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Sensitive fluorescent detection of DNA methyltransferase using nicking endonuclease-mediated multiple primers-like rolling circle amplification

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ABSTRACT

Sensitive and reliable detection of DNA methyltransferase (MTase) is of great significance for both early tumor diagnosis and therapy. In this study, a simple, label-free and sensitive DNA MTase-sensing method was developed on the basis of a nicking endonuclease-mediated multiple primers-like rolling circle amplification (RCA) strategy. In this method, a dumbbell RCA template was prepared by blunt-end ligation of two molecules of hairpin DNA. In addition to the primer-binding sequence, the dumbbell template contained another three important parts: 5′-CCGG-3′ sequences in double-stranded stems, nicking endonuclease recognition sites and C-rich sequences in single-stranded loops. The introduction of 5′-CCGG-3′ sequences allows the dumbbell template to be destroyed by the restriction endonuclease, HpaII, but is not destroyed in the presence of the target MTase—M.SssI MTase. The introduction of nicking endonuclease recognition sites makes the M.SssI MTase-protected dumbbell template-mediated RCA proceed in a multiple primers-like exponential mode, thus providing the RCA with high amplification efficiency. The introduction of C-rich sequences may promote the folding of amplification products into a G-quadruplex structure, which is specifically recognized by the commercially available fluorescent probe thioflavin T. Improved RCA amplification efficiency and specific fluorescent recognition of RCA products provide the M.SssI MTase-sensing platform with high sensitivity. When a dumbbell template containing four nicking endonuclease sites is used, highly specific M.SssI MTase activity detection can be achieved in the range of 0.008–50 U/mL with a detection limit as low as 0.0011 U/mL. Simple experimental operation and mix-and-detection fluorescent sensing mode ensures that M.SssI MTase quantitation works well in a real-time RCA mode, thus further simplifying the sensing performance and making high throughput detection possible. The proposed MTase-sensing strategy was also demonstrated to be applicable for screening and evaluating the inhibitory activity of MTase inhibitors.

1. Introduction

DNA methylation is an important epigenetic modification in both prokaryotes and eukaryotes, and plays crucial roles in regulating the growth, development, gene expression pattern and stability of genetic substances (Heithoff et al., 1999). DNA methylation is a chemical modification process catalyzed by DNA methyltransferase (MTase), which can transfer an active methyl group from the methyl donor S-adenosylmethionine (SAM) to special nucleobases in double-stranded DNA (dsDNA) (Cheng and Roberts, 2001; Jeltsch, 2002; Song et al., 2009). Studies have shown that aberrant MTase activity may lead to an abnormal DNA methylation pattern, which is linked to various diseases such as cancer initiation and progression (Baylin and Herman, 2000; Das and Singal, 2004; Heyn and Esteller, 2012; Jones and Laird, 1999; Shukla et al., 2010). As the abnormal expression of MTase activity usually occurs prior to other signs of malignancy, DNA MTase has been used as a promising biomarker for early diagnosis of cancer and a potential target in cancer therapy (Heithoff et al., 1999; Low et al., 2001). Therefore, the development of a simple, convenient, sensitive and reliable method to detect MTase activity is of great significance for

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both early tumor diagnosis and therapy.

Many methods, including radioactive labeling (Pradhan et al., 1999), gel electrophoresis (Rebeck and Samson, 1991), high-performance liquid chromatography (Reenila et al., 1995), electrochemical assay (Deng et al., 2014; Muren and Barton, 2013; Zhou et al., 2016), immune-based assay (Jiang et al., 2012; Wang et al., 2012), nanomaterial-based assay (Li et al., 2012; Ji et al., 2014; Liu et al., 2010; Song et al., 2009), and DNA-based biosensors (Bi et al., 2013; Li et al., 2007; Xing et al., 2014; Xue et al., 2015; Zhang et al., 2015b; Zhao et al., 2014a, 2014b), have been developed for MTase activity analysis. Of these methods, DNA biosensors have attracted considerable attention due to their advantages including cost-effectiveness, easy operation and flexibility in design. In addition, the detection sensitivity can be easily improved by the introduction of appropriate nucleic acid amplification strategies such as strand displacement amplification (SDA) (Du et al., 2016; Zhao et al., 2014a, 2014b), nicking enzyme signal amplification (NESA) (Xue et al., 2015; Zhang and Xu et al., 2015) and rolling circle amplification (RCA) (Bi et al., 2013; Zeng et al., 2013). As an isothermal nucleic acid amplification technique, RCA shows many unique advantages in biosensing applications (Ali et al., 2014; Kim and Easley, 2011), and is increasingly used in many types of enzyme activity assays (Jiang et al., 2014, 2015). Recently, the Jiang group reported an elegant design strategy for determining MTase activity on the basis of target MTase-protected dumbbell molecular probe-mediated cascade RCA, and achieved highly sensitive detection of MTase activity (Zhao et al., 2016). However, two rounds of RCA reactions are required. Between the two rounds, the reaction tube must be repeatedly opened to perform the experiments including the cleavage of long-stranded RCA products, heat-inactivation of restriction endonucleases, and preparation of new circular RCA templates. This increases not only the complexity of the experimental operation, but also the risk of product contamination.

In the present study, we developed a much simpler MTase activity-sensing method on the basis of nicking endonuclease-mediated RCA. In this method, the introduction of nicking endonuclease allows the MTase-dependent RCA reaction to be performed in a multiple primers-like mode, thus improving the signal amplification efficiency without increasing the complexity of the experimental operation. The introduction of a nucleic acid G-quadruplex structure in the RCA product to achieve product detection utilizing G-quadruplex-specific recognition by the fluorescent probe, thioflavin T (ThT), greatly decreases the background signal and thus increases the signal-to-background ratio. The proposed method was demonstrated to work well not only for the sensitive and specific detection of MTase activity, but also for screening and evaluating the inhibitory activity of MTase inhibitors.

2. Experimental section

2.1. Materials and reagents

Oligonucleotides (Table S1) used in this work were synthesized and purified by Sangon Biotech. Co. Ltd. (Shanghai, China). M.SssI MTase, Dam MTase, HpaII restriction endonuclease, T4 DNA ligase, phi29 DNA polymerase, Nb.BbvCI nicking endonuclease, and corresponding buffers were bought from New England Biolabs (Beijing, China). Thioflavin T (3, 6-dimethyl-2-(4-dimethylaminophenyl)benzo-thiazolium cation, ThT), 5-azacytidine (5-Aza) and 5-aza-2′-deoxycytidine (5-Aza-dC) were obtained from Sigma-Aldrich. GelRed nucleic acid gel stain was purchased from KeyGEN BioTECH. Healthy human serum was bought from Sorlabio (Beijing, China). All chemical reagents were of analytical grade and used without further purification.

2.2. Preparation of the dumbbell templates

Dumbbell templates were obtained by T4 DNA ligase-mediated blunt end ligation of two molecules of 5′-phosphorylated hairpin DNA oligonucleotides (Dumbbell-1 was prepared by Hairpin-1 and Dumbbell-2 was prepared by Hairpin-2). Reaction mixture was prepared by adding 750 nM hairpin DNA (Hairpin-1 or Hairpin-2) in 20 μL of 1× T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, pH 7.5). To ensure the formation of hairpin structure, the mixture was heated at 95 °C for 5 min and then incubated at 37 °C for 30 min. After addition of 40 U T4 DNA ligase, the mixture was allowed to incubate at 16 °C for 1 h. Lastly, the ligation reaction was terminated by a thermal treatment at 65 °C for 10 min. The prepared dumbbell templates were used immediately or stored at −20 °C until use.

2.3. Methylation and cleavage of the dumbbell templates

The methylation experiments were carried out at 37 °C for 2 h in 40 μL of reaction mixture containing various amounts of M.SssI MTase, 20 μL as-prepared dumbbell template, 1× NEB buffer2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9), and 160 μM SAM. Then, the cleavage of unmethylated dumbbell template was initiated by adding 1× cutsmart buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 100 μg/mL BSA, pH 7.9) and 2 U HpaII endonuclease. The total volume of the reaction mixture was 60 μL and the reaction was performed at 37 °C for 2 h.

2.4. M.SssI MTase activity detection

The above reaction mixture was added 1× phi29 polymerase reaction buffer (50 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, and 4 mM dithiothreitol), 50 nM Primer, 2 U phi29 DNA polymerase, 3 U Nb.BbvCI nicking endonuclease (Nb.BbvCI was not added in linear RCA reaction), 4 mM dNTPs and 5 μM ThT. The total volume was 100 μL. Then, RCA reaction was performed at 37 °C for specified time. For end-point detection, the fluorescence spectrum of the reaction system was recorded using a Shimadzu RF-5301 fluorescence spectrometer (Shimadzu Ltd., Japan) in the range of 435~615 nm under the excitation at 425 nm, and the fluorescence intensity at 485 nm was used for M.SssI MTase activity quantitation. As for real-time RCA detection mode, RCA reaction was performed at 37 °C in a commercial StepOnePlus™ Real-Time PCR instrument for 2.5 h. The fluorescence detection channel set for 6-carboxy-fluorescein (FAM) was used to monitor the fluorescence signal, and the fluorescence signal was collected at intervals of 1 min.

2.5. MTase inhibitor assay

To investigate the influence of 5-Aza or 5-Aza-dC on M.SssI MTase activity, the potential effects of the two drugs on HpaII endonuclease, phi29 DNA polymerase and Nb.BbvCI nicking endonuclease should be excluded firstly. Thus, the tested drugs were added after dumbbell template methylation process. That is, the methylation of the prepared dumbbell template was performed as above in the absence or presence of 10 U/mL M.SssI MTase. Before subsequent HpaII-mediated cleavage reaction, 5 μM 5-Aza or 5-Aza-dC was added. Then, the fluorescence signal of the reaction solution was detected after RCA reaction. Similar fluorescence signal levels given by the systems with or without 5-Aza (or 5-Aza-dC) demonstrated that these two drugs have no effects on HpaII, phi29 and Nb.BbvCI. In M.SssI MTase activity inhibition experiments, all procedures were the same as above except that different concentrations of 5-Aza or 5-Aza-dC were added together with M.SssI MTase before methylation reaction. Tested inhibitor-induced relative activity (RA) change of M.SssI MTase can be calculated by the following equation (Zeng et al., 2013; Zhao et al., 2016):