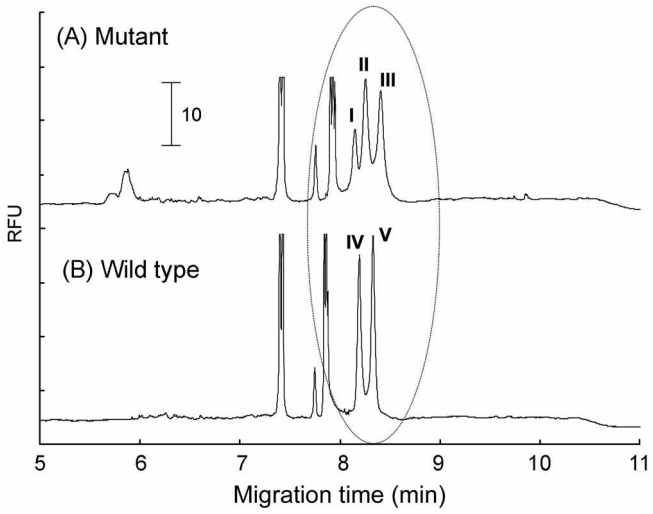

Figure 2. Representative SSCP-CE electropherograms of the mutant (A) and wild type (B) human obesity DNA under the optimum separation conditions. SSCP-CE conditions: sieving matrix and run buffer, 1.5% PVP (M_r 1 300 000), 1.0% PEO (M_r 600 000) and 5.0% wt glycerol in 0.5× TBE buffer with 0.5 ppm EtBr; capillary, 50 μm I.D. × 365 μm O.D. × 60 cm total length (30 cm effective length). The samples were prepared by heating them at 70 °C for 5 min and then chilling them in an ice bath (1 °C) for 3 min before injecting the sample into the CE (mixture of nuclease free water 9.0 μL and PCR sample 1.0 μL); electrokinetic injection, −15 kV for 60 s; applied separation electric field, 375 V/cm. RFU: Relative fluorescence unit. Indicators: I, II and III = specific peaks of mutant type. IV and V = specific peaks of wild type (From Kang, S. H.; Jang, S.; Yi, H.-K. *J. Korean Chem. Soc.* **2005**, *49*, 537.[32]).

Table 1. Effect of the hydroxide ion (OH^-) concentration on the detection sensitivity of the SSCP analysis by ME with base stacking (BS)

Mutant			Wild type		
NaOH (M)	Peaks	Peak height ratio[a]	NaOH (M)	Peaks	Peak height ratio[a]
0	I	1:1	0	IV	1:1
	II	1:1		V	1:1
	III	1:1			
0.010	I	1:2.3	0.010	IV	1:5.7
	II	1:5.5		V	1:6.7
	III	1:8.4			
0.025	I	1:2.8	0.025	IV	1:3.6
	II	1:5.6		V	1:7.1
	III	1:9.6			
0.050	I	1:2.8	0.050	IV	1:2.8
	II	1:4.2		V	1:3.7
	III	1:1.9			
0.100	I	1:0.4	0.100	IV	1:0.8
	II	1:0.8		V	1:0.3
	III	1:0.2			

[a]Peak height ratio: The ratio of the normal peak height-to-peak height with BS. SSCP-BS conditions: microchip, 85 mm long × 50 mm width × 20 μm depth, effective length, 50 mm; sieving matrix and run buffer, 1.75% PVP (M_r 1 300 000)/1.0% PEO (M_r 600 000)/5.0% wt glycerol in 0.5× TBE with 0.5 ppm EtBr; applied electric fields for BS, 533.3 V/cm at the gel buffer reservoir 1 and grounding the 0.000-0.100 M NaOH reservoir 4 for 20 s; sample injection, 480 V for 60 s; separation electric field, 500 V/cm.

mutations. The PCR amplification conditions were optimized using CE analysis with a laser-induced fluorescence (LIF) detector for the untagged PCR products (207-bp DNA fragment). Because a denaturing step is essential in SSCP analysis,[35,36] experiments were performed to test the effect of the denaturing conditions on the SSCP profiles in the CE-based analysis. The results of the CE-SSCP analysis were the same as those previously reported,[32] and were analyzed within 9 min (Figure 2). The potential provided by the SSCP-CE assay was improved by translating it to the microchip format for the ultra-fast detection of the point mutation in the human obesity gene, Leu72Met mutation (207-bp DNA).

Detection Sensitivity Enhancement with Base Stacking. The most significant advantage of ME in DNA analysis is its high speed compared with conventional slab gel electrophoresis and CE. However, although ME has certain advantages over slab gel electrophoresis, the small sample volume imposed by this method is a major limitation, because it results in low concentration sensitivity. The BS technique is generally desirable because making alterations to the microchip design in order to enhance the injection volume and the detection window length can create other problems such as band broadening.[34] The BS method was quite simple to perform, because it is only necessary to introduce OH^- ions before injecting the DNA sample. The peak height ratios of the DNA fragments increased with increasing OH^- concentration in the range of 0.01-0.025 M NaOH (Table 1). However, there was no further signal enhancement in the peak height, but rather a reduction in the resolution, at concentrations higher than 0.05 M NaOH. The overinjection of OH^- ions onto the microchip causes rapid neutralization, which can reduce the suppressed electro-osmotic flow. This in turn can trigger the adsorption of DNA molecules onto the inner surface of the microchannel,[34] which can lead to an abrupt increase in the DNA concentration at the beginning of the peak band with a long tail after the DNA peak. The average calculated signals (n = 5) according to the peak height measurements were enhanced 2.8-9.6-fold at an NaOH concentration of 0.025 M for all SSCP profiles (peaks I-V) in the BS-ME separation compared with those obtained using normal ME (Table 1). However, the resolutions of peaks I-III were better at an NaOH concentration of 0.05 M than at 0.025 M (data not shown). Enhancing the resolution of the DNA fragments is essential to improve the SSCP analysis by ME. In order to optimize the detection sensitivity and resolution, an injection time of 20 s and an NaOH concentration of 0.05 M were selected as the optimum injection time and OH^- concentration at a 100-μm offset, double-T microchip and a channel length of 85 mm under an applied electric field of 500 V/cm for the SSCP-ME separation with BS.

Optimizing the Conditions for the ME-based SSCP Analysis. The previous SSCP-CE method[32] was translated

to the microchip format, wherein the SSCP analysis was completed in several seconds, which nevertheless represents only a fraction of the reduction in the analysis time that can be achieved using microchip technology. The aim of SSCP

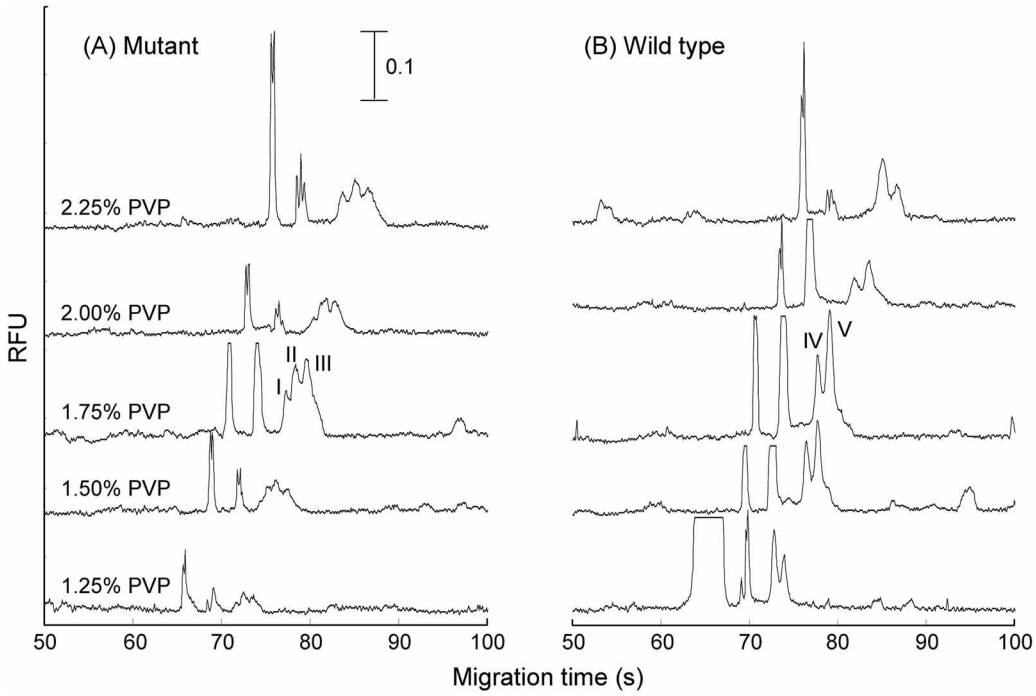

Figure 3. Representative SSCP-ME electropherograms showing the effect of PVP on the resolution. (A) Mutant. (B) Wild type. SSCP-ME conditions: microchip, 85 μm long × 50 μm width × 20 mm depth, effective length, 50 mm; sieving matrix and run buffer, 1.25-2.25% PVP (M_r 1 300 000), 1.0% PEO (M_r 600 000) and 5.0 % wt glycerol in 0.5× TBE with 0.5 ppm EtBr. The samples were diluted with 9.0 μL of nuclease free water, and 1.0 μL of each PCR sample was placed into a 200 μL PCR tube. The samples were heated at 95 °C for 5 min and cooled on ice (1 °C) for 3 min before the injection. 533.3 V/cm was applied to the gel buffer reservoir (1) and the 0.025 M NaOH reservoir (4) was grounded for 20 s; sample injection, electrokinetic injection, 500 V/cm for 60 s; separation electric field, 500 V/cm. RFU: Relative fluorescence unit. Indicators: I, II and III = specific peaks of mutant type. IV and V = specific peaks of wild type.

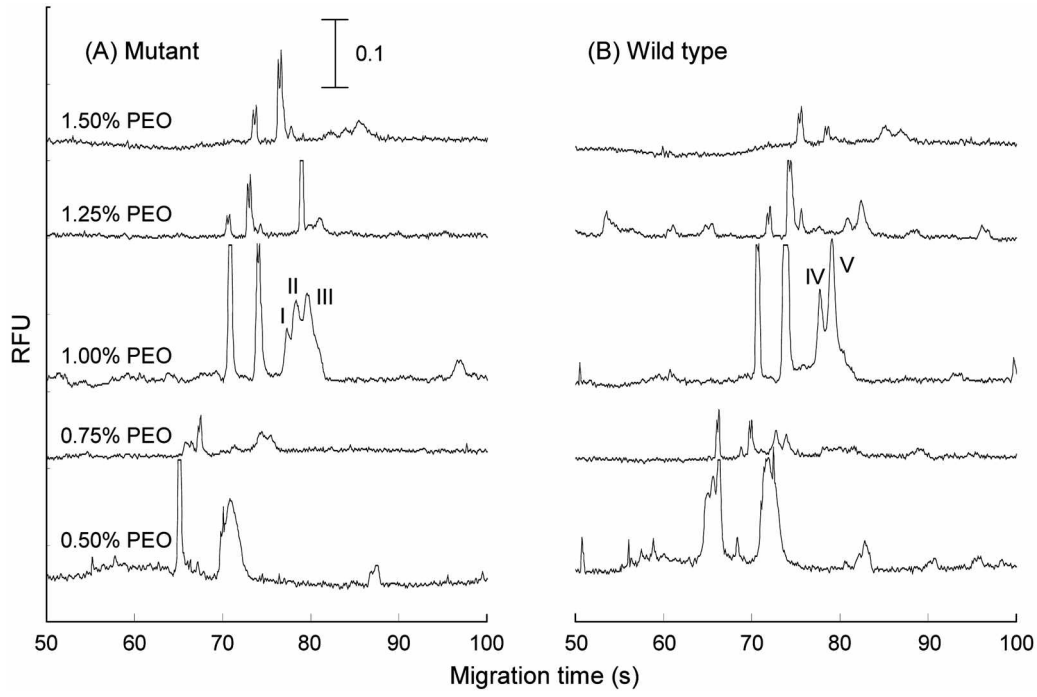

Figure 4. Effect of PEO on the resolution of the SSCP analysis by ME with base stacking. (A) Mutant. (B) Wild type. Sieving matrix and run buffer, 1.75% PVP (M_r 1 300 000), 0.50-1.50% PEO (M_r 600 000) and 5.0% wt glycerol in 0.5× TBE with 0.5 ppm EtBr. The other SSCP-ME conditions were the same as those described in Figure 3.

analysis by ME is to separate the ssDNA fragments (wild type and mutant) rapidly according to their conformations under native conditions. Conventional SSCP analysis involves polyacrylamide slab-gel electrophoresis in the presence of neutral additives, such as glycerol, urea, ethylene glycol, formamide, and sucrose, which are used to improve the resolution.[37] It is important to test the effect of each additive on the resolution when using ME-based SSCP, where various linear polymer solutions in the microchip are used for size-based DNA fragment separation, instead of using polyacrylamide gel. Therefore, in this study, we evaluated the effect of various additives on the rapid detection of the Leu72Met point mutation using SSCP-ME analysis, by varying the glycerol concentration and the electric field in solutions of polymers such as PVP and PEO.

Single-stand DNA (ssDNA) fragments, which are different from the original DNA fragments, were prepared in order to determine the SSCP profiles by directly immersing the original DNA fragments in a 1 °C ice bath after denaturing the amplified 207-bp human obesity double-strand DNA (dsDNA) sample at 95 °C. The neutral additives did not affect the detection or resolution of the ssDNA fragments (data not shown). Figures 3 and 4 show the effects of the PVP and PEO concentrations on the SSCP profiles of the amplified 207-bp DNA fragments, respectively. PVP and PEO were used as a sieving matrix for analyzing the PCR-amplified DNA containing the Leu72Met point mutation. Although the mutant and wild type ssDNA fragments were not resolved with the baseline, the SSCP profiles of the Leu72Met point mutation could be analyzed without difficulty using 1.75% PVP and 1% PEO in the sieving matrix and separation buffer solution, respectively.

Table 2. Effect of the glycerol concentration on the resolution of the SSCP-ME analysis with base stacking

Mutant				Wild type			
Glycerol (%)	Peaks	Migration time (s)	R_s	Glycerol (%)	Peaks	Migration time (s)	R_s
0.0	I	–	–	0.0	IV	68.313	0.607
	II	70.938	0.345		V	69.406	
	III	71.594					
2.5	I	71.813	0.447	2.5	IV	72.906	0.377
	II	72.907	0.058		V	74.000	
	III	73.089					
5.0	I	77.218	0.538	5.0	IV	77.828	0.656
	II	78.375	0.495		V	79.140	
	III	79.687					
7.5	I	87.453	0.339	7.5	IV	86.469	0.243
	II	88.657	0.216		V	87.453	
	III	89.641					
10.0	I	–	–	10.0	IV	92.047	0.345
	II	–	–		V	93.395	
	III	93.250					

SSCP-BS conditions: sieving matrix and run buffer, 1.75% PVP (M_r 1 300 000), 1.0% PEO (M_r 600 000) and 0.0-10.0% wt glycerol in 0.5× TBE with 0.5 ppm EtBr. The other conditions were the same as those described in Table 1.

The effect of varying the glycerol concentration in the mixed polymer solution containing 1.75% PVP (M_r 1 300 000) and 1.0% PEO (M_r 600 000) on the detection of the ssDNA fragments and their resolution by ME-based SSCP was also evaluated (Figure 5). Vidal-Puig *et al.* tested large-

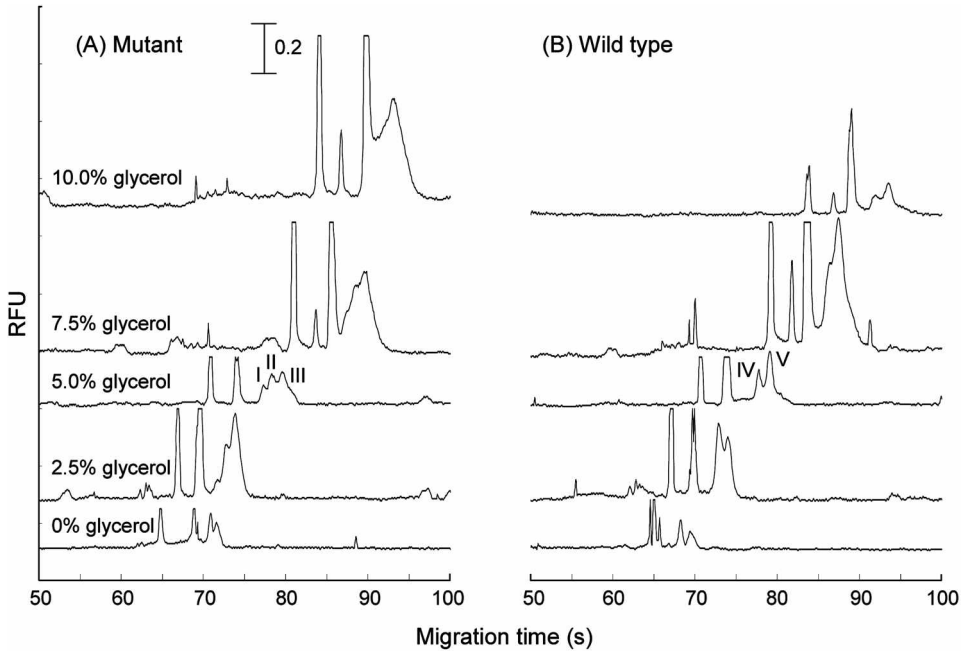

Figure 5. Effect of glycerol on the resolution of the SSCP analysis by ME with base stacking. (A) Mutant. (B) Wild type. SSCP-ME conditions: sieving matrix and run buffer, 1.75% PVP (M_r 1 300 000), 1.0% PEO (M_r 600 000) and 0.0-10.0% wt glycerol in 0.5× TBE with 0.5 ppm EtBr. The other SSCP-ME conditions were the same as those described in Figure 3.

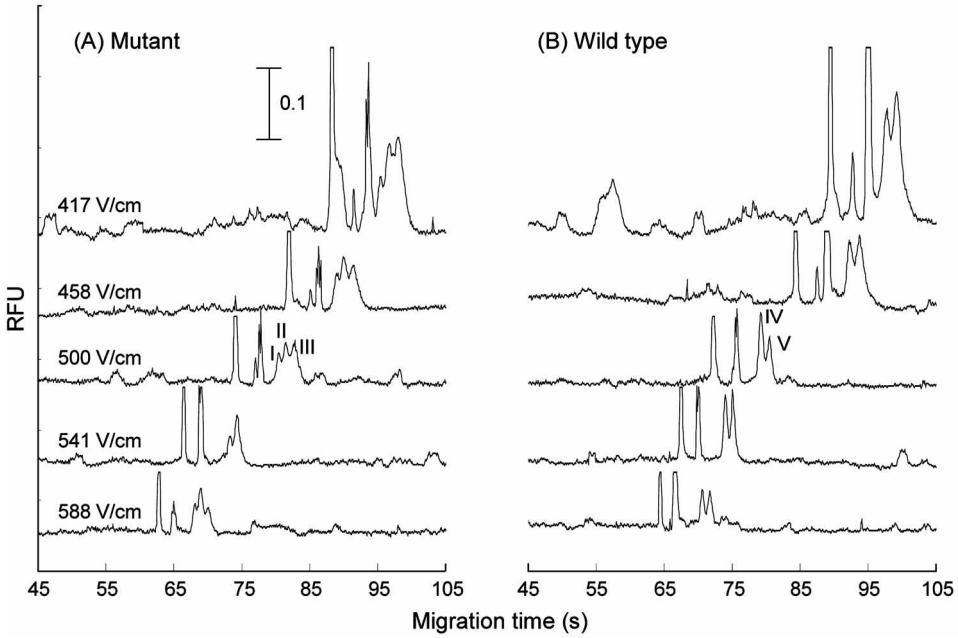

Figure 6. Representative SSCP-ME electropherograms showing the effect of the electric field. (A) Mutant. (B) Wild type. SSCP-ME conditions: separation electric field, 417-588 V/cm. The other SSCP-ME conditions were the same as those described in Figure 3.

format gels (5% polyacrylamide and Hydrolink-MDE™) with and without 10% glycerol in the SSCP analysis of 19 known human mutations.[38] They reported that all of these mutations could be detected with either polyacrylamide or Hydrolink®-MDE™ (AT Biochem, Malvern, PA, USA), regardless of whether or not glycerol was used in the SSCP analysis. The present study also found that glycerol did not affect the detection sensitivity in the SSCP analysis of the Leu72Met mutation. However, the use of glycerol improved the resolution of the SSCP peaks (Table 2). The presence of 2.5% glycerol in the sieving matrix and separation buffer solution was found to be beneficial for improving the resolution of both the mutant and wild type ssDNA fragments. The use of 5.0% glycerol was found to be optimal for both types with a separation time of less than 85 s, as shown in Figure 5.

Because the ultimate goal was to use SSCP analysis by CE to detect point mutations on an electrophoretic microchip, the differences between the two platforms need to be considered. On the other hand, the separation of DNA fragments by ME in a mixture matrix containing PVP and PEO is mainly affected by the electric field strength.[39] The SSCP-ME results were examined after injecting the PCR-amplified products (ssDNA fragments) from the Leu72Met point mutation directly without purification at electric field strengths ranging from 417 to 588 V/cm. The velocity of the DNA fragments increased and the migration time decreased with increasing electric field strength, which lead to shorter analysis times (Figure 6). However, higher voltages cause higher currents and, thus, more Joule heating. Increased heat in a microchip can lead to non-reproducible migration times, decomposition of the ssDNA fragments, and even boiling of the buffer, which will definitely decrease the resolution and efficiency. The SSCP peaks were easily analyzed within 85 s under a constant electric field strength of 500 V/cm (Figure 6 middle) and showed resolutions (R_s) of 0.423 (I and II), 0.515 (II and III) and 0.625 (IV and V).

Conclusion

The efficiency of SSCP-ME analysis with BS in the rapid detection of single base substitutions was tested on a point mutation in a human obesity gene, Leu72Met. The detection sensitivity was enhanced 2.8-9.6-fold when using an NaOH concentration of 0.025 M. However, 0.05 M NaOH was selected as the optimum concentration in the ME analysis with BS, because of the higher resolution and detection sensitivity that this afforded. Neutral additives such as urea and formamide had little or no effect on the detection and resolution of the SSCP peaks in the ME separation. The SSCP profiles were obtained in less than 85 s using ME with BS in a microchip with a sieving matrix consisting of 1.75% PVP, 1.0% PEO and 5.0% w/w glycerol, which resulted in a 10-70 times faster analysis time than that obtained in conventional CE and slab gel electrophoresis. The simplicity and speed of the ME-based SSCP methodology with BS for the rapid detection of Leu72Met point mutations makes this technique attractive for diagnosing childhood obesity in a clinical diagnostic laboratory.

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