Bioluminescence resonance energy transfer assay for cancer diagnosis

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Keywords: DNA methylation, Luciferase, Green fluorescent protein, BRET

Introduction

In human genomic DNA, DNA methylation at fifth position of the cytosine in cytosine-guanine sequence (CpG) is involved in regulation of gene expression. When CpG in the promoter region is methylated, the expression of the gene is repressed. In each tissue, tissue-specific DNA methylation patterns are established during development and the DNA methylation patterns are maintained in normal cells. In contrast, alterations in DNA methylation are observed in a variety of cancer cells. Hypermethylation at tumor suppressor genes and global DNA hypomethylation are common features of cancer genomic DNA. Therefore, alterations in DNA methylation can be utilized as biomarker for cancer diagnosis.

Previously, a method for measuring global DNA methylation level has been developed using bioluminescence resonance energy transfer (BRET) assay (*W. Yoshida, et al., Anal. Chem., 2016, 88, 9264*). In this assay, methyl-CpG binding domain (MBD)-fused *firefly* luciferase (MBD-Fluc) and fluorescent DNA intercalating dye are utilized. The MBD-Fluc binds to methylated CpG on genomic DNA to excite in the vicinity of the DNA intercalating dye, which bound to genomic DNA. The fluorescence intensity depends on amount of MBD-Fluc bound to methylated CpG; therefore, global DNA methylation level can be quantified. However, 30 minutes incubation is required to bind the DNA intercalating dye to the genomic DNA.

In this study, we aimed to develop a global DNA methylation level detection method by BRET assay using methyl-CpG binding domain (MBD)-fused *oplophorus* luciferase (MBD-Oluc) and MBD-fused green fluorescence protein (MBD-GFP). The principle in shown in Fig.1. GFP can be excited by luminescence of Oluc. When global DNA methylation level is high, MBD-Oluc and MBD-GFP simultaneously bind to methylated CpG regions, indicating that BRET signal between MBD-Oluc and MBD-GFP is detected depending on the global DNA methylation level. MBD specifically binds to methylated CpG on genomic DNA within 1 minute. We therefore, assumed that the global DNA methylation level is quantified by measuring the BRET assay within few minutes.

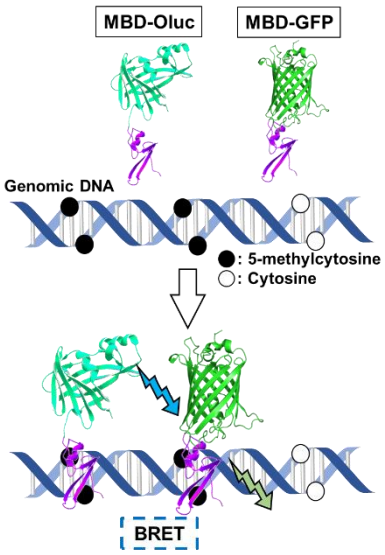

Fig.1 Schematic representation of quantification of global DNA methylation level by BRET assay using MBD-Oluc and MBD-GFP.

Methods

1. Preparation of the MBD-Oluc and MBD-GFP

pET30c-*Strep-tag II-MBD-Oluc* and pET30c-*Strep-tag II-MBD-GFP* was constructed for expressing these proteins. *Escherichia coli* (*E. coli*) BL21 (DE3) was individually transformed by pET30c-*Strep tag II-MBD-Oluc* or pET30c-*Strep-tag II-MBD-GFP*. Each BL21 (DE3) was cultured in 150 mL LB medium at 37°C. When the OD_{600} was 0.5, 1.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture medium The collection cells were lysed by Protein Extraction Reagent with Phosphate buffer. To purify MBD-Oluc and MBD-GFP, the water-soluble fractions obtained from the lysate was flowed to a Strep-Tactin column. In each fraction, fluorescence intensity of Oluc and GFP were measured using a microplate reader (SpectraMax iD5, Molucular Devices). The eluted fractions of MBD-Oluc and MBD-GFP were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2. Binding analysis of the MBD-GFP to methylated DNA

Biotin-labeled methylated or unmethylated DNA were synthesized and mixed to prepare 0, 50, 100% methylated DNA. These DNAs were fixed on streptavidin coated plate. The purified MBD-GFP was added on the plate. After washing the wells, the fluorescence intensity of GFP was measured to analyze binding analysis of MBD-GFP to methylated DNA.

3. Detection of global DNA methylation level by BRET

Human genomic DNA was extracted from HeLa cells. MBD-Oluc and MBD-GFP was added to these HeLa genomic DNA in PBS and then incubated at room temperature for 1 minute. After the incubation, emission spectra of MBD-Oluc and MBD-GFP was measured by addition of the luminescence substrate.

Results

1. Preparation of the MBD-Oluc and MBD-GFP

The fluorescence intensity of MBD-GFP and luminescence intensity of MBD-Oluc in each fraction were measured. The highest intensity was showed in the second eluted fraction in both cases. In SDS-PAGE analysis, a single band corresponding to MBD-Oluc and MBD-GFP was detected in the eluted fractions. These results demonstrated that MBD-Oluc and MBD-GFP were successfully expressed in BL21 (DE3) and purified by the Streptag/Strep-Tactin system.

2. Binding analysis of the MBD-GFP to methylated DNA

To examine whether MBD-GFP binds to methylated DNA, fluorescence intensity of MBD-GFP that bound to immobilized DNA was measured. The fluorescence intensity of MBD-GFP increased depending on the methylation level of the immobilized DNA, indicating that MBD-GFP specifically bound to methylated DNA.

3. Detection of global DNA methylation level by BRET

To investigate whether BRET signal between MBD-Oluc and MBD-GFP is detected on genomic DNA, emission spectra of MBD-Oluc and MBD-GFP was measured. The highest peak was detected at approximately 460 nm, which is the maximum emission wavelength of Oluc. After normalizing at 460 nm, the intensity at 510 nm, which is the maximum excitation wavelength of GFP, was measured. In the presence of genomic DNA, the intensity at 510 nm increased. These results indicated that BRET signal between MBD-Oluc and MBD-GFP was detected on genomic DNA.

Conclusion

In this study, MBD-Oluc and MBD-GFP were successfully expressed in *E. coli*. and purified by the Streptag/Strep-Tactin system. The BRET signal between MBD-Oluc and MBD-GFP was detected on genomic DNA, suggesting that the BRET assay may utilize for global DNA methylation level quantification.