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# Graphene as adsorbent for highly efficient extraction of modified nucleosides in urine prior to liquid chromatography-tandem mass spectrometry analysis

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ABSTRACT

RNA modifications have been involved in numerous biological processes, and aberrations of these modifications are tightly associated with various diseases including cancer. Herein, we developed graphene-based solid-phase extraction and robust ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) combined with stable isotope-dilution for simultaneous enrichment and accurate determination of 17 modified nucleosides in human urine. We found graphene could effectively adsorb various modified nucleosides in human urine samples. With this method, we identified and quantified these modified nucleosides in urine samples collected from lung cancer patients and healthy controls. We revealed that the levels of 12 modified nucleosides were all diminished in urine from lung cancer patients, compared with healthy controls. It is worth noting that we demonstrated, for the first time, the presence of 5,2′-*O*-dimethyluridine ($m^5U_m$) in human urine. Together, we established a robust analytical method for simultaneous determinations of 17 modified nucleosides in human urine, and our results revealed a close correlation between the concentrations of urinary modified nucleosides and the occurrence of lung cancer, implying the potential applications of these modified nucleosides as noninvasive biomarkers for the early detection of lung cancer. Moreover, this study will stimulate future investigations on the regulatory roles of RNA modifications in the initiation and progression of lung cancer.



Post-transcriptional modifications of RNA play important roles in a variety of biological activities and RNA epigenetics/epitranscriptomics has been proposed [1]. So far, more than 170 types of chemical modifications such as methylation and acetylation were identified in RNA and these modifications could regulate the structure and function of RNA [2]. Among these epigenetic modifications, RNA methylation has attracted great attention and tremendous efforts have been devoted to this hot spot [3]. Accompanied with the identification of regulatory proteins including methyltransferases, demethylases and reader proteins, the functions of RNA methylation were gradually revealed. Taking $N^6$-methyladenosine ($m^6A$) as an example, various cellular functions such as mRNA stability, degradation, translation, splicing and nuclear export were exerted under the regulation of methyltransferases (*e.g.*, METTL3, METTL14 and WTAP), demethylases (*e.g.*, FTO and ALKBH5), and reader proteins (*e.g.*, YTHDF1 and YTHDC1) [4–6]. In the past few decades, accumulating evidences have proved that RNA modifications were associated with the initiation and progression of human diseases including cancer [7–9].

Modified nucleosides derived from the degradation of RNA are excreted into the human urine, which is a favored body fluid for biomarker discovery since the urine samples are easy to obtain and the collection process is noninvasive. Therefore, determination of modified nucleosides in human urine could evaluate their potential as noninvasive biomarkers of diseases. In this vein, a variety of modified nucleosides including methylated and hydroxymethylated nucleosides were determined in human urine [10–14].

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Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a powerful analytical technique for biomarker discovery because of its great advantages in sensitivity, accuracy and selectivity, compared with other analytical techniques [15–19]. In the past few decades, LC-MS/MS has been widely used for determination of modified nucleosides in various biological samples such as urine, plasma and tissues [10–14,20–26]. Nevertheless, due to the low abundance of modified nucleosides in biological samples and the serious matrix interference of biological samples, it is still challenging for determination of modified nucleosides. For instance, urine contains a lot of inorganic salts and various organic compounds, these ingredients would suppress the ionization of modified nucleosides in MS detection. Thus, enrichment of modified nucleosides from complex biological samples is necessary to enhance the detection sensitivity of subsequent LC-MS/MS analysis.

Until now, boronate affinity adsorbents were widely utilized for enrichment of nucleosides since they could react with *cis*-diol group in nucleosides to yield cyclic esters [27,28]. Besides, metal-organic frameworks and metal oxides (*e.g.*, titanium dioxide) were also used for enrichment of nucleosides [29,30]. However, these methods could not enrich nucleosides those lack *cis*-diol group, such as 2′-*O*-methylated nucleosides ($N_m$). Recently, we found $Fe_3O_4$/graphene-based magnetic dispersive solid-phase extraction could effectively enrich methylated adenine nucleosides (*e.g.*, $N^6$-methyl-2′-deoxyadenosine, 2′-*O*-methyladenosine and $N^6$,2′-*O*-dimethyladenosine) in human urine [13], although there was no *cis*-diol group in the structures of these methylated adenine nucleosides. In this work, we established a solid-phase extraction (SPE) method with the use of graphene as adsorbent for simultaneous enrichment of various modified nucleosides. We also developed a robust ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method, in combination with the stable isotope-dilution strategy for accurate determination of these modified nucleosides in urine samples collected from lung cancer patients and healthy controls.

A UPLC-MS/MS method for simultaneous detection of 17 modified nucleosides was first established. The chemical structures of these modified nucleosides and stable isotope-labeled internal standards were illustrated in Fig. S1 (Supporting information). Among these modified nucleosides, there are four groups of isomeric modified nucleosides ($m^5C$ and $m^3C$; $m^6A$ and $m^1A$; $m^5U$ and $m^3U$; $m^1G$, $m^2G$ and $m^7G$) and two groups of modified nucleosides with similar molecular weights ($m^5C$, $m^3C$, $m^5U$ and $m^3U$; $C_m$ and $U_m$). Besides, the multiple-reaction monitoring (MRM) ion transition of $ac^4C$ and $G_m$ is also similar to that of $[D_3]m^6A$/$[D_3]m^1A$ and $[D_3]m^6A_m$, respectively. Nucleosides with same or similar MRM ion transitions need to be effectively separated under UPLC-MS/MS analysis in MRM mode to avoid interference from each other. Therefore, we optimized chromatographic separation conditions for these 17 modified nucleosides. As shown in Fig. 1, satisfactory separation was gained under optimized conditions, especially those with same or similar ion transitions were well discriminated from each other. Moreover, these 17 modified nucleosides could be rapidly separated within 8 min by using a BEH C18 column (2.1 × 100 mm, 1.7 μm), indicating the established method is competent for analysis of a great quantity of samples. The optimized MRM parameters is shown in Table S1 (Supporting information).

As a powerful sample pretreatment technique, SPE has been widely employed for sample cleanup [31,32]. We prepared cartridges filled with adsorbent of graphene, then evaluated and compared the enrichment efficiency of various modified nucleosides by using graphene-based SPE cartridges and other two types of SPE cartridges (Sep-pak C18 and Oasis MCX). We first optimized the experimental conditions of graphene-based SPE, including the amount of adsorbent, elution solvent, the percentage of acid in elution solvent, and elution volume. A mixture of modified nucleoside standards was used for optimization. As shown in Fig. 2A, the adsorption of most of these modified nucleosides increased gradually with increasing amounts of adsorbent up to 0.75 mg, whereas the extraction efficiency decreased with 1 mg of adsorbent. Therefore, 0.75 mg of adsorbent was used for subsequent experiments. Then, the types of elution solvents were optimized and several solvents including methanol, ethanol, isopropanol, acetonitrile and ethyl acetate were examined. The results revealed that methanol exhibited highest desorption capacity toward these modified nucleosides (Fig. 2B). Moreover, the presence of formic acid or acetic acid in methanol could improve the desorption of these modified nucleosides from the cartridge (Fig. 2C). Thus, methanol containing 0.1% acetic acid was selected as the elution solvent. We further optimized the elution volume and the results revealed that 1.5 mL of 0.1% acetic acid in methanol gave the best recovery (Fig. 2D).

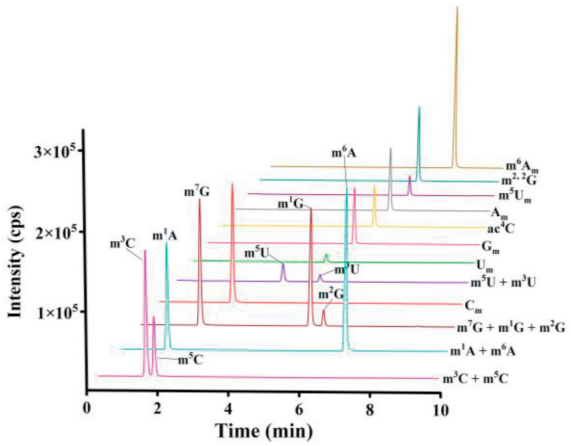


**Fig. 1.** The MRM chromatograms of 17 modified nucleosides standards. The concentration of modified uridine nucleosides was 250 nmol/L each due to their low ionization efficiency. For other modified nucleosides, the concentration was 50 nmol/L each. $m^3C$, 3-methylcytidine; $m^5C$, 5-methylcytidine; $C_m$, 2′-*O*-methylcytidine; $ac^4C$, $N^4$-acetylcytidine; $m^5U$, 5-methyluridine; $m^3U$, 3-methyluridine; $U_m$, 2′-*O*-methyluridine; $m^5U_m$, 5,2′-*O*-dimethyluridine; $m^1A$, $N^1$-methyladenosine; $m^6A$, $N^6$-methyladenosine; $A_m$, 2′-*O*-methyladenosine; $m^6A_m$, $N^6$,2′-*O*-dimethyladenosine; $m^1G$, $N^1$-methylguanosine; $m^2G$, $N^2$-methylguanosine; $m^7G$, $N^7$-methylguanosine; $G_m$, 2′-*O*-methylguanosine; $m^{2,2}G$, $N^2$,$N^2$-dimethylguanosine.

Under the optimized extraction conditions, we compared the performance of graphene-based SPE cartridges and other two types of SPE cartridges (C18 and MCX). We found that modified cytidine nucleosides (*e.g.*, $m^5C$ and $C_m$) and modified uridine nucleosides (*e.g.*, $m^5U$, $U_m$ and $m^3U$) could be washed off the C18 cartridge with $H_2O$ (Fig. 3A). Although modified cytidine nucleosides could be retained on the MCX cartridge, modified uridine nucleosides (*e.g.*, $m^5U$, $U_m$, $m^3U$ and $m^5U_m$) could be washed off the MCX cartridge with $H_2O$ or acetonitrile (Fig. 3B). Surprisingly, none of these modified nucleosides displayed substantial loss during the sample loading or washing process when graphene-based cartridge was used. These modified nucleosides were retained in the cartridge until they were eluted with 0.1% acetic acid in methanol (Fig. 3C). The retention of analytes in C18 cartridges mainly relies on the hydrophobic interaction. Modified cytidine or uridine nucleosides are of relatively high polarity, leading to weak hydrophobic interaction with C18 cartridges. In addition, modified uridine nucleosides are hard to be positively charged due to their relatively low proton affinity, resulting in the weak electrostatic interaction with cation-exchange group in MCX cartridges. Graphene-based cartridges can retain modified nucleosides by $\pi$-$\pi$ stacking and hydrophobic interactions, which can provide stronger retention for polar com-

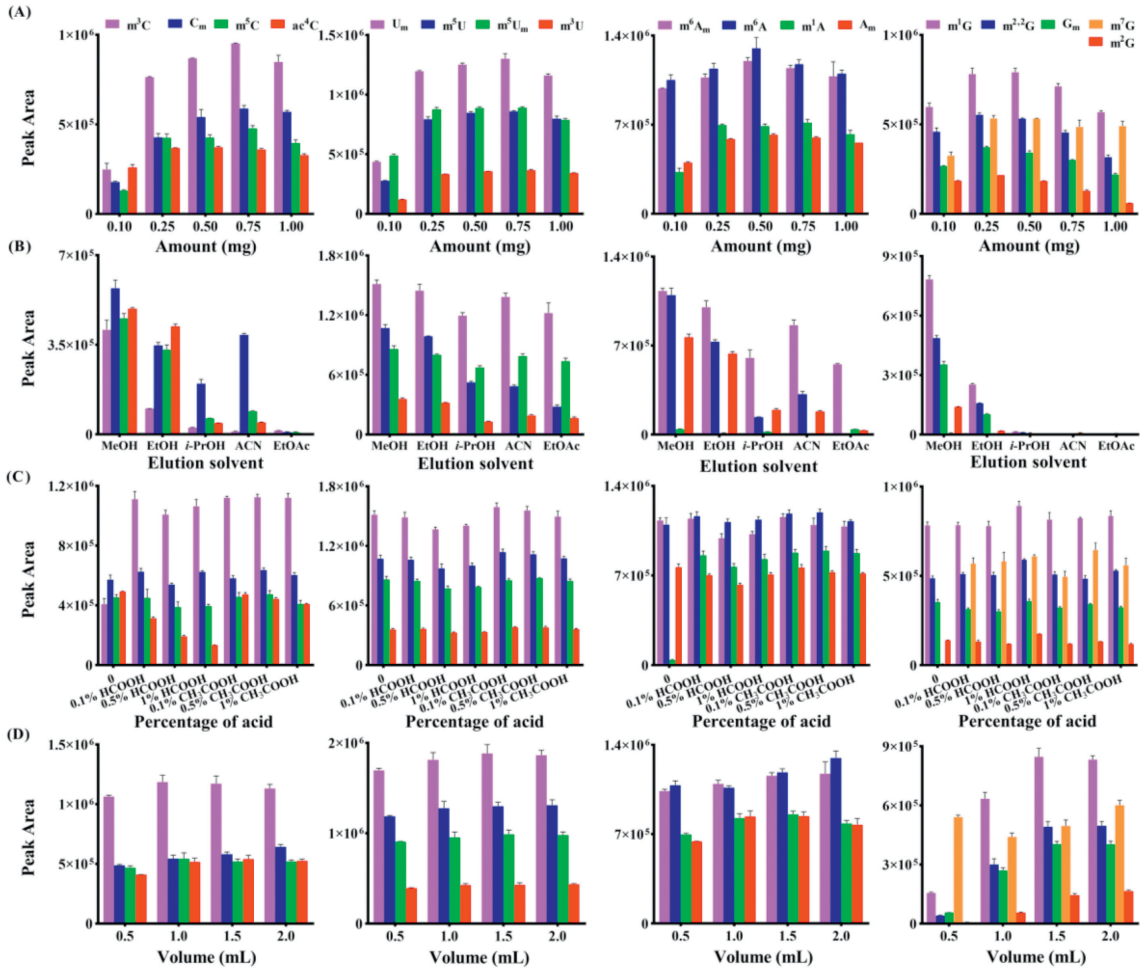


**Fig. 2.** Optimization of graphene-based SPE conditions, including (A) the amount of adsorbent, (B) elution solvent, (C) percentage of acid in elution solvent, and (D) elution volume.

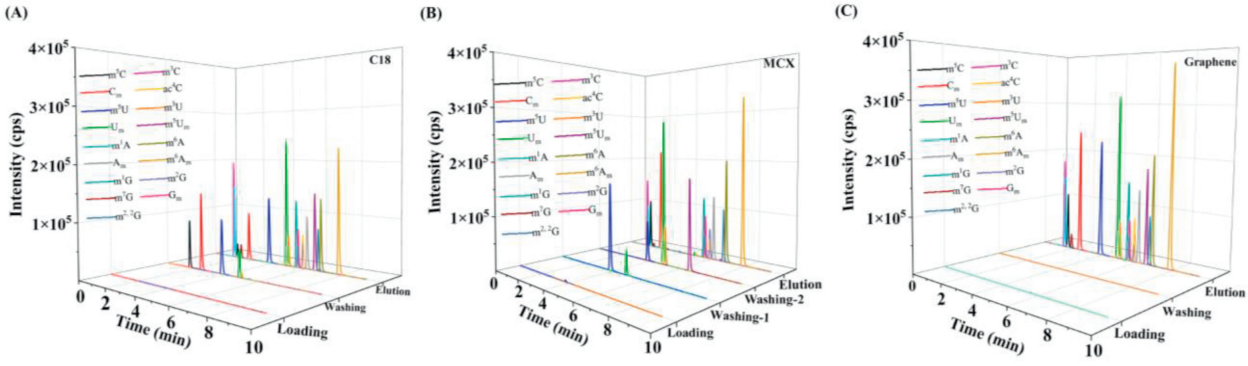


**Fig. 3.** The MRM chromatograms of each SPE fraction obtained from the mixture of modified nucleoside standards pretreated with (A) C18 cartridge, (B) MCX cartridge, and (C) graphene-based cartridge. Graphene-based cartridges have best recoveries of all these modified nucleosides.

pounds, leading to the best recoveries of all these modified nucleosides. Besides, the cost of graphene-based cartridges is much lower than that of C18 and MCX cartridges. Taken together, graphene-based cartridges were selected for the enrichment of various modified nucleosides from human urine samples.

We next assessed the feasibility of the established method. Parameters including linearity, limits of detection (LODs), limits of quantification (LOQs), intra- and inter-day precision, accuracy and recovery were evaluated. A series of nucleoside standards solution spiked with stable isotope-labeled internal standards were prepared and calibration curves were constructed. As shown in Table S2 (Supporting information), the calibration curves showed excellent linearity with correlation coefficients ($R^2$) being > 0.99. Besides, the matrix effect values were between 89.86 and 118.46%, indicating the matrix effect is negligible. The LODs and LOQs of these modified nucleosides were between 0.025 nmol/L and 0.25 nmol/L, and between 0.05 nmol/L and 0.5 nmol/L, respectively (Table S3 in Supporting information). As shown in Table S4 (Supporting information), the intra- and inter-day precision values were in the range of 1.14%–7.21% and 0.34%–7.78%, respectively. The accuracy of the

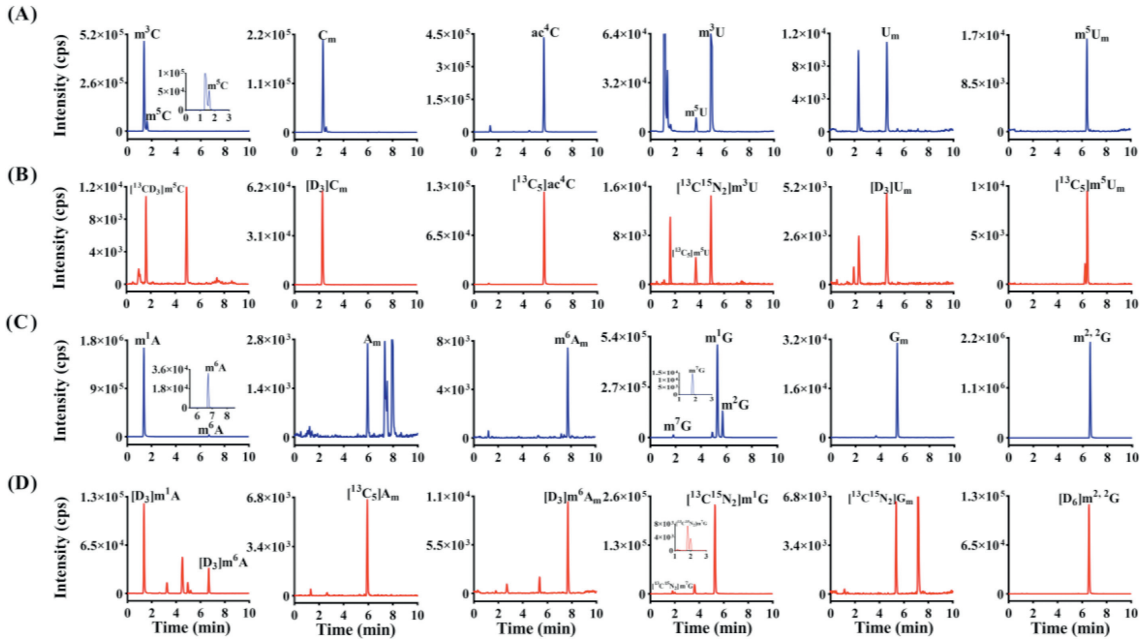


**Fig. 4.** Identification of modified nucleosides in human urine. (A, C) The MRM chromatograms of modified nucleoside in human urine sample. (B, D) The MRM chromatograms of spiked stable isotope-labeled internal standards in human urine sample.

intra- and inter-day analysis varied from 80.17% to 119.77%, indicating that the reproducibility and accuracy were satisfactory. Under the optimized extraction conditions, the recoveries at three different spiking levels of the developed method ranged from 80.31% to 118.20% (Table S5 in Supporting information), indicating the efficient extraction of modified nucleosides from human urine by graphene-based SPE. These results revealed that the established method was robust for determination of modified nucleosides in human urine.

By employing the developed method for enrichment using graphene-based SPE and accurate quantification using UPLC-MS/MS coupled with stable isotope-dilution, we measured these 17 modified nucleosides in urine samples from 56 lung cancer patients and 35 healthy controls. The information of these volunteers was listed in Table S6 (Supporting information). The results demonstrated that the retention time of 15 modified nucleosides (*i.e.*, $m^5C$, $C_m$, $ac^4C$, $m^5U$, $m^3U$, $U_m$, $m^5U_m$, $m^1A$, $m^6A$, $A_m$, $m^6A_m$, $m^1G$, $m^7G$, $G_m$, and $m^{2,2}G$) were identical to those of their corresponding isotope-labeled internal standards (Fig. 4), and the retention time of $m^3C$ and $m^2G$ were also identical to those of their standards (Fig. S2 in Supporting information), confirming the presence of these modified nucleosides in human urine samples. Of note, to the best of our knowledge, the presence of $m^5U_m$ was identified in human urine for the first time.

We next quantified these modified nucleosides in urine samples from lung cancer patients and healthy controls. The concentrations of modified nucleosides were normalized against the content of urinary creatinine. The results showed that the contents of $m^3C$, $C_m$, $ac^4C$, $m^5U$, $m^3U$, $U_m$, $m^5U_m$, $m^1A$, $m^6A$, $A_m$, $m^6A_m$, $m^1G$, $m^2G$, $m^7G$, $G_m$ and $m^{2,2}G$ were in the ranges of 91.01–783.11, 130.20–1316.68, 468.16–3193.25, 1.96–39.60, 47.58–283.43, 28.82–615.42, 1.76–178.93, 310.39–10,513.46, 3.17–446.44, 0.72–14.67, 1.05–24.03, 43.58–3041.97, 88.25–2091.62, 0.35–30.13, 49.08–480.64, and 654.81–4276.67 nmol per mmol creatinine, respectively. In most of urine samples, the signal-to-noise ratio of $m^5C$ was too low to reach the LOQ, and thus its level could not be accurately quantified. The average levels of these modified nucleosides in urine in these two groups are summarized in Table S7 (Supporting information), and the detailed levels of each individual are shown in Tables S8 and S9 (Supporting information).

Then, we compared the contents of these modified nucleosides in urine samples from lung cancer patients and healthy controls. As illustrated in Fig. 5A, the levels of $m^3C$, $C_m$, $ac^4C$, $m^3U$, $m^1A$, $A_m$, $m^6A_m$, $m^1G$, $m^2G$, $m^7G$, $G_m$ and $m^{2,2}G$ in urine were all significantly lower in lung cancer patients than healthy controls. However, there was no significant difference in levels of $m^5U$, $U_m$, $m^5U_m$ or $m^6A$ between lung cancer patients and healthy controls. Nevertheless, we found the mean concentrations of $m^5U$, $U_m$ and $m^5U_m$ were lower in lung cancer patients than healthy controls (Table S7). In addition, we found that the concentrations of $m^3C$, $ac^4C$, $m^3U$, $m^1A$, $A_m$, $m^1G$ and $m^{2,2}G$ were significantly lower in lung cancer patients in stage IV with distant metastasis of tumor than patients in stages I-III without distant metastasis of tumor (Fig. S3 in Supporting information).

We further performed receiver operating characteristic (ROC) curve analysis to assess the potential of these modified nucleosides as biomarkers for early detection and prognosis of lung cancer. As shown in Fig. 5B, the modified nucleosides exhibiting significant differences were highly effective for the detection of lung cancer with the area under the curve (AUC) ranging from 0.631 to 0.862. Hence, our results revealed the correlation between the levels of these modified nucleosides and the occurrence of lung cancer, indicating these modified nucleosides could be potential noninvasive biomarkers for lung cancer detection.

RNA modification plays a vital role in numerous biological activities and aberrant levels of RNA modification is tightly associated with cancer [33]. In the past few decades, the regulatory roles of RNA modification in the occurrence and progression of various types of cancer have been extensively investigated [7–9]. Urinary modified nucleosides derived from the degradation of RNA could reflect RNA turnover and have drawn great attention. In recent years, several novel modified nucleosides were identified and quantified in human urine [10–14]. Recently, the presence of $m^5U_m$ was identified in human serum [34]. Besides, a recent study revealed that the level of $m^5U_m$ in small RNA was significantly increased in thyroid carcinoma compared to the normal tissues

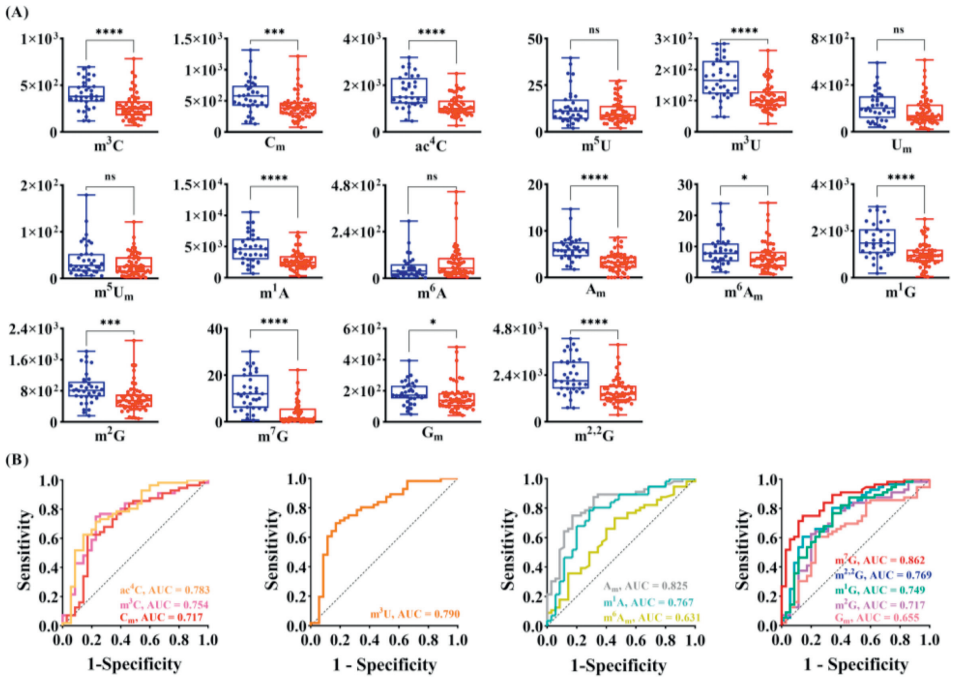


**Fig. 5.** Quantification results of these modified nucleosides in urine samples from healthy controls and lung cancer patients. (A) Levels of modified nucleosides. Blue and red represent the modified nucleosides in urine from healthy controls and lung cancer patients, respectively. The *y*-axis represents the levels of modified nucleosides (nmol per mmol creatinine). ns, $P > 0.05$; $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$. (B) ROC analysis for modified nucleosides in urine samples from healthy controls and lung cancer patients.

[22], although the function of $m^5U_m$ is not clear. By using the established graphene-based SPE coupled with UPLC-MS/MS method, we identified, for the first time, the presence of $m^5U_m$ in human urine, and accurately quantified the concentration of 16 modified nucleosides in urine samples from lung cancer patients.

The incidence and mortality of lung cancer is rather high worldwide, and early detection is crucial to improve the survival rate and decrease the treatment expense. Currently, low-dose computed tomography (LDCT) has been commonly used for lung cancer screening. However, LDCT is still radiative, costly and has a high false positive rate. Therefore, hunting for novel biomarkers for early detection of lung cancer is desirable. Our results revealed that the concentrations of 12 modified nucleosides were diminished in urine from lung cancer patients. This suggested that the decrease of these modified nucleosides in urine might be a common feature of lung cancer, and these modified nucleosides might act as potential noninvasive biomarkers for early detection and prognosis of lung cancer.

In summary, we established graphene-based SPE for enrichment of 17 modified nucleosides and a robust UPLC-MS/MS method, in combination with stable isotope-dilution for accurate quantitation of these modified nucleosides in human urine. By employing the developed method, we realized the simultaneous enrichment of 17 modified nucleosides from human urine samples. We also unambiguously identified the presence of these modified nucleosides and accurately quantified their concentrations in human urine using the stable isotope-dilution strategy. To the best of our knowledge, this is the first time that $m^5U_m$ was identified and quantified in human urine. We revealed that the levels of 12 modified nucleosides in urine from lung cancer patients were all diminished, compared with healthy controls, suggesting these modified nucleosides could serve as potential noninvasive biomarkers for early detection and prognosis of lung cancer. Besides, the present study will stimulate future investigations on the regulatory roles of RNA modifications in the initiation and progression of lung cancer.

## Declaration of competing interest

The authors declare no competing financial interests.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2023.108867.

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