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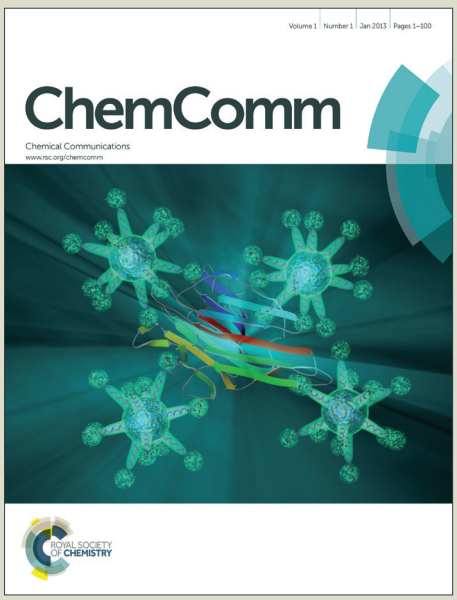

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Qualitatively and QuantitativelyDetection of Methylation at CpG Sites by Fluorescein-dGTP Incorporated Asymmetric PCR Assay Strategy

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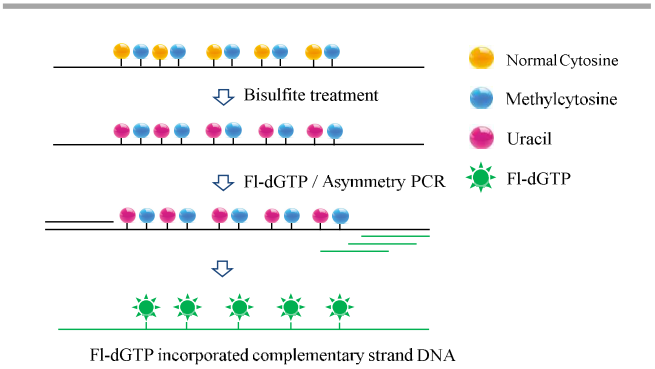
Methylation status of each CpG site can be monitored by Fl-dGTP Incorporated Asymmetric PCR. The ability of quantitative detection makes it a good choice for detecting partial methylation at CpG sites compared with others.And the monitoring is not limited to sites within PCR primers or restriction enzyme-recognition sites.

DNA methylation at CpG dinucleotides is an essential epigenetic modification that frequently occurs in higher-order eukaryotes. As a well-studied epigenetic alteration involved in the regulation of gene expression during cancer development, DNA methylation provides valuable biological information about tumorigenesis[1-3].Previous studies have shown that aberrant methylation of normal CpG islands, such as gross hypomethylation and hypermethylation of promoters, is closely associated with many types of cancer. Hypermethylation of CpG islands in the promoter regions of a tumor suppressor gene is the main cause of transcriptional inactivation of these genes and are the best characterized epigenetic changes in tumors[4-7].

To investigate the role of methylation in carcinogenesis, various methods have been developed to quantify methylation levels at CpG dinucleotides at specific sites in tumor suppressor genes. Most of these techniques take advantage of bisulfite conversion[8] or restriction enzyme treatment. Among these, MS-PCR[8] is one of the most widely used approaches. The key characteristic of this method is the design of specific primers. More than one CpG dinucleotide should be covered by the primers to efficiently discriminate among the original templates with different methylation statuses. However, this technique may not be a good choice for detecting partial methylation at CpG sites[9]. Other MS-PCR-based assays[10-12] also have the same drawback that the methylation status of only two CpG sites (the 3' ends of two PCR primers) and not all CpG sites in the promoter regioncan be analyzed[9]. Combined bisulfite restriction analysis (COBRA)[13] is based on digestion at newly formed restriction sites after bisulfite conversion. However, the limited availability of enzyme recognition sites is an obstacle to obtain the complete picture of CpG methylation status in the original genomic DNA. The same unsatisfied application also exists for many restriction enzyme-based methods[14, 15]. Most information about methylation patterns can be revealed by bisulfite-based sequencing[16]; however, the labor and intensive instrument[17] requirements hamper further development in typical laboratories. Therefore, a simple method for analyzing methylation status that is not limited to sites within PCR primers and restriction enzyme recognition sites is urgently needed.

Here, we report a novel fluorescein-dGTP incorporatedasymmetric PCR assay to qualitatively and quantitatively determine methylation status in a region of interest in DNA. The principle is illustrated in Scheme**1**. Initial modification by bisulfite treatment efficiently converts unmethylated cytosine into uracil, which is replicated as thymine during PCR amplification. In contrast, methylcytosine, which is not converted by bisulfite treatment, is maintained as cytosine during PCR amplification,creating sequence differences compared with unmethylated cytosine for detection.

Given that only methylcytosine in the original DNA remains intact and is matched to guanine in the complementary DNA strand,the issue of methylation detection can be transformed into one of



Scheme1. The principle of the fluorescein-dGTP incorporation asymmetric PCR assay to identify diverse methylation statuses in a region of interest in DNA.

guanine quantification in the complementary DNA strand.Thus, we have adapted asymmetric PCR to obtain the corresponding complementary DNA strand. Fluorescein-labeled dGTP is introduced into the dNTP mix and is incorporated into the complementary DNA strand by extension to obtain a fluorescent signal if methylcytosine is present in the original DNA. This method enables quantification of all CpG sites in a promoter regionat a time because every methylcytosine in the original DNA corresponds to one fluorescein-labeled dGTP in the complementary DNA strand,avoiding the cumbersome design of multiple primers. Furthermore, the primers used here do not cover CpG dinucleotides, so no specific primers are needed to discriminate between the original methylated and unmethylated templates, preventing false-positive or false-negative results caused by primer annealing.

Five DNA sequences containing diverse CpG sites were chosen to demonstrate the ability of this method to identify diverse methylation statuses in a region of interest. The sequences were subjected to bisulfite treatment using standard protocol[8] and asymmetric PCR. Firstly, the fraction of fluorescein-labeled dGTP was determined. Due to the relatively slow extension rate of fluorescein-labeled dGTP in the PCR reaction compared with normal PCR, a large fraction of fluorescein-labeled dGTP would lead to inefficient amplification. In contrast, only low-fluorescence products with small amounts of fluorescein-labeled dGTP would be produced if only a small fraction of fluorescein-labeled dGTP were contained in the dNTP mix (Figure **S2**), reducing the sensitivity of this method. Therefore, 75% fluorescein-labeled dGTP was finally chosen to mix with other dNTPs in the finalPCR reaction. Gel electrophoresis results are shown in Figure **1a**. Nofluorescent band can be observed for unmethylatedDNA PCR products because no fluorescein-labeled dGTP was incorporated into the complementary DNA strand (Lane 1).

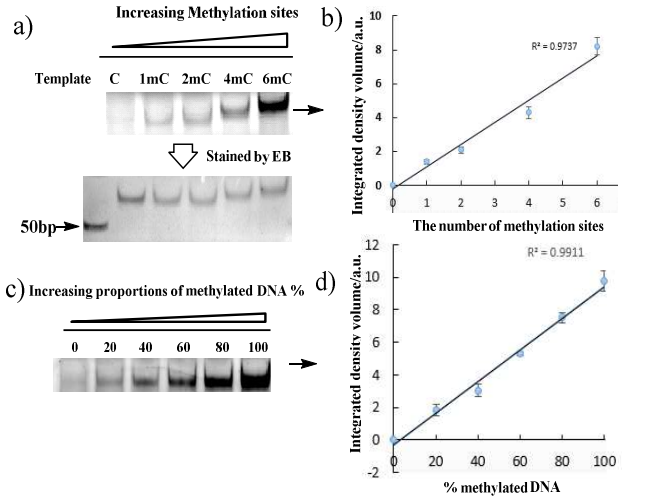


Fig.1 a) Non-denaturing polyacrylamide (12%) gel electrophoresis of DNA sequences with diverse methylation statuses. Lane 1, DNA template only contained unmethylated cytosine; Lanes 2-5, DNA template containing 1, 2, 4 and 6 methylation sites, respectively. The figure below was obtained by stained with EB, indicating successful amplification for all templates. b) Linear relationship between integrated density volume and number of methylation sites, $R^2 = 0.9737$. c) Non-denaturing PAGE (12%) analysis of mixed samples with diverse proportions of methylated DNA. d) Integrated density volumes of fluorescent bands plotted with respect to methylation level, and a linear model fits the experimental data well, $R^2 = 0.9911$.

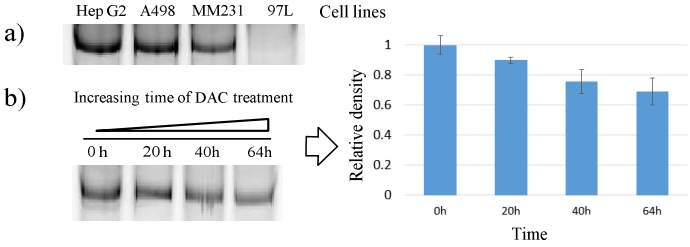


Fig. 2a) Detection of promoter hypomethylation in the E-cadherin suppressor gene in the human cancer cell lines HepG2, A498, MDA-MB-231 and 97L. b) Methylation reversal of the E-cadherin suppressor gene from MDA-MB-231 with increasing incubation times with 5-aza-2'-deoxycytidine (DAC).

However, with increasing methylation sites, the corresponding fluorescent bands become more intense (Lanes 2-5). The integrated density volume of the PCRproducts shows a linear relationship with the number of methylation sites (Figure **1b**). Therefore, quantitative information about methylation status at CpG sites in a region of interest can be revealed by the corresponding standard curves. Furthermore, because more fluorescein-labeled dGTP was incorporated into the complementary DNA strand for original templates containing multiple methylation sites, the corresponding PCR products possessed decreased electrophoretic mobility. Moreover, because the primers used here do not contain any CpG sites, there was no bias for original DNA templates with particular methylation statuses. DNA bands can also be observed for unmethylated DNA after stained with EB (ethidium bromide), indicating successful PCR amplification of both methylated and unmethylated DNA templates.

The fluorescein-dGTP-incorporated complementary DNA strand can be also measured by fluorescence spectra. The residual fluorescein-labeled dGTP was removed by filtration through a Millipore membrane three times. For unmethylated DNA, there is no fluorescein-labeled dGTP in the PCR product, so almost no fluorescence emission can be measured, whereas a strong increase in fluorescence was observed for the methylated DNA (Figure **3a**) due to the multiple fluorescein-labeled dGTP residues incorporated into the complementary DNA strand.

Sensitivity and accuracy were further verified by detecting mixed samples with diverse methylation levels. Samples of 0%, 20%, 40%, 60% 80% and 100% methylated DNA were diluted with unmethylated DNA for a total of 20 ng of input DNA. All DNA samples were treated with bisulfite and subjected to asymmetric PCR under the same conditions described above. Because more fluorescent PCR products were generated due to the increasing proportions of methylated DNA, the integrated density volume of the corresponding bands also increased in a linear fashion (Figure **1c** and **1d**). This quantitative phenomenon demonstrates the successful application of this fluorescein-dGTP incorporation asymmetric PCR assay for the quantitative detection of methylation changes.

To investigate the association between tumors and transcriptional silencing caused by aberrant hypermethylation, E-cadherin, a tumor suppressor gene, was further characterized in four human cancer cell lines (HepG2, A498, MDA-MB-231 and 97L). Approximately

120ng of genomic DNA extracted from these cell lines was subjected to

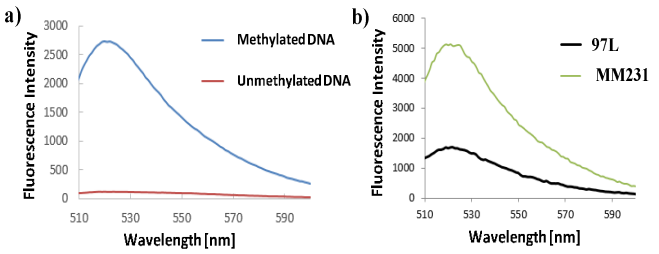


Fig.3 Fluorescence measurement of the fluorescein-dGTP incorporation asymmetric PCR assay products to determine methylation status. a) Blue line refers to methylated DNA containing six methylation sites; red line refers to unmethylated DNA without methylation sites. b) An approximately 3-fold enhancement in fluorescence can be observed for DNA from MDA-MB-231 cells (green line) compared with that from 97L cells (black line).

bisulfite treatment and the corresponding single-stranded DNA with the expected length was generated after the amplification using specific primers. As shown in Figure **2a**, fluorescent bands were clearly observed in HepG2, A498 and MDA-MB-231 cells, indicating that the E-cadherin tumor suppressor gene ishypermethylated in these cell lines. However, DNA from 97L shows no evidence of methylation in the fluorescein-dGTP incorporation asymmetric PCR assay, with no fluorescent bands observed by PAGE analysis. The methylation status of the E-cadherin promoter can also be monitored by fluorescence spectra (Figure **3b)**. The fluorescence of MDA-MB-231 at 520 nm was 3-4 times higher than that of 97L, which is consistent with the results obtained by PAGE analysis.

Previous studies indicate that activation of tumor suppressor genes by 5-aza-2'-deoxycytidine (DAC)[9, 18-20] treatment is associated with demethylation of CpG islands in the corresponding promoter. As an inhibitor of DNA methyltransferases, DAC can be incorporated into DNA during DNA replication, consequently reducing methylation levels. Therefore, we used DAC-treated cells (MDA-MA-231) to monitor methylation changes in the E-cadherin suppressor gene using our fluorescein-dGTP incorporated asymmetric PCR assay. MDA-MB-231 cells were cultured and treated with DAC for increasing times. After incubation for 0 h, 20 h, 40 h and 64 h, DNA was extracted from the DAC-treated cells and subjected to bisulfite conversion and asymmetric PCR amplification. Figure **2b** shows that the relative density compared with that of control DNA (0 h) gradually decreased, indicating that methylation levels decreased with increasing DAC treatment times. Partial methylation at CpG sites caused by DAC decreased the amount of fluorescein-labeled dGTP in the PCR products, which is consistent with results obtained from bisulfite sequencing[9].

Conclusions

In summary, the fluorescein-dGTP incorporated asymmetric PCR assay described here can provide accurate quantitative information about methylation changes at CpG sites. No specific regions with sufficient methylation density are needed for primer design, reducing false-positive and false-negative results compared with MSP-associated methods. Furthermore, taking advantage of fluorescein-labeled dGTP, the methylation status at each CpG site can be monitored in a single reaction and is not limited to sites within PCR primers or restriction enzyme recognition sites. Both PAGE analysis and fluorescence measurement can be used to determine methylation status in different cancer cell lines. Furthermore, avoiding the use of restriction enzymes, radioactive materials and labeled primers or probes facilitates the determination of methylation status at CpG sites.

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Notes and references

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Electronic Supplementary Information (ESI) available: Materials and general measurement, cell culture and 5-aza-2'-deoxycytidine (DAC) treatment. See DOI:10.1039/c000000x/

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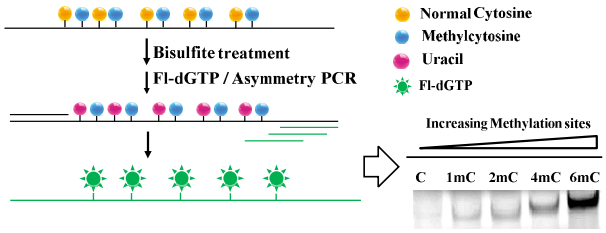

Quantitative information about DNA sequences with diverse methylation statuses can be provided through fluorescin-dGTP incorporated asymmetric PCR.