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Sensitive colorimetric biosensing for methylation analysis of *p16/CDKN2* promoter with hyperbranched rolling circle amplification

Yao Geng [a,b,1], Jie Wu [b,1], Lijia Shao [a], Feng Yan [a,*], Huangxian Ju [b,**]

[a] Department of Clinical Laboratory, Nanjing Medical University Cancer Hospital & Jiangsu Cancer Hospital, 42 Baiziting Road, Nanjing 210009, Jiangsu, China

[b] State Key Laboratory of Analytical Chemistry for Life Science, Department of Chemistry, Nanjing University, Nanjing 210093, China

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ABSTRACT

A simple, fast and sensitive colorimetric biosensing method for DNA methylation analysis was developed by combining methylation-sensitive endonuclease based digestion with hyperbranched rolling circle amplification (HRCA) induced signal enhancement. The assay was carried out on a DNA capture probe modified 96-cell microplate with four sequential steps of target recognition, endonuclease-based digestion, isothermal HRCA, and enzyme-catalyzed colorimetric readout within 3 h. The semi-quantitative and precise analysis of methylated DNA could be easily achieved by naked eye and absorbance measurements, respectively. The strategy exhibited excellent detection specificity and accuracy with a log-linear response to methylated DNA from 100 fM to 10 nM. As a proof of concept, the assay was applied to investigate the methylation status of *p16/CDKN2* promoter of breast cancer patients. The methylated *p16* concentration was not significantly associated with the clinical parameters. The proposed method allowed efficient methylation detection with simplicity, rapidness, low cost and high sensitivity, showing great promise for application in early diagnosis of methylation-related diseases.

1. Introduction

Methylation of DNA cytosines in the context of CpG dinucleotides is an essential part of epigenetic modification (Jaenisch and Bird, 2003). It influences the heritable state of gene expression and leads to the function loss of tumor-suppressor genes of almost every type of cancer (Holliday and Grigg, 1993). Because methylation-based gene inactivation occurs even before gene mutations (Schuebel et al., 2007), the accurate and sensitive analysis of DNA methylation has significant clinical and prognostic implications for early cancer detection.

Generally, DNA methylation analysis includes 3 steps: DNA extraction, DNA treatment, and methylation detection via PCR amplification. The most widely applied methods for analysis of DNA methylation use sodium bisulfite to treat genomic DNA for converting cytosines, but not 5-methylcytosines, to uracil. As the bisulfite conversion includes multiple chemical reactions such as denaturation of genomic DNA, deamination of unmethylated cytosines, and desulfonation, the treatment procedure requires rigorous conditions of temperature, pH and salt concentration (Frommer et al., 1992) and is time-consuming (Herman et al., 1996). As an alternative to bisulfite-based treatment, methylation-sensitive restriction endonuclease (MSRE) digestion, which are able to cleave DNA at specific unmethylated-cytosine residues and leave the methylated DNA intact, is more rapid, easier and cheaper and has lower risk of contamination. Hence, it has been used in highly efficient methylation analysis (Chan et al., 2008; Mao and Chou, 2010). For example, von Kanel et al. (2010) described a quantitative 1-step DNA methylation analysis by combining the rapid MSRE digestion and quantitative real-time PCR in a single reaction. This assay decreased both hands-on time and possible cross-contamination, and the whole assay could be accomplished within 1.5 h after DNA extraction in a closed tube.

Both bisulfite conversion and MSRE digestion used in sensitive quantification of DNA methylation are generally combined with a PCR amplification process. These assay methods include methylation-specific PCR (Ostrow et al., 2010), fluorescence-labeled real-time PCR (Eads et al., 2000; Lorente et al., 2008; Mao and Chou, 2010; von Kanel et al., 2010), methylation-specific fluorescence resonance energy transfer (Bailey et al., 2009), bisulfite sequencing (Leakey et al., 2008; Su et al., 2013), pyrosequencing (Shaw et al., 2006), and MALDI-TOF mass spectrometry (Schatz et al., 2006; van den Boom and Ehrich, 2009). They need a high-precision thermocycler, and most of them require expensive detectors, fluorescence-labeled probes, detection kits and other

* Corresponding author. Tel.: +86 25 83283401
** Corresponding author. Tel.: +86 25 83593593.
E-mail addresses: yanfeng2007@sohu.com (F. Yan), hxju@nju.edu.cn (H. Ju).
[1] These authors contributed equally to this work.

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consumables. Recently, Zhang et al. combined thermostable ligation and isothermal hyperbranched rolling circle amplification (HRCA) to propose a label-free DNA methylation assay method with a detection limit of 0.8 fM (Cao and Zhang, 2012). HRCA is an isothermal and exponential amplification through turn-by-turn cascade of primer extension and strand displacement (Lizardi et al., 1998; Cheng et al., 2009), and has been widely applied for sensitive detection of single nucleotide polymorphisms (Qi et al., 2001), viral RNA (Gilbert and O'Leary, 2005), and proteins (Zhang and Liu, 2003).

Here, we developed a simple, fast, sensitive and economical colorimetric biosensing method for methylation analysis of *p16/CDKN2* promoter by coupling a rapid endonuclease-based digestion with HRCA for signal enhancement. The *p16* gene encodes a protein of D-type cyclin-dependent kinase (CDK) inhibitor that blocks the ability of CDK4 to interact with cyclin D1 and stimulates the progression of eukaryotic cells through G1 phase of the cell cycle (Serrano et al., 1993). Methylation associated transcriptional block of *p16/CDKN2* has been observed frequently in the early stage of many primary tumors (Gonzalez-Zulueta et al., 1995; Merlo et al., 1995; Lim et al., 2009), including breast cancer with a rate of 31% of de novo methylation (Lim et al., 2009). The methylated *p16* promoter can be extracted conveniently from the cell-free DNA (cfDNA) (Alix-Panabieres et al., 2011; Schwarzenbach et al., 2011) isolated from plasma. The proposed assay was carried out on a DNA capture probe modified 96-cell microplate (Scheme 1), and could be accomplished within 3 h. Different from current strategies for DNA methylation quantitation, this work employed a biotin-streptavidin system to introduce an enzymatic reaction for colorimetric or naked eye readout. This method showed high sensitivity with a detection range of 5 orders of magnitude, indicating a great promise for application in early diagnosis of methylation-related diseases.

2. Experimental

2.1. Materials and reagents

Phi29 DNA polymerase, T4 DNA ligase, exonuclease I, exonuclease III and dNTP mixture were obtained from Fermentas. Horseradish peroxidase labeled streptavidin (SA-HRP) was obtained from Thermo. *Hpa*II in NEB buffer was purchased from New England Biolabs. Amino-coated microplate was obtained from Nunc. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was obtained from Sigma. TMB color development including 3,3′,5,5′-tetramethylbenzidine (TMB) was obtained from Beyotime. Other reagents were of analytical grade. All aqueous solutions were prepared using ultra-pure water (≥ 18 MΩ, Milli-Q, Millipore). Methylated DNA sequence was obtained from DNA methylation database (MethPrimer). All oligonucleotides used in this work were synthesized and purified by HPLC at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., and their sequences were listed as follows (from 5′ to 3′ end):

Methylated DNA: **CGAGCTGCCTGGAG**TTGCGTTCCAGGCGT$_m$CG-GC$_m$CC

CTGGGCCGTCACCGCG

Unmethylated DNA: **CGAGCTGCCTGGAG**TTGCGTTCCAGGCGT-CCGGCC

CCTGGGCCGTCACCGCG

Circular template: CACGCGATCCGCCCCACCCTCCGCGGTGACG-GCCCAG

GAATTCGTGTA***ACTACACGAATTC***CAACCGCCGAACG

Primer: ACCAAGAGCA***ACTACACGAATTC***

Capture probe: HOOC-AACCGTCTTCCAAGAGACCTT**CTCCAGG-CAGCTC G**

Biotinylated probe: GCGATCCGCCCCA-biotin

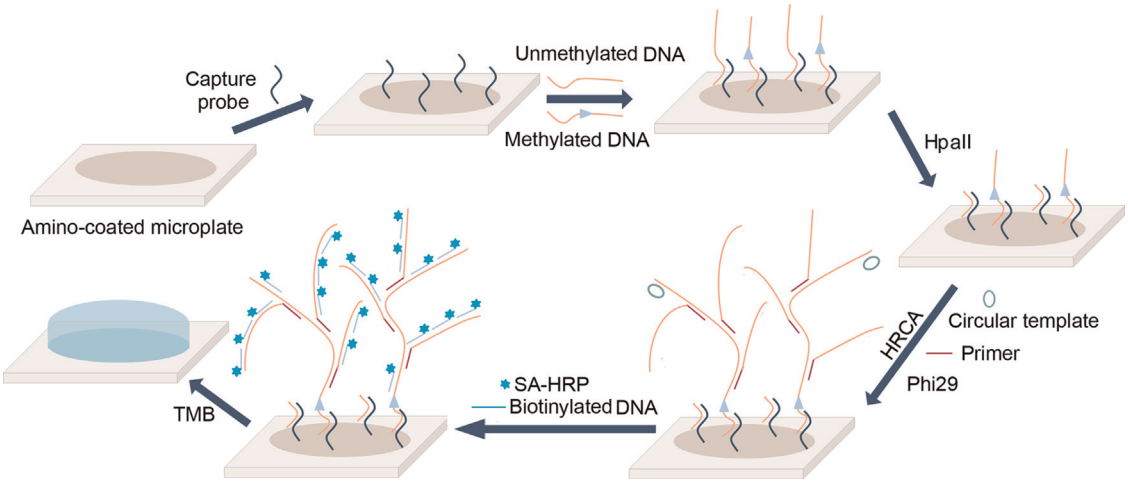
The binding regions between methylated (or unmethylated) DNA and capture probe as well as circular template were shown in bold and underlined, respectively. The region with the same sequences in circular template and primer was shown in bold italics.

2.2. Apparatus

CfDNAs were isolated from corresponding plasma of 72 breast cancer patients who were diagnosed by pathology section detection in Jiangsu Cancer Hospital from July 2008 to October 2010, and 43 samples from a control population by using Nucleospin® plasma XS Kit (Macherey-Nagel) according to the manufacturer's protocol. The total concentration of cfDNA was detected by spectrophotometry. The samples were stored at −80 °C before use. Clinical data of all patients were collected from the database of the hospital. Any change in a patient's treatment or progress was entered into the database. All study participants signed an informed consent form, which was approved by the Ethics Committee of Jiangsu Cancer Hospital.

2.3. Circularization of DNA template

Circular template DNA was prepared according to the method reported previously (Cheng et al., 2010). Briefly, 100 μL of ligation



Scheme 1. Schematic representation of the colorimetric methylation analysis.

buffer (50 mM pH 7.5 Tris–HCl buffer, 10 mM $MgCl_2$, 10 mM dithiothreitol and 0.5 mM ATP) containing 10 μM circle forming cfDNA template was incubated at 37 °C for 30 min, followed by the addition of 160 U T4 DNA ligase and incubation at 22 °C for 1 h. Afterwards, T4 DNA ligase was inactivated by heating the reaction mixture at 65 °C for 10 min. After 400 U exonuclease I and 2000 U exonuclease III were added in the mixture to digest the residue linear DNA at 37 °C for 50 min, the circular template DNA was extracted sequentially with phenol/chloroform (1:1) and chloroform, and precipitated with ethanol. The circular template DNA was dissolved in 100 μL of pure H_2O and stored at −20 °C before use.

2.4. Assay protocol

Firstly, 100 μL of 1 μM capture probe containing 50 mM EDC was dropped in each cell of the amino-coated microplate slide and incubated for 3 h at room temperature in a humidified chamber. After washing with pure water, the capture probe modified microplate was obtained for following assay.

As shown in Scheme 1, 50 μL of *p16* gene or cfDNA was dropped in the cell and incubated for 20 min at room temperature. Following a washing step, 10 U HpaII was added in the cell to digest the unmethylated DNA at 37 °C for 30 min. After washing, 50 μL of HRCA reaction mixture (containing 1 × polymerase buffer, 0.05 μM primer, 0.1 μM circular template DNA, 400 μM dNTP, and 8 U Phi29 DNA polymerase) was added to carry out the HRCA process, which was performed at 63 °C for 60 min and terminated by incubation at 95 °C for 10 min. Then, the cell was washed with pure water and incubated with 50 μL mixture of SA-HRP (50 ng mL^{-1}) and biotinylated probe (0.1 μM) at room temperature for 20 min. After another washing, 200 μL TMB color development solution was dropped in the cell for colorimetric reaction, which took 30 min and was terminated by the addition of 100 μL 0.5 M H_2SO_4. Finally, the microplate was measured at 450 nm by a commercial microplate reader (Bio-rad 680). The whole assay could be carried out within 3 h, which was much shorter than 18 h in the most widely DNA methylation method including 4 steps of denaturation, deamination, desulfonation, and PCR-based amplification and detection (Herman et al., 1996; Eads et al., 2000).

2.5. Statistical analysis

The concentrations of methylated *p16/CDKN2* promoter in the samples from breast cancer patients and healthy control were compared with the Wilcoxon signed rank test. A log rank test was used to determine the correlation between methylated *p16/CDKN2* concentration and the clinical parameters of cancer patients. All analyses were performed with SPSS 16.0 for Windows. *P* values less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Optimization of detection conditions

The proposed colorimetric methylation analysis employed an isothermal HRCA process to enhance the signal and improve the detection sensitivity. In order to obtain high amplification efficiency, some important parameters, such as the concentrations of primer and circular template DNA, used in HRCA were first optimized. With the increasing primer concentration from 0.01 to 0.5 μM, the optical density (OD) of 0.5 nM methylated DNA increased and trended to a plateau at 0.05 μM, while the control OD value obtained with 0.5 nM unmethylated DNA showed slight change (Fig. 1A), indicating the HRCA reached an equilibrium state with primer at the concentration of 0.05 μM. According to the high signal-to-noise ratio, 0.05 μM was selected as the optimum concentration of primer in the subsequent research. Similar variation tendencies of OD value were observed with the increasing concentration of circular template DNA from 0.05 to 1.0 μM (Fig. 1B). The OD value reached the maximum signal-to-noise ratio at 0.1 μM, which was selected as the optimum concentration of circular template DNA.

3.2. Signal amplification by HRCA

The proposed methylation analysis was carried out on a capture probe modified microplate. A MSRE procedure was employed to digest the unmethylated DNA, and the remaining methylated DNA initiated the HRCA process to generate hyperbranched DNA structure that was capable of loading high amount of HRP labeled SA through the complementary biotinylated probe (Scheme 1). The loaded HRP catalyzed the colorimetric reaction for quantification of methylated DNA. Thus, the detection sensitivity of the assay depended on the amplification efficiency of the HRCA process. In order to evaluate the amplification ability of HRCA, the colorimetric signals of 0.5 nM methylated DNA obtained by HRCA and RCA were compared. As shown in Fig. 2A, the HRCA process produced 1.5 times higher OD response than RCA, along with the similar noise, indicating that the HRCA process created more binding sites for biotinylated probe as well as AS-HRP.

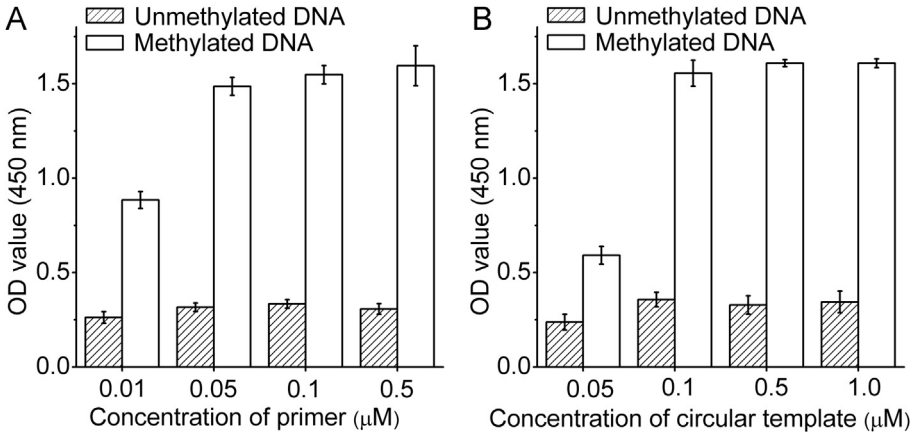


Fig. 1. Effects of primer (A) and circular template (B) concentration. Results are expressed as the average of 3 independent experiments at 0.5 nM methylated and unmethylated DNA. Error bars represent SDs.

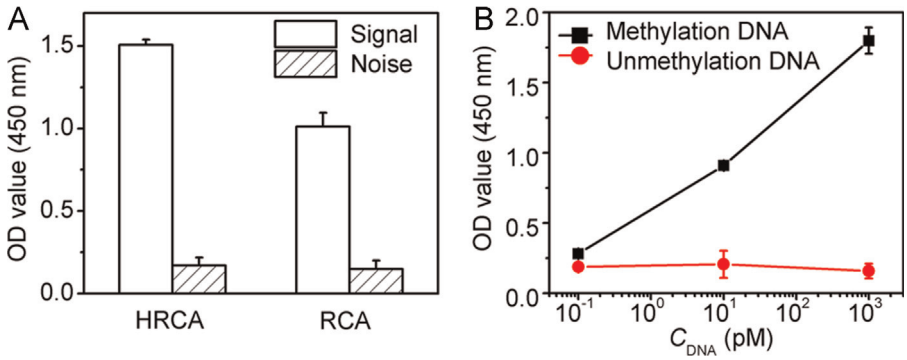


Fig. 2. Performance of HRCA compared with RCA (A) and specificity of methylated DNA detection (B). Results are expressed as the average of 3 independent experiments. Error bars represent SDs.

3.3. Specificity

In order to validate the feasibility of the proposed method, both methylated and unmethylated DNA at different concentrations were detected (Fig. 2B). The methylated DNA exhibited obvious OD signal even at 100 fM, and the signal increased correspondingly with the increasing concentration. In contrast, the unmethylated DNA did not produce detectable signal, even at the concentration of 1 nM, indicating excellent detection specificity of this method for methylated DNA.

3.4. Analytical performance for detection of methylated DNA

As shown in Fig. 3A, under optimal conditions the color turned gradually to deep blue (I) or yellow (II) with the increasing concentration of methylation DNA, and the enzymatic color difference from 100 fM to 1 nM could be distinguished easily by the naked eye. The OD value was log-linear correlation with the concentration of methylation DNA over a range of 5 orders of magnitude from 100 fM to 10 nM (Fig. 3B). The correlation equation was $OD = 0.3746 \log C + 0.6298$ with a correlation coefficient of 0.9967. A detection limit of 93 fM was obtained by evaluating the average response of the blank plus three times of standard deviation. The detection limit was much lower than those obtained by single-base extension reaction based SERS (Hu and Zhang, 2012) and microarray based Ms-SNuPE (Wu et al., 2008). Moreover, the higher sensitivity could be obtained by extending the HRCA process in connection with nanomaterial-based amplification.

Accurate assessment of methylation in serum or plasma was significant for the early diagnostic of cancer. A recovery experiment was used to verify the validity of the proposed assay after a certain amount of methylated *p16* gene was added into different cfDNA samples isolated from the cancer patients. Both the concentrations of methylated *p16* in samples without and with spiked *p16* were detected and the results were summarized in Table 1. Although the background levels of methylated *p16* in each sample were different, all recoveries were higher than 90.8%, indicating good accuracy of the proposed method for sample detection.

3.5. Methylation of P16/CDKN2 promoter in cfDNA

The methylation status of the *p16/CDKN2* promoter was evaluated in cfDNA isolated from 72 breast cancer patients and 43 healthy individuals. Promoter methylation of *P16* gene was observed in 57 (79.2%) of 72 patients and 4 (9.3%) of 43 healthy individuals with the median concentrations of 1.2×10^{12} and 5.5×10^{11} copies L^{-1}, respectively. The difference of methylated *p16* concentrations between the two groups was statistically significant ($P < 0.0001$, Wilcoxon test). The correlation between

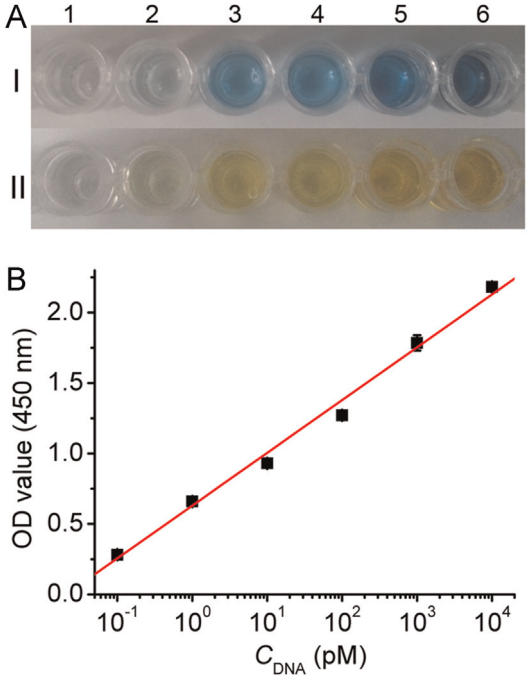


Fig. 3. Methylation analysis by naked eye (A) and microplate reader (B). The TMB color development is terminated without (I) and with (II) 0.5 M H_2SO_4. 1–6 represent the concentrations of methylated DNA of 0, 100 fM, 1 pM, 10 pM, 100 pM and 1 nM. Results in (B) are expressed as the average of 3 independent assays. Error bars represent SDs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Methylated *p16/CDKN2* promoter in cfDNA tested using the proposed method.

Sample no.	Added (pM)	Found (pM)	Recovery (%)[a]
1	0	0.589	90.8 ± 0.324
	1.0	1.497	
2	0	0.167	92.7 ± 0.267
	1.0	1.094	
3	0	0.174	91.1 ± 0.241
	1.0	1.085	
4	0	1.269	94.1 ± 0.281
	1.0	2.21	

[a] Results were obtained from 3 independent experiments.

p16/CDKN2 promoter methylation in cfDNA and the clinical parameters, such as age, menopausal, grade, histology grade, stage and lymph node metastasis of 72 breast cancer patients was assessed in Table 2. The results of log-rank test did not show significant

Table 2
Correlations of *p16/CDKN2* promoter methylation in cfDNA with different clinical parameters.

Parameters	Variable	Number of cases	*P16/CDKN2* methylation		
			+	−	*P**
Age	< 50 years	27	23	4	NS
	≥ 50 years	45	34	11	
Menopausal	Premenopausal	29	22	7	NS
	Postmenopausal	43	35	8	
Grade	I	26	19	7	NS
	II	17	16	1	
	III,IV	29	22	7	
LNM	No	43	32	11	NS
	Yes	29	25	4	
Cancer size	< 2 cm	32	25	7	NS
	≥ 2 cm	40	32	8	
Histology grade	I	28	22	6	NS
	II,III	44	35	9	

NS: not significant.

* Log-rank test. LNM: lymph node metastasis.

association between the methylated *p16* concentration and the clinical outcome of the patients. This phenomenon was similar to that observed in colorectal cancer patients (Nakayama et al., 2002).

4. Conclusions

A sensitive colorimetric biosensing method for DNA methylation analysis has been proposed by combining the endonuclease-based digestion with HRCA-based signal enhancement on a DNA capture probe modified microplate. The quantification and semi-quantitative detection of methylation level can be easily achieved by a cost-effective commercial microplate reader and by naked eye, respectively. Combining specific DNA recognition, endonuclease-based rapid digestion with isothermal amplification of HRCA, the proposed assay can avoid the limitations of PCR-based methylation analysis and possesses the advantages of good specificity, high sensitivity, wide detection range, short test time (3 h) and easy operation. The use of 96-cell microplate for colorimetric analysis greatly improves the detection throughput and makes it easier to design a miniaturizable and automatable detection system. In addition, the assay method has been applied in accurate quantification of DNA methylation and copy number in serum. The results show statistic difference of methylated *p16* concentrations between breast cancer patients and healthy control group, indicating great promise for further application in early diagnosis of methylation-related diseases.

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