Method

A novel method to analyze 5-hydroxymethylcytosine in CpG sequences using maintenance DNA methyltransferase, DNMT1

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Abstract

Hydroxymethylcytosine has been shown to be involved in DNA demethylation and gene expression. Although methods to determine the position of hydroxymethylcytosine at single-base resolution have been reported, these methods involve some difficulties. Here, we report a simple method to analyze hydroxymethylcytosine in the CpG sequence utilizing the maintenance DNA methylation activity of DNMT1, which selectively methylates hemi-methylated but not hemi-hydroxymethylated CpG sequences. The method enables monitoring of the dynamics of the hydroxymethylation state of a specific genome site.

1. Introduction

In mammals, cytosine in the CpG sequence is often methylated by DNA methyltransferase (Dnmt) [1–3]. Methylated cytosine (5mC) plays crucial roles in gene silencing, genomic imprinting, X-chromosome inactivation and the stability of genomic DNA. Aberrant DNA methylation causes embryonic lethality and cancer [6,7]. Recently, 5-hydroxymethylcytosine (5hmC) was discovered to be a new modification of cytosine, being an intermediate of the demethylation process, and thus implicated in the pluripotency of stem cells, development, and disease [10,11]. 5hmC, which is relatively abundant in brain and embryonic stem (ES) cells [12,13], is produced through hydroxylation of 5mC by oxygenase, ten-eleven translocation (Tet), Tet1, Tet2, and Tet3 [14–16]. 5hmC plays a role in gene expression regulation during cell differentiation and maintenance of pluripotency, and thus regulation of the hydroxylation of 5mC to 5hmC is essential.

The macroscopic distribution of 5hmC in nuclei has been examined by using a specific antibody against 5hmC [17], 5hmC-specific restriction endonucleases [18,19], or labeling of 5hmC with biotin by the click reaction [20]. For detailed analysis of 5hmC, a method to determine the position of 5hmC at the single-base level is necessary. Bisulfite (BS) sequencing, which now is a standard method to analyze 5mC, identifies 5hmC as 5mC [21]. To determine 5hmC at single-base resolution, five techniques, oxBS-seq [22], Tet-assisted BS sequencing (TAB-seq) [23], oxidation of 5hmC with a peroxytungsten catalyst [24], Pvu-seal-seq [25], and glucosylation coupled with restriction-enzyme digestion [26] have been reported. However, each method has advantages and disadvantages. Perruthenate used in oxBS-seq method tends to damage DNA and the oxidized product of 5-formylcytosine is difficult to deaminate, and TAB-seq requires a highly active Tet enzyme [20]. The other three methods have limitations in the sequences they can analyze.

In the present study, we have developed a novel method to determine 5hmC in the CpG sequence utilizing DNA methyltransferase 1 (DNMT1), which scarcely methylates hemi-hydroxymethylated DNA [27]. We named this method DNMT1 methylation activity-assisted BS sequencing (DMAB-seq), which involves a different tool from those previously reported. This method can be used to easily monitor the 5hmC modification status at a specific genomic locus and is a useful technique to examine the dynamic change of 5hmC during a biologically important process.
2. Material and methods

2.1. Preparation of recombinant DNMT1

Full-length DNMT1 was prepared as described elsewhere [28]. Briefly, His- and GSH-tagged DNMT1 CDNA in baculovirus was expressed in insect cells Sf9 and purified. The protein concentration was determined from the absorbance at 280 nm using the molar extinction coefficient.

2.2. Analysis of 5hmC using synthetic DNA

Annealed DNA (Supplemental Table S1) was methylated with DNMT1 in buffer A comprising 5 mM EDTA, 12 mM NaCl, 2.7 M glycerol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 20 mM Tris–HCl (pH 7.4), and the indicated concentrations of KCl in the presence of 1 mM S-adenosyl-l-methionine (SAM). BS treatment was performed with Epitect bisulfite kit (Qiagen) according to the manufacturer’s instructions with an extra one hour’s BS treatment at 60 °C. After the BS treatment, DNA was amplified with EpiTaq (Takara) and specific primers (Supplemental Table S1), and then purified with MinElute PCR purification kit (Qiagen). The purified DNA was subcloned into pBlueScriptII, and then its sequence was determined by the dideoxy method.

2.3. In silico analysis of CpG dinucleotide density in the mouse and human genomes

The distances between CpGs and the numbers of CpG repetitions were calculated using an in-house perl script, which is available on request. Genome assembly versions b19 and mm10 for man and mouse, respectively, were used for the whole-genome analysis. A list of CpG islands for man and mouse was downloaded from the UCSC genome browser.

2.4. Cell culture

E14Tg2a mouse ES cells were cultured on gelatin-coated dishes in the presence of 1000 U/ml of LIF (in house) containing 20% (v/v) Knockout Serum Replacement (including vitamin C (Invitrogen) in DMEM supplemented with non-essential amino acids, sodium pyruvate, 2-mercaptoethanol, penicillin/streptomycin, and two kinase inhibitors, 2i (3 μM CHIR99021 and 1 μM PD0325901). As for the vitamin C and 2i minus conditions, 20% fetal bovine serum (FBS) was used instead of KSR. After switching the culture medium, ES cells were maintained for 10 days before DMAB-seq.

2.5. Preparation of spike DNA

The Morc1 upstream region with an inserted AT sequence (Supplemental Fig. S1) was amplified by PCR with specific primers (Supplemental Table S1) using LA Taq polymerase (Takara). Fully methylated, hydroxymethylated, or unmodified DNA was amplified in the presence of 5-methyl-dCTP (ZymoResearch), 5-hydroxymethyl-dCTP (ZymoResearch), or dCTP, respectively, and dATP, dTTP, and dGTP. The amplified DNA was purified with AMPure XP beads (Beckman Coulter). The concentrations of the fragments were determined from the absorbance at 260 nm.

2.6. Application of DMAB-seq to genomic DNA

Genomic DNA (1 μg), prepared from E14Tg2a mouse ES cells with Wizard DNA purification kit (Promega), was digested with EcoRI. To the digested DNA, 300 fg of spike DNA and 10 pmol of biotin-labeled Morc1 primer (Supplemental Table S1 and Fig. S1) were mixed in 6.6 μl of annealing buffer comprising 50 mM NaCl, 1 mM EDTA, and 10 mM Tris–HCl (pH 8.0). The mixture was incubated at 95 °C and then gradually cooled down to 15 °C, and then the primer was extended with 5 units of T4 polymerase (Toyobo) at 37 °C for 10 min. Excess biotin-labeled primer was removed with AMPure XP beads. The purified genomic DNA was methylated with 0.5–3 μg of recombinant DNMT1 at 37 °C for 1 h in 25 μl of buffer A supplemented with 108 mM KCl, 12 mM NaCl and 1 mM SAM. After the methylation, the reaction mixture was digested with Proteinase K, and then BS-treated as described above. The biotin-labeled DNA fragment was purified with Dynabeads M-280 Streptavidin beads (Invitrogen). The beads were suspended with 20 μl of 10 mM Tris–HCl (pH 8.0), and then one-fifth of the mixture was PCR amplified with the primer set (Supplemental Table S1). The amplified DNA was subcloned into pBlueScriptII and then its sequence was determined as described above.

3. Results

Two powerful techniques, TAB-seq and oxBs-seq, to analyze 5hmC at single-base resolution have been reported [22,23]. In the present study, we have developed an alternative method utilizing DNMT1. DNMT1 is reported to preferentially methylate the hemi-methylated state of CpG, but to scarcely methylate its hemi-hydroxymethylated state [27]. This unique methylation property of DNMT1 was utilized for the analysis of 5hmC in the CpG sequence. The experimental outline is shown in Fig. 1A. Firstly, the target sequence to be analyzed is primer extended with a specific primer to produce hemi-methylated and hemi-hydroxymethylated states (Step 1). Secondly, the synthesized DNA is treated with DNMT1 to produce fully-methylated DNA except for unmodified and hemi-hydroxymethylated sites (Step 2). As DNMT1 scarcely methylates hemi-hydroxymethylated DNA [27], not the hydroxymethylated but only the methylated CpGs in the parent strand are copied to the newly synthesized strand. Finally, the DNA is BS-treated to determine the methylation sites (Step3). Comparison of the methylation states of parent and DNMT1-treated DNA provides the 5hmC sites, which are expected to be converted to T (U).

3.1. Optimization of DNMT1 reaction conditions

To optimize the conditions of the methylation reaction of DNMT1, we utilized 100bp_6CpG (Supplemental Table S1) comprising two unmodified (C1 and C2), two hemi-methylated (M1 and M2), and two hemi-hydroxymethylated (H1 and H2) CpGs (Fig. 1B). Under 12 mM NaCl and 108 mM KCl conditions, 88% and 96% of hemi-methylated site of M1 and M2, respectively, of DNA (62 ng) were methylated with DNMT1 (0.92 μg). Under these conditions, about 10%, respectively, of unmodified C1 and C2, and 23% and 7% of the hemi-hydroxymethylated sites H1 and H2, respectively, were methylated (Fig. 1C and Supplemental Fig. S2A). When the amount of DNMT1 in the reaction mixture was increased from 0.92 to 3 μg, 88% and 100% of hemi-methylated sites M1 and M2, respectively, were determined to be methylated, while 41% and 31% of hemi-hydroxymethylated sites H1 and H2, respectively, were determined to be methylated (Fig. 1C). The ratio of DNMT1 to DNA is the crucial factor for the substrate specificity of DNMT1.

Since DNA methyltransferases are sensitive to salt concentrations, and the DNA methylation activity is inhibited at above physiological salt concentrations [29], we next examined the salt concentration-dependent DNA methylation activity of DNMT1 towards DNA containing hemi-methylated, unmodified, and...
hemi-hydroxymethylated CpG. The KCl concentration was changed from 0 to 200 mM with the constant concentration of 12 mM NaCl derived from the DNMT1 preparation (Fig. 1D and Supplemental Fig. S2B). With 38 mM KCl, 80% and 53% of the hemi-hydroxymethylated sites of H1 and H2, respectively, were methylated. With 108 mM KCl, 23% and 7% of the hemi-hydroxymethylated sites of H1 and H2, respectively, and about 10% of the unmodified C1 and C2 sites were methylated. Under identical salt conditions, 87% and 100% of M1 and M2, respectively, were methylated. With the higher concentration of KCl (188 mM), more than 85% of all the CpG sites remained unmethylated. From these findings, we set the conditions for the methylation reaction.
as 12 mM NaCl and 108 mM KCl at 37°C with 15 (w/w) DNMT1/DNA for synthesized DNA.

Under the above conditions, three independent DNA methylation reactions were performed (Fig. 1E). The two hemi-methylated CpG sites were 82 ± 10 (M1) and 86 ± 1% (M2) methylated, the two hemi-hydroxymethylated CpG sites were 13 ± 7 (H1) and 15 ± 1% (H2) methylated, and the un-methylated CpG sites were 14 ± 10 (C1) and 4 ± 4% (C2) methylated. This indicates that the method can discriminate hemi-hydroxymethylated CpG from hemi-methylated CpG with an eightfold preference.

3.2. Effect of density of DNA methylation or hydroxymethylation on DNMT1 methylation activity

As dense CpG methylation affects the de novo methylation activity of mouse Dnmt1 [30], we next examined the effect of the CpG density on DNMT1 methylation. We designed another synthetic DNA substrate, 100bp_7CpG, with a higher CpG density (Fig. 2A, upper panel, and Supplemental Table S1). The DNA methylation activity of DNMT1 towards hemi-hydroxymethylated sites H1 and H2 was significantly lower than towards those hemi-methylated sites M1 and M3. The hemi-methylated site, M2, however, was not efficiently methylated compared to the neighboring two hemi-methylated sites, M1 and M3 (Fig. 2A, lower panel). To further evaluate the effect of the local density of methylated and hydroxymethylated CpG on the activity of DNMT1, we designed the synthesized DNA 70bp_10CpG (Fig. 2B, upper panel, and Supplemental Table S1), DNMT1 efficiently methylated the hemi-hydroxymethylated sites H1 and H3, and H4, the one next to hemi-methylated M2 (Fig. 2B, lower panel). DNA methylation of the hemi-methylated sites, M3-5, was not affected by the density. These results indicate that the maintenance DNA methylation activity of DNMT1 is strongly affected by neighboring CpG methylation and hydroxymethylation, and thus DMAB-seq may not be applicable to a DNA sequence with a high CpG density and with high levels of methylation or hydroxymethylation. However, when the distance between two CpG sequences was more than 5 bp, DNMT1 properly recognized the modification status (Figs. 1 and 2).

3.3. Application of DMAB-seq to genomic DNA

Since DNMT1 methylation activity was affected by the 5mC and 5hmC densities, we analyzed the CpG distribution in both mouse and human genomes in silico. For the distances between the two CpGs that are less than 150 bp apart from each other, the medians are 27 and 33 bp, and the averages are 40 and 46 bp for human and mouse genomes, respectively (Supplemental Fig. S3A). Furthermore, the population of distances of more than 5 bp between two CpGs is 87% and 89% in human and mouse genomes, respectively.

Analysis of the consecutive CpG sequences in human and mouse genomes indicated that tandem CpG is observed with an about one-tenth probability compared to an orphan CpG (Supplemental Fig. S3B). The population of orphan CpGs in both human and mouse whole genomes is 98% (Supplemental Fig. S3B). Even in CpG islands, there is a one-tenth probability of a tandem CpG compared to an isolated single CpG (Supplemental Fig. S3C).

Therefore, DMAB-seq can be applied to most human and mouse genome regions.
3.4. Application of DMAB-seq to genomic DNA prepared from mouse ES cells

In the genome of mouse ES cells, cytosine bases at chr16:48431084 (mm9) located upstream of Morc1 gene are reported to be highly hydroxymethylated [23]. We used this region for evaluation of DMAB-seq. Firstly, we performed BS sequencing of the four CpG sequences, CpG1-4, of the upstream region of Morc1 gene (Supplemental Fig. S1). We found 44 ± 5% (three independent experiments; n = 3) and less than 5% methylation or hydroxymethylation at CpG1 and CpG2-4, respectively (Supplemental Fig. S4A). This result is consistent with the previous report that methylation or hydroxymethylation is found at CpG1 by BS sequencing [31].

To monitor the methylation by DNMT1, unmodified, fully-methylated, or fully-hydroxymethylated spike DNA with an AT sequence was mixed with genomic DNA as internal standards (Fig. 3A). Genomic DNA was mixed with one of the three spike DNAs, and primer extended with the biotinylated primer, and then treated with DNMT1. The methylated, hydroxymethylated, and unmodified spike DNA were determined to be 94 ± 3, 6 ± 6, and 9 ± 2% methylated, respectively (Fig. 3B). The CpG1 of the parent genomic DNA by DMAB-seq showed apparently 16 ± 3% methylation, while BS-seq showed 44 ± 5% methylation (Fig. 3B). By subtracting the average of methylation of hemi-hydroxymethylated and unmodified spike DNA, that is, 8% of the apparent genome methylation levels obtained by DMAB-seq or BS-seq, CpG1 was estimated to be 8% methylated, 36% hydroxymethylated, and 56% un-methylated (Fig. 3C, and Supplemental Fig. S4B).

Fig. 3. Culture condition-dependent changes of DNA modifications at specific CpG sites in Morc1. (A) Illustration of the position of the AT spike. The four CpG sites in Morc1 5' upstream region (vertical lines), the gene body (gray box), and the DNA sequences around the AT spike are shown. (B) DNA modifications in ES cells cultured with KSR in the presence of 2i were determined. The methylation state of the CpG1 site in the Morc1 gene was determined by BS-seq and DMAB-seq, and representative results for experiment 1 on the CpG1 are shown (left panel). The CPs sites determined to be T and C are shown as open and closed circles, respectively. The results of more than three independent experiments are shown as a bar graph with averages ± S. D. (n = 3 or 9) (right panel). The background (BG) of DMAB-seq (dotted line) was estimated to be approximately 8% (average of two backgrounds, 5.6% and 9.3%, for 5mC and C in spike DNA, respectively). (C) DNA modifications in ES cells cultured with KSR and absence of 2i were determined and are shown. The background (BG) of DMAB-seq (dotted line) was estimated to be approximately 5% (average of two backgrounds, 2% and 8%, for 5hmC and C in spike DNA, respectively). All the raw data for the calculation are shown in Supplemental Fig. S4. (D) The amounts of methylated, hydroxymethylated, and unmodified CpG at the CG1 site of Morc1 gene of the genome prepared from cells cultured in the presence (KSR) or absence (FBS) of vitamin C and 2i.

be un-methylated (Supplemental Fig. S5). Taken together, the results indicate that DMAB-seq can be applied to determine 5hmC at CpG sites.

It was reported that addition of vitamin C to the culture medium increases the global 5hmC level [32], and two kinase inhibitors (CHIR99021 and PD0325901; 2i) decrease the global 5mC level [33]. The above analyses were performed in the presence of vitamin C contained in KSR and 2i. We then analyzed the DNA modification state at the CpG1 site of Morc1 gene in the absence of both vitamin C and 2i by DMAB-seq (Fig. 3C). By calculating the modification level as in Fig. 3B, 5mC, 5hmC, and unmethylated cytosine at the CpG1 site in the absence of vitamin C and 2i were estimated to be 57, 26 and 17%, respectively (Figs. 3C, and Supplemental Figs. S4C and D). As shown in Fig. 3C, CpG1 in ES cells cultured in the presence of KSR and 2i showed hypomethylation compared to those cultured in the absence of vitamin C and 2i in FBS. In addition, by changing the medium including KSR and 2i to the one with FBS, the level of 5mC significantly increased while that of 5hmC moderately decreased at the CpG1 site (Fig. 3D). These results show that DMAB-seq is a valuable tool to analyze the changes of the 5hmC and 5mC levels at the CpG sequence in a specific genomic region, and may contribute in understanding of the biological function of dynamic changes of 5hmC.

4. Discussion

In the present study, we have developed a novel method to analyze the position of 5hmC in the CpG sequence by utilizing the unique activity of DNMT1, which selectively methylates hemi-methylated but not hemi-hydroxymethylated CpG sites. Compared with conventional BS treatment, this method involves only two extra steps, primer extension and methylation with DNMT1.

The enzymatic property of recombinant DNMT1, which possesses de novo-type DNA methylation [30], indicates that an excess amount of DNMT1 may over-introduce de novo CpG methylation in addition to maintenance methylation. Under the optimized conditions, however, we did not detect significant de novo methylation activity using synthesized DNA (Fig. 1E) and spike DNA (Fig. 3B and C). In addition, theoretically, as the parent CpG methylation status is used as a standard, the bias due to de novo methylation activity may be excluded. Another crucial point of the method is the densities of 5mC and 5hmC in genome, which affect the selective DNA methylation activity of DNMT1 (Fig. 2). In the human and mouse genomes, however, about 90% of the CpG sites are separated not to be affected by the density (Supplemental Fig. S3). More importantly, regulatory elements in CpG-poor sequence contexts undergo extensive and dynamic methylation and demethylation [34].

Methylation on non-CpG sites does occur in the genome, even if it is at a low level, and these 5hmC sites may suffer from hydroxymethylation. DMAB-seq cannot analyze these 5hmC sites as DNMT1 is specific for CpG methylation. However, non-CpG methylation is rare and thus so is 5hmC at non-CpG sites. Adult brain is reported to contain a high level of non-CpG methylation, about 18.9% of total 5mC, and non-CpG 5hmC is only 2.5% of the total 5hmC level [35]. Thus, failing to measure 5hmC at non-CpG sites may not be significant in most cases.

It was reported that DNA methyltransferases possess dehydroxymethylation activity in vitro [36,37]. Because of this there might be an argument that DNMT1 used in the present study catalyzed dehydroxymethylation during copying of methylation state. The dehydroxymethylation activities were detected in the absence of cofactor SAM in both the reports. Since the dehydroxymethylation activity is the reverse reaction of methylation, it is reasonable to assume that dehydroxymethylation activity should be detected only in the absence of methyl-group donor SAM. In DMAB-seq, however, an excess of SAM was used for the methylation reaction by DNMT1. Therefore, it seems unlikely that dehydroxymethyla-
tion occurred during the methylation reaction.

As an example, we have chosen the CpG1 site of the 5’ upstream region of Morc1 gene, which is reported to be 44% hydroxymethylated [23]. By DMAB-seq, we successfully determined the CpG1 site to be 35% hydroxymethylated. However, one disadvantage of DMAB-seq is that when the basal 5mC content is high, the accuracy of 5hmC determination is reduced. Similar to DMAB-seq, oo-BS-seq is also affected as to the accuracy of 5hmC quantification by a high level of 5mC.

During the course of this study, we found that DNMT1 methylated hemi-hydroxymethylated sites when the sites were densely surrounded by 5mC. It was reported that dense CpG methylation enhances de novo methylation [30]. Both results may suggest that densely methylated CpG islands are resistant to replication-dependent demethylation, which may occur at hydroxymethylated CpG sites [27]. This provides a new concept that densely methylated CpG islands are resistant to Tet-dependent demethylation due to a unique methylation property of DNMT1.

Author contributions

ShoT and IS conceived and designed the experiments, and supervised the work. SaT and IS performed the experiments. SaT and IS analyzed the data. JE performed the genome analysis. SaT, IS, and ShoT wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2015.09.003.

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