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Quantification of Regional DNA Methylation by Liquid Chromatography/Tandem Mass Spectrometry

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Abstract

Promoter hypermethylation associated tumor suppressor genes (TSGs) silencing has been explored as a therapeutic target for hypomethylating agents. Promoter methylation change may serve as a pharmacodynamic endpoint for evaluation of the efficacy of these agents and predict the patient's clinical response. Herein, a LC-MS/MS assay has been developed for quantitative regional DNA methylation analysis using the molar ratio of 5-methyl-2'-deoxycytidine (5mdC) to 2'-deoxycytidine (2dC) in the enzymatic hydrolysate of fully methylated bisulfite-converted PCR amplicons as the methylation indicator. The assay can differentiate 5% of promoter methylation level with an intra-day precision ranging from 3.00 to 16.0% using two TSGs: HIN-1 and RASSF1A. This method was applied to characterize decitabine-induced promoter DNA methylation changes of these two TSGs in a breast cancer MCF-7 cell line. Promoter methylation of these TSGs was found to decrease in a dose-dependent manner. Correspondingly, the expression of these TSGs was enhanced. The sensitivity and reproducibility of the method make it a valuable tool for specific gene methylation analysis, which could aid characterization of hypomethylating activity on specific genes by hypomethylating agents in a clinical setting.

Keywords

Regional DNA Methylation; LC-MS/MS; Quantification

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Introduction

DNA methylation of the cytosine residues within the 5'-Cytosine-phospho-Guanosine (CpG) dinucleotides is an epigenetic event that regulates gene transcription, genetic imprinting and perhaps genome stability [1]. In malignant cells, the aberrant DNA methylation pattern has been manifested as genomic DNA hypomethylating and CpG island hypermethylation. Hypermethylation of CpG islands in promoters of tumor suppressor genes (TSGs) in cancer cells is associated with their transcriptional silencing and contributes to malignant transformation at various stages [2; 3; 4]. Therefore, methylation status has been explored as a potential clinical biomarker for cancer classification, diagnostics, prognostics, and proposed as a chemotherapeutic target in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) [5; 6]. Additionally, TSG hypomethylation has been advocated as pharmacodynamic endpoints of hypomethylation agents. To meet this demand, various methylation methods including specific gene methylation methods [7; 8; 9; 10; 11; 12] have been developed and applied in the aforementioned purposes [13; 14; 15; 16; 17; 18]. It was found that different quantitative high-throughput DNA methylation methods have resulted in inconsistent results [19]. This might be due to the limitation of the covered range of analyzed sequence of these TSGs. Therefore, there is a need for a screening technique that will allow for the rapid and reliable evaluation of DNA methylation and to provide quantitative information on the methylation density of the entire amplified region, not only of a few CpG Loci that are covered by PCR primers. Ideally, this method should be robust and adaptable to large sample sets in clinical methylation analysis of particular biomarkers.

Recently, we adapted, improved and validated a reported LC-MS/MS method [20] for determination of global DNA methylation as a pharmacodynamic endpoint of hypomethylating agents. This method was applied to characterize the *in-vitro* and *in-vivo* hypomethylating activity of decitabine in AML. This result demonstrated that our method is both robust and sensitive, and easily be adapted to high throughput platform. In this paper, we adapted this LC-MS/MS method to establish a practical method for quantitative analysis of regional DNA methylation level by incorporation with bisulfite-treated PCR. The method enabled us to characterize two TSGs (HIN-1 and RASSF1A) promoter methylation changes in a primary human breast cancer cell line. This result demonstrated that our LC-MS/MS method is effective to measure the basal level of regional DNA methylation and its alteration induced by hypomethylating agents.

Materials and Methods

Incubation of MCF-7 cells with decitabine and isolation of DNA and RNA

MCF-7 breast carcinoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech; Herndon, VA) or MV4-11 and Kasumi-1 cells maintained in RPMI supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco; Grand Island, NY) and 1% (v/v) penicillin/streptomycin (Invitrogen Life Technologies; Carlsbad, CA) antibiotic solution at 37 °C supplemented with 5% CO₂. Cells were seeded at 10% confluency, and treated with decitabine at three different concentrations (0, 0.10, and 0.75 μM) for 72 h. Then, the cell pellets were collected in 15 ml tubes. Genomic DNA was isolated from these pellets using DNeasy tissue kit (Qiagen, Minneapolis, MN), according to the manufacturer's instructions. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Generation of DNA methylation standards and bisulfite conversion of genomic DNA

The genomic DNA isolated from normal peripheral blood lymphocytes (PBL) was methylated and purified as described previously [20]. The fully methylated and non-methylated genomic DNAs from PBL were concentration-adjusted to 20 ng/ml and mixed in ratios of 0:100, 5:95, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, 97.5:2.5 and 100:0, respectively, to give solutions of the following methylation level of 0, 5, 20, 30, 40, 50, 60, 70, 80, 90, 97.5 and 100 %, respectively. One μ g aliquots of these mixtures or genomic DNAs isolated from MCF-7 cells treated with +/- decitabine were bisulfite converted using Active Motif methylation-Kit (Zymo Research Orange, CA) according to the manufacturer's instruction. The residue was diluted to a final volume of 300 μ l with ddH₂O.

Combined bisulfite restriction analysis (COBRA) and methylation of PCR amplicons with M. SssI

A 10 μ l aliquot of the above bisulfite-converted solutions at various ratios (0:100, 5:95, 10:90, 30:70, 50:50, 70:30, 90:10, and 100:0) of methylated genomic DNA (representing 100% methylated DNA) and non-methylated genomic DNA (representing 0% methylated DNA) from PBL was amplified by PCR using appropriate primers (HIN-1F: 5'-GTA GGG ATT AGG GAG TTA GGA ATT G-3'; HIN-1R: 5'-TAA AAC CCT CTA AAA ACA AAC AAA C-3'; RASSF1AF: 5'-TTA TTT AGT GGG TAG GCC AAG TGT GTT-3'; RASSF1AR: 5'-CCT AAA TAC AAA AAC TAT AAA ACC C -3' designed to amplify both methylated and unmethylated alleles of bisulfite-converted DNA). PCR amplifications were performed and purified as described before. One part of these purified PCR amplicons was digested by *Bst*UI (CG↓CG) restriction enzyme (NEB, Ipswich, MA) and the resulting digested fragments were separated on an 8% polyacrylamide gel. The rest was methylated with M. SssI and purified according to procedure as described before [20].

DNA Hydrolysis

DNA hydrolysis was performed as previously described [20]. Briefly, 1 μ g of genomic DNA was first denatured by heating at 100 °C for 3 min and then chilled on ice. After adding a 1/10 volume of 0.1 M ammonium acetate (pH 5.3) and 2 units of nuclease P1, the mixture was incubated at 45 °C for 2 h. One-tenth volume of 1 M ammonium bicarbonate and 0.002 unit of venom phosphodiesterase I were added, and the mixture was incubated at 37 °C for 1 h. Next, 0.5 unit of alkaline phosphatase was added, and the mixture was incubated at 37 °C for 1 h.

Instrumentation

For quantification, the LC-MS system used consisted of a Perkin-Elmer Sciex API 300 triple-quadrupole mass spectrometer (Thornhill, Ontario, Canada) coupled to a Shimadzu HPLC system (Shimadzu, Columbia, MD). The HPLC system was equipped with an SCL-10A system controller, a LC-10AD pump and a SIL-10A auto-sampler (Shimadzu, Columbia, MD).

HPLC chromatographic and mass spectrometric conditions

5mdC and the internal standard (I.S.) 2dC were separated on a 250 × 2.1 mm Hypersil Aquasil C₁₈ 5 μ m stainless steel column (Thermo Hypersil-Keystone, Bellefonte, PA), which was coupled to a 2 μ m Aquasil pre-column (Thermo Hypersil-Keystone, Bellefonte, PA), using the mobile phase consisting of 30% methanol in 10 mM ammonium formate at the flow rate of 0.2 ml/min. Pure methanol was then added to the flow at 0.2 ml/min via a separated HPLC pump and mixed post-column prior to the entrance to the ion-source. The LC elute was introduced into the API source at 20 μ l/min after a 95:5 (LC/MS) split. The

mass spectrometer (Sciex API 300) was operated under electrospray ionization (ESI) with an ion-spray voltage of +4700 V. The positive ion multiple reaction-monitoring (MRM) mode analysis was performed using nitrogen as the collision gas. The curtain gas (nitrogen) and the nebulizer gas (nitrogen) flow rates were set at 0.6 l/min and 1.1 L/min, respectively. The pressure in the collision cell was set at 0.29 Pa. The orifice voltage and ring voltages were set to +30 and +300 V, respectively. A dwell time of 600 ms and a pause time of 5 ms between scans were used to monitor the following precursor/product ion pair of m/z 228.1/112.2 for 2dC and m/z 242.1/126.1 for 5mdC. The mass spectrometer was tuned to its optimum sensitivity and mass accuracy by infusion of a fresh standard solution of 5mdC at 5 ng/ml. Data acquisition was performed using the PE Sciex software Sample Control 1.2 and the data were analyzed by PE Sciex software MacQuan 1.4.

Sample preparation and method validation

Stock solution of 5mdC and 2dC were prepared by dissolving the accurately weighed nucleoside in 10 ml of methanol to a final concentration of 1 mg/ml. These solutions were stored in a glass vial at -80°C . Working solutions were freshly prepared daily by diluting the stock solution with methanol. Appropriate volumes of 5mdC working solution were added into working solution of 2dC (1000 ng/ml) to prepare calibration standards at the following concentrations: 2 (8), 5 (20), 10 (40), 20 (80), 50 (200), 100 (400), 200 (800), and 500 (2000) ng/ml (nM). The enzymatic digestion solutions of the fully-methylated PRC amplicons of RASSF1A or HIN-1 promoters, or genomic DNA of MCF-7 cells were reconstituted in 200 μl water at 4°C and analyzed immediately by LC-MS/MS. The method was validated in mobile phased spiked with indicated concentration of 5mdC and constant concentration of 2dC. Then, the method was validated using various ratios of fully-methylated and non-methylated PCR amplicons of RASSF1A and HIN-1 promoters. The between-day precision was determined for three quality controls (QCs, 5%, 30%, 70%, 90%) samples at six different days and the mean concentrations and % coefficients of variation (CV) were calculated. The accuracy of the assay was determined by comparing the calculated concentrations with the nominal concentrations.

Real Time RT-PCR assays

Quantitative RT-PCR was used to quantify the expression of RASSF1A, and HIN-1 (HIN-1F: 5'-GAG CAT CTA CCT GAG GAC AAG AC-3', HIN-1R: 5'-TTT TGC TCT TAA CCA CGT TTA TTG A-3', RASSF1AF: 5'-GTT CTT GGT GGT GGA TGA CC-3', SSF1AR: 5'-CCT TCA GGA CAA AGC TCA GG-3'). Untreated cells were used as a negative control. Sybergreen real-time RT-PCR was performed using 2 μg of total RNA extracted with Trizol reagent (Invitrogen, CA) according to the manufacturer's instruction. Data were analyzed according to the comparative CT method using the internal control (18S) transcript levels to normalize differences in sample loading and preparation. Results represent the n-fold difference of transcript levels between different samples and are expressed as the mean $\pm$ SD of triplicate reaction wells.

Results

The strategy of the LC-MS/MS method for regional DNA methylation (RGM) level determination—The strategy of this assay for RDM was depicted schematically in Figure 1. The starting point of the assay is bisulfite conversion of genomic DNA to convert cytosine (C) into uracil (U), but to retain 5-methylcytosine (^mC) as its original form. This results in methylation dependent sequence changes in the genomic DNA template. PCR with specific primers for any length regional DNA is used to amplify this template, while preserving the induced sequence changes. Then, the PCR amplicons are methylated with M. SssI to recover the original methylated CpG moiety in the genomic DNA followed by

digestion to nucleosides. 2-Dexoycytidine (2dC) and 5mdC will be monitored using precursor/product ion pairs of m/z 228.2/112.0, and 242.0/126.0, respectively. Conversion of C to U in the genomic DNA, and sequentially to thymine (T) in the PCR amplicons results in dependency of the content of G in PCR amplicons on methylation level and independency of the content of C in PCR amplicons on methylation level of regional DNAs. Therefore, the ratio 5mdC/2dC will be used to express the DNA methylation level, which can easily be converted to actual DNA methylation level in the regional DNA. Assuming there are M Gs and N Cs in the sense single strand promoter of specific gene, the DNA methylation level of the promoter should be $5mdC/2dC * (M/(M+N))$.

Mass spectrometric characterization of 5mdC and 2dC

A mixture of 5mdC (10 μ g/ml) and the I.S. 2dC (10 μ g/ml) in 50% methanol and 5 mM ammonium formate solution was infused into the triple quadrupole mass spectrometer at a flow rate of 10 μ l/min for 1 min. The average mass spectrum as acquired under a positive ion ESI exhibited two major ions at m/z 242.1 and 228.1 (Figure 2A). These ions correspond to the protonated molecular ions (MH^+) of 5mdC and 2dC, respectively. It is similar to the mass spectra of 5mdC and 2dC reported before [20]. The collision assisted dissociation (CAD) spectrum (Figure 2B) of the MH^+ of 5mdC at m/z 242.2 exhibited a base fragment ion at m/z 126.1, corresponding to the protonated 5-methylcytosine generated by glycosidic cleavage of the protonated 5mdC. The CAD spectrum of the MH^+ of 2dC at m/z 228.1 (Figure 1C) exhibited a base fragment ion at m/z 112.2, corresponding to the protonated cytosine formed by glycosidic cleavage of the protonated 2dC. Therefore, the ion transition at m/z 242.1>126.1 and the ion transition at m/z 228.1>112.2 were selected for monitoring 5mdC and 2dC, respectively. Under the HPLC condition for GDM analysis [20], 5mdC and 2dC was eluted at 5.1 and 4.7 min, respectively as shown in Figures 3A and B.

Validation of the LC-MS/MS method

For the assay validation, we utilized serial dilutions of 5mdC into 2dC standards. The assay was found to be linear from 40 fmol to 200 pmol (1 ng/ml to 500 ng/ml) of 5mdC on column using aqueous buffer solution. The precision and accuracy of the assay were assessed within the physiologic range of RGM from 80 fmol to 40 pmol. Levels of methylation were calculated using a calibration curve where the area ratios of the mass signal of 5mdC to 2dC was plotted against known weight/weight ratio of 5mdC and 2dC, which allows conversion to % DNA methylation (40 fmol 5mdC is equivalent to approximately 5% DNA methylation of 1 ng DNA) [21].

The inter-day and intra-day precision and accuracy of using the ratio of 5mdC and 2dC as the indicator of regional DNA methylation at 0.2%, 2%, 10% and 20% under the LC-MS/MS conditions are summarized in Table I. The intra-day precision expressed as CV ranged from 2.68% to 10.2% and the inter-day precision values ranged from 0.91% to 17.6% at the lower limit of quantitation (LLOQ, 0.2% of 5mdC/2dC) of the method. The accuracy values of the assay varied from 87.9% to 102.6%. All these values were within the commonly accepted FDA guidelines for analytic methods validation (Guidance for Industry Bioanalytical Method Validation, p.5, available from the website: <http://www.fda.gov/cder/guidance/4252fnl.pdf>).

Proof of Concept—To evaluate the strategy depicted in Figure 1, the methylation level of two TSGs, RASSF1A and HIN-1, in the genomic DNA isolated from PBL and its corresponding fully-methylated genomic DNA was evaluated using Combined Bisulfite Restriction Analysis COBRA). First, the natural genomic DNA and the fully-methylated genomic DNA were bisulfite-converted. Then, the promoters of RASSF1A (269 bp, 167 to 435) and HIN-1 (182 bp, 611 to 792) were amplified using primers including no CpG locus

and non-methylated or fully-methylated bisulfite-converted genomic DNA as template, respectively. Partial PCR amplicons were digested with a methylation sensitive restriction enzyme, BstUI. The resulting mixtures were applied to gel electrophoresis in an 8% polyacrylamide gel and visualized by ethidium bromide staining. As shown in Figure 4, single bands in lanes 2 (269bp) and 10 (182 bp) were detected, representing 0% methylation of RASSF1A and HIN-1 promoter genes, respectively, as expected. These bands essentially disappeared with appearance of several bands following treatment with methylation sensitive restriction enzyme (lanes 9 and 17, respectively), also as expected. The next step is to evaluate their methylation levels using our strategy. The single strand sequences of these two promoters are depicted in Figure 5. As shown, the selected single strand oligonucleotide of RASSF1A promoter contains 104 2-dGs, 78 2-dCs and 18 CpG moieties, after PCR amplification of the bisulfite-converted fully methylated genomic DNA, the amplicon should contain 36 2dCs in CpG from 5mdC and 104 2dCs from 2-dG in the original methylated genomic DNA. After full methylation of the amplicon by M. SssI, 36 2dCs in CpG will be converted to 36 5mdC in the amplicon. Hence, the methylation level of the fully-methylated amplicon expressed as 5mdC/2dC should be $36/104=34.6\%$. The selected single strand of HIN-1 promoter contains 72 2-dGs, 67 2-dCs and 15 CpG moieties, the amplicon of bisulfite-converted fully methylated genomic DNA should contain 30 2dCs in CpG from 5mdC and 72 2dCs from 2-dG. After full methylation of the amplicon by M. SssI, 30 2dCs in CpG will be converted to 30 5mdC in the amplicon. Hence, the methylation level of the amplicon expressed as 5mdC/2dC should be $30/72=41.7\%$. LC-MS/MS analysis of the hydrolysates of fully methylated HIN-1 amplicons demonstrated that 4.4% and 37.9% of 5-mdC/2dC were in its amplicons from genomic DNA and fully-methylated genomic DNA. The 4.4% of 5-mdC/2dC detected in the non-methylated HIN-1 promoter suggested that the methylation level of HIN-1 promoter is consistent with genomic DNA methylation level (3–5%), which is not detectable using COBRA. The 37.9% of 5mdC/2dC detected in the fully-methylated HIN-1 promoter, corresponding to 90 % of theoretic value of 41.7%, suggested that most of methylated CpG Loci can be recovered from the original genomic DNA, in spite of a slight difference between the theoretic value and measured values.

Based on these encouraging results, various ratios (0%, 20%, 40%, 60%, 80% and 100%) of HIN-1 amplicons amplified from bisulfite-treated non-methylated and full-methylated genomic DNAs were mixed followed by full methylation using M. SssI and enzymatic digestion sequentially. LC-MS/MS analysis of these samples demonstrated that the ratio of 5mdC and 2dC reflecting the ratio of non-methylated and full-methylated genomic DNAs is linear with R^2 of 0.9952 as shown in Figure SM1 in the Supplemental Material (SM) section. This result suggests that the detected ratio of 5mdC/2dC can reflect the original methylation level in the genomic DNA and provided the evidence to further develop our method for specific gene methylation quantification.

Determination of sensitivity, reproducibility and accuracy of our LC-MS/MS method—The sensitivity of LC-MS/MS method was determined by testing the methylation gradient using the following mixtures of 0, 5%, 10%, 30%, 50%, 70%, 90% and 97.5% of fully-methylated HIN-1 amplicon amplified from fully-methylated genomic DNA. As shown in Figure SM1B in SM, the ratio of 5mdC/2dC reflecting the ratio of the methylation level in the range of 5% to 97% is also linear with a R^2 of 0.9947. Similarly, linearity was also observed for RASSF1A gene promoter methylation (**Data Not Shown**). These results demonstrated that the method can differentiate 5% methylation of specific genes. To validate our method, the within-day accuracy and precision of our LC-MS/MS method was assessed using various ratios of two amplicons for HIN-1 or RASSF1A in six different runs of the same digest for each of two genes and the validation parameters are shown in Table I. As shown in Table I, the method is quite robust and reproducible. In parallel, the above methylation gradient (5% to 100 %) was also generated by mixing *in-vitro* methylated DNA

with PBL DNA (see Materials and Methods) and evaluated by COBRA. As shown in Figure 4, our LC-MS/MS generates result consistent with that obtained by COBRA. However, COBRA only gives qualitative methylation level of these mixtures and the indicated methylation levels only reflect the methylation level of several specific CGCG moieties in specific amplicons.

Characterization of hypomethylation activity of decitabine in MCF-7 cells—To test the applicability of our LC-MS/MS for characterization of the hypomethylation activity of decitabine, MCF-7 cells were exposed to decitabine (0, 0.10, 0.75, 2.0 μ M) for 72 h. The genomic DNA extracted from these cells was bisulfite-converted. The PCR amplicon of the promoter of HIN-1 using bisulfite-converted genomic DNA as a template was fully methylated with M. SssI followed by enzymatic digestion into nucleosides. The promoter methylation level was measured using the above LC-MS/MS method and the global DNA methylation level was determined using our published LC-MS/MS method [20]. As shown in Figure 6A, for GDM, the methylation level decreased to about 23% at 0.1 μ M decitabine, and to 70% at both 0.75 and 2.0 μ M decitabine. For promoter of RASSF1A (Figure 6B), decreases of 50% and 71% in methylation were detected at 0.1 μ M and 0.75 μ M decitabine, respectively. For promoter of HIN-1 (Figure 6D), a slight decrease in methylation (10%) and a 68–62 % decrease was detected at 0.1 and 0.75 μ M of decitabine, respectively, consistent with the global DNA methylation change. These results support that our method can be used to characterize the regional DNA methylation perturbation by hypomethylation agents. Additionally, the expression levels of HIN-1 and RASSF1A were measured using Sybergreen real-time (RT)-PCR. Consistent with their hypomethylation change, the expression level of RASSF1A and HIN-1 were found to be elevated about 2–3 fold at 0.75 μ M of decitabine as shown in Figure 6C and Figure 6E, respectively.

DISCUSSION

There is a large body of evidence that indicates DNA methylation may be an early event in tumor development [2; 3; 4], since aberrantly methylated DNA molecules can be found in secretions and body fluids of individual's years in advance to the clinical diagnosis of cancer [12; 13; 14; 15]. In addition, pharmacological modulation of aberrant DNA methylation patterns using hypomethylating agents, recently proven to be an effective therapy for hematological malignancy, has now been expanded in solid tumors, such as non small cell lung cancer [22; 23; 24]. However, many issues surrounding the strategy remain to be fully addressed. Among these, identification and quantification of effective DNA methylation biomarkers for various tumors, establishment of pharmacodynamic endpoints for *in-vitro* and *in-vivo* characterization of hypomethylating activities of hypomethylating agents are two most important areas. Although many methods for characterization of DNA methylation, such as determination of global DNA methylation level [20], regional DNA methylation level [7; 8; 9; 10; 11; 12], including bisulfite conversion of DNA, direct sequencing (bisulfite sequencing), COBRA [8], methylation-specific PCR (MSP) [7; 12], and more recently MALDI-TOF (MassArray) [11] for assessment of gene specific DNA methylation have been developed. Most have limitations, especially for quantitative analysis as it pertains to sensitivity, specificity, and practical applicability.

Although it is used as the gold standard for methylation analysis, bisulfite sequencing has many limitations, e.g. labor intensive, time consuming, relatively expensive, and not high-throughput. Recognition of these limitations has led to the development of highly efficient semi-quantitative MSP, and now evolvement into the Taqman-based Quantitative (Q)-MSP and Quantitative-multiplexed QM-MSP [12] for specific CpG loci methylation analysis. However, the stringent requirement of primers and probes designed to avoid false positive, condition optimization, and rigidity in detection of fully complementary sequence require

different protocols for different genes. Although integration of pyrosequencing method into DNA methylation permitted a more accurate locus-specific quantitative method of DNA methylation, based on actual sequences rather than fluorescence data from PCR-based amplification [10], its reliability is limited to less than 75 bp sequence length or less [10], and the method requires dedicated and expensive equipments. The most promising method for DNA methylation is combination of base-specific cleavage and MALDI-TOF MS analysis (MassArray), which is high throughput. However, this method needs four sets of cleavage for full characterization of all CpG sites methylation, and seems incapable of characterization of methylation of region generated oligonucleotide with MW over 10000 Da and a length over 400 bases [11].

All these techniques but MassArray can only analyze a restricted set of CpG sites in their target regions, and one must use extrapolation to estimate the degree of methylation in the whole region. Misinterpretation of the methylation status has been reported [19], and is caused, for example, by incomplete bisulfite conversion, improper primer design in methods based on selective amplification or partial digestion, when restriction-based methods are applied. A further complication arises for those methods restricted to selected CpGs, when their methylation within the examined genomic region is not representative [19].

Recently, a robust, specific LC-MS/MS method [20] for determination of GDM level as the ratio of 5mdC to 2dG was developed and used for high throughput characterization of the pharmacodynamics of decitabine in a clinical setting. Here, we combined the LC-MS/MS method with bisulfite-treated PCR to successfully quantify the promoter DNA methylation levels of two TSGs RASSF1A and HIN-1 and their changes induced by decitabine in MCF-7 cells. The frequent observation of RASSF1A and HIN-1 hypermethylation [26; 27] in various cancers, including breast cancer and other biological matrices, therefore provides an ideal model for testing our method effectiveness and examining the modulation effect on methylation of hypomethylating agents *in-vivo*.

This method is a practical and comprehensive approach for regional DNA methylation analysis (major for TSG's promoters). It overcomes those aforementioned limitations. This method provides information on the methylation levels of the examined region is based on the ratio of 5mdC/2dC as absolute quantitative numbers, regardless of individual ratio of methylation and unmethylation of specific CpG site(s), primer/probe annealing for specificity and partial methylation of CpG sites. In addition, it is a sensitive, accurate, and reliable technique. Importantly, our method allows for a rapid, accurate and cost-effective determination of absolute DNA methylation that enables the comparison of these values across large sample sets. As demonstrated, the data generated by this method is highly reproducible, experimental values can be converted to actual methylation values in a single step without the need of the use of DNA methylation standard. The tested dynamic range ensures that virtually any PCR product from 20 ng to 1 µg [20] can be digested without having to adjust the DNA concentration of any sample as demonstrated in the published GDM method [20]. It should also be noted that the bisulfite DNA conversions performed in this study used 1 µg of genomic DNA as substrate. Recently, bisulfite conversion of DNA has been successfully carried out using much smaller amounts of starting material [28]. The approach can also be adapted to various needs in DNA methylation analysis, including quantitative analysis of the relative methylation of any target region, e.g. LINE-1 for methylation biomarker identification in various biofluids or tissue and characterization of hypomethylation activity of hypomethylating agents. A conventional high-throughput tandem mass spectrometer is capable of processing 200 samples per day. The implementation of this technique will provide an excellent tool for cost-effective high throughput methylation analysis and, therefore, will help our understanding of epigenetic modifications and facilitate the clinical scheme optimization by study the correlation of

pharmacokinetics and pharmacodynamics of hypomethylating agents, e.g. decitabine. Most importantly, LC-MS/MS systems now are widely accepted as routine tools in most pharmaceutical and academic laboratories.

In conclusion, to our knowledge this is the first robust, sensitive, specific LC-MS/MS method for determination of TSG's promoter CpG island DNA methylation level of any length. It is easily adaptable for high throughput analysis of large set of DNA methylation samples for potential DNA methylation biomarker identification and clinical pharmacodynamic characterization in clinical trials and for discovery of novel hypomethylating agents.

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Glossary

TSGs	tumor suppressor genes
5mdC	5-methyl-2'-deoxycytidine
2dC	2'-deoxycytidine

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Figure 1. The scheme of the LC-MS/MS method for regional DNA methylation (RGM) level determination

Bisulfite treatment of genomic DNA converts cytosine (C in blue) into uracil (U in blue) and retains 5-methylcytosine (^mC in red) unchanged, which was PCR amplified with specific primers to yield PCR amplicons with U (in blue) converted to T in blue and ^mC (in red) converted to C (in red). The PCR amplicons are methylated with M. SssI to recover the original methylated CpG moiety in the genomic DNA followed by digestion to nucleosides. The concentration of 2dC and 5mdC in the hydrolysates is determined using precursor/product ion pairs of *m/z* 228.2/112.0, and 242.0/126.0, respectively, and the molar ratio of 5mdC/2dC will be used to express the DNA methylation level of this amplified regional DNA.

Figure 2. The mass spectra and tandem mass spectra of 2dC and 5mdC

The 1 min average mass spectrum (A) of the mixture 2dC and 5mdC (10 $\mu\text{g/ml}$, each) showed two peaks at m/z 228.10 and 242.12, corresponding to the protonated molecules of 2dC and 5mdC, respectively, and the collision assisted dissociation (CAD) spectra of the protonated molecules of 2dC (B) of m/z 228.10 and 5mdC (C) of m/z 241.12 showing a fragment ion at m/z 112.16 and 126.21, corresponding to protonated cytosine and 5-methylcytosine, respectively. The chemical structures of 2dC and 5mdC and their postulated fragment pathways are inset in B and C.

Figure 3. The extracted ion chromatogram (XIC) for 5mdC at m/z 242.10/126.21 (A), and 2dC at 228.10/112.16 (B) in Mobile Phase A spiked with 1 ng/ml 5mdC, 1000 ng/ml 2dC, respectively.

Figure 4. The COBRA analysis of DNA methylation levels for RASSF1A (left) and HIN-1 (Right) in different methylation ratios

Fragment sizes are indicated to the right of the gel and percentage ratio of fully-methylated amplicon and non-methylated amplicons of RASSF1A (269 bp, 167 to 435) and HIN-1 (182 bp, 611 to 792) for each lane are indicated at the top. The mixtures were digested with a methylation sensitive restriction enzyme, BstUI. The resulting mixtures were applied to gel electrophoresis in an 8% polyacrylamide gel and visualized by ethidium bromide staining. Lanes **2** and **9** for RASSF1A amplicons from non-methylated genomic DNA, lanes **10** and **17** for HIN-1 amplicons from full-methylated genomic DNA, intact PCR products at the top corresponding to the non-methylated promoter were detected in Lanes **2** to **9** and the lack of full-length PCR fragments, in other words, complete digestion of the PCR products in the 100% methylated samples were detected in Lane **10** to **17**. As the methylation percent of the sample increases, there is a decrease in the top band.

A: Sequence of one strand template for PCR amplification of RASSF1A (167-435)

CCACCCAGTGGGTAGGCCAAGTGTGTTGCTTCAGCAAAC**CG**GACCAGGAGGGCC
 AGGGC**CG**GATGTGGGGACCCTCTTCCTCTAGCACAGTAAAGCTGGCCTCCAGAA
 ACA**CG**GGTATCTC**CGCG**TGGTGCTTTG**CGGT****CGCG**T**CG**TTGTGGC**CGTC****CGGG**
 GTGGGTGTGAGGAGGGGA**CGA**AGGAGGGAAGGAAGGGCAAGG**CGGGGGGG**
 CTCTG**CG**AGAG**CGCG**CCCAGCCC**CG**CCTT**CGGG**CCCCACAGTCCCTGCACCCAG
GCG

CG%=182/269=67.66% Gs 104, Cs 78 CGs:18, 36/182=19.78% (100% methylation)

Conversion and PCR 5mdC/2dC: 36/104=34.6%

B: Sequence of one strand template for PCR amplification of HIN-1(611-792)

TGGGGCCCTCTGAGAGCAGGCAGGCC**CG**GCCTTTGTCTC**CGCG**AGGCCCA
 CCC**CG**GCC**CG**CACCTT**CG**CTTTG**CGGT**CTGACCCCA**CGCG**CCCCCCTGCA
 GGGCTGGGCC**CG**GGTGAGGGGAGCTTCCCT**CGCG**CCAGGGCAGGGG**CGGG**
 GG**CGGCG**CAGTTCCTGGCTCCCTGGTCCCTGCCTCTGA

CG%=139/182=76.39% Gs 73, Cs 69 CGs:15, 30/139=21.6% (100% methylation)

Conversion and PCR 5mdC/2dC: 30/73=41.67%

Figure 5. Sequence of a one strand template for PCR amplification of RASSF1A (Upper panel) and HIN-1 (bottom panel) and the theoretic methylation level as indication of 5mdC/2dC of their fully-methylated counterparts.

Figure 6. Modulation on global and promoter DNA methylation of two tumor suppressor genes and their re-expression in MCF-7 cells by decitabine

Global DNA methylation was found to be decreased in MCF-7 cells with 0.1, 0.75, and 2.0 μ M of decitabine for 72 h compared to the untreated control (A); the promoter methylation levels of RASSF1A (B) and of HIN-1 (D) were found to decrease and the expression of RASSF1A (C) and HIN-1 (E) genes were found to increase, when MCF-7 cells were exposed to indicate concentrations of decitabine (0.1 and 0.75 μ M) for 72 h. DNA (1 μ g) from decitabine treated or untreated MCF-7 cells were treated with bisulfite followed by PCR amplification and M. SssI methylation. The methylated amplicons or genomic DNA were digested to nucleosides. 5mdC, 2dC and 2dG in the hydrolysates were measured using the LC-MS/MS method. The ratio of 5mdC to 2dC was used as a DNA methylation indicator for promoter methylation, whereas the ratio of 5mdC to 2dG was used for global DNA methylation; RASSF1A and HIN-1 gene expressions were measured by Real-Time RT-PCR. * indicates that alteration is statistically significant ($p < 0.05$).

Table I

Within-day and Between-day Validation Parameters

% 5-mdC/2dC	Within-day*		Between-day*	
	C.V.(%)	Accuracy (%)	C.V. (%)	Accuracy (%)
0.2%	10.2	87.9	17.6	95.4
2%	2.68	101.3	0.91	100.3
10%	6.11	102.6	1.21	100.3
20%	6.84	101.7	4.21	102.3
Gene	HIN-1		RASSF1A	
% M [#]	C.V.(%)	Accuracy (%)	C.V. (%)	Accuracy (%)
5%	16.5	114.9	7.13	120.9
30%	16.5	87.1	5.21	98.0
70%	9.96	105.7	3.40	98.9
90%	3.54	98.5	3.00	103.4

* All n=6

[#] Methylation