

Detection of 5-Carboxyl Cytosine in Mammalian Cells DNA

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Abstract

5-carboxylcytosine (5caC) is an important derivative of cytosine modification in DNA demethylation pathway. However, the accurate quantification of 5caC is a challenge, especially in mammalian tissue. This plight probably results from the trace amount of 5caC in mammalian and the inadequate sensitivity of the existing technologies. Herein, we report a novel LC-MS/MS method to precisely quantify 5caC in mammalian cells. The 5caC standard reagent was synthesized, and the genomic DNA was hydrolyzed with formic acid and the target compound 5caC was detected by HILIC LC-MS/MS. The results showed 5caC generally exist in mice organs and cancer tissues. The content of 5caC in mice brain was higher than in lungs and liver, and was obviously decreased in colorectal cancer tissues compared with the adjacent tissue. This result suggests that 5caC probably associates with tumorigenesis and plays a dominated role in epigenetic control of neuronal function.

Keywords: Hydrophilic interaction liquid chromatography-Tandem mass spectrometry; DNA Demethylation; 5-Carboxylcytosine; Cancer tissues; Organs

Introduction

Cytosine (Cyt) modification in DNA is an important epigenetic marker that plays regulatory roles in a variety of cellular processes, such as embryogenesis, carcinogenesis and gene depression [1-4]. Mammalian DNA cytosine modification is a dynamic process (Figure 1). 5-methylcytosine (5mC) is generated by an addition of methyl group at the 5-position of Cyt with the help of DNA methyltransferases (DNMTs), and also can be converted back into unmodified Cyt in the absence of DNMTs. Furthermore, ten-eleven translocation (TET) protein family oxidizes 5mC to 5-hydroxymethylcytosine (5hmC) [5, 6]. The latest studies have shown that 5hmC can be further oxidized by TETs to create 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which can be removed by thymine DNA glycosylase (TDG) to restore the unmethylated Cyt through base excision repair (BER) pathway (Figure 2) [7,8]. And 5caC was suggested as one of the most important intermediates in DNA demethylation pathway.

Due to the importance of DNA demethylation process in mammalian, the intermediates including 5mC, 5hmC and 5fC have been consecutively quantified in different biological samples to explore their function [9-12]. However, the quantification of 5caC is extremely difficult. So far, quantification of 5caC is mainly reported in embryonic stem cells (approximately 3/10⁶ cytosines) [7]. Pfaffeneder *et al* attempted to perform an HPLC-MS assay for simultaneous determination of 5fC and 5caC. Unfortunately, none 5caC signal was detected [10]. Considering the fact that 5caC had not been detected in mammals cell, He *et al.* [7] deduced that 5caC might be actively removed from genomic DNA immediately after its generation in cells. Encouragingly, 5caC was localized in brain tissues and follicular cells of axolotl ovary by immunohistochemistry and immunofluorescence [13-15], though not confirmed by more robust approaches such as mass spectrometry. Due to the trace amount of 5caC *in vivo* as well as interference from the high amount of normal nucleosides, quantification of 5caC is an extreme challenge. To achieve precise analysis of 5caC in mammalian genomes, a highly sensitive detection method is required.

Materials and Methods

For the purpose of qualification and quantification, a standard substance of 5caC was synthesized according to reported method [16]. As shown in Scheme 1, the synthesis of 5caC started with ethyl ureidomethylene cyanacetate (A), which was prepared from urea, ethyl ortho-formate and cyanoacetic acid ethyl ester under reflux.

Heterocyclization of A in the presence of sodium ethoxide produced 5-ethoxycarbonylcytosine (B). Subsequent treatment with NaOH furnished the conversion of B to the target compound (5caC).

Then we developed a LC-MS/MS method for accurately detection of 5caC, using synthetic 5caC material as standard. Specially, genomic DNA was directly hydrolyzed by formic acid. LC separation of nucleobases was performed on a bridged ethylene hybrid (BEH) HILIC column, and tandem MS analysis was conducted with isotope standard Cyt¹³C¹⁵N₂ [17].

Discussion and Results

Qualitative test based on the synthetic 5caC standard was performed with Agilent 6430A LC-MS/MS using nitrogen as the nebulizer gas. The MS was operated at unit mass resolution for both precursor and product ions. Qualification of 5caC was achieved in multiple-reaction monitoring (MRM) mode, which could be seen from full-scan mass spectrum of 5caC shown in Figure 3a. The ion signal (*m/z* 156) was in excellent agreement with the calculated exact mass of protonated molecular ion [M+H]⁺ of 5caC. And the product ions *m/z* 138.1, 95.1 and 67.9 were generated from the precursor ion *m/z* 156.0. Therefore, the product ions at *m/z* 156.0/138.1, 156.0/95.1 were chosen for 5caC as qualitative ions (Figure 3b). To improve the reliability, an ion at *m/z* 67.9 was also selected to qualify 5caC in real samples. The results showed that samples were consistent with the 5caC standard in ions mass, abundant scale and the retention time (Figure 4c-4d), illustrating that 5caC was detected in mammalian samples.

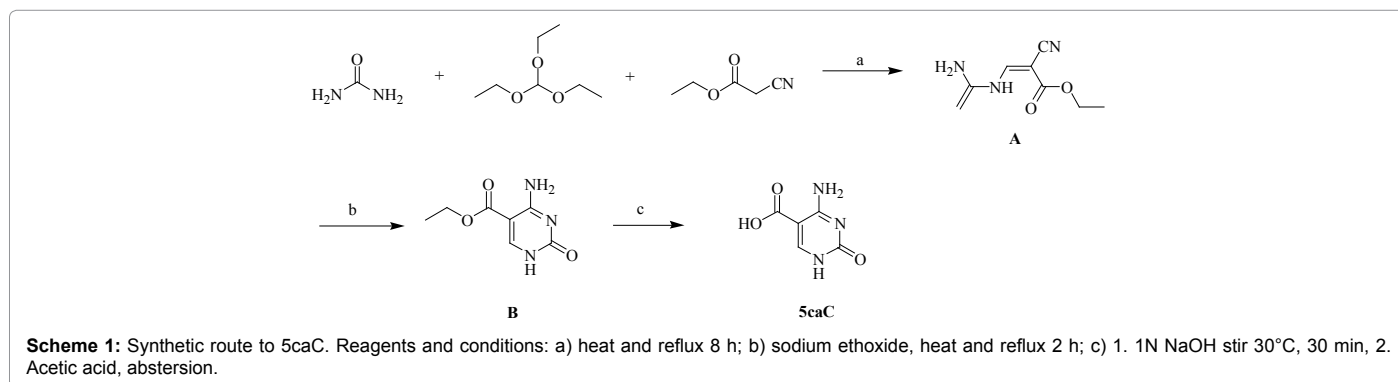
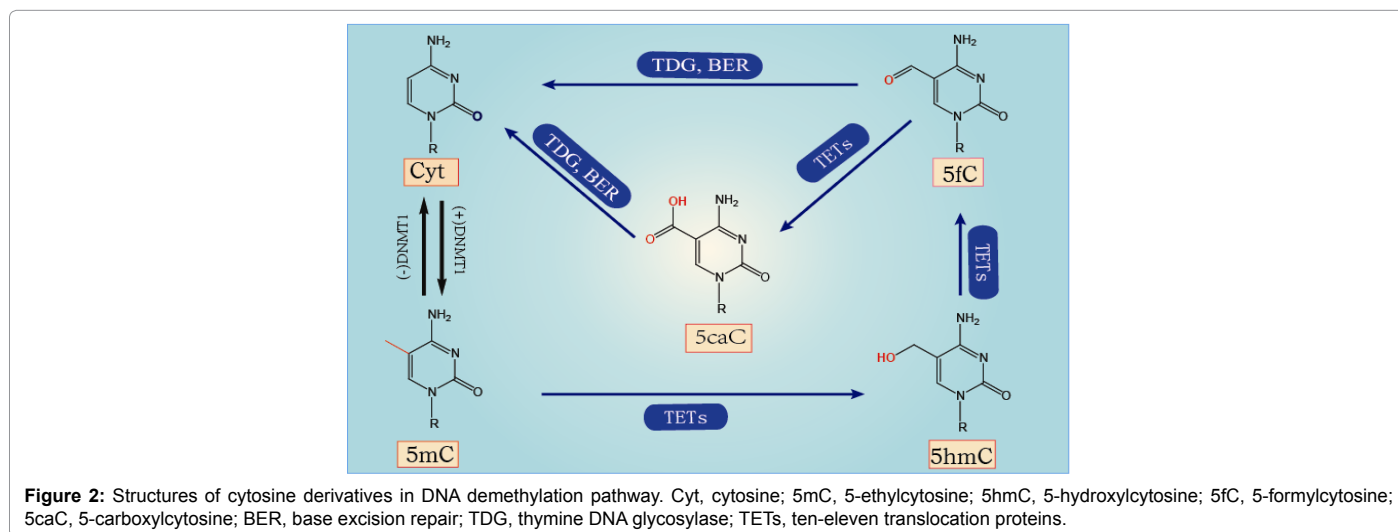
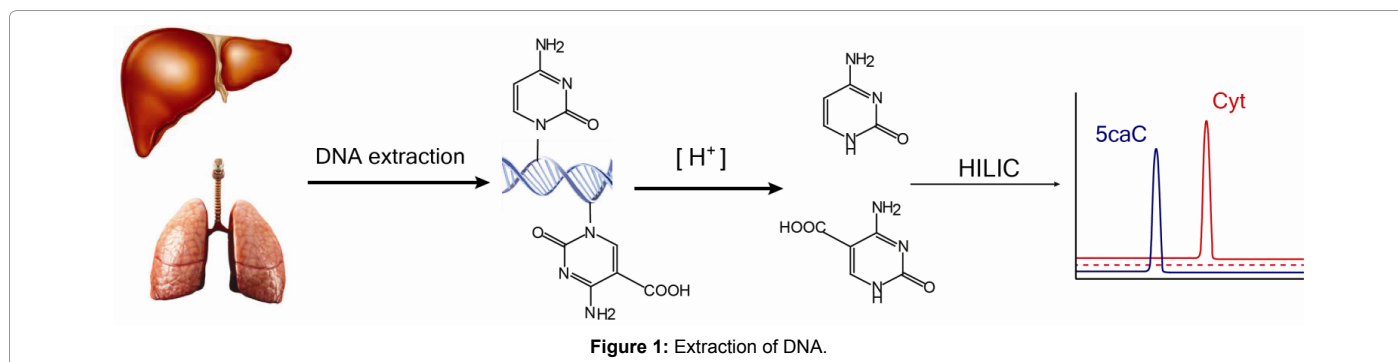
For separation and quantification of the Cyt and 5caC, a WATERS BEH HILIC column (50 × 2.1 mm id, 1.7 μm) was used. The mobile phase was composed of (A) 7 mM ammonium formate and 0.1% ammonia in Milli-Q water and (B) 0.1% ammonia in acetonitrile with a proportion of A:B=17:83 (v/v). As can be seen from Figure 3c, 5caC and Cyt are separated from each other within a retention time of 1.5 min.

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We utilized high concentration of organic phase to achieve excellent LC separation of HILIC for target compounds and increase desolvation and reduce surface tension to enhance sensitivity of mass spectrometry. In this analysis conditions, the synthesized 5caC was used to generate mass spectrometry calibration curves to enable precise LC-MS quantification [11]. The calibration curves of Cyt (Linear range 50 ng/mL to 3500 ng/mL) and 5caC (Linear range 2 ng/mL to 80 ng/mL) were obtained and both the correlation coefficients were higher than 0.99, and the LOD of Cyt and 5caC were 0.05 ng/mL and 0.1 ng/mL, respectively. The average recovery was 89.51% (n=6, RSD=4.23%). Compared with the mass spectrometry methods, the excellent sensitivity was acquired by the following two points: First, the DNA was hydrolyzed to nucleobase rather than nucleoside, which obtained by enzyme. It can overcome the inconsistencies in the activity and stability of enzyme, which maybe led to the incompleteness of DNA hydrolysis. Second, as hydrophilic

separation column, the mobile phase of HILIC was composed of high percentage of organic solvent so that it was benefit for the ionization and high sensitivity of the analyte by LC-MS/MS.

To quantify 5caC in different organs, we collected the brain, lungs and liver from 6 male Sprague-Dawley (SD) rats weighing 170 g to 200 g, which were purchased from the Animal Center at Guangdong Medical University and housed in a pathogen-free environment with a 12 h to 12 h light-dark cycle. Food and water were available ad libitum. The animals were euthanized by 'method of sacrifice 1', i.e., increasing the concentration of carbon dioxide. The animal experiments in this study were approved by the Committee on the Ethics of Animal Experiments of Guangdong Medical University. All efforts were made to minimize animal suffering. The samples were stored at -80°C until analysis. And the colorectal tissue and adjacent normal tissue in six

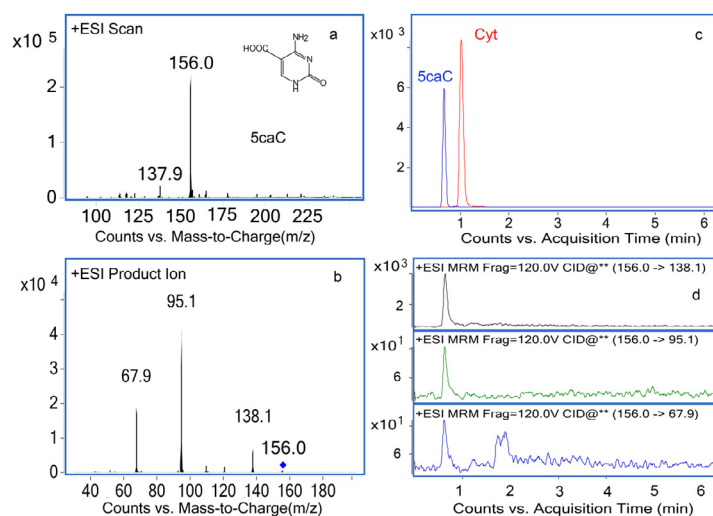


Figure 3: Mass spectrometric and liquid chromatographic characterization of 5caC. a) the full scan of 5caC; b) the product ion scan of 5caC; c) the quantitative chromatogram of 5caC and Cyt; d) the multiple-reaction monitoring (MRM) chromatogram of 5caC from biological sample.

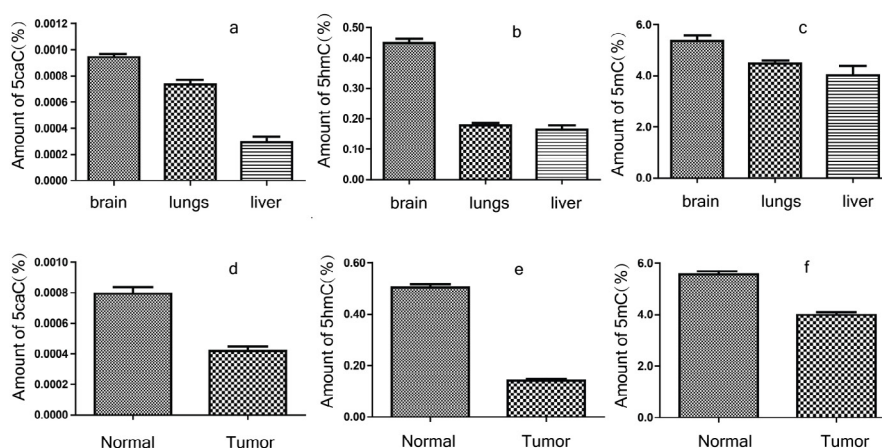


Figure 4: The percentages of 5caC, 5hmC and 5mC in brain, lungs and liver of mice (a, b, c), Tumor-adjacent normal tissues and tumor tissues of colorectal cancer (d, e, f).

patients with colorectal cancer were obtained from the affiliated hospital of Guangdong Medical University. The samples were stored at -80°C until analysis. About $30\ \mu\text{g}$ DNA was isolated using commercial DNA extraction kit. After removing the RNA with RNAase, the acquired pure DNA was tested with Agarose gel electrophoresis. The DNA with an $A_{280\text{nm}}/A_{260\text{nm}}$ value at 1.8-2.0 was precipitated by 1/10 volume of sodium acetate (pH 5.4; 3 mol/L) and 2.5-fold volume of ethanol. The residue was mixed with 0.2 mL of $\text{Cyt}^{13}\text{C}^{15}\text{N}_2$ (50 ng/mL) and dried by nitrogen. The residual was dissolved in 88% formic acid and hydrolyzed at 140°C for 90 min. When cooled down to room temperature, the solution was dried under nitrogen. The further residue was dissolved in 0.2 ml acetonitrile-water (83:17, v/v) and centrifuged at 15 000 g for 5 min. The final supernatant was extracted for LC-MS/MS analysis. Importantly, the whole analysis process of nucleobases reported here could be finished within 2 hours, while enzymatic digestion costs more than 24 hours to fulfill identical process.

Then we quantified 5caC in DNA samples using this HILIC-MS/MS method. As shown in the MRM chromatograms of a sample from mice organs (Figure 3d), the signals of the quantifier m/z 156.0/138.1 and two qualitative ions m/z 156.0/95.1 and m/z 156.0/67.9 were detected at the identical retention time of synthetic 5caC standard,

supporting the feasibility of our assay and the expected existence of 5caC in these subjects. The carboxylation percentage was calculated using the formula as follow: $5\text{caC}\% = Q_{5\text{caC}} / Q_{\text{Cyt}} \times 100\%$. $Q_{5\text{caC}}$ and Q_{Cyt} stand for the molar quantity of 5caC and Cyt detected in DNA samples. As can be seen from Figure 3a/d, the content of 5caC in mice organs ranges from $3/10^6$ to $1/10^3$, which is in accordance with that in mouse embryonic stem cells [7]. And the content of 5caC in brain is highest, followed by that in liver and lungs in sequence. Notably, the distribution of 5caC observed in mice organs exhibits a biological implication that 5caC plays an important role in the epigenetic control of neuronal function. Meanwhile, the variations of 5mC and 5hmC in corresponding organs were detected by our reported method [18], and the highest level of 5hmC and 5mC was also found in brain among the organs. We presumed that a relatively high expression of TET proteins in brain promoted the biosynthesis of the derivatives in the demethylation pathway.

After clarifying distribution of 5caC in normal mammal organs, we sequentially focused on the 5caC variations in abnormal individuals, such as tumor tissues. The DNA samples from colorectal cancer tissues and its adjacent congeners were analyzed. The results showed that contents of 5caC were approximately 0.0008% and 0.0004% in tumor-

adjacent tissues and colorectal cancer tissues respectively (Figure 4d). In agreement with earlier studies, an average level of 5hmC%=0.50% was measured in tumor-adjacent tissues and decreased to 0.15% in colorectal cancer tissues (Figure 3e). And about 1.0% declination of 5mC was also observed from normal tissues to tumor ones (Figure 4f). By contrast to normal tissues, 5caC degree in colorectal cancer tissues was apparently decreased, which was identical with the variation tendency of 5hmC and 5mC. Lots of studies have confirmed that the declination of 5hmC and 5mC was closely associated with occurrence and growth of various malignant tumors [11,19,20]. Thus, 5caC is a potential biomarker to indicate cancer development as well as its precursors.

In summary, we have confirmed and quantified the 5caC in mammalian tissues with excellent accuracy, and found that 5caC was normally presented in mammalian organs and the content was higher in tumor-adjacent tissues than colorectal cancer congeners. In accordance with the results, 5caC was probably suggested to participate in regulation of neuronal activities and cancer development. In our method, the sample preparation was simple, and the nucleobases could be fully hydrolyzed by formic acid within a short time and directly used for LC-MS analysis. In addition, the modified cytosine instead of its derivatives was determined, which made the qualification and quantification of target compounds more direct and accurate. This study will provide a firm foundation for the exploration of the functions of 5caC in the DNA demethylation process in mammals.

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