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Highly sensitive determination of the methylated *p16* gene in cancer patients by microchip electrophoresis

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Abstract

The *p16* tumor suppressor gene is inactivated by promoter region hypermethylation in many types of tumor. Recent studies showed that aberrant methylation of the *p16* gene is an early event in many tumors, especially in lung cancer, and may constitute a new biomarker for early detection and monitoring of prevention trials. We detected tumor-associated aberrant hypermethylation of the *p16* gene in plasma and tissue DNA from 153 specimens using a modified semi-nested methylation-specific PCR (MSP) combining plastic microchip electrophoresis or slab gel electrophoresis, respectively. Specimens were from 79 lung cancer patients, 15 abdominal tumor patients, 30 positive controls and 30 negative controls. The results showed that the positive rate obtained by microchip electrophoresis was more than 26.6% higher and the same specificity was kept when compared with slab gel electrophoresis. The microchip electrophoresis can rapidly and accurately analyze the PCR products of methylated DNA and obviously improve the positive rate of diagnosis of cancer patients when compared with gel electrophoresis. This method with the high assay sensitivity might be used for detection of methylation of *p16* gene and even to facilitate early diagnosis of cancer patients.

Keywords: Microchip electrophoresis; DNA methylation; Cancer diagnosis

1. Introduction

Alterations in normal DNA methylation patterns are frequently observed in cancer cells and hypermethylation in the promoter regions of tumor suppressor genes is associated with an epigenetically mediated gene silencing, which is a common feature in human carcinomas [1,2]. Aberrant hypermethylation of the *p16* gene, which is a tumor suppressor gene and has a key role in cell cycle regulation, has been reported to be an early event in lung cancer and a potential biomarker for early diagnosis [3]. Previous studies have shown that hypermethylation of *p16* gene could be detected not only in cancer tissues but also in serum or plasma of patients with several types of carcinomas because it has been reported that tumor DNA could be released into the circulation and enriched in serum and plasma [4–9]. Recently, an improvement of the methylation-specific PCR (MSP) procedure incorporating a nested PCR approach has made the detection of the methylation of *p16* gene more sensitive, which would facilitate the detection of this epigenetic alteration in the sera and of NSCLC patients [10,11].

PCR is widely used in molecular biology and medical diagnosis. Conventionally, slab gel electrophoresis has been used for the analysis of PCR products for sizing, mutations, or

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polymorphisms, but the technique is time-consuming, labor-intensive, and nonquantitative. Because of the superior separation efficiency and speed in an automated format, capillary electrophoresis (CE) is rapidly becoming an important tool for PCR analysis. However, as the CE technique advances to microchip devices, the use of microchip electrophoresis for DNA analysis is emerging as a more promising method. The manipulation and transport of analytes in microchip devices is based on electrokinetic phenomena, e.g., electrophoretic and electroosmotic effects. Buffer and sample flow within the channel network can be precisely controlled through high voltages applied at the buffer/sample reservoirs. The technique allows the manipulation of picoliter volumes with high precision that ultimately leads to analysis performance equivalent to or exceeding current techniques. In contrast to conventional CE, the higher surface-to-volume ratio in microchip devices results in better heat dissipation, therefore, microchip electrophoresis allows separations at higher field strengths. The separation time is generally 10-fold shorter than CE and 100-fold shorter than traditional gel electrophoresis [12]. Most of the reports on microfabricated electrophoretic chips have utilized glass or silica as the basal materials for chip fabrication [13–15], whenas the polymer substrates are considered as promising alternatives for fabrication of microfluidic systems [16,17] because they are less expensive and easier to manipulate than quartz, glass or silica based substrates. In addition, the inherent hydrophilic nature of the polymer substrates allows direct use of the channel for clinical analysis of biomolecules without the need of surface modifications to reduce wall adsorption [18].

The aim of the present study was to develop a novel PMMA microchip electrophoresis method for rapidly and highly sensitive detection and diagnosis of cancer. The performance of the new method was verified by comparing with the conventional agarose gel electrophoresis method in detection of modified MSP products from cancer tissues and plasma specimens according to the blinded-assay trail.

2. Experimental

2.1. Materials

1.5% Hydroxyprolymethyl cellulose (HPMC, 50 cps. Sigma Chemical Co., St. Louis, MO) TBE buffer (100 mmol/L Tris (tris(hydroxymethyl)methylamine, Sigma)/100 mmol/L boric acid/2 mmol/L EDTA buffer (TBE) with a pH 8.5) was used as running buffer. The labeling reagent was prepared by dissolving SYTOX Orange dye (Molecular Probes, Eugene, OR) to a concentration of l μmol/L in running buffer in order to label DNA on-line. A 50 ng/μL φX-174/HaeIII digest DNA restriction fragments ranging in size from 72 to 1353 base pairs (11 fragments) was purchased from the TaKaRa Biotechnology (Dalian, China) Co., Ltd.

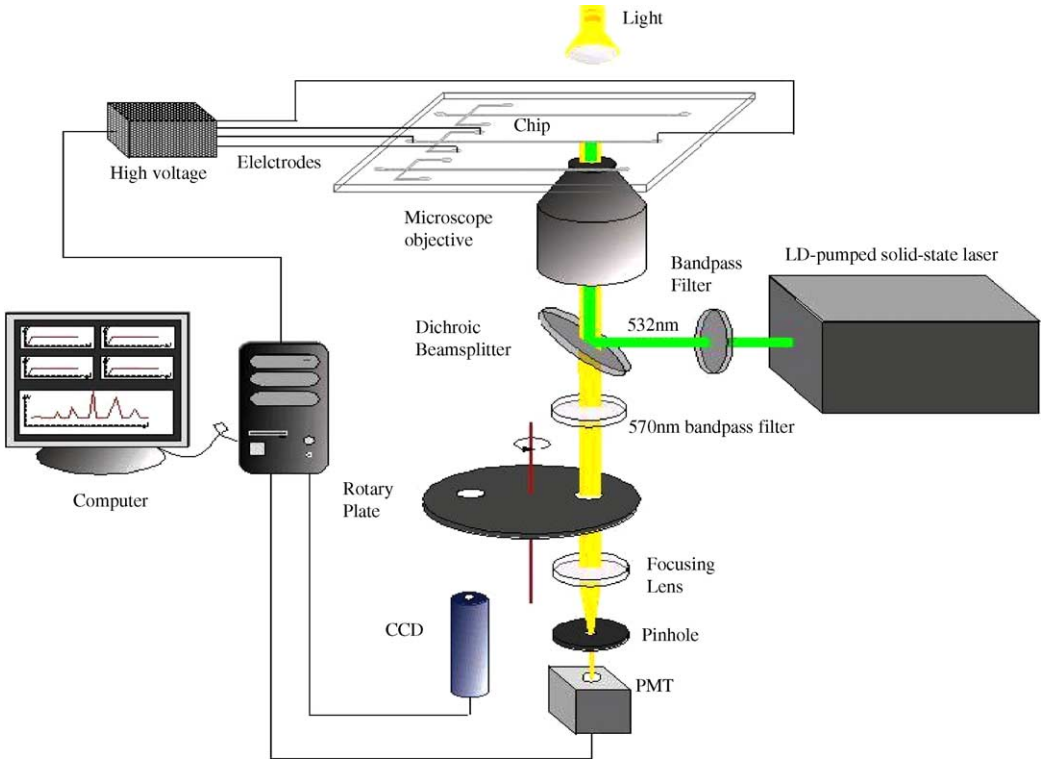

Fig. 1. Schematic representation of the experimental instrument and the laser confocal detection arrangement.

All solutions were prepared with doubly distilled water and filtered (0.22 μm filters) before use.

2.2. Apparatus

Fig. 1 shows schematically the layout and dimensions of a micro-CE device with integrated confocal laser fluorescence detector and auto micro-manipulation stage. The output radiation (532 nm) from an air-cooled LD-pumped solid-state laser (20 mW) (Mektec Seiwa Corporation, Beijing, China) passed through a 532 nm filter (Omega Optical, Brattleboro,VT). The laser beam was reflected by a dichroic beamsplitter (Omega Optical) and focused on the channel through a 20× microscope objective (0.4 N.A). The emission fluorescence was collected by the same objective and transmitted back through the dichroic beamsplitter. The emission beam passed through a 570 nm bandpass filter (Omega Optical), and was focused by a focusing lens through a 400 μm pinhole. The photomultiplier tube (Hamamatsu Photonics R212, Japan) was mounted in an integrated detection module including high voltage power supplies, voltage divider, and amplifier. A charge coupled device (CCD) camera was fixed at the same board as the photomultiplier tube in order to focus and observe the channel surface. The whole optical system was installed on a *X-Y-Z* translational stage (3-D micro-manipulator, which adjusting precision is l μm).

2.3. Fabrication of plastic microchip

A multistep fabrication process was developed for the preparation of three-microchannel electrophoresis chips. Initially, the silicon template was fabricated according to a reported method [19]. Briefly, the schematic of three channels shown in Fig. 2a was first drawn using a standard CAD software package. The image was reproduced on a chromium glass, which was subsequently used as a mark for photolithography. An oxide silicon layer was produced on the surface of a 4-in. silicon wafer. A layer of photoresist was spin-coated over the surface of the oxide and subsequently exposed to UV light through the glass image aligned to the wafer flat. The photoresist was then developed, and revealed the transferred image. The exposed silicon oxide layer was removed with HF solution, and then the wafer was thoroughly rinsed with doubly distilled water, followed by the removal of the photoresist with acetone. The wafer was etched in KOH solution to produce three "negative" 3-D images of the channels. A layer of metal was plated on the surface of the silicon template with three channels to prepare electroform. The silicon template was soaked in the electroforming solution to electroform and produce a "positive" nickel mold. Metal columns of 1.5 mm height, 4 mm diameter were mounted at the terminal of every channel to produce the holes of the reservoirs. The nickel mold was then machined to be the injection mold insert, which was inserted in a metal mold. After the metal mold was installed on the injection machine, the plastic substrates (poly(methyl methacrylate), PMMA) were molded to make chips.

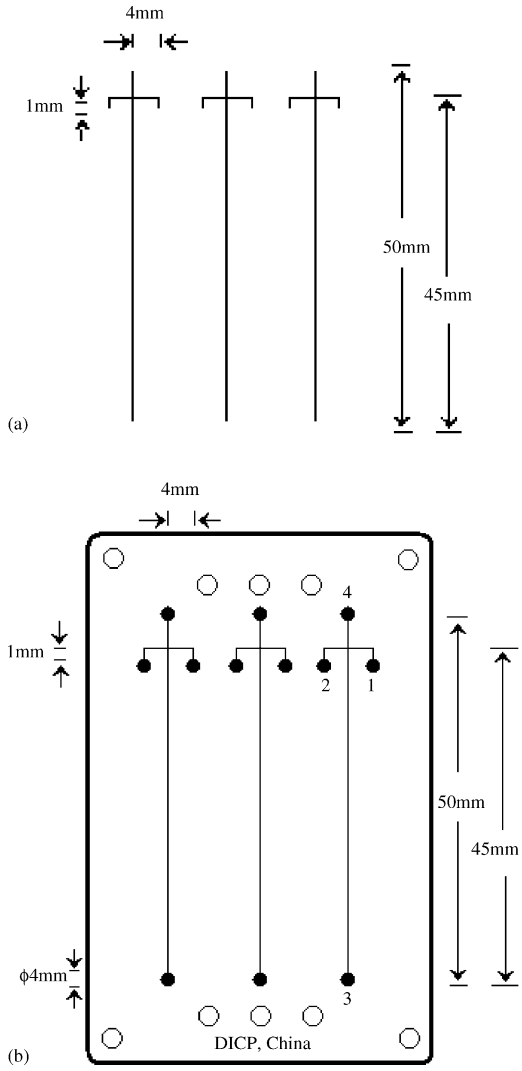

Fig. 2. Dimensions and layout of the injection-molded PMMA chips used in this work: (a) is the schematic of three channels, (b) in black spots indicate the reservoir numbers: (1) injection waste; (2) sample; (3) waste; (4) buffer. The small figures denote the channel lengths and device dimensions (in mm).

The dimension of an injection-molded microchip was of 54 mm by 85 mm. Individual chip was prepared for channel-seal by casting 4 mm diameter of holes in the chip to serve as buffer reservoirs at the terminal of the channels. Each separation channel was 50 mm long, and each injection channel was 5 mm away from the cross (Fig. 2b). The microchip was cleaned with the diluted surfactant to weed out the attached oil on the surfaces of microfluidic chip, and then with distilled water, followed by dry before sealing the channel with a 50 μm cover PMMA film (Goodfellow Corporation, England). The film seal forms the fourth wall of the separation channel and the bottoms of the four wells used as reservoirs. Sealing was done by a mixed organic solvent, which is mainly composed of alcohol and ketone.

2.4. Samples

The 93 specimens of modified MSP products of the aberrant methylation of the *p16* gene in plasma and tissue DNA from different cancer patients (48 plasma specimens and 30 tissue specimens from lung cancer patients, 15 plasma specimens from abdominal tumor patients including breast cancer, hepatoma, stomach cancer, intestinum cancer) were analyzed. 30 samples of methylation (DNA from the prostate cancer cell line TSU-PR1, which has *p16* methylation [20]), were used as the positive control for MSP, and 30 samples of non-methylation were used as the negative control were also analyzed. The specimens described above were from Department of Chemical Etiology and Carcinogenesis, Cancer Institute (Hospital), Peking Union Medical College & Chinese Academy of Medical Sciences.

2.5. DNA preparation, bisulfite conversion and PCR procedure

DNA preparation, bisulfite conversion of DNA, and the modified semi-nested PCR were performed according to the literature [10]. Briefly, DNA was prepared as follows, the cells derived from tumor tissues were digested with SDS/proteinase K, and DNA was extracted by phenol–chloroform, and then ethanol-precipitated using standard protocols. DNA from plasma samples was extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. For bisulfite conversion of DNA, plasma DNA (50 μL) or 1–2 μg tumor DNA was treated with sodium bisulfite (Sigma) and purified using the Wizard DNA purification resin (Promega) according to the manufacturer's instruction. The purified DNA was mixed with 3 M NaOH to a final concentration of 0.3 M and incubated for 10 min at 37 °C, and then DNA was ethanol-precipitated and resuspended in 20 mL of Tris EDTA buffer (TE). All bisulfite-treated DNA was stored at −20 °C until the subsequent PCR.

For semi-nested MSP, we used a modified semi-nested MSP in a 25 μL reaction volume to facilitate the detection of *p16* hypermethylation. Two stages of PCR reactions were carried out in a UNO II thermocycler (Biometra). The PCR products were checked by both agarose gel electrophoresis and microchip electrophoresis, and each PCR amplification was repeated at least once to confirm the result. DNA from the prostate cancer cell line TSU-PR1, which has *p16* methylation, was used as a positive control for MSP.

2.6. Electrophoretic procedures

The injection channel, separation channel and the reservoirs of the used microchip were filled with running buffer except the sample reservoir. Every MSP product was first loaded into the sample reservoir and then was injected by applying a voltage of 400 V/cm at the sample waste and grounding the sample reservoir for 30 s. The buffer and waste reservoirs had no potentials applied since the low diffusion coefficients of DNA fragments in the polymer sieving medium resulted in no substantial increase in the injection plug length or deterioration of separation channel. During separation the relative potentials were switched to 0, 0.65, 0.3, and 0.3 kV at the buffer, waste, sample, and sample waste reservoirs, respectively. The microchannels were rinsed with distilled water after each run. No pretreatment of the channel walls was done before separation.

MSP products were also analyzed by the 2% agarose slab gel electrophoresis after electrophoretic separation by soaking in a solution of ethidium bromide, a dye that displays enhanced fluorescence when intercalated between stacked nucleic acid bases.

3. Results and discussion

3.1. Performance characteristics of microchip electrophoresis

For characterization of the microchip electrophoretic system, we performed a separation of PCR product from a

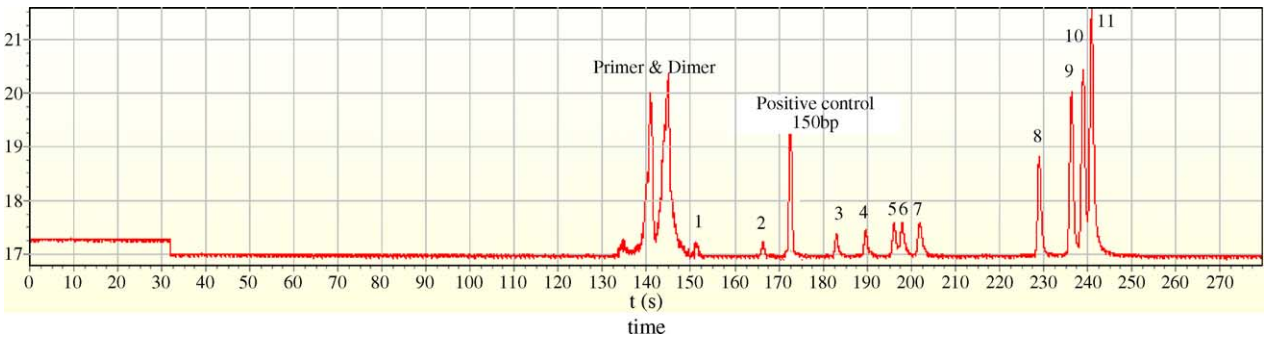

Fig. 3. Microchip electrophoresis (210 V/cm) after co-injection of MSP product from a methylation-positive control with ϕ X-174/HaeIII digest DNA. The individual DNA fragment sizes of the ϕ X-174/HaeIII digest in order of increasing fragment sizes are 72 (1), 118 (2), 194 (3), 234 (4), 271(5), 281(6), 310 (7), 603 (8), 872 (9), 1078 (10) and 1353 (11) bp. The additional peak between the 118- and 194-bp ϕ X-174/HaeIII fragments, corresponds to the expected 150-bp MSP product. The migration times are indicated in seconds; the signal intensity obtained with laser-induced fluorescence detection is given in relative fluorescence units (RFU) and s, seconds.

methylation-positive control specimen. The expected size of the MSP product was 150-bp, whereas the φX-174/HaeIII digest DNA marker contained DNA fragments from 72 to 1353-bp with good fragment coverage in the size range of the expected PCR product (150-bp). PCR product (5 μL) from a 1:3 diluted methylation-positive control specimen was mixed with 5 μL of the diluted φX-174/HaeIII digest with a final plasmid DNA concentration of 0.5 ng/μL. The microchip electropherogram resulting from co-injection is shown in Fig. 3. The fragments of the plasmid digest ranging from 72 to 1353-bp were clearly baseline separated in 250 s at the field strength of 210 V/cm. The peak was visible between the 118- and 194-bp fragments. The migration time for the MSP product in the microchip electrophoresis was 141 s under the separation conditions. The MSP product in size was verified to be 150-bp by comparing the migration time of the standard DNA marker with the migration time of the MSP product. The analyses of methylation-positive and -negative specimens by microchip electrophoresis are shown in Fig. 4A and B.

The use of the PMMA chip increased the separation speed and decreased separation channel pretreatment such as channel coated, which is not needed in DNA analysis. For the PMMA microchip, many repetitive injections appear to be acceptable. In addition, mass production of disposable devices is possible because of the low cost of PMMA material. These peculiarities are well suited for clinical analysis, which requires the handling of a large amount of biological fluids. The polymer microchip, with advantages that include fast processing time, simple operation, and disposable use, holds great potential for clinical analysis.

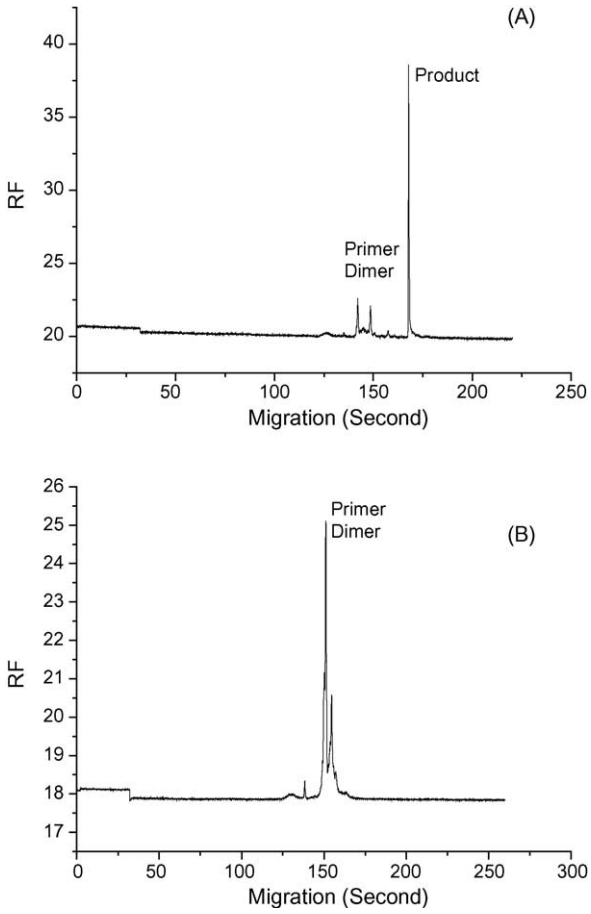

Fig. 4. Microchip electrophoresis of separation of *p16* gene MSP positive (A) and negative (B) plasma specimens of lung cancer patients by microchip electrophoresis.

3.2. Detection limit of the microchip electrophoretic system

The detection limit of Rhodamine 6G dye for the microchip electrophoretic system was 6.67×10^{-13} mol/L ($S/N > 3$). The linear range of detection was 4.02×10^{-6}–4.02×10^{-9} mol/L ($r = 0.9996$). The detailed information was referenced to the literature [21]. A 50 ng/μL φX-174/HaeIII digest DNA marker, which fragments ranging in size were from 72 to 1353 base pairs (72, 118, 194, 234, 271, 281, 310, 603, 872, 1078, 1353 bp), was diluted to serial solutions, and then the serial solutions were respectively determined on the system. When the signal-to-noise ratio was larger than 3 or up to 3, the 603-bp fragment corresponding concentration was 0.2 ng/μL (the mass of 603-bp fragment detected was 3.36 fg, according to injection volume). The comparison of the detection limits of microchip electrophoresis and slab electrophoresis for the detection of DNA methylation PCR products using serial dilutions of a known positive specimen is shown in Fig. 5. The microchip electrophoretic system is able to detect a 1:2000 dilution of DNA methylation PCR product. The signal observed with the slab gel

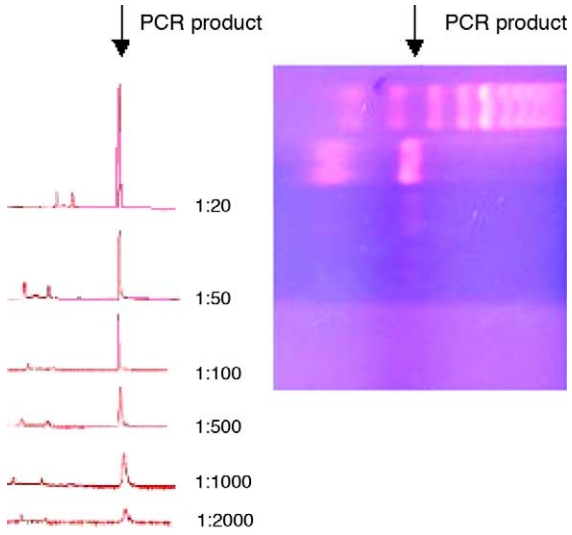

Fig. 5. Detection limit of the microchip electrophoresis system for the MSP products compared with the slab gel electrophoresis method. The microchip electrophoresis is able to detect the MSP product in a 1:2000 dilution. A very faint signal is observed with the slab gel electrophoresis assay in the 1:50 dilution. The signals corresponding to the MSP product on the slab gel electropherograms and the microchip electropherograms are indicated by arrows.

Table 1
Comparison of results for MSP product detection obtained with gel electrophoresis vs. microchip electrophoresis

Method	Positive		Negative
	Positive control (30 cases)	Abdominal tumor patients (9 cases)	Negative control (30 cases)
Gel electrophoresis	30	9	30
Microchip electrophoresis	30	9	30

electrophoresis method for the 1:50 dilution was extremely faint and could easily be overlooked when examining routine clinical assay. These data showed that this system had a high sensitivity, which was over 40-fold higher than that of the slab gel electrophoresis in the MSP assay. We considered that the high sensitivity of microchip electrophoresis was mainly related to the laser-induced fluorescence detection assay with the high sensitivity and to the optimized optical system. The high assay sensitivity is very important for both on early diagnosis of diseases and early identifying the suspected patients with cancer.

3.3. Comparison of the specificity of both microchip electrophoresis and agarose gel electrophoresis

MSP products obtained from a total of 69 specimens from nine abdominal tumor patients, 30 positive controls and 30 negative controls were split into two aliquots and analyzed by both detection methods, slab gel electrophoresis and microchip electrophoresis. The results of the comparison are shown in Table 1. The slab gel electrophoresis was interpreted as positive for methylated DNA in 39 cases and negative in 30 cases. Microchip electrophoresis was able to unequivocally identify all positive, negative cases correctly. The specificity of the microchip electrophoresis was, therefore, 100% when compared with agarose gel electrophoresis.

In addition, we had clearly demonstrated that MSP product detection and identification could be achieved simultaneously by microchip electrophoresis using a coinjection of the MSP product with an internal DNA sizing. Furthermore, SYTOX Orange dye used in this study is an intercalated nucleic acids stain, which specially stains DNA. Hence, the specificity of the assay can be fully ensured in methodology.

3.4. Deterimination of MSP products

The 48 plasma specimens and 30 cancer tissue specimens from lung cancer patients were tested negative by gel electrophoresis analysis of MSP product and then, positives were tested in 13 (27.08%) of 48 plasma specimens and in 8 (26.67%) of 30 cancer tissue specimens by microchip electrophoresis of MSP product. The 15 plasma specimens from abdominal tumor patients were tested 9 positive (60.00%) by gel electrophoresis analysis of MSP product, and then 13 specimens, which included the corresponding 7 positive and 2 weakly positive tested by slab gel electrophoresis analysis, gave positive (86.67%) by microchip electrophoresis of MSP product. These results are shown in Table 2. The results showed that the positive rate obtained by microchip electrophoresis was 26.67% higher than that attained by slab gel electrophoresis. The positive rates obtained by two methods were significantly different ($P < 0.005$) by χ^2 test (chi-square test). The data showed that the microchip electrophoresis could apparently improve the positive rate of methylated *p16* gene in cancer patients. On the other hand, the assay is simple, rapid and inexpensive because of the use of the low cost of PMMA chip. The experimental results showed that the positive rate of the MSP products analysis was apparently increased (>26.67%) by microchip electrophoresis.

Table 2
Comparison of results for MSP product detection obtained from slab gel electrophoresis vs. microchip electrophoresis

Sample/result	Method	
	Slab gel electrophoresis	Microchip electrophoresis
48 Plasma specimens from the lung cancer patients	Negative	13 Positive (27.08%)
30 Tissue specimens from the lung cancer patients	Negative	8 Positive (26.67%)
15 Plasma specimens from the abdominal tumor patients	9 (Positive + weakly positive) (60%)	13 Positive (86.67%)

For a new analytical assay, fast, relatively simple operation and inexpensiveness play an important role in wide application in clinical diagnosis. In this report, microchip electrophoresis reduced analysis time while maintaining full diagnostic capacity and improved apparently the positive rate of the methylated *p16* gene. It will become a new, highly sensitive assay for clinical cancer diagnosis. In addition, the disposable and cheap PMMA microchips might be extensively used in some other clinical analysis.

4. Conclusions

In summary, highly sensitive, rapid analysis of the methylated *p16* gene in cancer patients was attained by microchip electrophoresis. These results showed the feasibility and value of this method to detect the methylated *p16* gene. This study demonstrated that the microchip electrophoresis method in methylation analysis has many advantages over the conventional gel electrophoresis method. The most im-

portant point is high sensitivity and the substantial savings in time and labor. This is the important step toward accepting this technology as a new paradigm in cancer diagnosis.

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