Sensitive and Label-Free DNA Methylation Detection by Ligation-Mediated Hyperbranched Rolling Circle Amplification

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ABSTRACT: Sensitive and specific detection of DNA methylation in CpG sites of genomic DNA is imperative for rapid epigenetic evaluation and early cancer diagnosis. Here, we employ for the first time the thermostable ligation for methylated DNA discrimination and hyperbranched rolling circle amplification (HRCA) for signal enhancement, without the need for restriction enzymes, PCR amplification, or fluorescence-labeled probes. After bisulfite treatment of methylated DNA, the methylation-specific linear padlock probe can be circularized only in the presence of methylated DNA and serves subsequently as a template for HRCA, whose products are easily detected using SYBR Green I and a standard fluorometer. While in the presence of unmethylated DNA, the linear padlock probe cannot be circularized because of the defectively matched substrate, and no HRCA occurs. This ligation-mediated HRCA-based method exhibits excellent specificity and high sensitivity with a detection limit of 0.8 fM and a detection range of 4 orders of magnitude, and it can even distinguish as low as 0.01% methylation level from the mixture, which is superior to most currently used methods for DNA methylation assay. This method can be further applied to analyze genomic DNA in human lung cancer cells.

However, these methods need a high-precision thermocycler, and most of them require expensive fluorescence-labeled probes. Combined bisulphite restriction analysis (COBRA) offers an alternative to analyzing the aberrant methylation in a sensitive and quantitative way, but the prerequisite for COBRA is the accessibility of restriction sites to the methylation-sensitive enzymes. Recently, a simple DNA methylation assay has been developed on the basis of surface enhanced Raman spectroscopy and single-base extension reaction. However, it lacks sensitivity for the detection of low-abundance methylated DNA.

Herein, we develop a simple, highly sensitive and label-free method for analyzing the methylation status of specific CpG sites on the basis of ligation-mediated hyperbranched rolling circle amplification (HRCA). HRCA is an isothermal and exponential amplification through turn-by-turn cascade of primer extension and single-base extension reaction, and it has been widely applied for the detection of single nucleotide polymorphisms, viral RNA, and proteins owing to its high sensitivity. However, the detection of DNA methylation with HRCA has never been reported so far. In this research, we employ for the first time the thermostable ligation for methylated DNA discrimination and the HRCA for signal enhancement, without the need for restriction enzymes, PCR amplification, or fluorescence-labeled probes. This ligation-mediated HRCA method has distinct advantages of excellent specificity and high sensitivity with a high-throughput potential.
Experimental Section

Materials. All oligonucleotides used in this research (Table 1) were synthesized and purified by HPLC at Takara Biotechnology Co., Ltd. (Dalian, China). Human lung cancer cell lines H157 and H209 were obtained from Xiamen Biovision Biotechnology Co. Ltd. (Xiamen, China). All other chemicals were of analytical reagent grade and obtained from Sigma Chemical Co., USA.

Preparation of Genomic DNA. Both H157 and H209 cells were cultured in Dulbecco’s modification of Eagle’s medium (DMEM, Cellgro) supplemented with 10% fetal bovine serum in a humidified incubator containing 5% CO2 at 37 °C. Genomic DNA was extracted by a universal genomic DNA extraction kit Ver.3.0 (TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer’s instruction. The concentrations of genomic DNA were determined by measuring the absorbance at 260 nm with a spectrophotometer. Genomic DNA was fragmented by digestion using PstI and BstE II for 60 min and used as the target in the subsequent experiments.

Bisulfite Treatment of DNA. Bisulfite treatment of DNA was performed according to the reported method. First, 1 μg DNA was denatured in 0.35 M NaOH at 37 °C for 20 min. Bisulfite reaction was carried out in 3.2 M sodium bisulfite and 0.5 mM hydroquinone (both were freshly prepared) at 50 °C for 16 h ~ 18 h. Then DNA was recovered by a desalting column (DNA cleanup system, Promega Inc., USA) and the modification was completed in 0.3 M NaOH at 37 °C for 15 min, followed by neutralization with ammonium acetate, precipitation with ethanol, and drying. The resulting DNA was resuspended in water and used immediately or stored at −20 °C.

Ligation and Exonuclease Treatment. Hybridization of the target with the linear padlock probe was carried out in 20 μL buffer containing 20 mM Tris–HCl (pH 7.6), 25 mM potassium acetate, 10 mM dithiothreitol, 10 μM magnesium acetate, 1 mM nicotinamide adenine dinucleotides, 0.1% Triton X-100, 100 nM linear padlock probe, different-concentration DNA target, and 12 U of Taq DNA ligase at 95 °C for 5 min, and then the mixture was incubated at 65 °C for 60 min. After ligation, 10 μL products were added to 10 μL exonuclease mixture containing 1 mM DTT, 6.7 mM MgCl2, 67 mM glycine-KOH (pH 9.5), 10 U

Experimental Section

Table 1. Sequences of Target Oligonucleotides, Padlock Probe, and Primers¹

<table>
<thead>
<tr>
<th>note</th>
<th>sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>methylated DNA</td>
<td>GAG GGT GGG GCG GAC GTG GCG TGC G</td>
</tr>
<tr>
<td>unmethylated DNA</td>
<td>GAG GGT GGG GCG GAC GTG GCG TGC G</td>
</tr>
<tr>
<td>linear padlock probe</td>
<td>CAC GCG ATC GCC CCC ACC CTC ATT AGG TTA</td>
</tr>
<tr>
<td>HRCA primer 1</td>
<td>CTT GTG CTA ATC GCA GTA ACC TAA T</td>
</tr>
<tr>
<td>HRCA primer 2</td>
<td>ACC AAG AGC AAC TAC ACG AAT TC</td>
</tr>
</tbody>
</table>

¹The underlined regions of methylated and unmethylated DNA indicate the base difference between them. In the linear padlock probe, the binding region for the HRCA primer 1 is shown in boldface, and the region with the same sequence as the HRCA primer 2 is shown in bold italics.

Scheme 1. Scheme of DNA Methylation Assay Based on Ligation-Mediated HRCA²

²This assay involves following steps: (1) bisulfite treatment, (2) hybridization of methylated DNA with the linear padlock probe and the subsequent ligation of linear padlock, (3) purification of obtained circular padlock probes by digestion, and (4) hyperbranched rolling circle amplification, whose products can be assessed by SYBR Green I. As a result, in the presence of unmethylated DNA, neither HRCA nor distinct fluorescence signal is observed (A), while in the presence of methylated DNA, both HRCA and fluorescence enhancement are observed (B).
Exonuclease I, and 20 U Exonuclease III. The mixture was incubated at 37 °C for 2 h, followed by inactivation at 95 °C for 10 min.

HRCA Reaction. After ligation and purification, a following step was performed to amplify the circular probes in the presence of Bst DNA polymerase. The HRCA reaction was performed in 30 μL buffer containing 1× polymerase buffer, 0.05 μM of each HRCA primers, 400 μM deoxynucleoside triphosphate mixtures, 10 μL of postdigestion mixture, and 8 U Bst DNA polymerase. The circular probes were amplified at 63 °C for 60 min and terminated by incubation at 95 °C for 10 min. Gel electrophoresis images were acquired with a Kodak Image Station 4000MM.

Fluorescence Detection. Aliquots of 30 μL HRCA product and 1 μL SYBR Green I (20×) were added into 70 μL deionized water, followed by incubation at room temperature for 10 min. Fluorescence spectra were obtained using a Hitachi F-4500 fluorometer (Tokyo, Japan) equipped with a xenon lamp as the excitation source. The excitation wavelength was 488 nm and the spectra were recorded in the range from 500 nm to 650 nm. The fluorescence intensity was measured at the emission wavelength of 520 nm.

RESULTS AND DISCUSSION

Principle of DNA Methylation Assay Based on Ligation-Mediated HRCA. The principle of DNA methylation assay is presented in Scheme 1. Upon the treatment of sodium bisulfite, cytosine is converted to uracil, but C5-methylcytosine (mC) remains unchanged.19,20 Therefore, a distinguishable difference in the DNA sequence is generated between methylated and unmethylated DNA. For the purpose of both methylated DNA discrimination and the initiation of HRCA, the linear padlock probe with 83 nucleotides (nt) consists of following distinct regions: two target-complementary sequences located at the 5′ and the 3′ ends and two special regions used to initiate HRCA. When the two ends of linear padlock probe perfectly hybridize with methylated DNA, the 5′ and the 3′ ends are brought into proximity, generating a circular padlock probe in the presence of Taq DNA ligase. In order to reduce the ligation-independent amplification, the obtained circular padlock probes are further purified by digestion of the unreacted linear padlock probes and the excess oligonucleotides using Exonucleases I and III.21 Subsequently, the primer extension and strand displacement will take place in the presence of circular padlock probes, two primers, and Bst DNA polymerase, producing a large number of double-stranded DNA (dsDNA) fragments in variable length, which can be assessed using SYBR Green I as the fluorescent dye (Scheme 1B).22 While in the presence of unmethylated DNA, the linear padlock probes cannot be circularized due to the defectively matched substrate; neither HRCA nor fluorescence enhancement is observed (Scheme 1A). Consequently, sensitive quantification of methylated DNA with excellent specificity can be achieved in a homogeneous way.

To improve the specificity of DNA methylation assay, asymmetric sequences are designed for the two ends of linear padlock probe with 21 nt at the 5′ end and 13 nt at the 3′ end.21 The 3′ end of the linear padlock probe is designed to be guanine, which is complementary to the methylated DNA site, making the DNA ligase join the linear padlock only in the presence of methylated DNA.23 It should be noted that the methyl group at

![Figure 1. Illustration of base paring and hydrogen bond formation. 5-Methylcytosine can hybridize with guanine, but unmethylated cytosine upon bisulfite treatment is changed to uracil, which is not complementary to guanine.](image_url)
5-position of pyrimidine in methylated DNA does not affect the hybridization of methylcytosine with guanine because of the presence of three hydrogen bonds between them (Figure 1). However, upon treatment of sodium bisulfite, the amino group at 4-position of pyrimidine ring in unmethylated DNA is displaced by carbonyl because of the hydrolytic deamination, i.e., cytosine is converted to uracil. Unfortunately, uracil is incapable of hybridizing with guanine (Figure 1). As a result, the selectivity of the DNA methylation assay is achieved not only by the specific hybridization of the linear padlock probe with methylated DNA but also by the highly discriminative nick closure activity of the Taq DNA ligase toward the perfectly matched substrate.

**Optimization of HRCA Experimental Condition.** To obtain high amplification efficiency of HRCA, the concentrations of primers and dNTP substrates are experimentally optimized (Figure 2). With the decrease in primer concentration from 1 μM to 0.05 μM, the difference between the fluorescence intensity induced by methylated DNA and that by unmethylated DNA increases (Figure 2A), suggesting that low primer concentration leads to improved discrimination against the methylated DNA. This can be explained by the fact that high-concentration exogenous primers might adversely cause primer dimerization and nonspecific amplification. Therefore, 0.05 μM is selected as the optimum concentration of primers in the subsequent research. In addition, when the concentration of dNTP substrates increases from 2 μM to 400 μM, the fluorescence intensity in the presence of methylated DNA gradually increases, but the fluorescence intensity in the presence of unmethylated DNA remains unchanged (Figure 2B). Thus, 400 μM is selected as the optimum concentration of dNTP substrates in the following research.

**Detection of Methylated DNA.** Previous research demonstrated that hypermethylation of CpG island in p16 tumor suppressor gene was associated with the complete loss of gene expression in a variety of cancers. To validate the feasibility of the proposed method, we detect a DNA sequence homologous to part of p16 tumor suppressor gene under the optimum reaction condition. As shown in Figure 3A, the fluorescence signal produced by 10 nM methylated DNA is 698.8 ± 25.3, which is as much as 23 times higher than that produced by 10 nM unmethylated DNA (30.2 ± 5.5), suggesting that the proposed method can be used to distinguish methylated DNA from unmethylated DNA successfully.

In order to further verify the validity and specificity of ligation reaction, the ligation products are analyzed by 10% polyacrylamide gel electrophoresis (PAGE) and a standard silver-staining method (Figure 3B). In the presence of 10 nM methylated DNA, the linear padlock is ligated into a circular form (lane 3 in Figure 3B), which moves slower than the products in the presence of the target-free control (lane 1 in Figure 3B) and 10 nM unmethylated DNA (lane 2 in Figure 3B). After exonuclease treatment, the linear oligonucleotides in the ligation products are totally digested (lanes 1 and 2 in Figure 3C) except for the circular padlock probes (lane 3 in Figure 3C). Therefore,
exonucleolysis is a necessary process to remove the excess oligonucleotides and to reduce the ligation-independent amplification. The products of HRCA are further electrophoresed in a 2% agarose gel, and the ladder-type bands are observed in the presence of 10 nM methylated DNA (lane 3 in Figure 3D), but no ladder-type band is observed in the presence of the target-free control (lane 1 in Figure 3D) and 10 nM unmethylated DNA (lane 2 in Figure 3D). These results are consistent with those of fluorescence detection (Figure 3A), suggesting that this method can be used to detect DNA methylation with excellent specificity.

Detection Sensitivity. Quantitative detection of DNA methylation with high sensitivity is critical for early diagnosis of methylation-related diseases. To demonstrate the high sensitivity of the proposed method, we investigate the variance of fluorescence intensity with the concentration of methylated DNA. In the absence of methylated DNA, no observable fluorescence signal is detected (control in Figure 4A) because there is no HRCA amplification and no dsDNA-SYBR Green I formation. In contrast, methylated DNA can hybridize with the methylation-specific linear padlock probe, which can be circularized in the presence of Taq DNA ligase to obtain concatemeric dsDNA through HRCA, leading to the formation of dsDNA-SYBR Green I complexes and consequently observable fluorescence signals. As shown in Figure 4A, the fluorescence signal improves with the increasing concentration of methylated DNA, and an exponential curve is obtained between the fluorescence intensity and concentration of methylated DNA. Notably, the fluorescence intensity is log-linear correlation with the concentration of methylated DNA over a range of 4 orders of magnitude from 1 fM to 10 pM (Figure 4B). The correlation equation is $I_f = 34.54 + 102.98 \log_{10} C$ with a correlation coefficient of 0.9972, where $I_f$ is the fluorescence intensity, and $C$ is the concentration of methylated DNA (fM), respectively. The detection limit is calculated to be 0.8 fM by evaluating the average response of the blank plus three times

Figure 4. (A) Variance of the fluorescence intensity as a function of the concentration of methylated DNA. (B) The fluorescence intensity is a log–linear correlation with the concentration of methylated DNA in the range from 1 fM to 10 pM. Error bars show the standard deviation of three experiments. (C) Variance of the fluorescence intensity as a function of input methylation level in the mixtures of methylated and unmethylated DNA. (D) Correlation of the measured and the actual input methylation level in the artificial mixtures of methylated and unmethylated DNA. Error bars show the standard deviation of three experiments.

Figure 5. Variance of the fluorescence intensity with the amount of genomic DNA extracted from human lung cancer cells H157 (slash column) and H209 (solid column). Error bars show the standard deviation of three experiments.
standard deviation. The sensitivity of this method has improved by as much as 8 orders of magnitude as compared with the gold nanoparticle-based colorimetric assay and 3 orders of magnitude as compared with single-base extension reaction-based SERS as well. Moreover, in comparison with the gold nanoparticle-based colorimetric assay, this method provides an unlimited platform for DNA methylation detection because of the initial use of bisulfite treatment rather than the restriction enzymes.

It is of great importance to evaluate the capability of the proposed method for the detection of aberrant methylation in real samples. To this end, we prepare the artificial mixtures composed of methylated and unmethylated DNA at different ratios for the measurement of methylation level. The total concentration of methylated and unmethylated DNA is 10 pM, and the mixture contains 0.01%, 0.05%, 0.5%, 5%, 50%, and 100% methylated DNA. As shown in Figure 4C, with the increase in the amount of input methylated DNA in the mixture, there is a corresponding increase in the fluorescence intensity. The measured methylation level is calculated as the quantity of methylated DNA measured by the proposed method divided by the total concentration of methylated and unmethylated DNA. Figure 4D reveals a linear relationship between the measured methylation level and the actual input methylation level. It is worth noting that this assay can even distinguish as low as a 0.01% methylation level, which is superior to most currently used methods for DNA methylation analysis, such as MALDI-MS spectrometry (5%), quantum dot-based FRET method (1%), cationic conjugated polyelectrolyte method (1%), and MSP (0.1%). It is even comparable with that of MS-qFRET (0.01%). However, the synthesis process of quantum dots is very strict and very complicated, and the labeled primers used in the MS-qFRET method are quite expensive. Therefore, this ligation-mediated HRCA might provide a simple, sensitive, and universal platform for DNA methylation assay.

**Real Sample Analysis.** We further apply this method to investigate the methylation status of six CpG sites in the p16 promoter region of human lung cancer cell lines H157 (non-small-cell lung cancer cell line) and H209 (small-cell lung cancer cell line). The CpG sites have been reported to be highly methylated in H157 cells but unmethylated in H209 cells. Different-concentration genomic DNA extracted from the above cell lines was analyzed by the proposed method under the optimum condition. In real sample analysis, a restriction digestion was performed before the bisulfite treatment to prevent DNA from supercoiling and decircularization during the heating step. As shown in Figure 5, with the increase in the amount of genomic DNA, the fluorescence intensity increases in H157 cells but remains unchanged in H209 cells, suggesting that this method can be used to sensitively analyze the methylation status in the cancer cells. Moreover, the detection limit of this method can be down to 2 ng, which is much lower than that of MS-qFRET and is comparable with that of MSP and CE-LIFP immunoassay.

Significant advantages of the proposed method are its convenience, excellent specificity, and high sensitivity as well as low cost. Unlike the PCR-based methods such as CCP-based assay, and MS-qFRET, this method is based on a robust isothermal amplification of HRCA without the need for high-precision temperature cycling and additional separation steps. Moreover, the following features endow this method with excellent specificity and high sensitivity: (1) specific hybridization of the linear padlock probe with methylated DNA, (2) highly discriminative nick closure activity of Taq DNA ligase toward the perfectly matched substrate, (3) purification of obtained circular padlock probes by Exonucleases I and III, and (4) exponential amplification capability of HRCA. In addition, this method is extremely low cost because of the use of SYBR Green I as the fluorescent dye instead of expensive fluorescence-labeled probes.

**CONCLUSIONS**

In summary, the proposed method combines for the first time the high fidelity of DNA ligation for methylated DNA discrimination with the isothermal and exponential amplification of HRCA for signal enhancement. As a result, this ligation-mediated HRCA-based method exhibits excellent specificity and high sensitivity with a detection limit of 0.8 fM, and it can even distinguish as low as 0.01% methylation level from the mixtures, which is superior to most currently used methods for DNA methylation assay. This method holds great promise for further applications in the study of epigenetic modification and early diagnosis of methylation-related diseases.

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**


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