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Formation and Determination of Endogenous Methylated Nucleotides in Mammals by Chemical Labeling coupled with Mass Spectrometry Analysis

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ABSTRACT: 5-Methylcytosine (5-mC) is an important epigenetic mark that plays critical roles in a variety of cellular processes. To properly exert physiological functions, the distribution of 5-mC needed to be tightly controlled in both DNA and RNA. In addition to methyltransferase-mediated DNA and RNA methylation, pre-methylated nucleotides can be potentially incorporated into DNA and RNA during replication and transcription. To exclude the pre-modified nucleotides into DNA and RNA, endogenous 5-methyl-2'-deoxycytidine monophosphate (5-Me-dCMP) generated from nucleic acids metabolism can be enzymatically deaminated to thymidine monophosphate (TMP). Therefore, previous studies failed to detect 5-Me-dCMP or 5-methylcytidine monophosphate (5-Me-CMP) in cells. In the current study, we established a method by chemical labeling coupled with liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS/MS) for sensitive and simultaneous determination of 10 nucleotides, including 5-Me-dCMP and 5-Me-CMP. As *N*,*N*-dimethyl-p-phenylenediamine (DMPA) was utilized for labeling, the detection sensitivities of nucleotides increased by 88-372 folds due to the introduction of a tertiary amino group and a hydrophobic moiety from DMPA. Using this method, we found that endogenous 5-Me-dCMP and 5-Me-CMP widely existed in cultured human cells, human tissues, and human urinary samples. The presence of endogenous 5-Me-dCMP and 5-Me-CMP indicates that deaminases may not fully deaminate these methylated nucleotides. Consequently, the remaining pre-methylated nucleosides could be converted to nucleosides triphosphate as building blocks for DNA and RNA synthesis. Furthermore, we found that the contents of 5-Me-dCMP and 5-Me-CMP exhibited significant decreases in renal carcinoma tissues and urine samples of lymphoma patients compared to their controls, probably due to the more reutilization of methylated nucleotides in DNA and RNA synthesis. This study is, to the best of our knowledge, the first report for detecting endogenous 5-Me-dCMP and 5-Me-CMP in mammals. The detectable endogenous methylated nucleotides indicates the potential deleterious effects of pre-modified nucleotides on aberrant gene regulation in cancers.

INTRODUCTION

DNA cytosine methylation (5-methylcytosine, 5-mC) is the best characterized epigenetic mark that plays critical roles in a variety of cellular processes, including regulation of gene expression, genomic imprinting and X-chromosome inactivation.[1] Aberrant DNA methylation is associated with many human diseases.[2] In addition to DNA methylation, reversible RNA modification recently has been proposed to represent another realm for biological regulation.[3,4] 5-mC has long been known present in RNA and recent studies demonstrated that 5-mC is widespread in both coding and noncoding RNA of mammals, implicating 5-mC in RNA could be critical for regulating gene transcription and protein translation.[5] For example, 5-mC modification in rRNA is prevalent and play critical roles in translational fidelity and tRNA recognition.[6] In tRNA, 5-mC has been shown to stabilize tRNA secondary structure.[7]

Due to the important regulatory roles of DNA and RNA methylation, it is vital to maintain proper DNA and RNA methylation status for the normal functions of cells.[8,9] Abnormal DNA and RNA methylation can cause many human diseases, such as diabetes,[10,11] neurological disorders [12] and cancers.[13-15] In mammals, DNA methylation is mediated by DNA methyltransferase (DNMT) family of enzymes (DNMT1, DNMT3A and DNMT3B) that catalyze the transfer of a methyl group from S-adenosyl-L-methionine to DNA.[16] On the other hand, 5-mC can be generated in RNA of mammals by enzymes including DNMT2, NSun2 and NSun4.[17]

In addition to enzymatically methylated DNA and RNA, methylated nucleotides can be incorporated into DNA and RNA during replication and transcription.[18] Previous study reported that, in Chinese hamster ovary (CHO) cells treated with 5-methyl-2'-deoxycytidine triphosphate

(5-Me-dCTP), certain genes can be silenced by the incorporation of 5-Me-dCTP into DNA.[19] DNA and RNA metabolism can lead to formation of endogenous nucleoside monophosphates, including the modified cytidines of 5-methyl-2'-deoxycytidine monophosphate (5-Me-dCMP) and 5-methylcytidine monophosphate (5-Me-CMP). In this respect, DNA and RNA methylation could be potentially generated by converting 5-Me-dCMP and 5-Me-CMP to 5-Me-dCTP and 5-methylcytidine triphosphate (5-Me-CTP) respectively and the subsequent incorporation into DNA and RNA.

To properly exert the biological functions, the distribution of 5-mC in DNA and RNA needs to be tightly controlled.[20] Thus, it is essential that these pre-modified nucleotides are not re-incorporated into DNA and RNA. Otherwise, their random positions would alter the normal distribution of 5-mC in DNA and RNA, eventually leading to the dysregulation of gene expression. To prevent them from being introduced into DNA and RNA, it was believed that endogenous 5-Me-dCMP can be enzymatically deaminated to thymidine monophosphate (TMP).[21] Supported by this explanation, previous studies have reported none of these endogenous 5-Me-dCMP and 5-Me-CMP were detected in cells.[22]

In the current study, we established a chemical labeling method coupled with liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS/MS) for sensitive and simultaneous determination of 10 nucleotides, including 5-Me-dCMP and 5-Me-CMP. With the method, we found that 5-Me-dCMP and 5-Me-CMP widely existed in cultured human cells, human tissues, and human urinary samples. The detectable levels of 5-Me-dCMP and 5-Me-CMP indicated that deaminases may not fully deaminate these methylated nucleotides. These pre-methylated nucleotides could therefore be potentially converted to nucleosides triphosphate and became building blocks for DNA and RNA synthesis. The utilization of pre-methylated nucleotides in DNA and RNA synthesis would have deleterious effects on gene regulation.

EXPERIMENTAL SECTION

Chemicals and reagents

Adenosine 5'-monophosphate (AMP), uridine 5'-monophosphate (UMP), cytidine 5'-monophosphate (CMP), guanosine 5'-monophosphate (GMP), 2'-deoxyadenosine 5'-monophosphate (dAMP), thymidine 5'-monophosphate (TMP), 2'-deoxycytidine 5'-monophosphate (dCMP) and 2'-deoxyguanosine 5'-monophosphate (dGMP) were purchased from Sigma-Aldrich (Beijing, China). *N*,*N*-Dimethyl-p-phenylenediamine (DMPA), imidazole and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Aladdin Reagent Co. (Shanghai, China). 5-Methyl-2'-deoxycytidine 5'-monophosphate (5-Me-dCMP) was purchased from Carbosynth (San Diego, USA). 5-Methylcytidine 5'-monophosphate (5-Me-CMP) was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The structures of 5-Me-dCMP and 5-Me-CMP were shown in Figure 1A. The structures of other nucleotides were shown in Figure S1 in Supporting Information.

Chromatographic grade methanol and acetonitrile (ACN) were purchased from Tedia Co. Inc. (Fairfield, OH, USA). The water used throughout the study was purified by a Milli-Q apparatus (Millipore, Bedford, MA). Stock solutions of dAMP, TMP, dCMP, dGMP, AMP, UMP, CMP, GMP, 5-Me-dCMP, and 5-Me-CMP were prepared in water at a concentration of 10 mM. DMPA was prepared in ACN at a concentration of 140 mM. Imidazole was prepared in water at a concentration of 10 mM (pH 6).

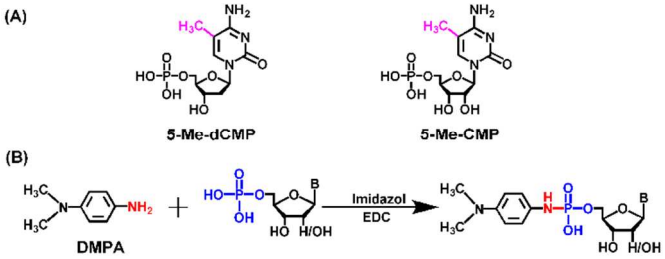

Figure 1. (A) The chemical structures of 5-Me-dCMP and 5-Me-CMP. (B) Chemical labeling of nucleotides by DMPA. "B" in nucleotides represents nucleobase.

Biological and clinical samples

The first morning urine samples from lymphoma patients and healthy controls were collected from Hubei Cancer Hospital, China. Each group contains 10 urine samples from 5 males and 5 females. A total of 18 tissue samples from renal carcinoma patients, including 9 pairs of renal carcinoma tissues and matched tumor adjacent normal tissues, were collected from Hubei Cancer Hospital. Detailed information can be found in Table S1 in Supporting Information. All the patients were diagnosed with cancer for the first time and had not been given any treatment at the time point of urine sample collection. Healthy controls were selected based on medical history and physical examination. The healthy controls and cancer patients were not detected with other diseases. An approval was granted by the Hubei Cancer Hospital Ethics Committee and met the declaration of Helsinki. All the experiments were performed in accordance with Hubei Cancer Hospital Ethics Committee's guidelines and regulations.

Human 293T and HeLa cells were obtained from the China Center for Type Culture Collection and maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Sample pretreatment

The pretreatment of the urine samples were performed according to previously described method.[23,24] Briefly, the first morning urine samples were collected and then centrifuged immediately at 5,000 × g for 10 min under 4°C twice. The resulting supernatant was filtered by a PRECLEANTM Syringe Filter Nylon membrane (13 mm × 0.22 μM, ANPEL Scientific Instrument Co., Shanghai, China). At last, the supernatant was collected and stored at -80°C.

The tissue samples were extracted by homogenization in pre-chilled 80% aqueous methanol (0°C, 1 mL). After centrifugation at 14,000 × g for 15 min to remove precipitated protein, supernatants were collected and dried under nitrogen gas and stored at -80°C.[25]

The human 293T and HeLa cells, at a density of approximate 7.5×10^5 cells/mL (10 mL), were collected by centrifugation at 2,000 × g under 4°C for 5 min and then washed twice with ice-cold phosphate-buffered saline (PBS) to remove the fetal bovine serum. Cells were rapidly quenched with pre-chilled 80% aqueous methanol (0°C, 2 mL) and incubated at -20°C for 30 min according to previous study.[25] Then the cell extracts were centrifuged at 14,000 × g for 15 min at 4°C to remove precipitated protein. Supernatants were collected and then dried under nitrogen gas and stored at -80°C.

Enrichment of nucleotides

The nucleotides were isolated from urines, tissues, and cultured cells by solid phase extraction (SPE) using Cleanert NH_2 cartridge (200 mg/mL, Weltech Co., Ltd., Wuhan, China). To achieve good recovery, several extraction conditions were optimized, including the percentage of ACN in loading solution, the percentage of ACN in desorption solution and desorption volume.

Chemical Labeling

DMPA was used to label nucleotides (Figure 1B). To achieve the best labeling efficiency, we optimized the labeling conditions, including reaction temperature and time, the concentration of imidazole, the molar ratio of DMPA and EDC versus nucleotides. All the reactions were performed in 100 μL imidazole solution (1 mM, pH 6.0).

After DMPA labeling, 300 μL of water and 300 μL of a dichloromethane-hexane (2:1, v/v) solvent (4°C) were added to the 100 μL of reaction solution followed by vortexing and centrifugation at 13,000 × g for 5 min to remove excessive DMPA. Then 400 μL of the upper aqueous phase was collected and mixed with 600 μL of water. Strata X SPE cartridge (30 mg/mL, Phenomenex, Guangzhou, China) was used to remove excessive EDC (Figure 2).

Analysis of DMPA-labeled nucleotides by LC-ESI-MS/MS

Analysis of the DMPA-labeled nucleotides was performed on the LC-ESI-MS/MS system consisting of an AB 3200 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) with an electrospray ionization (ESI) source (Turbo Ionspray) and a Shimadzu LC-20AD HPLC (Tokyo, Japan). Data acquisition and processing were performed using AB SCIEX Analyst 1.5 Software (Applied Biosystems, Foster City, CA, USA). The HPLC separation was performed on an Inertsil ODS-3 column (250 mm × 2.0 mm i.d., 5 μm, Tokyo, Japan) at 35□. Water containing 2 mM NH_4HCO_3 (solvent A) and ACN (solvent B) was employed as the mobile phase. A gradient of 0 - 15 min from 5% to 30% B, 15 - 20 min 30% B, 20 - 22 min from 30% to 70% B, and 22 - 30 min 70% B was used. The flow rate of the mobile phase was set at 0.2 mL/min. The DMPA-labeled nucleotides were monitored under multiple reaction monitoring (MRM) positive ion mode. The optimal MRM parameters were listed in Table S2 in Supporting Information.

Statistical analysis

Statistical data were processed with Origin 8.0 software (Electronic Arts Inc). Independent *t*-test was performed to evaluate the differences of nucleotides concentrations in urine samples obtained from lymphoma patients and healthy controls. Paired *t*-test was performed to evaluate the concentration differences of nucleotides between renal carcinoma tissues and tumor adjacent normal tissues. All *p* values were two-sided, and generally, *p* values < 0.05 were considered statistically significant.

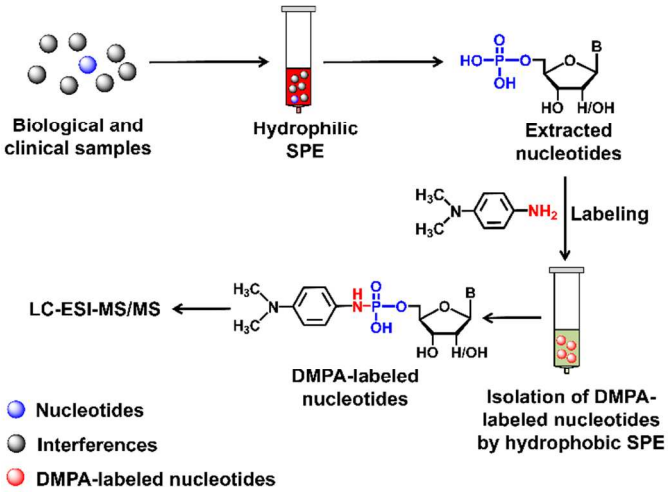

Figure 2. The schematic illustration of the developed method for the determination of nucleotides by chemical labeling coupled with LC-ESI-MS/MS analysis. "B" in nucleotides represents nucleobase.

RESULTS AND DISCUSSION

Strategy for determination of methylated nucleotides by chemical labeling coupled with LC-ESI-MS/MS analysis

DNA and RNA metabolism could lead to the formation of endogenous 5-Me-dCMP and 5-Me-CMP, which are likely to be converted into 5-Me-dCTP and 5-Me-CTP and then incorporated into DNA and RNA (Figure S2 in Supporting Information). Although 5-Me-dCMP can be enzymatically deaminated to TMP, so far no enzyme has

been reported to be capable of converting 5-Me-CMP, which could be potentially phosphorylated to 5-methylcytidine diphosphate (5-Me-CDP) and 5-Me-CTP. Therefore, we suspected that endogenous methylated nucleotides may exist. However, identification and quantification of endogenous methylated nucleotides in mammalian cells has not been realized, possibly due to their low in-vivo abundance as well as the poor ionization efficiencies in detecting nucleotides using mass spectrometry.

Introduction of an easily ionizable group to targeted analytes could enhance the ionization efficiency in mass spectrometry analysis.[26] Our group recently established chemical labeling methods for sensitive detection of endogenous low-abundant compounds by LC-MS.[27-30] Along this line, here we used DMPA that harbors a hydrophobic phenyl group and an easily chargeable tertiary amine group to simultaneously label the phosphate group in 10 nucleotides including 5-Me-dCMP and 5-Me-CMP (Figure 1 and Figure S1 in Supporting Information). As a consequence, the detection sensitivities of these DMPA-labeled products increased on LC-ESI-MS/MS owing to their conjugation to the pre-charged tertiary amine group on DMPA. In addition, the introduced hydrophobic phenyl group in DMPA elongated the retention of these nucleotides on reversed-phase LC, which further improved the detection sensitivities. Using the developed method, we were able to distinctly identify and quantify endogenous 5-Me-dCMP and 5-Me-CMP in mammalian cells.

Extraction of nucleotides from biological and clinical samples

Due to the complex matrix of biological and clinical samples, it is essential to extract nucleotides and remove the matrix interferences. Here we used hydrophilic Cleanert NH_2 SPE cartridge to extract nucleotides from pretreated samples. The extraction conditions were optimized to obtain good extraction efficiencies. The optimized conditions were as follow: loading solution and washing solution, 3.0 mL of 80% ACN (ACN/0.25% NH_4OH, 80/20, v/v); desorption solution, 3.0 mL of 10% ACN in water. More details could be found in the text and Figure S3 in Supporting Information.

Under the aforementioned conditions, we evaluated the recoveries of 10 nucleotides in water and in urine samples spiked with nucleotides. The results showed that the recoveries of 10 nucleotides ranged from 90.3% to 101.2% and 88.3% to 103.3% in water and urine sample, respectively, suggesting good extraction recoveries towards nucleotides (Table S3 in Supporting Information).

Identification of DMPA-labeled nucleotides

We examined the DMPA-labeled nucleotides by LC-ESI-MS/MS. The labeled products can be produced through the reaction of the amine group in DMPA with the phosphate group in nucleotides. The product ion spectra demonstrated that the fragment ions generated by the derivatives were identical to their corresponding theoretical values (Figure 3), suggesting that the desired DMPA-labeled dAMP, TMP, dCMP, dGMP, AMP, UMP, CMP, GMP, 5-Me-dCMP, and 5-Me-CMP were successfully formed.

DMPA labeling

To obtain good labeling efficiencies by DMPA, we optimized the labeling conditions, including reaction temperature and time, concentration of imidazole buffer, molar ratio of DMPA and EDC versus nucleotide. dCMP, UMP and CMP were used as the analytes for optimization.

The reaction temperature was first optimized ranging from 4°C to 70°C. The reaction was incubated for 1.5 h with shaking at 1,500 rpm. Our results demonstrated that the peak area of nucleotide derivatives peaked at 50°C (Figure 4A). Therefore, 50°C was used for the following experiments. Next, the reaction time was optimized ranging from 10 to 120 min, and the reaction was performed at 50°C with shaking at 1,500 rpm. The peak areas of nucleotide derivatives increased with increasing time from 10 to 90 min and slightly decreased over 90 min (Figure 4B). Thus, 90 min was chosen for the labeling reaction.

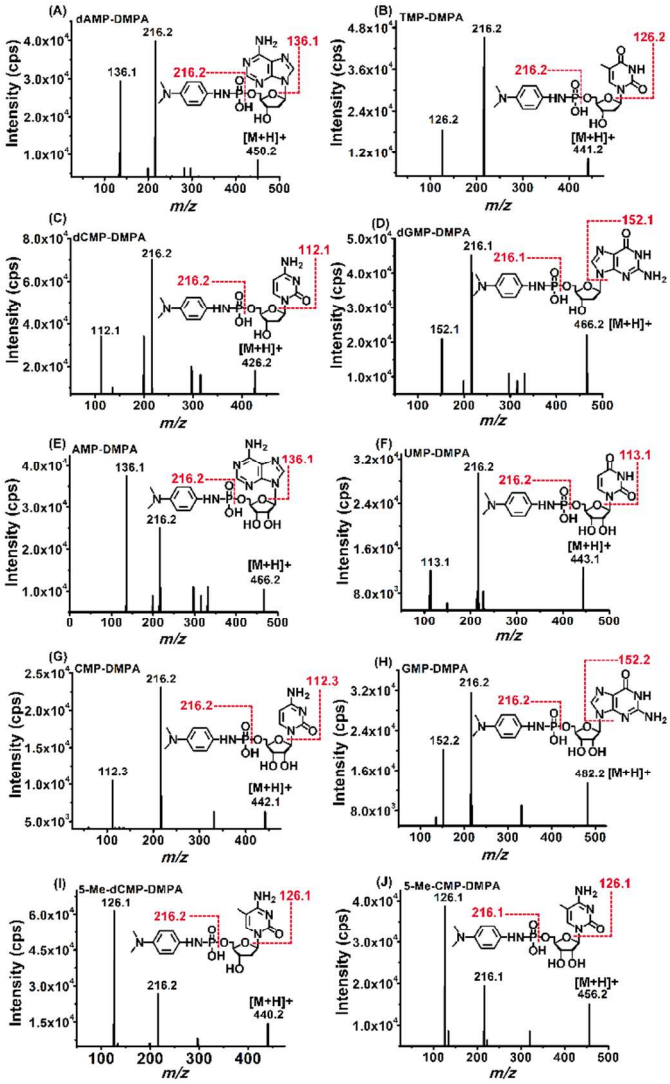

Figure 3. Product ions spectra of DMPA-labeled dAMP (A), TMP (B), dCMP (C), dGMP (D), AMP (E), UMP (F), CMP (G), GMP (H), 5-Me-dCMP (I), 5-Me-CMP (J).

In the labeling reaction, imidazole was used as buffer to provide a weak acidic environment (pH 6.0). To assess the influence of imidazole buffer on labeling, we investigated imidazole buffer concentrations ranging from 0 to 10 mM (pH 6.0). The results showed that the largest peak areas of nucleotide derivatives were achieved at 1 mM of imidazole (pH 6.0) (Figure 4C). Therefore, 1 mM imidazole (pH 6.0) in the reaction solution was used for the following experiments.

In addition, the optimal concentration of DMPA for the labeling of nucleotides was investigated. The results showed that the peak areas of nucleotide derivatives reached plateau when the molar ratio of DMPA/nucleotides was greater than 40,000 (Figure 4D). Therefore, the molar ratio of 40,000 of DMPA/nucleotides was used for the labeling reaction. Finally, the optimal concentration of activator EDC was investigated. The results showed that the largest peak areas of nucleotide derivatives were achieved when the molar ratio of EDC/nucleotides was greater than 5,000 (Figure 4E). Therefore, the molar ratio of 5,000 of EDC/nucleotides was used for the labeling reaction.

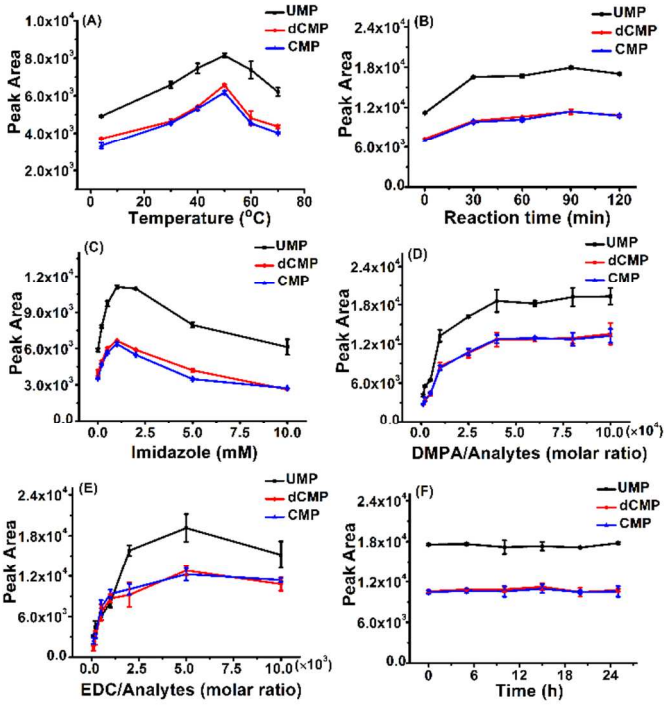

Figure 4. Optimization of DMPA labeling conditions. (A) Optimization of reaction temperature. (B) Optimization of reaction time. (C) Optimization of the concentration of imidazole buffer. (D) Optimization of the molar ratio of DMPA/nucleotides. (E) Optimization of the molar ratio of EDC/nucleotides. (F) Evaluation of the stabilities of the DMPA-labeled nucleotides.

Taken together, DMPA labeling were conducted at 50°C for 1.5 h in 1 mM imidazole buffer (pH 6.0), and the molar ratios of DMPA and EDC over nucleotides were set as 40,000 and 5,000, respectively. Under these optimized reaction conditions, the labeling efficiencies of the 10 nucleotides by DMPA ranged from 84.4% to 100.3% (Table S4 in Supporting Information). We also evaluated the stability of DMPA-labeled nucleotides. The results showed that DMPA-labeled nucleotides were stable at room temperature for at least 24 h (Figure 4F), which is sufficient for the subsequent processing and analysis. The reaction solution was then further proceeded to remove excessive DMPA and EDC and the general recoveries of nucleotide derivatives ranging from 90.3% to 103.0% were achieved (Table S5 in Supporting Information).

Improvements of detection performance upon DMPA labeling

The main purpose for chemical labeling is to improve the detection sensitivities of nucleotides during LC-ESI-MS/MS analysis. Here we investigated the detection sensitivities of 10 nucleotides upon DMPA labeling under their own optimized mass spectrometry conditions. The extracted-ion chromatograms showed that the retention of 10 native nucleotides was relatively weak on the reversed-phase chromatographic column even under optimized separation conditions (Figure 5A). However, after DMPA labeling, their retention dramatically increased (Figure 5B), owing to the increased hydrophobicity of these labeled products endowed by the hydrophobic phenyl group of DMPA.

The limit of detection (LOD) defined as the amount of analyte at a signal-to-noise ratio (S/N) of 3 was employed to evaluate the improved detection sensitivities of nucleotides by DMPA labeling. The LODs of 10 nucleotides with and without DMPA labeling were shown in Table 1. We found the detection sensitivities of these nucleotides labeled by DMPA increased by 88 to 372 folds. We reason that the tertiary amino group in DMPA can increase ionization efficiency during mass spectrometry analysis. In addition, increased hydrophobicity of these DMPA-labeled products results in longer retention time on the LC column and thus delayed in elution with a higher ratio of organic solvent. In this way, the analytes could be ionized more effectively during electrospray ionization because of higher spraying and desolvation efficiency in higher ACN content. In comparison with previously reported methods, the detection limits for nucleotides acquired by our method were reduced by at least 20 folds (Table S6 in Supporting Information). Collectively, compared to the native forms of 10 nucleotides, DMPA labeling could dramatically increase the detection sensitivities.

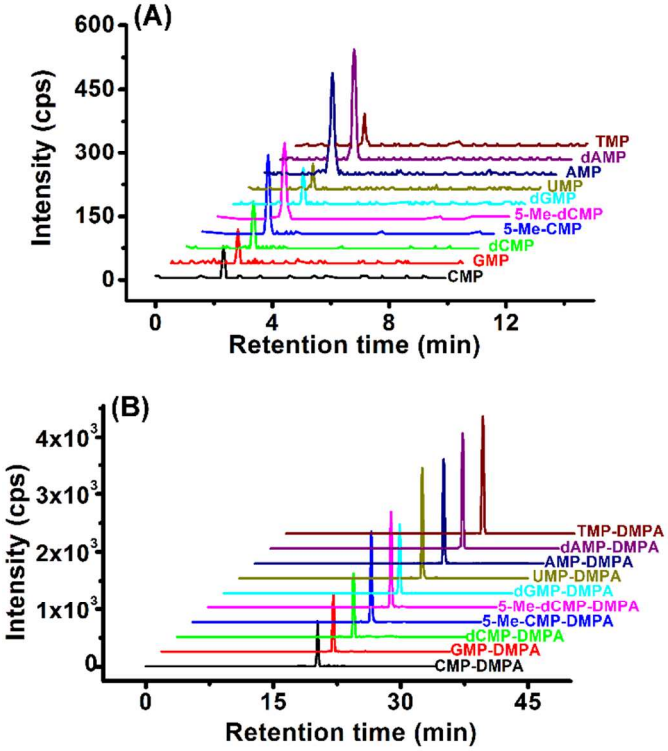

Figure 5. Extracted-ion chromatograms of 10 nucleotides before (A) and after (B) DMPA labeling.

Method validation

To evaluate the linearity of the method, 10 nucleotide standards at concentrations of 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 800,1000 pg/μl and fixed amount of deuterated DMPA (DMPA-d_4)-labeled nucleotides as internal standards (IS) were used to construct calibration curves by plotting peak area ratio (analyte/IS) against the concentrations of DMPA-labeled nucleotides with triplicate measurements. The results showed that good linearities within the 3 orders of magnitude in dynamic range of nucleotides were obtained with the coefficients of determination (R^2) being greater than 0.9939 (Table S7 in Supporting Information).

The accuracy and precision of our method were assessed by the relative errors as well as intra- and inter-day relative standard deviations (RSDs). Both relative errors and intra- and inter-day RSDs were calculated by spiking nucleotide standards in urine samples at three different concentrations. For each concentration, triplicate measurements were performed. Intra-day variation was evaluated by repeating the process for three times within one day, and the inter-day variation was investigated on three successive days. The accuracy and reproducibility of the developed method were evaluated with the REs and RSDs being less than 15.0% and 15.3%, respectively, demonstrating good accuracy and reproducibility (Table S8 in Supporting Information).

Table 1. Limits of detection of 10 nucleotides with and without DMPA labeling followed by LC-ESI-MS/MS analysis.

Nucleotides	LODs (fmol)		Detection sensitivities improved folds
	Unlabeled	DMPA labeling	
dAMP	25.7	0.13	198
TMP	48.4	0.13	372
dCMP	44.2	0.42	105
dGMP	33.6	0.38	88
AMP	28.7	0.12	239
UMP	96.0	0.26	369
CMP	56.2	0.47	120
GMP	53.0	0.43	123
5-Me-dCMP	32.3	0.30	108
5-Me-CMP	34.6	0.36	96

Determination of the methylated nucleotides in mammals

With the established method, we investigated whether methylated nucleotides (5-Me-dCMP and 5-Me-CMP) existed in human tissues. Shown in Figure 6 are the typical extracted-ion chromatograms of 10 nucleotides in human renal tissue before and after DMPA labeling. The results demonstrated that, while canonical nucleotides (dAMP, TMP, dCMP, dGMP, AMP, UMP, CMP and GMP) can be detected without DMPA labeling, 5-Me-dCMP and 5-Me-CMP were undetectable (Figure 6A; shown in Figure 6C is the zoomed-in chromatograms of Figure 6A). On the contrary, all the 10 nucleotides, including 5-Me-dCMP and 5-Me-CMP can be distinctly detected by our developed strategy of DMPA labeling combined LC-ESI-MS/MS analysis (Figure 6B; shown in Figure 6D is the zoomed-in chromatograms of Figure 6B).

To examine whether 5-Me-dCMP and 5-Me-CMP are widely present in vivo, we further analyzed 5-Me-dCMP and 5-Me-CMP in various biological samples, including cultured human 293T cells, HeLa cells, and human urine samples. As shown in the extracted-ion chromatograms (Figure 7), DMPA-labeled 5-Me-dCMP and 5-Me-CMP in human 293T cells, HeLa cells, and human urine samples had similar retentions as the DMPA-d_4-labeled 5-Me-dCMP and 5-Me-CMP standards, suggesting that the detected compounds from these human samples were expected methylated nucleotides, i.e., 5-Me-dCMP and 5-Me-CMP. It is noted that the retention times of DMPA-d_4-labeled 5-Me-dCMP and 5-Me-CMP standards were slightly shorter than those of the corresponding DMPA-labeled compounds (Figure 7). The phenomenon was frequently observed in plenty of studies, i.e., the deuterated compounds typically eluted earlier than their corresponding protiated compounds in C18 stationary phase.[31,32] Since C–H bond has a higher oscillation frequency than the C–D bond, C–H bond induces greater forces of attraction between itself and the C18 stationary phase. Therefore, pro-

tiated compounds have stronger binding to the C18 stationary phase than deuterated ones.[31,32] Collectively, the study confirmed the wide existence of 5-Me-dCMP and 5-Me-CMP in mammals.

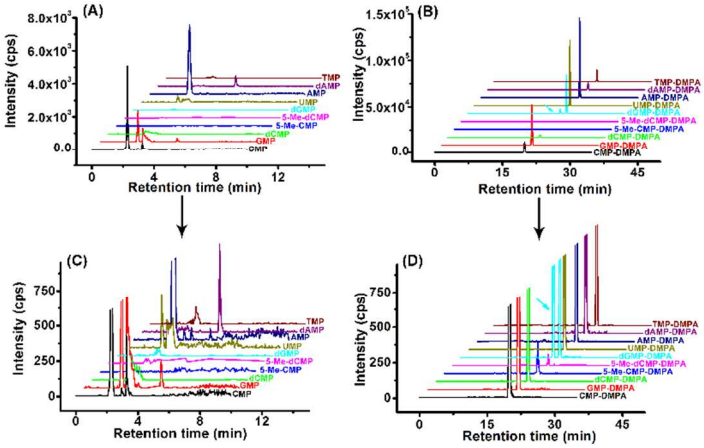

Figure 6. Extracted-ion chromatograms of 10 nucleotides detected in human renal tissue by DMPA labeling coupled with LC-ESI-MS/MS analysis. (A) Analysis without DMPA labeling. (B) Analysis with DMPA labeling. (C) Zoomed-in chromatograms from (A). (D) Zoomed-in chromatograms from (B).

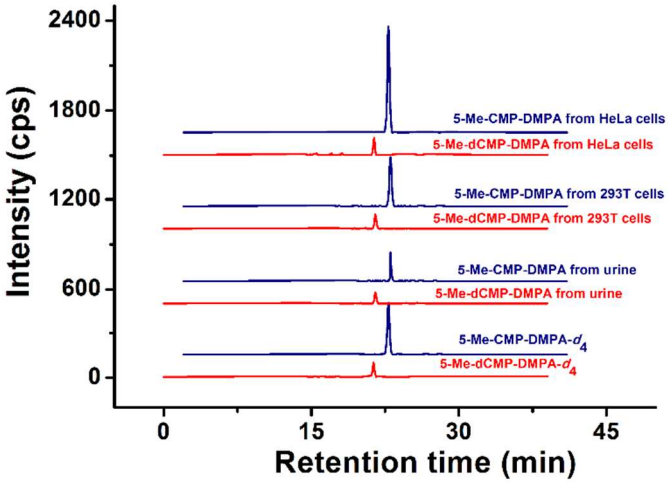

Figure 7. Extracted-ion chromatograms of DMPA-d_4-labeled 5-Me-dCMP and 5-Me-CMP standards and DMPA-labeled 5-Me-dCMP and 5-Me-CMP from the human urine, 293T cells and HeLa cells.

Our quantification data showed that the contents of 5-Me-dCMP and 5-Me-CMP in human renal tissues ranged from 0.002 to 0.010 and 0.004 to 0.013 pmol/mg protein, respectively (Table S9 in Supporting Information); the contents of 5-Me-dCMP and 5-Me-CMP in 293T cells were 0.0025 and 0.010 pmol/mg protein, respectively (Table S10 in Supporting Information); the contents of 5-Me-dCMP and 5-Me-CMP in HeLa cells were 0.0030 and 0.020 pmol/mg protein, respectively (Table S10 in Supporting Information); the contents of 5-Me-dCMP and 5-Me-CMP in human urine samples ranged from 0.01 to 0.08 and 0.03 to 0.29 pmol/mg creatinine, respectively (Table S11 in Supporting Information).

Contents change of nucleotides in tissues and urine samples of cancer patients

Having demonstrated the presence of 5-Me-dCMP and 5-Me-CMP in various human samples, we next asked whether the levels of these methylated nucleotides differ in human carcinoma tissues. In this respect, a total of 18 tissue samples derived from renal carcinoma patients, including 9 paired tumor tissues and tumor adjacent normal tissues, were analyzed by our method. The results showed that the mean contents of 5-Me-dCMP in renal carcinoma tissues and tumor adjacent normal tissues were 0.0039 and 0.0068 pmol/mg protein, respectively; while the mean contents of 5-Me-CMP in renal carcinoma tissues and tumor adjacent normal tissues were 0.0058 and 0.0084 pmol/mg protein, respectively. The results revealed significant decreases in the levels of 5-Me-dCMP and 5-Me-CMP in human renal carcinoma tissues compared to tumor-adjacent normal tissues (p = 0.001 for 5-Me-dCMP; p = 0.001 for 5-Me-CMP, Figure 8A and 8B; Table S8 in Supporting Information).

The mean ratios of 5-Me-dCMP/dCMP and 5-Me-CMP/CMP in human renal tissues were 0.78% and 0.04%, respectively, which were only several folds lower than the typical cytosine methylation in DNA (~ 3%) and RNA (~ 0.1%). Therefore, it is very likely that the endogenous 5-Me-dCMP and 5-Me-CMP could be incorporated into DNA and RNA after converting to triphosphate nucleosides.

We also compared the contents of 5-Me-dCMP and 5-Me-CMP in urine samples from human lymphoma patients and healthy controls. The results showed that the mean contents of 5-Me-dCMP in urine samples of human lymphoma patients and healthy controls were 0.025 pmol/mg creatinine and 0.048 pmol/mg creatinine, respectively; while the mean contents of 5-Me-CMP in urine samples of human lymphoma patients and healthy controls were 0.077 pmol/mg creatinine and 0.176 pmol/mg creatinine, respectively. The results also showed significant decreases in the levels of 5-Me-dCMP and 5-Me-CMP in urine samples from human lymphoma patients compared to those from healthy controls ($p = 2.0 \times 10^{-4}$ for 5-Me-dCMP; $p = 2.2 \times 10^{-4}$ for 5-Me-CMP, Figure 8C and 8D; Table S10 in Supporting Information). We reason that the significant decrease of 5-Me-dCMP and 5-Me-CMP in urine samples of lymphoma patients may be due to the more reutilization of methylated nucleotides in DNA and RNA synthesis in lymphoma patients. On the other hand, global genome hypomethylation is known to be frequently accompanied with tumor development.[33] In this respect, the lower levels of 5-Me-dCMP and 5-Me-CMP in tumor tissues and urine samples of cancer patients suggest that they may arise from the excision repair products of nucleic acids or from the decomposition of nucleic acids of dead cells. However, the mechanisms require further investigation.

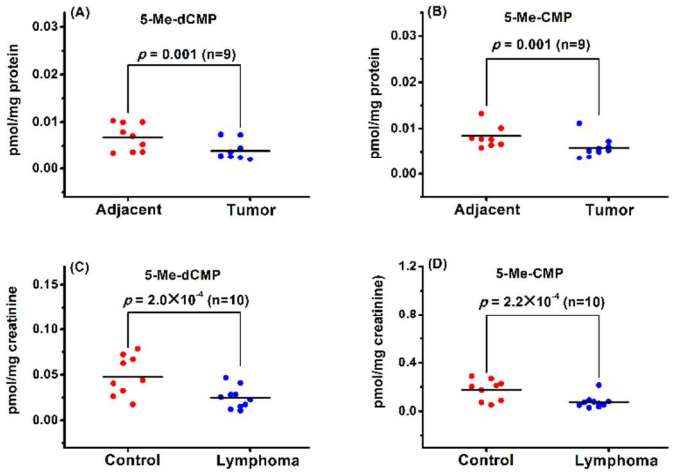

Figure 8. Quantification and statistical analysis of methylated nucleotides in human renal tissues and urine. (A) 5-Me-dCMP in human renal carcinoma tissues and tumor adjacent normal tissues. (B) 5-Me-CMP in human renal carcinoma tissues and tumor adjacent normal tissues. (C) 5-Me-dCMP in human urines from healthy controls and lymphoma patients. (D) 5-Me-CMP in human urines from healthy controls and lymphoma patients.

Deamination of 5-Me-dCMP is an important mechanism to exclude 5-Me-dCMP from incorporation into DNA. However, deamination of 5-Me-dCMP to TMP may not be always highly efficient, as demonstrated by our current study that endogenous 5-Me-dCMP and 5-Me-CMP can be unambiguously detected in mammals. The second mechanism that could prevent 5-Me-dCTP formation and its incorporation into DNA is the proposed absence of a kinase that can act on 5-Me-dCMP converting it to 5-methyl-2'-deoxycytidine diphosphate (5-Me-dCDP).[22] However, previous study demonstrated that there was not a complete absence of kinase activity since the radioactive labeled 5-[^{3}H]-Me-dC was measurable in DNA upon treatment of CHO cells with 5-[^{3}H]-Me-dC,[34] suggesting that incorporation of 5-Me-dCTP originated from 5-Me-dCMP is possible.

On the other hand, 5-Me-CMP from RNA metabolism could be phosphorylated to 5-Me-CDP, a substrate for ribonucleotide reductase to produce 5-Me-dCDP. The formed 5-Me-dCDP can be further phosphorylated to 5-Me-dCTP since the kinase converting nucleotides diphosphates to nucleotides triphosphate is known not to be specific.[35,36] In addition, 5-Me-CDP could be phosphorylated to 5-Me-CTP that can be used for RNA synthesis. Therefore, even deaminase may convert 5-Me-dCMP to TMP, 5-Me-dCTP can be generated from 5-Me-CDP by ribonucleotide reductase and then phosphorylated by nucleoside diphosphate kinases. The method developed in our study is also suitable for the detection of nucleoside triphosphates. Because nucleoside triphosphates are more hydrophilic than nucleoside monophosphates, further optimization of SPE conditions is required to remove the excessive EDC by Strata X cartridge. Determination of endogenous modified nucleoside triphosphates will be performed in the future study.

This study is, to the best of our knowledge, the first report for detecting endogenous 5-Me-CMP and 5-Me-dCMP in human urine, tissues and cultured cells. 5-Me-dCMP and 5-Me-CMP can be potentially converted to their triphosphate forms and became the substrates for DNA and RNA synthesis. The biological significance of endogenous 5-Me-CMP and 5-Me-dCMP, however, need further investigation.

CONCLUSIONS

In the current study, we established a sensitive method to determine endogenous nucleotides in mammals based on DMPA labeling coupled with LC-ESI-MS/MS analysis. The detection sensitivities for nucleotides increased by 88 to 372 folds upon DMPA labeling. Using the developed method, we were able to identify and quantify endogenous 5-Me-dCMP and 5-Me-CMP in multiple human samples, including cultured human cells, human tissues, and human urine samples. The results showed that 5-Me-dCMP and 5-Me-CMP were widely present in human samples, which, for the first time, confirmed their existence in vivo. The quantification results also demonstrated significant depletion of 5-Me-dCMP and 5-Me-CMP in renal carcinoma tissues and urine samples of lymphoma patients compared to controls, indicating the potential reutilization of methylated nucleotides in DNA and RNA synthesis. The biological consequence of endogenous 5-Me-dCMP and 5-Me-CMP still requires further investigation.

ASSOCIATED CONTENT

Supporting Information

Supporting Information Available: Optimization of the extraction conditions for nucleotides; Extraction of DMPA-labeled nucleotides; Table S1 – S11; Figure S1 – S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. †These authors contributed equally.

Notes

The authors declare no competing financial interest.

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