ANALYTICA CHIMICA ACTA 603 (2007) 199–204

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/aca

Allele-specific extension on microarray for DNA methylation analysis

Zhixiang Wu[a], *Junfeng Luo*[a], *Qinyu Ge*[b], *Dingdong Zhang*[a], *Yan Wang*[a], *Chao Jia*[a], *Zuhong Lu*[a,b,*]

[a] *State Key Laboratory of Bioelectronics, Southeast University, Nanjing 210096, China*
[b] *Key laboratory of Child Development and Learning Science, Ministry of Education, Southeast University, Nanjing 210096, China*

ARTICLE INFO

Article history:
Received 26 June 2007
Received in revised form
4 September 2007
Accepted 5 September 2007
Published on line 22 September 2007

Keywords:
Methylation
Microarray
Allele-specific extension
Methylation-specific PCR

ABSTRACT

Aberrant DNA methylation of CpG site in the gene promoter region has been confirmed to be closely associated with carcinogenesis. In this present study, a new method based on the allele-specific extension on microarray technique for detecting changes of DNA methylation in cancer was developed. The target gene regions were amplified from the bisulfite treated genomic DNA (gDNA) with modified primers and treated with exonuclease to generate single-strand targets. Allele-specific extension of the immobilized primers took place along a stretch of target sequence with the presence of DNA polymerase and Cy5-labeled dGTP. To control the false positive signals, the hybridization condition, DNA polymerase, extension time and primers design were optimized. Two breast tumor-related genes (P16 and E-cadherin) were analyzed with this present method successfully and all the results were compatible with that of traditional methylation-specific PCR. The experiments results demonstrated that this DNA microarray-based method could be applied as a high throughput tool for methylation status analysis of the cancer-related genes, which could be widely used in cancer diagnosis or the detection of recurrence.

1. Introduction

DNA methylation, which covalently adds a methyl group to position five of the cytosine ring within most CpG dinucleotides in the mammalian genome, is an epigenetic modification that can play an important role in gene expression of mammalian cells [1]. Normal methylation patterns are frequently disrupted in tumor cells with global hypomethylation accompanying region-specific hypermethylation, which is now recognized as an important and early event in carcinogenesis, so, quantitative methylation analysis of cancer-related genes may be useful for cancer diagnosis or the detection of recurrence [2–7]. Until now, many molecular biology methods have been developed to determine the methylation status of a CpG island [8–10], among which methylation-specific PCR (MSP) [11–14] is the most commonly used method to qualitative and quantitative DNA methylation analysis. MSP, as a classic method, is sensitive and easy to use but can analyze only a very limited number of dinucleotide cytosine–guanosine (CpG) dinucleotides (~3–10, depending on the primers/probes used) [15]. Furthermore, this method could not provide precise information about the methylation status of single CpG site. Recently, Gitan et al. [10] have developed a novel technique called methylation-specific

* *Corresponding author at*: State Key Laboratory of Bioelectronics, School of Biological Science and Medical Engineering, Southeast University, Nanjing 210096, China. Tel.: +86 25 83793779; fax: +86 25 83793779.
E-mail address: zhlu@seu.edu.cn (Z. Lu).
0003-2670/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2007.09.027

oligonucleotide (MSO) microarray that combines bisulfite DNA assay and oligonucleotides microarray for DNA methylation analysis. The targets were derived from PCR products of bisulfite-modified DNA, whereas the probes used a series of arrayed oligonucleotides that can discriminate between converted and unconverted nucleotides, that is, unmethylated and methylated cytosines, at CpG sites. The MSO microarray potentially allows rapid screening of multiple CpG sites in many gene promoters however, cross-hybridization between imperfect-match probes and targets could be observed in this method and it either cannot provide precise information about the methylation status of single CpG sites if there are more than one CpG site in the oligonucleotide probe. CpG island hypermethylation has been reported to be linked to the silencing of more than 100 cancer-related genes [16]. So a high throughput analysis tool is highly needed, which could analyze all of related genes for cancer diagnosis or the detection of recurrence.

In this study, allele-specific extension [17–22] on microarray, which can be generated to contain hundreds of oligonucleotides designed to discriminate methylated and unmethylated sequences in these gene promoters were developed. A series of synthesized oligonucleotides corresponding to eight investigated targets were immobilized on the aldehyde-coated glass slide to fabricate a DNA microarray for detecting methylation status of P16 and E-cadherin genes and all the results were validated by traditional MSP. The result shows that this method allows potential quantification for almost all CpG methylation alleles within the selected genes.

2. Material and method

2.1. Cell line

Total cell lines genomic DNA MNK7, SK-PR-3, ZR-75-30-1, ZR-75-30-2, MDA-MB-231, Bcap-37, HBL-100, MDA-MB-435S, L02, and HuvEc (number from C1 to C10) were obtained from shenerg biocolor, Shanghai, China. Whole blood cells of healthy human were obtained from ZhongDa hospital, Nanjing, China. Genomic DNA was extracted from whole blood cells by standard phenol/chloroform method (number N1 and N2). All the Genomic DNA was suspended in TE buffer and the DNA concentrations were calculated according to their OD_{260} readings.

2.2. Bisulfite analysis

Bisulfite treatment was carried out following the modified procedure described by Frommer et al. [23] briefly, 5 μg of genomic DNA was digested with EcoRI (NewEngland Biolabs) and denatured with 0.3 M NaOH for 30 min at 42 °C in the final volume of 20 μL. Three hundred and eighty microliters freshly prepared solution of sodium bisulfite (2.5 M, pH 5.0) and hydroquinone (100 mM) was added to the denatured DNA, and the mixture was incubated at 53 °C for 14–18 h. After desalting (Wizard Clean-UpSystem, Promega), the DNA was desulfonated with 0.3 M NaOH for 30 min at 37 °C. The solution was neutralized with 5 M ammonium acetate (pH 7.0), neutralized by ammonium acetate, alcohol precipitated, dried, and then resuspended in Tris–EDTA (pH 7.5) for use. After bisulfite processing, the all unmethylated cytosine residues converted to uracil, whereas the methylated ones remained unchanged.

The targets P16 gene and E-cadherin gene were amplified with 20 pmol of each forward and backward primer by PCR as described previously [16]. The profile consisted of an initial melting step of 5 min at 95 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 62 °C (54 °C for E-cadherin), and 30 s at 72 °C and a final elongation step of 10 min at 72 °C. The PCR mixtures contained 20 mM Tris–HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 400 μmol each dNTP, and 1.25 units of Taq DNA polymerase (TIANWEI), and 2 μL bisulfite-modified DNA (equivalent to 100 ng genomic DNA) in 50 μL. PCR was preformed in a PTC-225 thermocycler (MJ Research, Watertown, MA). PCR products were purified using a QIAquick column (Qiagen). The eluted PCR products were dried in vacuum and resuspended in deionized water for use.

2.3. Generation of DNA target

Single-strand generation: to generate single-strand DNA, we modified the 5′-end of one primer with phosphorylation, which could activate the exonuclease and be digested, and the other end with phosphorothioate to resist being digested. Primers for amplification P16 gene product were P16-L (5′-PO4-AAAGAGGAGGGGTTGGTTGGTTATTA) and P16-R (5′-T-s-ACCTAATTCCAATTCCCCTACAAACT); for E-cadherin gene product, E-cad-L (5′-PO4-GGTAGGTGAATTTTTAGTTAATTAG) and E-cad-R (5′-A-s-ATACCTACAACAACAACAACAAC). The purified double-stranded PCR product, resuspending in deionized water with 1× lambda exonuclease reaction buffer (67 mM glycine–KOH, 2.5 mM $MgCl_2$, 50 μg/mL, BSA, pH 9.4), 5 U lambda exonuclease (New England Biolabs) was added and mixture incubated for 2 h at 37 °C and 10 min at 75 °C for inactivation. The digested product was used to hybridize directly.

2.4. Allele-specific oligonucleotide probes and microarray preparation

All the CpG site-specific primers were synthesized with a 5′-amino group that facilitates covalent immobilization on the glass slide. A spacer sequence of 12 T residues was included at the 5′-end of the site-specific sequence. The site-specific sequences of the extension primers were designed to have a Tm of 63 ± 2 °C with the 3′-nucleotide hybridizing at the variant position. Each site has two primers which are different from each other for the last base at the 3′-end. One is C corresponding methylated CpG site, the other is T corresponding unmethylated site. If there is more than one CpG site in a primer size, we used the Y (C or T) to replace the ascertained base (C or T), such as the primer P16-13. In this experiment, the date showed that such a design had little effect on the hybridization and extension (data not showed). The methylation-specific and unmethylattion-specific oligonucleotide probes used in this study were synthesized by Invitrogen™ (Shanghai, China) and are summarized in Table 1. The oligonucleotides, diluted in carbonate buffer (0.1 mol/L, pH 9.0) to the final concentration of 10 μM, were spotted on the slides which were modified with aldehyde group, using a PixSys5500 microarrayer (Cartesian Technology). The spotted slides were roasted at 80 °C for 1 h. After

Table 1 – Allele-specific oligonucleotide sequence

Name	Sequence	Tm
P16-05	MNH_2-$(T)_{12}$GATTTAGGTGGGTAGAGGGTTTGTAGC; U NH_2-$(T)_{12}$GATTTAGGTGGGTAGAGGGTTTGTAGT	64.5
P16-13	M NH_2-$(T)_{12}$GGGTAGAGGAGGTGYGGGC; U NH_2-$(T)_{12}$GGGTAGAGGAGGTGYGGGT	63.9
P16-20	M NH_2-$(T)_{12}$GGCGGGGAGTAGTATGGAGTTTTC; U NH_2-$(T)_{12}$GGCGGGGAGTAGTATGGAGTTTTT	64.6
P16-26	M NH_2-$(T)_{12}$CGGAGAGGGGGAGAGTAGGTAGC; U NH_2-$(T)_{12}$CGGAGAGGGGGAGAGTAGGTAGT	64.7
P16-33	M NH_2-$(T)_{12}$GGTTATTAGAGGGTGGGGC; U NH_2-$(T)_{12}$GGTTATTAGAGGGTGGGGT	65
E-cad-01	M NH_2-$(T)_{12}$GGTAGGTGAATTTTTAGTTAATTAGC; U NH_2-$(T)_{12}$GGTAGGTGAATTTTTAGTTAATTAGT	63
E-cad-08	M NH_2-$(T)_{12}$CGGAATTGTAAAGTATTTGTGAGTTTGC; U NH_2-$(T)_{12}$ CGGAATTGTAAAGTATTTGTGAGTTTGT	64.4
E-cad-20	M NH_2-$(T)_{12}$TCGGTTAGTTATGGGTTTTTGGAGTC; U NH_2-$(T)_{12}$TCGGTTAGTTATGGGTTTTTGGAGTT	64.3

further treatment with a $NaBH_4$ solution for 30 min, the slide was respectively rinsed and washed at room temperature with 2× SSC-0.5% SDS 5 min × 2, 0.1× SSC-0.1% SDS 5 min × 2 and ddH_2O 5 min × 2. The slides were ready for hybridization after being dried with nitrogen.

2.5. *Hybridization and elongation*

Hybridization of the microarray was performed in a moist hybridization chamber and warmed-up at 72 °C for 10 min. The digested product was suspended in Unihybridization solution (1:3 dilution v/v; Telechem) and added to the prepared microarray. The hybridization was done at 72 °C for 5 min and cooled to room temperature more than 30 min. After hybridization, the slide was respectively rinsed and washed at room temperature with 2× SSC-0.1% SDS 5 min × 2, 0.1× SSC-0.1% SDS 5 min × 2 and ddH_2O 5 min × 2. The slides were ready for extension after dried with nitrogen.

After the hybridization, the microarray was warmed-up at 72 °C for 15 min and the extension was carried out in moist hybridization chamber under a cover slip at 72 °C for 3 min. Reaction conditions were 20 mM Tris–HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 10 pmol Cy5-dGTP and 0.5 units of Taq DNA polymerase (Tianwei) in 10 μL. The reaction terminated by rinsing with 2× SSC-0.5% SDS followed by washing at room temperature with 2× SSC-0.5% SDS 5 min × 2, 0.1× SSC-0.1% SDS 5 min × 2 and ddH_2O 5 min × 2. The slides were ready for scanning after being dried with nitrogen.

2.6. *Image scanning and data processing*

The microarray slide was scanned with Tecan Scanner after the above treatment. The images acquired by the scanner were analyzed with software Array-Pro Analyzer 4.5. Each spot was defined by the positioning of a grid of circles over the array image. For each fluorescent image, the average pixel intensity within each circle was determined and a local background using mean pixel intensity was computed for each spot. The net signal was determined by subtraction of this local background from the mean average intensity for each spot. Statistical analyses were conducted using Microsoft excel®.

3. Result

3.1. *Strategy of this assay*

Fig. 1 outlines our strategy for the DNA methylation analysis, which has some similarity to classic MSP. To distinguish methylated and unmethylated states of the CpG sites in target sequence relying on the power of the Taq polymerase, DNA from all samples was first treated with bisulfite, converting unmethylated cytosine to uracil and nonconversion of 5-methylcytosine. The region of interest was then amplified by PCR with modified primers, converting originally unmethylated CpG dinucleotide to TpG while conserving originally methylated CpG. After treatment with lambda exonuclease (NEB), single-strand target DNA was then hybridized to the arrayed oligonucleotide probes, followed by extension with Cy5 labeled dGTP. In order to control the false positive signal,

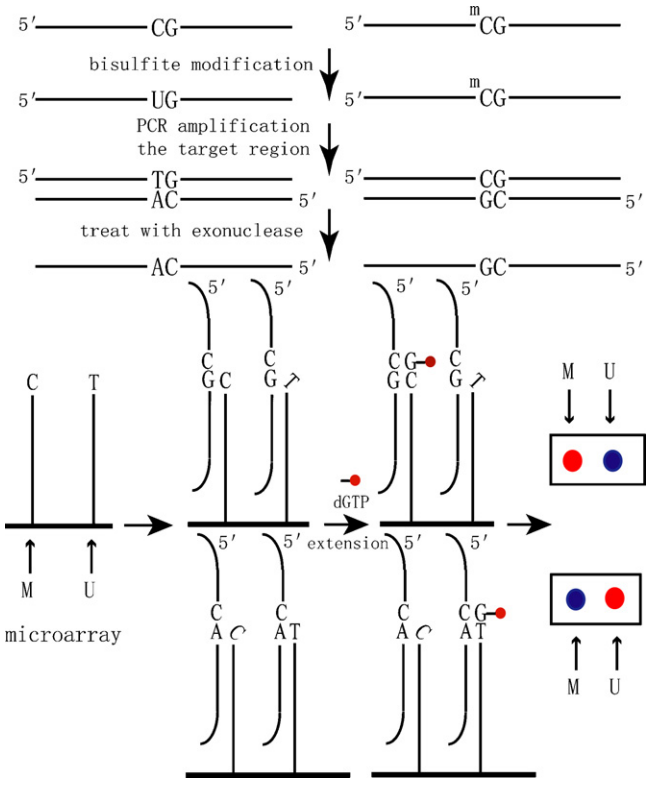

Fig. 1 – Principle of the allele-specific extension on microarray. After the bisulfite modification, target gene regions are amplified by PCR. The products are digested to single strand which serves as templates in a Taq polymerase-catalyzed extension reaction of immobilized oligonucleotide primers. The pair of match and mismatch primers differs at their free 3′-ends by C or T base, which is discriminated by the enzyme. Cy5 labeled dGTP was used to stead normal dGTP for the signal detection in the extension reaction.

Fig. 2 – Nucleotide sequences of the 5′-untranslated region and the first exon of the p16 gene (Genbank accession no. U12818.1GI:533724). (A) Is the original sequence of the p16 gene. (B) Is the sequence after the bisulfite modification. Y means C or T, which is determined by the methylation status. The CpG sites tested by oligonucleotide microarray are labeled with number and the site-specific probes sequences are underlined. The primer sites used to amplify the target are labeled with red font. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

we used only Cy5 labeled dGTP without normal dNTP in this assay.

Taking P16 gene as an example (Fig. 2), a 336 bp segment was selected in the 5′-untranslated regions and the first exon of the p16 gene, as the investigated target, which contains 33 CpG sites. Five sets of oligonucleotide probes were designed to test 5 CpG sites within the island, each set contained a pair of methylated and unmethylated oligonucleotides for the corresponding CpG site (Table 1). We examined the feasibility of our strategy by assessing the methylation status of 5 CpG sites located in p16 gene and 3 CpG sites located in E-cadherin gene. First, control DNA targets were used to test the accuracy and reproducibility of probes designed for microarray hybridization and extension. We used fully methylated and unmethylated clones as positive and negative controls. The positive control generated in this way remained 100% cytosine in the tested CpG sites, whereas the negative control had all cytosine residues converted into thymine in the tested CpG sites. Next, a series of microarray hybridization and extension were performed with mixtures of positive and negative DNA targets at different proportions representing 0%, 25%, 50%, 75%, and 100% of DNA methylation to test the linearity of the protocol (Fig. 3).

3.2. *Hybridization and extension*

In this study we compared two way of hybridization. One was according the traditional way, briefly, hybridization of the microarray was performed in a moist hybridization chamber at 37 °C for at least 3 h, and the other one was described above. There was no obvious different between this two different way of hybridization, but the later can save a lot of time.

Then to evaluate the performance of allele-specific extension on DNA microarrays, we compared different enzyme and different extension time. Here we used three kinds of DNA polymerase to compare with each other. The Taq DNA polymerase enzyme showed the best result among Taq polymerase, Bst polymerase, and Therminator polymerase. The result demonstrated that Therminator DNA polymerase nearly could not differentiate the match or mismatch base at the 3′-end of the primers (Fig. 4A). The intensity of false positive signal increased with the increase of extension time (from 3 min to 30 min). Good signal-noise ratio could be obtained through extension time of 3 min long (Fig. 4B)

3.3. *Verification by methylation-specific PCR (MSP)*

To further validate the allele-specific extension findings, MSP was conducted in these samples. The primers P16 MA, MS, and E-cadherin MA, MS for MSP were designed to conform probes

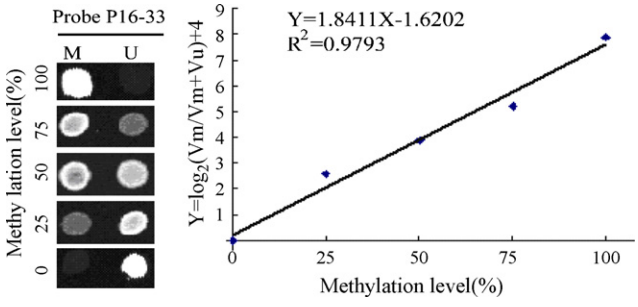

Fig. 3 – Preparation of the calibration curve. Fully methylated and unmethylated control DNA prepared and mixed (0%, 25%, 50%, 75%, or 100%) and then amplified by PCR using bisulfite primers for target gene CpG islands. Here, take the probe P16-33 as an example. The calibration curve for measuring methylation changes at the probe P16-33 CpG sites. The linear distribution shows that measurements of the different mixtures are well distinguished and can be used to determine the methylation status for test samples. Vm represents fluorescence signal intensities of methylation-specific probe, while, Vu represents fluorescence signal intensities of unmethylation-specific probe.

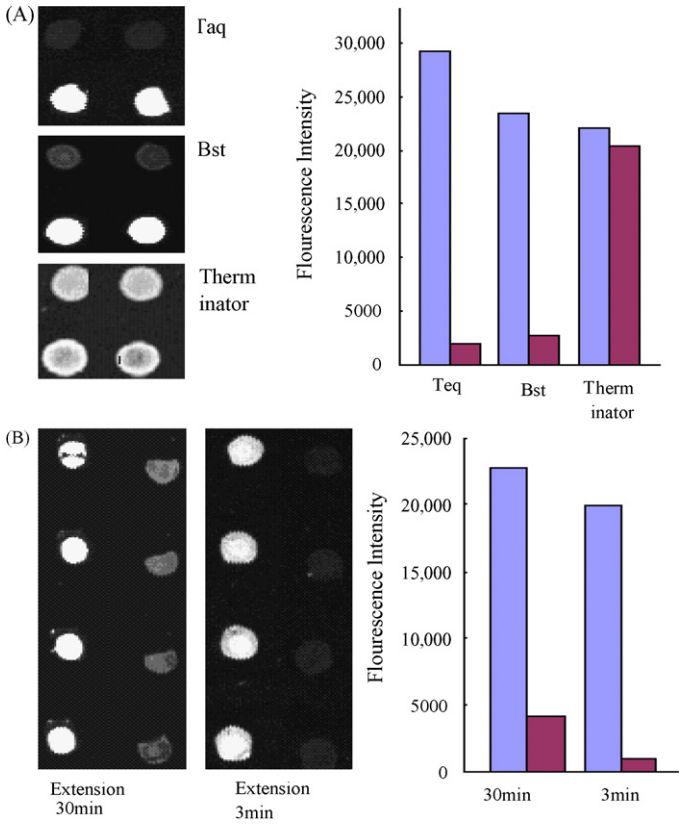

Fig. 4 – False positive singles under different conditions. (A) Right: from the top to the bottom was the result of Taq, Bst, Therminator DNA polymerase used in this study. Right: data analysis by Microsoft excel. The Taq DNA polymerase played the best in the discrimination of match or mismatch at the primers' free 3′-end. (B) The intensity of false positive signal differed with increased extension time (from 3 min to 30 min).

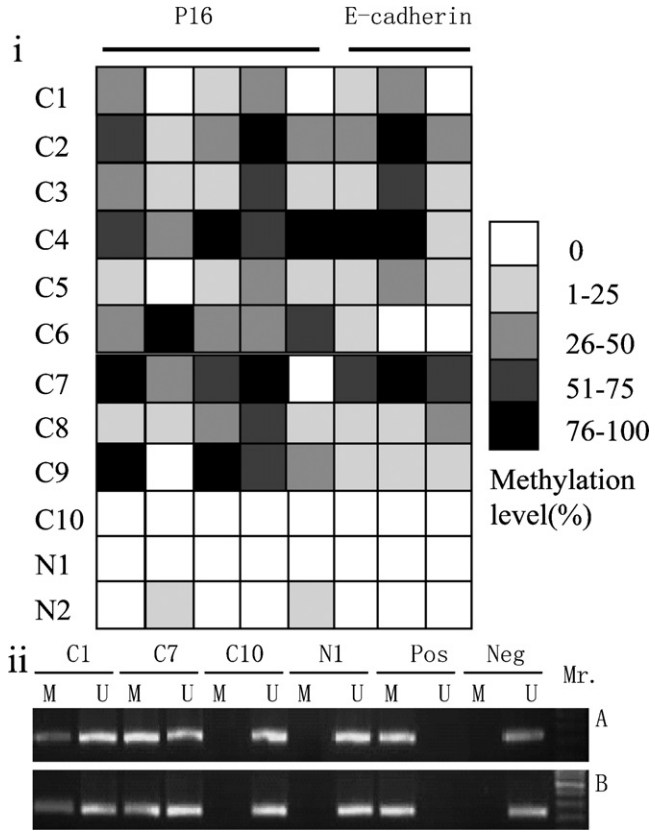

Fig. 5 – Methylation analysis of p16 gene and E-cadherin gene CpG islands by allele-specific oligonucleotide microarray and validated by MSP. (i) Summaries of the microarray results are shown for 12 samples. Gray scale shown at right represents the methylation levels in percentage determined from the calibration curve for the test CpG sites. (ii) MSP analysis of the p16 gene and E-cadherin gene CpG island in 12 samples. M and U indicate amplification using methylated and unmethylated sequence-specific primers, respectively. (Pos) positive control; (Neg) negative control; (Mr.) DNA marker; (A) P16 gene and (B) E-cadherin gene.

E-cadherin-01, E-cadherin-20, and P16-05, P16-33 (Table 2). A representation of the MSP analysis is shown in Fig. 5. According to this approach, C10 and N1 were completely unmethylated (C10 was the normal cell line DNA while N 1 was the DNA extracted from normal person's blood). MSP results of the nine-breast tumor cell line completely matched with microarray results. The above results indicated that microarray assay could potentially increase the frequency of detecting

Table 2 – Primers for methylation-specific PCR (MSP)

Primer sets	Sequences (5′ → 3′)	Size (bp)	Annealing temperature (°C)
P16 MS	TTGGTTATTAGAGGGTGGGGC	252	61
P16 MA1	TACAAACCCTCTACCCACCTAAATCG		
P16 US	TTGGTTATTAGAGGGTGGGGT	252	59
P16 UA1	TACAAACCCTCTACCCACCTAAATCA		
E-cadherin MS	GGTAGGTGAATTTTTAGTTAATTAGC	222	54
E-cadherin MA1	GACTCCAAAAACCCATAACTAACG		
E-cadherin US	GGTAGGTGAATTTTTAGTTAATTAGT	222	53
E-cadherin UA1	GACTCCAAAAACCCATAACTAACA		

P16 gene and E-cadherin gene island CpG site methylation from tumor samples more than MSP could do.

4. Discussion

Allele-specific has been proved that it can be used in SNP typing by many groups [17–21,24]. In this assay, we attempted to use this traditional method in DNA methylation analysis. The results showed that this method was successfully used to map methylated CpG sites within the p16 gene and E-cadherin CpG islands in samples. The derived methylation information for samples was assessed quantitatively and independently validated by classic MSP.

At present time, most methylation assays are limited to analyzing CpG islands of a few known genes and also restricted in throughput for a genome-wide analysis. Such as MSP, which is a simple, sensitive, and specific method for determining the methylation status of virtually any CpG-rich region. Nevertheless, methylation could not be detected when some CpG sites were not methylated in region of MSP primers by MSP. The issue could be easily overcome by using microarray assay. Recently, a novel technique called MSO microarray, that combines bisulfite DNA assay and oligonucleotides microarray for analysis of DNA methylation, have developed by Gitan et at. [10] The MSO microarray potentially allows rapid screening of multiple CpG sites in many gene promoters. But cross-hybridization between imperfect-match probes, targets and some probes inherently diminish hybridization signals, probably due to decreased duplex stability of targets and probes [25], can be observed in this method. On the other hand for each site to be detected a probe labeled with fluorescence has to be designed which is so expensive. As a result, it is costly for the methylation CpG sites analysis in the genomic wide scale.

So this microarray-based allele-specific extension to analysis of DNA methylation is expected to provide a novel tool for research in this field, and easy to realize the throughput analysis of multiplex genes and multiplex CpG sites. CpG island hypermethylation has been reported to be associated with the silencing of many cancer-related genes [26], accordingly it is necessary for us to analyze genome-wide DNA methylation status. Bisulfite treated genomic DNA from each of these loci can be amplified to generate multiple targets for analysis.

Compared to other methylation-related oligonucleotide microarray, it is so important to control false positive signals in the process of allele-specific extension. Fikert Erdogan and his collogues had compared the false signals under different experimental conditional [25]. In our assay, we also compared three DNA polymerase. Under standard condition in this study, we can control the false positive signals effectively. The last result demonstrated that this microarray-based method could be applied as a powerful tool to the assessment of selected CpG dinucleotides and quantification of methylation at each site.

In conclusion, this microarray-based technique could be applied for the analysis of DNA methylation in high throughput manner and it also will contribute significant information to our understanding of CpG island methylation in cancer. In addition, it would be a potential useful research tool in the academic research and clinical application.

Acknowledgement

This work was supported by project 60701008 and 60121101 (National Natural Science Foundation of China).

REFERENCES

[1] R.A. Martienssen, E.J. Richards, Curr. Opin. Genet. Dev. 5 (1995) 234.
[2] M.T. Valenzuela, R. Galisteo, A. Zuluaga, M. Villalobos, M.I. Nunez, F.J. Oliver, J.M. Ruizde Almodovar, Eur. Urol. 42 (2002) 622.
[3] P.A. Jones, Ann. NY Acad. Sci. 983 (2003) 213.
[4] H. Cedar, Cell 53 (1988) 3.
[5] M. Esteller, Adv. Exp. Med. Biol. 532 (2003) 39.
[6] S.B. Baylin, J.G. Herman, J.R. Graff, P.M. Vertino, J.P. Issa, Adv. Cancer Res. 72 (1998) 141.
[7] K.D. Robertson, P.A. Jones, Carcinogenesis 21 (2000) 461.
[8] S.E. Cottrell, Clin. Biochem. 37 (2004) 595.
[9] M.L. Gonzalgo, P.A. Jones, Methods (San Diego, CA) 27 (2002) 128.
[10] R.S. Gitan, H. Shi, C.M. Chen, P.S. Yan, T.H. Huang, Genome Res. 12 (2002) 158.
[11] J.G. Herman, J.R. Graff, S. Myohanen, B.D. Nelkin, S.B. Baylin, Proceedings of the National Academy of Sciences of the United States of America, 93 (1996) 9821.
[12] C. Goessl, M. Muller, K. Miller, Prostate Cancer Prostatic Dis. 3 (2000) S17.
[13] H. Ohashi, Methods Mol. Biol. (Clifton, NJ) 192 (2002) 91.
[14] T. Swift-Scanlan, A. Blackford, P. Argani, S. Sukumar, M.J. Fackler, BioTechniques 40 (2006) 210.
[15] K. Brakensiek, L.U. Wingen, F. Langer, H. Kreipe, U. Lehmann, Clin. Chem. 53 (2007) 17.
[16] P. Hou, J.Y. Shen, M.J. Ji, N.Y. He, Z.H. Lu, World J. Gastroenterol. 10 (2004), 3553A. 12.
[17] A. Russom, H. Ahmadian, P. Andersson, G. Nilsson, Stemme, Electrophoresis 24 (2003) 158.
[18] O. Ericsson, A. Sivertsson, J. Lundeberg, A. Ahmadian, Electrophoresis 24 (2003) 3330.
[19] A. Ahmadian, B. Gharizadeh, D. O'Meara, J. Odeberg, J. Lundeberg, Nucleic Acids Res. 29 (2001) E121.
[20] A. Ahmadian, A. Russom, H. Andersson, M. Uhlen, G. Stemme, P. Nilsson, BioTechniques 32 (2002) 748.
[21] D. O'Meara, A. Ahmadian, J. Odeberg, J. Lundeberg, Nucleic Acids Res. 30 (2002) e75.
[22] K.L. Gunderson, F.J. Steemers, G. Lee, L.G. Mendoza, M.S. Chee, Nat. Genet. 37 (2005) 549.
[23] M. Frommer, L.E. McDonald, D.S. Millar, C.M. Collis, F. Watt, G.W. Grigg, P.L. Molloy, C.L. Paul, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 1827.
[24] J.G. Hacia, Nat. Genet. 21 (1999) 42.
[25] S.B. Baylin, J.G. Herman, Trends Genet. 16 (2000) 168.
[26] F. Erdogan, R. Kirchner, W. Mann, H.H. Ropers, U.A. Nuber, Nucleic Acids Res. 29 (2001) E36.