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Detection and discrimination of maintenance and de novo CpG methylation events using MethylBreak

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ABSTRACT

Understanding the principles governing the establishment and maintenance activities of DNA methyltransferases (DNMTs) can help in the development of predictive biomarkers associated with genetic disorders and diseases. A detection system was developed that distinguishes and quantifies methylation events using methylation-sensitive endonucleases and molecular beacon technology. MethylBreak (MB) is a 22-mer oligonucleotide with one hemimethylated and two unmethylated CpG sites, which are also recognition sites for *Sau96*I and *Sac*II, and is attached to a fluorophore and a quencher. Maintenance methylation was quantified by fluorescence emission due to the digestion of *Sac*II when the hemimethylated CpG site is methylated, which inhibits *Sau96*I cleavage. The signal difference between *Sac*II digestion of both MB substrate and maintenance methylated MB corresponds to *de novo* methylation event. Our technology successfully discriminated and measured both methylation activities at different concentrations of MB and achieved a high correlation coefficient of R^2=0.997. Additionally, MB was effectively applied to normal and cancer cell lines and in the analysis of enzymatic kinetics and RNA inhibition of recombinant human DNMT1.

1. Introduction

DNA methylation plays an important role in creating epigenetic signatures that contribute to the transcriptional regulation and repression of repetitive elements (Schubeler, 2015). This chemical modification exists in two-component system consisting of an enzyme that methylates unmethylated DNA (*de novo*) and another that methylates hemimethylated sites (maintenance). Each methylation system has its corresponding DNA methyltransferases (DNMTs) that catalyzes each reaction. Maintenance methylation reactions utilize DNMT1, while *de novo* methylation uses DNMT3a and 3b (Robertson, 2001). There has been considerable research interests in investigating the contributions of DNA methyltransferase activity to mammalian development (Okano et al., 1999), and it has been shown that disrupting the catalytic domain of DNMTs lead to progressive loss of DNA methylation (Feng et al., 2010; Liao et al., 2015).

Methylated DNA is an important regulator in many biological processes like X-chromosome inactivation, genomic imprinting and gene expression (Csankovszki et al., 2001; Li et al., 1993; Razin and Cedar, 1991). Methylation anomalies was known to play a direct role in tumorigenesis and genetic diseases. The epigenetic effect of DNA methylation on oncogenesis is believed by either the activation of proto-oncogenes due to hypomethylation or the inactivation of tumor suppressor genes due to hypermethylation (Baylin et al., 1998; Ehrlich, 2002). Recent studies have stated that DNMTs are potential predictive biomarkers and therapeutic targets in the diagnosis and treatment of different types of cancer (Belinsky et al., 1996; Issa et al., 1993; Kobayashi et al., 2011; Mutze et al., 2011) since aberrant DNMT events occur earlier than other signs of malignancy (Baylin and Herman, 2000).

Essential to these efforts are the ability to quantify and distinguish cell-type–specific DNA methylation activities. Conventional methods used in the screening of DNMT methylation events include high-performance liquid chromatography (HPLC) (Boye et al., 1992),

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methylation-specific polymerase chain reaction (PCR) (Eads et al., 1999), gel electrophoresis (Bergerat et al., 1991), high performance capillary electrophoresis (HPCE) (Fraga et al., 2000), and isotopically labelled S-adenosyl-L-methionine (Adams et al., 1991; Yokochi and Robertson, 2004). However, these technologies employ bulky and expensive equipment, require complicated sample preparations and undesirable amount of time for data analysis (Poh et al., 2016). A more straightforward and sensitive detection procedure is fluorescent DNMT assay which involves the absorption of light which excite fluorophores to promote electrons from ground state to excited states. Some studies have designed hairpin probes with fluorophores and quenchers attached to their ends as the substrate DNA for real-time and direct detection of DNMT activity (Frauer and Leonhardt, 2009; Li et al., 2007; Wood et al., 2010). In addition, methylation-dependent restriction enzymes can convert the number of methyl-moieties transferred onto hemimethylated CpG dinucleotides by DNA methyltransferase into fluorescence signal (Wood et al., 2010). The characterization of DNMT1 activity using hemimethylated DNA substrate requires further purification of DNMT1 which potentially experience interferences from *de novo* methyltransferase in samples containing a mixture of DNMTs, such as crude extracts. Even though numerous technologies have been developed, none is capable of discriminating *de novo* and maintenance events.

Here, we propose a novel strategy in measuring and discriminating both maintenance and *de novo* methylation using a molecular beacon probe coupled with methylation sensitive endonuclease technology, termed as MethylBreak (MB). This technology can measure both methylation events based on the protection or promotion of enzymatic cleavage of a fluorescence-labelled oligonucleotide (Fig. 1). A 22-bp DNA substrate (designated as MethylBreak or MB) was designed to include a hemithylated and two unmethylated CpG sites. Also, the designed oligonucleotide has a long and overlapping recognition sites for *Sau96*I and *Sac*II restriction enzymes. *Sau96*I has the ability to cleave the DNA substrate which was used as a background corrector or blank. The maintenance methylation of the hemimethylated site would inhibit the *Sau96I* digestion. Therefore, after *Sac*II digestion of the oligonucleotide, a fluorescence emission can be measured which corresponds to the maintenance methylation quantification. Moreover, the *de novo* methylation event can be measured using the signal difference between the MB substrate and maintenance methylated MB after *Sac*II digestion only. This technology was successful in measuring and discriminating the two methylation events in normal and cancer cell lines. MethylBreak was effectively utilized on the analysis of enzymatic kinetics of recombinant human DNMT1 and inhibitory effects of non-coding RNAs. Our technology can be used in studying the DNA methylation signatures of genetic disorders and diseases, such as cancer, and compared with normal tissues. This may lead to the development of DNMT activity assays for molecular biology and clinical practice.

2. Material and methods

2.1. Maintenance-specific MethylBreak assay

Maintenance-specific MethylBreak assays were carried out in a 20 µl reaction mixture containing 1 mM S-adenosylmethionine (AdoMet; Sigma), 27.3 nM DNMT1 (New England Biolabs, Taiwan) methyltransferase, and a 22-mer MB oligonucleotide (upper strand: 5'-GGACTXGGGCCGCGGATTAAAG-3', where X represents a methylated cytosine) with 50 mM Tris-HCl (pH 7.8), 1 mM 2-mercaptoethanol, 5% glycerol, and 100 µg/ml bovine serum albumen (BSA) at 37 °C for 60 min. The methylation reaction was stopped and diluted by the addition of a digestion buffer (10 mM Tris-HCl pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$) to a final volume of 100 µl. Then, restriction enzymes, *Sau96*I (New England Biolabs, Taiwan) and *Sac*II (New England Biolabs, Taiwan), were added separately to digest the unmaintained MB oligonucleotide and to increase the fluorescence intensity by separating the 6-FAM and diacyl group label at the 5'-end of the upper strand and 3'-end of the lower strand of the MB oligonucleotide. The fluorescence released by 6-FAM was detected with a fluorescence reader (Biotek HT Synergy).

Fig. 1. Designed Sequence for MethylBreak oligonucleotide probe. MethylBreak (MB) is a 22-mer oligonucleotide with one hemithylated and two unmethylated CpG sites with overlapping recognition sites for *Sau96*I (red line) and *Sac*II (orange line), and a fluorophore and quencher attached to its ends. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2. De novo-specific MethylBreak assay

For *de novo*-specific MethylBreak assays, the methylation reaction was prepared as described for the maintenance-specific protocol, but the methylation reaction was stopped by heat inactivation. The subsequent cleavage step for distinguishing *de novo*-methylated DNA from maintenance-methylated and unmethylated DNA was carried out using only one enzyme, *Sac*II.

2.3. DNMT1 kinetic analysis

The enzymatic properties of recombinant human DNMT1 were characterized by performing standard DNMT1 activity assays with increasing MB oligonucleotide concentrations (0.2–1.2 µM) in the presence of 5 µM AdoMet and 40 nM DNMT1 methyltransferase at 37 °C for 10–30 min in triplicates. The reactions were stopped by adding the digestion buffer and processed with *Sau*96I and *Sac*II. Final results were obtained by fluorescence detection with a fluorescence reader. Rate was plotted as a function of DNA concentration, and the data were fitted with nonlinear regression by non-Michaelis Menten kinetics which indicates cooperative binding of hemimethylated DNA to DNMT1 methyltransferase.

2.4. DNMT1 inhibition assay

DNMT1 inhibition assays were carried out at 37 °C for 30 min in triplicates with a total volume of 25 µl. The reaction contained 1 µM MB oligonucleotide and 2.5 pmol recombinant DNMT1 in an assay buffer (50 mM Tris-HCl pH 7.8, 1 mM 2-mercaptoethanol, 5% glycerol, 100 µg/ml BSA), and with or without 2.5 pmol RNA. Methylation reactions were immediately stopped by adding the digestion buffer and were processed by *Sau*96I and *Sac*II. Fluorescence of the processed reactions were measured with a fluorescence reader.

2.5. Cell culture

Different cell lines were purchased from BCRC (Bioresource Collection and Research Center) of Taiwan and were grown as indicated in Supplementary Table S1 with the presence of penicillin/streptomycin.

3. Results

3.1. The design of MethylBreak system

The nucleotide sequence of the MethylBreak (MB) technology was designed to have a long, overlapping, and hemimethylated CpG cleavage sites for the methylation-sensitive endonucleases, *Sau*96I and *Sac*II (Fig. 1). The principle of this technology is brought by the 22-mer oligonucleotide substrate wherein the cleavage sites are blocked by maintenance or *de novo* methylation. The lack of methyla-