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A quantitative homogeneous assay for global DNA methylation levels using CpG-binding domain- and methyl-CpG-binding domain-fused luciferase

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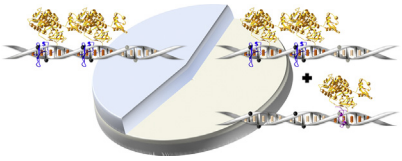
HIGHLIGHTS

- Unmethylated CpG binding domain (CXXC domain)-fused luciferase was constructed.
- A homogeneous assay for measuring the unmethylated CpG content of genomic DNA by CXXC-luciferase was developed.
- Global DNA methylation was quantified by MBD- and CXXC-luciferase without any calibration curve.

GRAPHICAL ABSTRACT



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ABSTRACT

Global DNA methylation levels have been considered as biomarkers for cancer diagnostics because transposable elements that constitute approximately 45% of the human genome are hypomethylated in cancer cells. We have previously reported a homogeneous assay for measuring methylated CpG content of genomic DNA based on bioluminescence resonance energy transfer (BRET) using methyl-CpG-binding domain (MBD)-fused luciferase (MBD-luciferase). In this study, a homogeneous assay for measuring unmethylated CpG content of genomic DNA in the same platform was developed using CXXC domain-fused luciferase (CXXC-luciferase) that specifically recognizes unmethylated CpG. In this assay, CXXC-luciferase recognizes unmethylated CpG on genomic DNA, whereby BRET between luciferase and the fluorescent DNA intercalating dye is detected. We demonstrated that the BRET signal depended on the genomic DNA concentration ($R^2 = 0.99$) and unmethylated CpG content determined by the bisulfite method ($R^2 = 0.97$). There was a significant negative correlation between the BRET signal of the CXXC-luciferase-based assay and that of the MBD-luciferase-based assay ($R^2 = 0.92$). Moreover, we demonstrated that the global DNA methylation level determined using the bisulfite method was dependent on the ratio of the BRET signal in the MBD-luciferase-based assay to the total BRET signal in the MBD-luciferase- and CXXC-luciferase-based assays ($R^2 = 0.99$, relative standard deviation < 2.2%, and analysis speed < 35 min). These results demonstrated that global DNA methylation levels can be quantified by calculating the BRET signal ratio without any calibration curve.

1. Introduction

DNA methylation at the 5′-position of cytosine in CpG dinucleotides has important biological roles including the regulation of gene expression, silencing of retrotransposons, and X-

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inactivation [1]. In mammals, tissue-specific DNA methylation patterns are established during development and further maintained in somatic cells [2]. In contrast, aberrant DNA methylation patterns are detected in abnormal cells. In cancer cells, aberrant DNA methylation states have been reported at cancer-related gene promoters and hypomethylation at transposable elements that constitute approximately 45% of the human genome [3–7]. Therefore, gene-specific DNA and global DNA methylation levels have been considered as biomarkers for cancer diagnostics [8–12].

To quantify the DNA methylation level, both methylated and unmethylated CpG are to be detected. For global DNA methylation level quantification, bisulfite-based analysis against transposable elements [13–15] and high-performance liquid chromatography (HPLC)-based analysis [16–20] have been widely utilized because they can simultaneously detect methylated and unmethylated CpG. Sodium bisulfite rapidly converts unmethylated cytosine to uracil, while methylated cytosine is protected from the reaction. During polymerase chain reaction (PCR) amplification of bisulfite-treated DNA, uracil and methylated cytosine are converted to thymine and cytosine, respectively; therefore, both methylated and unmethylated CpG can be detected by sequence-based analysis of the PCR products. In the HPLC-based analysis, genomic DNA is enzymatically digested, and then, the amount of methylated and unmethylated cytosine is determined by HPLC. Recently, electrochemical oxidation-based analyses have been reported [21–25]. The methods are based on the principle that oxidation peaks are different among bases; therefore, similar to HPLC-based analyses, genomic DNA has to be enzymatically digested.

In the bisulfite-, HPLC-, and electrochemical oxidation-based analyses, global DNA methylation levels can be accurately quantified; however, detection requires several steps. To detect the global DNA methylation level, we previously developed a homogeneous assay, which can perform the binding and detection reactions in the same vessel, based on bioluminescence resonance energy transfer (BRET) using methyl-CpG-binding domain (MBD)-fused luciferase (MBD-luciferase) [26]. MBD-luciferase recognizes methylated CpG on genomic DNA; thus, BRET between luciferase and the fluorescent DNA intercalating dye is detected. The intensity of the BRET signal depends on the methylated CpG content of the genomic DNA. To quantify global DNA methylation levels by the assay, a calibration curve is required as the assay does not detect unmethylated CpG content of the genomic DNA. We have reported BRET-based DNA detection system using zinc finger-fused luciferase [27], indicating that the assay can be applied for unmethylated CpG detection by replacing MBD with a protein that recognizes unmethylated CpG.

We focused on the CXXC domain found in many unmethylated CpG-binding proteins. CXXC domains have been identified in several chromatin-associated proteins, including MLL, DNMT1, MBD1, and CGBP [28–32]. Among them, the CXXC domain of MLL has the highest affinity to unmethylated CpG [31]. It has been reported that the MLL CXXC domain binds to 52 bp DNA containing six unmethylated CpG sites with a dissociation constant (K_d) of 33 nM but the K_d increased to more than 1 mM by methylation [28], suggesting that the MLL CXXC domain is suitable for the unmethylated CpG-binding protein. In this study, we aimed to develop a homogeneous assay for measuring unmethylated CpG content of genomic DNA based on BRET using MLL CXXC-fused luciferase (CXXC-luciferase) and then a quantitative assay for global DNA methylation using MBD-luciferase with CXXC-luciferase without any calibration curve (Fig. 1). In this assay, global DNA methylation level can be simply and rapidly quantified, as methylated and unmethylated CpG contents of the genomic DNA can be detected by simply adding the DNA intercalating dye, either fusion protein and luminescent substrate, to the sample without any sample preparation, such as bisulfite conversion and enzymatic digestion.

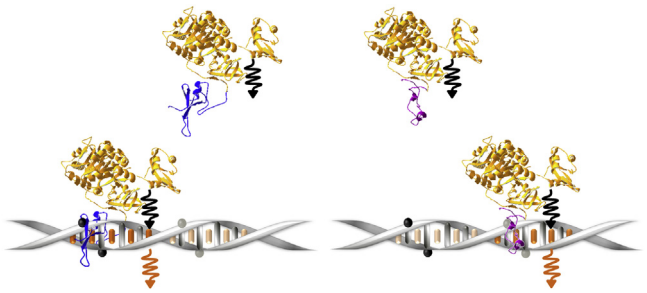

Fig. 1. A quantitative homogeneous assay for global DNA methylation levels using CXXC- and MBD-fused luciferase. The CXXC- and MBD-luciferase binds to unmethylated and methylated CpG on genomic DNA, respectively; thus, the BRET signals depend on the unmethylated and methylated CpG content. By calculating the ratio of the BRET signal in the MBD-luciferase-based assay to the total BRET signals, the global DNA methylation level can be quantified without any calibration curve. Luciferase, MBD, and CXXC are shown as yellow, blue, and purple, respectively. Methylcytosine, cytosine, and DNA intercalating dye are shown as a black circle, a gray circle, and an orange square, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Material and methods

2.1. Plasmid construction

The synthetic DNA containing *Nde*I site, Streptag coding sequence, 57 amino acids of the CXXC domain (1147–1203 of human MLL) coding sequence, and *Eco*RI site was purchased from Integrated DNA Technologies. The DNA was amplified by PCR using KOD DNA polymerase (Toyobo). The PCR product was digested by *Nde*I (NEB) and *Eco*RI (Nippon Gene). pET30c-*Streptag-MBD-luciferase* [26] was digested with *Nde*I and *Eco*RI to remove the *Streptag-MBD* and then the *Streptag-CXXC* was ligated with the pET30c-*luciferase* by Ligation High Ver.2 (Toyobo). *Escherichia coli* (*E. coli*) DH5α (Toyobo) was transformed by the ligated product, and the plasmid was purified. The plasmid was sequenced using a 3730xl DNA analyzer (Thermo Fisher Scientific).

2.2. Expression and purification of CXXC-luciferase and MBD-luciferase

MBD-luciferase was expressed in *E. coli* BL21 (DE3) (Biodynamics) and purified as previously reported [26]. To prepare CXXC-luciferase, *E. coli* BL21 (DE3) was transformed by pET30c-*Streptag-CXXC-luciferase*. The cells were cultured in 150 mL LB medium at 37 °C. When the OD_{600} reached 0.5, 1.2 mM IPTG (Wako) was added and the cell culture was incubated at 20 °C for 16 h to induce CXXC-luciferase expression. The cells were harvested by centrifugation at 2500*g* for 10 min, and the cell pellets lysed with BugBuster Protein Extraction Reagent (Millipore). The water-soluble fraction was prepared by centrifugation at 16,000×*g* and 4 °C for 20 min. The water-soluble fraction was 10-fold diluted with PBS buffer [137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4 (pH = 7.4); Thermo Fisher Scientific] and then filtered with a 0.45 μm filter (Millipore). The filtered sample was loaded onto a Strep-Tactin Superflow Plus Cartridge (1 ml; Qiagen) to purify CXXC-luciferase. The column was washed with 10 mL wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH = 8.0). CXXC-luciferase was eluted using 10 mL elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 2.5 mM desthiobiotin, pH = 8.0). In all steps, 1 mL of each fraction was collected. Purified samples were analyzed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)