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Determination of the heterogeneity of DNA methylation by combined bisulfite restriction analysis and capillary electrophoresis with laser-induced fluorescence

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ABSTRACT

The methylation of the promoter region of DNA is an important regulatory mechanism for the downstream gene expression, and the extent of methylation has been linked to cancer formation. In this study, we report a simple method to screen for the degree of DNA methylation by combined bisulfite restriction analysis (COBRA) and capillary electrophoresis with laser-induced fluorescence (CE-LIF). After treating genomic DNA with sodium bisulfite, nested-PCR amplification and endonuclease (*Taq* I) digestion were performed. The digested DNA fragments were then separated by capillary electrophoresis using 1.5% poly(ethylene) oxide (M_{ave}, 8,000,000 g/mol) in the presence of electroosmotic flow. The improvement for DNA amplification using the nested PCR described here corresponded to approximately ten cells. In addition, the level of DNA methylation shown in the electropherograms obtained corresponded to the original percentage of DNA methylation from commercial available standard sample (0–100%). The electrophoretic patterns demonstrated that the six cancer cell lines tested displayed different degrees of DNA methylation and could be differentiated by hierarchical cluster analysis. Furthermore, the DNA methylation level was eliminated after treating the cells with an anti-cancer drug (5′-aza-2′-deoxycytidine). Together, these results suggest that CE-LIF is a potentially useful and cost-effective tool for cancer diagnosis or prognosis based on the heterogeneity in a patient's DNA.

1. Introduction

Epigenetic modifications, including promoter DNA methylation and histone protein modification, modulate chromatin structures to control downstream gene expression and, thereby, regulate critical biological functions. Unlike DNA mutation or single nucleotide polymorphism, epigenetic modulation alters gene transcription without changing the original DNA sequence. DNA methylation usually occurs at the repeated dinucleotide CpG sequences located in the promoter region, referred to as CpG islands. A hypermethylated promoter typically suppresses the expression of its downstream gene. Therefore, if the DNA methylation occurs in the promoter of an oncogene for example, the methylation of CpG islands will result in a tumor suppressor function. In contrast, DNA methylation may enhance tumorigenesis, if the silenced gene is a tumor suppressor gene. Recently, the level of DNA methylation has been found to be closely related to cancer transformation, early development and inflammatory diseases [1–3]. Therefore, developing methods to determine the extent of DNA methylation could provide an important tool for disease diagnosis.

Reverse phase high-performance liquid chromatography is traditionally used to determine the presence of 5′-methylcytosine (^{5m}C) [4]. Recently, Yu et al. reported a high-throughput method for the rapid screening of DNA methylation when the green fluorescence protein fused methyl binding domain proteins was used to bind to CpG dinucleotide of DNA on the biochip [5]. By manufacturing the chip into the format of microarray, the multiple spot could be detected by the platform based on surface plasmon resonance. To quantify the methylation level of a specific gene, polymerase chain reaction (PCR) generally combined with bisulfite restriction analysis (COBRA) is usually performed [6]. PCR is commonly used to determine the extent of DNA methylation not only because the method is highly sensitive but also because PCR can provide excellent specificity. For all PCR-based techniques, it is necessary to treat the genomic DNA with sodium bisulfite, which converts cytosine to uracil but leaves the 5′-methylcytosine intact [7]. After PCR amplification, the amplicons may contain thymine or cytosine, depending on the original state of methylation. The difference in

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bases enables researchers to evaluate the DNA methylation by a number of specific techniques. For example, methods to determine the DNA methylation of specific genes include methylation-specific PCR [8], high-resolution melting analysis [9] and bisulfite genomic sequencing [10]. Recently, gold nanoparticle-based biosensors [11] and even the direct detection of methylation from synthetic DNA by real-time PCR have been reported [12]. Until now, COBRA has been one of the most useful methods in the biochemical laboratory [6]. However, this gel electrophoresis-based method has several drawbacks, including being time-consuming, requiring a large quantity of sample and having limited separation power. This makes COBRA difficult to automate for cancer diagnosis.

Capillary electrophoresis with laser-induced fluorescence has emerged as a powerful method for DNA sequencing [13–18], proteomics studies [19–21] and other applications in bioanalysis [22–25]. This method has many advantages, such as good separation efficiency, high sensitivity, and small sample sizes. This method has the added advantage that it can be automated to facilitate high-throughput screening. Recently, researchers have developed several novel approaches for DNA methylation studies based on CE-LIF. Schmitz et al. first reported a method to detect DNA adducts in genomic DNA by CE-LIF [26]. Krais et al. applied CE-LIF to the determination of the global N^6-methyladenine [27] and 5-hydroxymethyl-2′-deoxycytidine [28] in DNA. Suzuki et al. reported a methylation-sensitive, single-strand conformation analysis, based on the electrophoretic migration difference between two denatured, single strands of DNA, that quantifies the level of methylation [29]. In a recent report, Brena et al. integrated a commercial electrophoresis chip with COBRA to evaluate the percentage of DNA methylation with good linearity [30]. Related studies and discussion of COBRA and CE-LIF have been published by the Wunsch group [31,32]. They used polyvinyl alcohol-coated capillary to separate DNA restriction fragments in the presence of the use of 2-hydroxyethylcellulose as a sieving matrix. The baseline separation of three DNA fragments (PCR products: 394 bp, digested products: 237 bp and 157 bp) was accomplished by CE-LIF in the absence of electroosmotic flow. Until now, most methods have focused on determining the level of DNA methylation; however, the percentage of ^{5m}C in DNA may not reflect the importance of epigenetics in cancer biology because DNA is extracted from a large number of cells. In other words, the distribution of DNA methylation in CpG islands may vary between tissues, or even from cell to cell in the same tissue type. To obtain a more clear relationship between ^{5m}C and biological phenomena, it is necessary to clone the heterogeneous PCR products into bacteria and sequence the inserted DNA from individual colonies. If thousands of individual DNA sequences are required to obtain a sufficient population for statistical analysis, a researcher must perform many colony selections, DNA extractions and DNA sequencing to assess the methylation state. Screening a large number of genes using this process is therefore costly, time-consuming and laborious. Thus, a method to determine the heterogeneity in DNA methylation that is fast, cost-effective, and automated is highly desirable for epigenetic studies.

Recently, the separation of the restriction endonuclease-digested PCR products with DNA size as small as 10 bp has been successfully achieved when these PCR products migrate against the electroosmotic flow and enter the poly(ethylene oxide) (PEO) sieving matrix [33]. The presence of EOF can overcome the needs of desalting of PCR products and removal of primers. The aim of this work is to combine this method and COBRA for the determination of multiple DNA methylation site within specific amplified PCR products in small quantity of biological sample. A nested PCR step was introduced during the initial template preparation stage to enhance the sensitivity and to reduce the interference of primer-dimers.

2. Experimental

2.1. Materials and methods

Poly(ethylene oxide) (PEO, average M_w 8,000,000 g/mol) and the chemicals used to prepare the electrolytes were obtained from Sigma–Aldrich (St. Louis, MO, USA). Customized synthetic oligonucleotides for PCR were purchased from Integrated DNA Technologies (San Diego, CA, USA). Ethidium bromide and DNase/RNase-free water were obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture and DNA extraction

Cell lines from lung cancer (NCIH1299), NPC (TW02, TW04) breast cancer (MCF7), oral cancer (OECM-1), liver cancer (J7) and cervical cancer (HeLa) were gifts from the Molecular Medicine Research Center of Chang Gung University. The NPC cells (HK-1) and the EBV-positive NPC cells (C666-1) were kindly provided by Prof. Sai-Wah Tsao from the Department of Anatomy at the University of Hong Kong. The cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640) at 37 °C in 5% CO_2 for 3 days. Cells were treated with 5′-aza-2′-deoxycytidine for 0, 1, 3, and 5 days. DNA was extracted from cell lines using QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen, Germany). DNase/RNase-free water was used to adjust the concentration of the synthetic DNA, and all DNA samples were stored at −20 °C.

2.3. Sodium bisulfite treatment, PCR and restriction enzymatic digestion

The extracted DNA samples were treated with EZ DNA Methylation-Gold Kit according to the user manual provided by the manufacturer (Zymo Research Corporation, CA, USA). Briefly, 1 μg of genomic DNA (20 μL) was added to a microtube that contained 130 μL of CT conversion reagent. After brief mixing, the tube was placed on a PCR thermal cycler (Astec, Japan) for an incubation of 10 min at 98 °C, 2.5 h at 65 °C and 20 h at 4 °C. The mixture was then transferred to a Zymo-Spin™ IC Column that contained 600 μL of M-Binding Buffer and was centrifuged at 10,000 × *g* for 30 s. After another wash with 200 μL of M-Wash Buffer, 200 μL of M-Desulfonation Buffer was added to the collection tube, and it was incubated at ambient temperature for 20 min. The collection tube was then washed twice with 200 μL of M-Wash Buffer (10,000 × *g*, 30 s). Finally, the microtube was transferred to a new 1.5-mL centrifuge tube, and the genomic DNA was eluted with double-deionized water by centrifugation (30 s at 10,000 × *g*). All of the reagent/buffer denominate described above are according to encase of vials within the EZ DNA Methylation-Gold kit. All bisulfite-treated samples were further investigated by PCR and endonuclease digestion. Two internal primers (miR9-1-1F: GGATTAGAGATTATTTAGGGTTGTGAA and miR9-1-1R: TAAAAACAAAACAACCTCCTCTACC) were chosen for traditional PCR amplification. Briefly, the 25 μL PCR mixtures contained 2.5 μL of 10× PCR buffer, 0.5 μL of primers (10 μM), 2 μL of dNTPs (2.5 mM), 2.0 units of FastStart *Taq* DNA polymerase (Roche, Indianapolis, IN) and 1 μL of the DNA template, with double-deionized H_2O added to give a final volume of 25 μL. Thermocycling of the reaction mixtures was performed in a model 818A Thermocycler (Astek, Japan) programmed for denaturation for 4 min at 95 °C, followed by 35 amplification cycles (30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C) and an extra-elongation step for 7 min at 72 °C. The length of the amplified PCR product for miR9-1 was 342 bp. For nested PCR, the following external primers were designed for the first amplification: miR9-1F (GAGATTATTTAGGGTTGTGAAAATG) and miR9-1R (AAAAACTAAAAATCAACACAAAAAC). Following 30

amplification cycles (30 s at 95 °C, 30 s at 50 °C, and 60 s at 72 °C), 1 μL of the initial amplified products (638 bp) was transferred to another microtube that contained a new PCR mixture and the internal primers (miR9-1-1F and miR9-1-1R). The conditions for the second PCR cycle were the same as traditional PCR. Products from both traditional and nested PCR were 342 bp. For enzymatic digestion, 5 μL of the amplified PCR products was added to a mixture containing 0.2 μL of *Taq* I endonuclease (2 units) (Roche, Indianapolis, USA) and 2.5 μL of restriction buffer (10×). The mixture was brought to a total volume of 25 μL with double-deionized water. After gentle mixing, the mixtures were incubated for 60 min at 37 °C for *Taq* I digestion.

2.4. Capillary electrophoresis with laser-induced fluorescence

In this study, a CE-LIF system, which was built in-house with a slight modification from previous reports, was used for DNA methylation analysis [33–35]. Briefly, a high-voltage power supply (Gamma High Voltage Research Inc., Ormond Beach, FL, USA) was used to drive electrophoresis. The entire detection system was enclosed within a black box with a high-voltage interlock. The high-voltage end of the separation system was placed in plastic housing for safety. A 543-nm He–Ne laser from Uniphase (Milpitas, CA, USA) with a 4-mW output was used for excitation. Fluorescence was monitored with a 10× objective (numerical aperture = 0.25), and a 610-nm interference filter was arranged after the objective to reject the scattered light before the emitted light reached the photomultiplier tube (R3896, Hamamatsu Photonics, Hamamatsu, Japan). The amplified current was transferred directly through a 10-kΩ resistor to a 24-bit A/D interface at 10 Hz, controlled using the Clarity software (DataApex, Prague, Czech Republic). Data were stored on a personal computer. Bare fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ, USA) with a 75-μm internal diameter was used as purchased for separation without any further coating. The capillary length was 40 cm, and the effective length from the detector was 33 cm. Anodic and cathodic vials were filled with a 1.5% PEO solution prepared in 100 mM Tris–boric acid buffer (pH 9.0) containing 5 μg/mL ethidium bromide. The capillary was filled with 1.5 M Tris–boric acid buffer (pH 10.0). The DNA samples were introduced into the capillary from the inlet (anodic) end by hydrodynamic injection at a height of 20-cm (the difference between the specimen vial and the cathodic vial) for 10 s. At an applied positive voltage of 15 kV, the DNA fragments migrating against the electroosmotic flow (EOF) entered the neutral (non-ionic) PEO solution from the anodic end and were separated according to the sieving mechanism at ambient temperature.

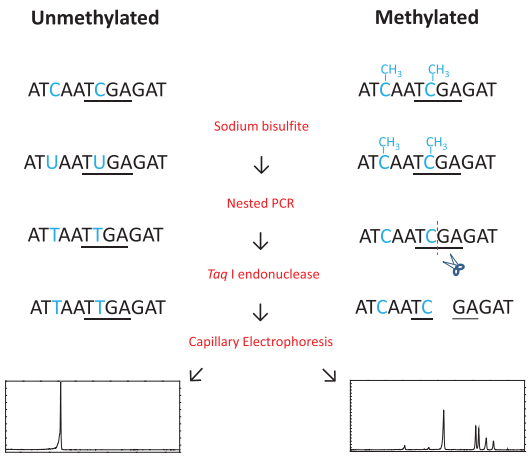
2.5. Data analysis

Peaks were identified by spiking a 10-bp (Invitrogen, Carlsbad, CA, USA) or 20-bp DNA ladder (Genepure, Taichung, Taiwan) into the digested DNA samples prior to capillary electrophoresis (CE) analysis. To normalize the difference in peak areas caused by the number of intercalated EtBr units in the digested PCR products, each identified peak area was divided by the length (in base pairs) of the PCR product. The normalized area of each peak was imported into the Partek Genomics Suite software (Partek Incorporated, MO, USA) for hierarchical clusters analysis. The color scale range was set from 0 to 20, with a middle point set at 3.

3. Results and discussion

3.1. Improvement of nested PCR for bisulfite treated genomic DNA

Previously, we reported a series of studies on the separation of small fragments of DNA based on capillary electrophoresis in



Scheme 1. Schematic representation of nested PCR combined with bisulfite restriction analysis followed by capillary electrophoresis (nCOBRA-CE).

the presence of electroosmotic flow [33,34]. Compared to the use of polymer-filled capillary, this method had several advantages including increased sensitivity (less DNA adsorbed on the wall of capillary), cost-effectiveness (longer life time for capillary), speed (without wash step), and a higher resolution of small DNA fragments (DNA migrate against EOF) [33–38]. In the present work, we evaluated the utility of CE-LIF in differentiating the heterogeneity of DNA methylation by analyzing the CpG islands of a microRNA gene 9 (miR9-1). A restriction endonuclease, *Tag* I, which recognizes the TCGA sequence, was used to digest amplified DNA into small fragments, generating a complex DNA pattern. As with most epigenetic studies, bisulfite treatment is necessary to discriminate the C and ^{5m}C in genomic DNA. As shown in Scheme 1, the unmethylated cytosine of genomic DNA was converted to uracil after bisulfate treatment. Following PCR or nested PCR, the uracil was converted to thymidine, but the ^{5m}C remained unchanged. The methyl group of ^{5m}C was not transferred to the amplicons during DNA amplification. Therefore, the T and C in the final PCR products (342 bp) represent the original C and ^{5m}C, respectively. If the original DNA template was methylated, the resulted PCR products will be cleaved into smaller DNA fragment after specific endonuclease (*Taq* I in this study) digestion.

Initially, we designed a primer pair for PCR amplification. However, due to the high GC content of the sequence, which led to poor amplification efficiency, the final PCR product was dominated by primer-dimers (Fig. S1A). Thus, nested PCR (i.e., double amplification) was chosen to improve PCR efficiency. In nested PCR, another outer primer pair covering half of the ^{5m}CpG islands region of the miR9 promoter was used for primary amplification. The reaction products were transferred to another microtube for secondary amplification. As shown in Fig. S1B, only a single sharp peak was present in the electropherogram, and primer-dimer formation was minimized, indicating a clean and efficient PCR amplification. By serially diluting the DNA in H_2O, as shown in Fig. S2, the minimum detectable DNA concentration that improved by nested PCR was calculated to be 30 pg before enzyme digestion, approximately equal to ten cells. These results indicate that nested PCR substantially improved the efficiency for bisulfite-treated DNA amplification, which is in agreement with the performance of nested PCR in a previous report [35].

3.2. Determinantion of the DNA methylation level for anti-cancer drug treated cancer cells

Previous studies have shown that the methylation levels of specific genes may play an important role in cancer development.

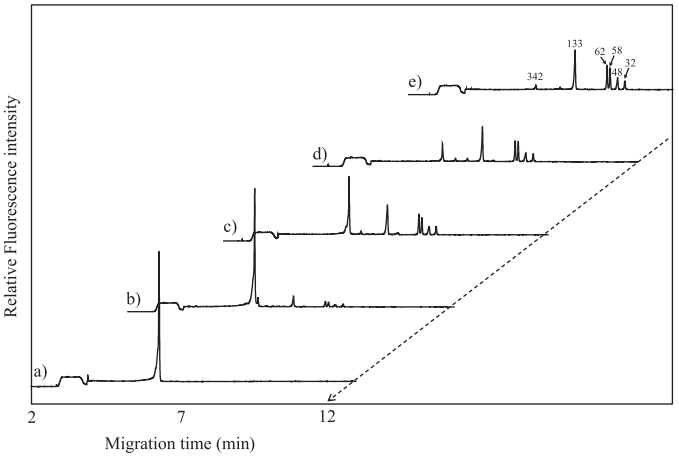


Fig. 1. The electropherograms of samples prepared by mixing fully methylated and unmethylated DNA in various proportions: (a) 0%, (b) 25%, (c) 50%, (d) 75%, and (e) 100% methylation.

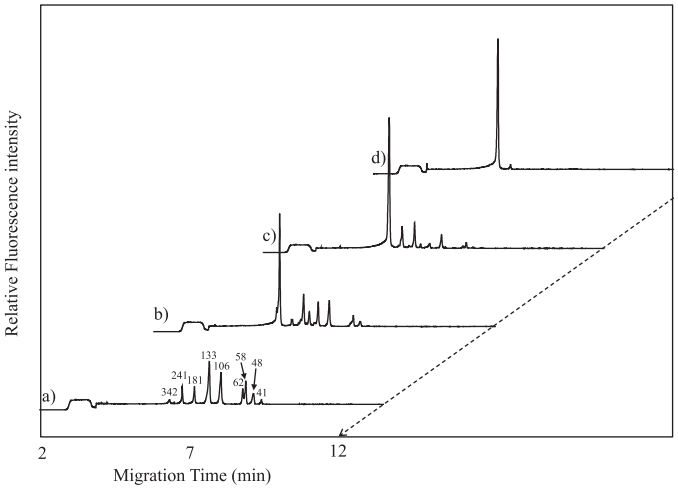


Fig. 2. nCOBRA-CE patterns obtained by treating cells (nasopharyngeal carcinoma cells, HK-1) with the anti-cancer drug 5′-aza-2′-deoxycytidine for (a) 0, (b) 1, (c) 3 and (d) 5 days.

COBRA-based techniques are widely used in biochemical laboratories due to ease of operation and cost effectiveness. Recently, Brena et al. reported a rapid method that evaluated the methylation state of SALL3, TWIST2 and C/EBPα genes based on chip electrophoresis [30]. To investigate the power of CE in separating methylated from unmethylated DNA, we mixed fully methylated and unmethylated DNA in the appropriate ratio (0%, 25%, 50%, 75%, and 100%) and the mixture underwent bisulfite conversion, PCR and endonuclease digestion. As shown in Fig. 1a, the peak corresponding to the DNA fragment that is 342 bp in length belongs to the PCR products that were amplified from unmethylated DNA without the bisulfite-induced *Taq* restriction site in the CpG islands. That is, the cytosines of this region were converted to uracils, thus eliminating the sequence required for endonuclease digestion. Conversely, the fully methylated DNA sample was not converted to uracil by sodium bisulfite due to the resistant methylated cytosine. Therefore, five smaller peaks were detected in the electropherogram (Fig. 1e) representing the fragments resulting from the *Taq* (TCGA) restriction digestion in the ^{5m}CpG islands of the fully methylated genomic DNA. These results indicate that nested PCR COBRA-CE (nCOBRA-CE) could serve as a useful tool in the evaluation of the level of DNA methylation from cancer candidates, as previously reported [30].

Esteller and Herman reported that the O(6)-methylguanine DNA methyltransferase (MGMT) is a DNA repair enzyme that may influence a patient's sensitivity to chemotherapy [39]. Therefore, in addition to rapid screening of the DNA methylation state, we tested whether nCOBRA-CE can also be used to monitor the efficacy of chemotherapy. In Fig. 2, HK1 cells were treated with 5′-aza-2′-deoxycytidine, a DNA methyltransferase inhibitor used to treat mylodysplasia, for 1, 3 and 5 days. The DNA methylation level began to decrease on the first day (Fig. 2b), and the highly methylated region (i.e., fragments less than 342 bp) was almost completely converted to unmethylated DNA (342 bp PCR products) after 5 days (Fig. 2e), leaving only one nPCR product peak in the electropherogram. This result suggests that the methyl groups in the genomic DNA were removed after several cell division cycles and, thus, could be converted to U by sodium bisulfite. Therefore, this method has the potential for use in prognosis or pharmacological applications by evaluating the total methylation level.

3.3. Determination of cancer restriction fragment patterns by nCOBRA-CE

However, measuring the methylation levels of a single CpG island of a specific gene alone may not be very useful for cancer diagnosis, due to the heterogeneity of DNA methylation in cancer cells. Therefore, six cancer cell lines were tested to demonstrate the feasibility of detecting DNA methylation heterogeneity based on nCOBRA-CE. Out of the six cancer types, lung cancer (Fig. 3a) displayed the lowest methylation level, and the TW02 cell line (Fig. 3f) was highly methylated in part of the CpG islands of the miR9 gene. As shown in Scheme 2, the 342 bp fragments of the PCR products (green line) used in this study had 4 *Taq* restriction sites when fully methylated DNA was used as the template, leading to five smaller DNA fragments (133, 62, 58, 48 and 41 bp). There were only five peaks present (red line in Scheme 2) when the DNA methylation level in cancer cells was homogeneous (like artificial 100% methylated DNA) (Fig. 1). Therefore, the unexpected peaks (other than 342, 133, 62, 58, 48 and 41 bp) that appeared in the electropherograms (Fig. 3f) may reflect the heterogeneity in DNA methylation. Scheme 2 illustrates the likely combination of digested DNA fragments caused by heterogeneous methylation. For instance, in addition to the five expected fragments (i.e., 133, 62, 58, 48 and 41 bp), the TW02 cells (Fig. 3f) contained additional peaks corresponding to fragments of 209, 181, 168 and 106 bp. Scheme 2 shows all of the digested DNA fragments from the 342 bp PCR products predicted if DNA methylation was incomplete. Following the

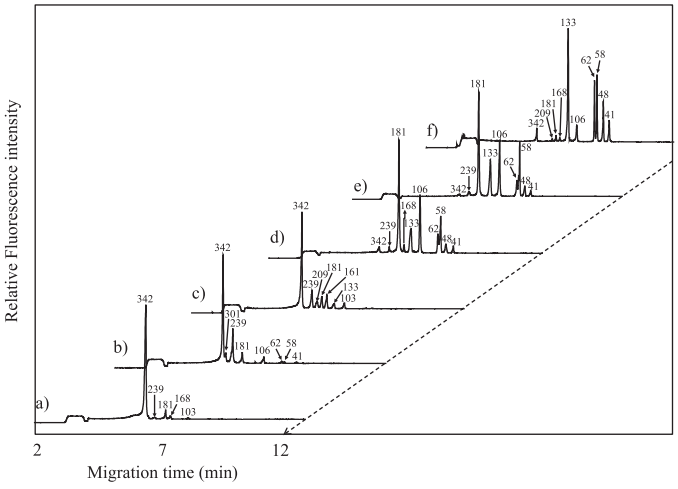
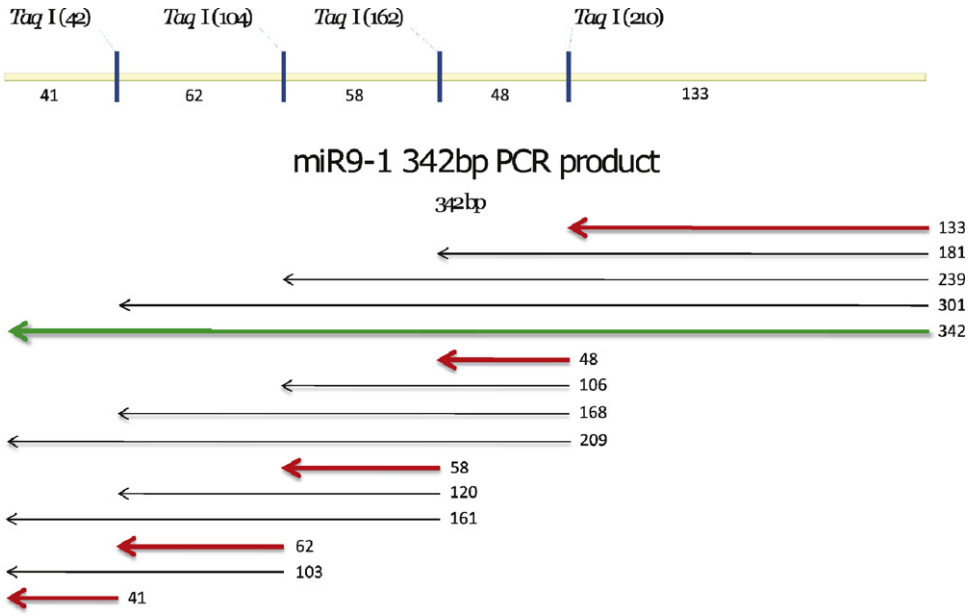


Fig. 3. Electropherograms of samples derived from six cancer cell lines analyzed by nCOBRA-CE: (a) NCIH1299 (lung cancer), (b) MCF7 (breast cancer), (c) OECM-1 (oral cancer), (d) J7 (liver cancer), (e) Hela (cervical cancer) and (f) TW02 (NPC).



Scheme 2. The predicted restriction fragments of miR9-1 nPCR products produced by *Taq* I endonuclease digestion. The green line denotes the length of the PCR products (342 bp), and the red lines indicate the five digestion products, if the amplicons of the fully methylated genomic DNA template are completely digested. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

illustrated restriction map, the four peaks at 209, 181, 168 and 106 bp should be the products of incomplete digestion resulting from heterogeneity in DNA methylation. Based on the data shown above, the main differences in the electropherograms (Fig. 3a–f) indicate that the identification of cancer is difficult to accomplish by measuring the total DNA methylation level. For instance, the total methylation levels normalized to the peak area ratio of the nPCR products before and after digestion of these cancer cell lines are as follows: NCIH1299 (lung cancer) at 16.5%, MCF7 (breast cancer) at 54.9%, OECM-1 (oral cancer) at 62.9%, J7 (liver cancer) at 99.4%, HeLa (cervical cancer) at 78.5% and TW02 (NPC) at 99.0%. The methylation level of MCF7 was similar to OECM-1, and J7 was similar to TW02, corresponding to over 99% methylation. These data also showed that the methylation of promoter DNA in cancer tissue is not homogenously distributed among all of the CpG islands of a gene. That is, methylation occurring in any CpG islands is not homogeneous, thus increasing the complexity of nCOBRA-CE electropherograms. Studies have clearly indicated that methylation heterogeneity of DNA is a result of complex interactions between DNA, histones and other epigenetic modulatory machinery [40]. Traditionally, to explore the heterogeneity of DNA methylation in a particular cell line, the PCR products were cloned into a plasmid and transfected into *Escherichia coli*. Following single-colony collection, DNA extraction and sequencing were performed if a certain heterogeneity level of DNA methylation was desired. This technique is time-consuming and costly, which limits its application in population-wide screening. However, with the sensitivity of nested PCR and the excellent separation efficiency of CE, our data indicated that nCOBRA-CE could provide better insights into the heterogeneity of DNA methylation. A simple CE-LIF approach could be used to rapidly distinguish cancer type based on the heterogeneity of DNA methylation.

3.4. Determination of the heterogeneity of DNA methylation by hierarchical cluster analysis

If nCOBRA-CE is performed on a high-throughput CE-LIF instrument [17,41–44], the collection of vast amounts of irrelevant data may waste time through excessive data processing and cancer identification due to the enormous amounts of electropherograms that are generated simultaneously by capillary array electrophoresis. Hierarchical cluster analysis software is a statistical tool used to quickly group large datasets into specific similar clusters. In this study, the peak data obtained from each cancer type were

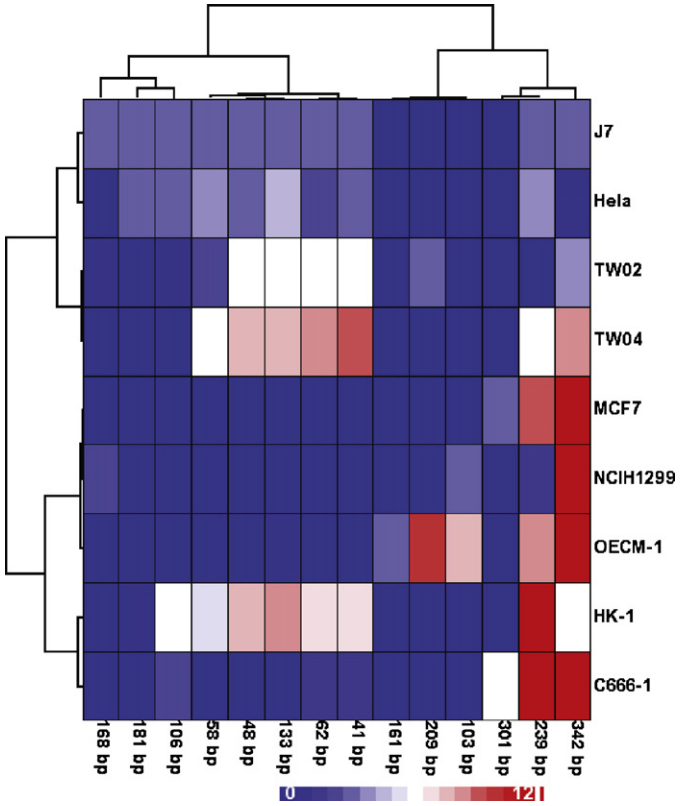


Fig. 4. Hierarchical clustering of the miR-9-1 methylation pattern in the nine cancer cells. The column dendrogram indicates the length of the restriction fragments in bp, while the row dendrogram indicates the clusters of cancer cells. The color scaling was set to range from 0 to 20, with a midpoint at 3.

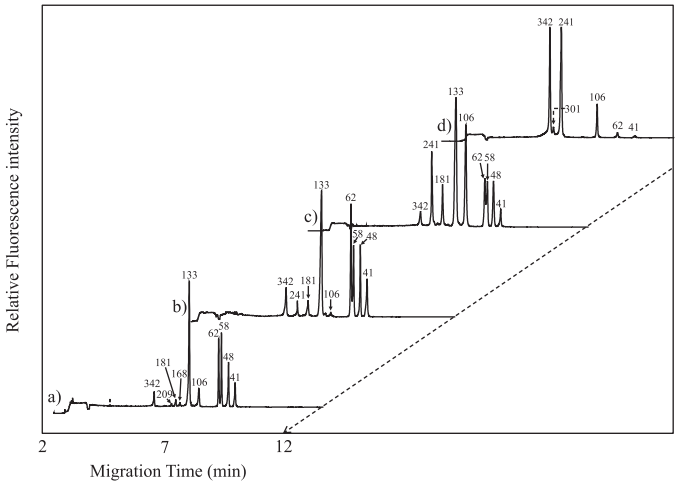


Fig. 5. nCOBRA-CE electropherograms of four nasopharyngeal carcinoma cells: (a) TW02, (b) TW04, (c) HK-1 and (d) C666-1.

imported into the software for hierarchical cluster analysis. Fig. 4 shows the result of hierarchical clustering of the six cell lines based on the normalized peak areas (i.e., the original peak area divided by the base-pair length of the restriction fragments). Compared to the electropherograms shown in Fig. 3, hierarchical clusters analysis discriminated among the different cancer types better than the percentages obtained from their level of total methylation. For example, MCF7 (54.9%) and OECM-1 (62.9%) have similar levels of total methylation, but could easily be differentiated by their 103, 209, 161 and 133 bp fragments. Similarly, both J7 and TW02 had over 99% total methylation, but the 239 bp fragment could serve as an identification marker between the two cells. On the other hand, two NPC cells (TW02 and TW04) were classified into one group in this plot, while the C6661 cells clustered into another group. The four NPC cells had the same total methylation level, except for C666-1. The level of total methylation for cells in the TW02, TW04, HK-1 and C666-1 groups corresponded to 99.0%, 99.8%, 99.4% and 75.1%, respectively. Among the four nasopharyngeal carcinomas, TW02 (Fig. 5a) represented the maximum level of methylation, and C666-1 (Fig. 5d) displayed a different restriction pattern. The C6661 cell line was derived from an EBV-positive cultured NPC cell and could serve as an excellent investigative tool for the viral latency pattern [45]. The presence of EBV-encoded genes could alter the methylation state of the C6661 genome and result in a different methylation pattern from other NPC cell lines. These results suggest that the heterogeneous DNA methylation patterns in different cell lines from the same cancer type can be used to distinguish between one cell and another through nCOBRA-CE-LIF with hierarchical clustering analysis.

4. Conclusions

In this study, a fast, simple and cost-effective method for determining the heterogeneity of DNA methylation was proposed. In addition to quantifying the total level of DNA methylation in a sample, nCOBRA-CE can also elucidate the pattern of DNA methylation. Although CE-LIF is not a new technology, to the best of our knowledge, this is the first study to use it to detect differences in DNA methylation between various cell lines. In this study, we used a single restriction endonuclease to digest the nPCR products, and due to the specificity of the *Taq* I endonuclease, there were only four restriction sites in this amplicon. Therefore, only four of the twenty-three ^{5m}CpG elements were recognized. The discriminatory power of this method would be greatly improved by combining more digestive enzymes (i.e., *Aci* I, *Bst* UI and *Hha* I) and using a high-resolution sieving medium [46–50] to separate the small DNA fragments. Therefore, the next challenge will be to select the appropriate restriction enzyme, or combination of enzymes, to generate more complex restriction patterns that will provide more detailed information for cancer diagnosis or prognosis based on the difference in DNA methylation patterns. However, our data indicated that the detectable genomic DNA could be lowered to approximately 30 pg by double amplification technology (i.e., nested PCR), even when the genomic DNA is CG-rich. Thus, we believe that this method may be used in biomedical applications, such as NPC diagnosis, in which the size of the tumor sample is small and only a limited tissue sample may be available.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.01.049.

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