ChemPubSoc Europe

DOI: 10.1002/open.201600103

Open Access ChemistryOPEN Full Papers

Urinary Measurement of Epigenetic DNA Modifications: A Non-Invasive Assessment of the Whole-Body Epigenetic Status in Healthy Subjects and Colorectal Cancer Patients

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Active mechanism of DNA demethylation can be responsible for the activation of previously silenced genes. Products of 5-methylcytosine oxidation are released into the bloodstream and eventually excreted with urine. Therefore, whole-body epigenetic status can be assessed non-invasively on the basis of the urinary excretion of a broad spectrum of epigenetic modifications: 5-hydroxymethylcytosine (5-hmCyt), 5-formylcytosine (5-fCyt), 5-carboxycytosine (5-caCyt), and 5-hydroxymethyluracil (5-hmUra). We have developed a specific and sensitive, isotope-dilution, automated, online, two-dimensional ultra-performance liquid chromatography system with tandem mass spectrometry (2D UPLC–MS/MS) to measure 5-hmCyt, 5-fCyt, 5-caCyt, and their deoxynucleosides in the same urine sample. Human urine contains all of the modifications except from 5-formyl-2′-deoxycytidine (5-fdC) and 5-carboxy-2′-deoxycytidine (5-cadC). A highly significant difference in the urinary excretion of 5-(hydroxymethyl)-2′-deoxycytidine (5-hmdC) was found between healthy subjects and colorectal cancer patients (3.5 vs. 7.8 nmol mmol^{-1} creatinine, respectively), as well as strong correlations between the majority of analyzed compounds.

1. Introduction

Cytosine methylation, usually at CpG dinucleotides, is one of the most important epigenetic modifications, which has a profound impact on gene repression, cellular identity, and organismal fate. However, the opposite process (i.e. DNA demethylation) is equally important, contributing to the activation of previously silenced genes.[1,2] The most plausible mechanisms of active 5-methylcytosine (5-mCyt) demethylation include involvement of ten-eleven translocation (TET) proteins in the oxidation of 5-mCyt to form 5-hydroxymethylcytosine (5-hmCyt), which can be further oxidized to 5-formylcytosine (5-fCyt) and 5-carboxycytosine (5-caCyt). Then, a base excision repair (BER) pathway is activated, owing to the involvement of thymine-DNA glycosylase (TDG), to replace these base modifications (5-fCyt, 5-caCyt) with cytosine in order to demethylate DNA (as reviewed in Ref. [1]). Some experimental evidence suggests that 5-hydroxymethyluracil (5-hmUra) can be also generated by TET enzymes and may also exhibit some epigenetic functions.[3,4] Many previous studies have centered around the determination of the 5-hmCyt level in DNA, and only a few authors have analyzed its concentrations in various tissues.[4–6] The low abundance of 5-fCyt, 5-caCyt, and 5-hmUra (approximately 3–4 orders of magnitude lower than that for 5-hmCyt) makes their accurate determination somehow challenging, and experimental data clearly demonstrate substantial inter-tissue variance in 5-hmCyt levels.

Following their excision from DNA, modified bases/nucleosides are released into the bloodstream and eventually appear in urine.[7] Therefore, the whole-body epigenetic status can be assessed non-invasively on the basis of the urinary excretion of a wide spectrum of epigenetic modifications, such as 5-hmCyt, 5-fCyt, 5-caCyt, 5-hmUra, and deoxynucleosides thereof. Only individual compounds were quantified in previous studies;[8,9] therefore, we used isotope-dilution, automated, online, two-dimensional ultra-performance liquid chromatography with tandem mass spectrometry (2D UPLC–MS/MS) to measure all of the above-mentioned modifications in the same urine sample. As epigenetic changes may contribute significantly to carcinogenesis,[10] we analyzed the urinary levels of the modifications in both healthy controls ($n = 24$) and colorectal cancer (CRC) patients (N = 34). 8-Oxo-7, 8-dihydroguanine (8-oxoGua) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) are the most extensively studied modifications, and their urinary levels are generally considered as markers for repair-enzyme activity.[7,11] Therefore, the urinary excretion rate of these well-char-

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Supporting Information and the ORCID identification number(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/open.201600103.

acterized compounds (8-oxoGua and 8-oxodG) has been determined, and also represents the reference point for the concentrations of the epigenetic modifications studied. Taking into account the low sensitivity of the hereby used method for 5-hmUra and 8-oxoGua, their levels were determined by means of LC/GC–MS, as previously described.[12]

To fully understand the results presented herein, a key question about the origin of the analyzed urinary lesions needs to be answered. Similar to 8-oxoGua and 8-oxodG, the most plausible source of the analyzed modifications seems to be DNA repair. 5-FCyt and 5-caCyt may inhibit DNA replication, which results in genome instability and mutagenesis.[13,14] Therefore, specific effective enzymatic systems are needed to remove these modifications from DNA. Indeed, TDG was demonstrated to exhibit a robust excision activity toward 5-fCyt or 5-caCyt in DNA.[15,16] Recent evidence suggests that the main enzymes involved in the removal of 5-hmUra from DNA are SMUG1 and TDG.[17] Therefore, the activity of the above-mentioned enzymes (as a part of BER pathway) may contribute to the presence of the modified bases in urine.

2. Results and Discussion

Until now, there have only been a few publications in which single (individual) compounds are quantified.[8,9] Our methodology is suitable for the detection of a broad spectrum of DNA epigenetic modifications in human urine (bases and deoxynucleosides). However, the level of 5-(hydroxymethyl)-2′-deoxycytidine (5-hmdC) in our work (25–70 nM) is very similar to previous results (5–51 nM).[9] The mechanisms responsible for the presence of active demethylation products in DNA and urine are still unclear. Nevertheless, it seems that oxidation of 5-mCyt by TET proteins is a source of 5-hmCyt, 5-fCyt, and 5-caCyt in DNA. Then, the BER pathway is activated, owing to the involvement of TDG glycosylase in replacing these base modifications (5-fCyt, 5-caCyt) with cytosine to demethylate DNA. Interestingly, the levels of 5-fCyt and 5-caCyt in cellular DNA are about two orders of magnitude lower as compared to 8-oxodG,[5] although the urinary excretion rates of the aforementioned bases are quite similar (Figure 1). No 5-fCyt or 5-caCyt deoxynucleosides were detected. Altogether, this evidence points to the high efficiency of the BER pathway in the removal of these epigenetic markers from cellular DNA. It should be stressed that the hereby analyzed modified deoxynucleosides were not the products of glycosylase, as their glycosidic bonds were cleaved; consequently, some other DNA repair systems were apparently involved in their formation. Perhaps, 5-fCyt, 5-caCyt, and 5-hmUra initiate processive demethylation of DNA, as proposed by Franchini et al.[18,19] In line with this hypothesis, an alternative pathway exists, the so-called processive DNA demethylation, aside from the active process involved in local and specific DNA demethylation. According to the authors of this hypothesis, a single initiating event (such as certain mismatch) may trigger the processive demethylation of numerous 5-mCyts (and perhaps also 5-hmCyts) on the same locus via long-path BER, DNA mismatch repair (MMR), or the nucleotide excision repair (NER) pathway. Recent experiments with cell-free extracts and circular heteroduplex DNA substrates have demonstrated that 5-hmUra may trigger the removal of distant epigenetic modifications (5-mCyt and 5-hmCyt) on MMR- and long-path BER-dependent pathways.[20] This, in turn, may explain the presence of 5-hmCyt and 5-mCyt deoxynucleosides in urine. The lesion-containing oligomers from NER-/MMR-/long-path BER may be a subject of intra-/extracellular 5′-3′ exonucleolytic digestion, which eventually results in the synthesis of 6-to-7-nucleotide-long oligomers. However, the latter may be further degraded, and this poorly characterized post-excision processing is eventually reflected by the modified deoxynucleotide yield.[21]

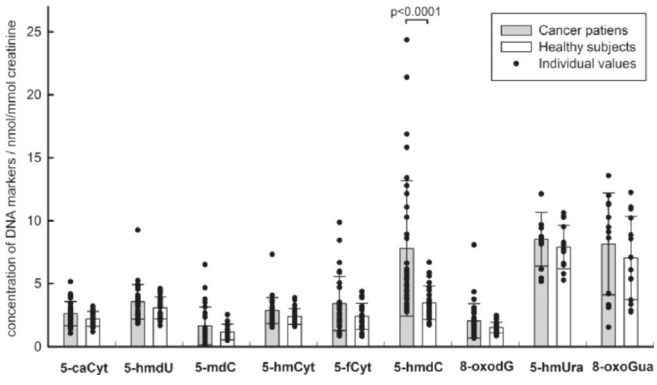

Figure 1. Urinary levels of DNA damage markers and active demethylation products of 5-metylcytosine in CRC patients and controls.

The mechanism involved in the recognition and excision of 5-hmCyt has been reported;[22] furthermore, Spruijt et al. suggested that 5-hmCyt may be recognized by Neil glycosylases.[3] Aberrant methylation of DNA is postulated to play a significant role in cancer development. An increasing body of evidence from recent experimental studies implicates active DNA demethylation, involving enzymatic oxidation and deamination of 5-methylcytosine with subsequent formation of 5-hmCyt and its derivatives (5-fCyt, 5-caCyt, 5-hmUra), as the key event in epigenetic reprogramming (as reviewed in Ref. [23]). Disruption of this process, in turn, may contribute to the aberrant DNA methylation pattern that is commonly observed in cancer.

In our present study, CRC patients presented higher levels of analyzed epigenetic modifications than the controls (Figure 1). However, probably owing to the small sample size, the intergroup difference was statistically significant only for 5-hmdC ($p < 0.001$). Higher levels of the epigenetic modifications in CRC patients may reflect the above-mentioned systemic disturbances of demethylation process during the course of carcinogenesis. As urinary excretion of the analyzed modifications is likely a marker of DNA repair, a question arises about their interrelationships. To address this issue, we analyzed correlations between the individual modification levels. The evidence of strong significant linear correlations between the majority of the modifications found in urine (Figure S1 a–e) further supports the hypothesis that their true sources are BER/NER/MMR pathways. The most evident difference in the urinary excretion rate was demonstrated for 5-hmdC, the level of which was

more than two-fold higher in cancer patients compared to healthy controls (Figure 1).

Several recently published studies have shown that the level of 5-hmCyt in cancer tissue is always lower than in matched non-malignant specimens.[24,25] However, the reasons behind the decreased level of 5-hmCyt in cancerous tissues, as well as the underlying mechanisms thereof, are still unclear. Perhaps this phenomenon may reflect the decreased activity of TET proteins.[26] Another potential explanation is a passive loss of 5-hmCyt during replication, as an inverse relationship was observed between the 5-hmCyt level and cell proliferation.[25] In our opinion, however, it is increased/aberrant activity of the repair pathways (MMR, long-path BER) that likely contributes to the decreased level of 5-hmCyt in cancerous tissues.

3. Conclusion

Our methodology is suitable for the detection of a broad spectrum of DNA epigenetic modifications in human urine. We found a highly significant difference in the urinary excretion of 5-hmdC in healthy subjects and CRC patients, as well as strong correlations between the majority of the modifications. As mentioned above, a large body of evidence suggests that the level of 5-hmCyt in many human malignancies is substantially reduced.[24,25,27,28] Moreover, recently published data suggest that the decrease in the 5-hmCyt level may serve as a biomarker for early carcinogenesis and can be used as a prognostic factor in cancer patients.[27,29] Consequently, the hereby described urinary modification may find applications as a potential risk and response marker. Owing to difficulties in obtaining the specimens of cancer tissues, determination of epigenetic DNA modifications in human urine may serve as an attractive non-invasive diagnostic option. Furthermore, the non-invasiveness of the test constitutes a strong argument for its application to a large-scale basic research and clinical studies dealing with the role of active demethylation in carcinogenesis.

Experimental Section

Urine samples from healthy subjects and CRC patients were spiked with a mixture of internal standards in a 4:1 volumetric ratio. The structures of the used internal standards are presented in Figure 2. Chromatographic separation was performed with a Waters Acquity 2D UPLC, consisting of a binary solvent manager (BSM) pump, flow-through-needle autosampler (FTN), and a photodiode array detector (PDA) for the first dimension chromatography, and quaternary solvent manager (QSM) and a Xevo TQ-S tandem quadrupole mass spectrometer for the second-dimension chromatography. Both dimensions were coupled with a column manager equipped with two programmable column heaters and a 2-position 6-port switching valve. An at-column dilution technique was used between the first and the second dimension to improve the retention at a trap/transfer column. The sample molecules were then adsorbed onto the packing material as very narrow bands that could be eluted as well-resolved, small-volume peaks. A diluting stream of water (0.25 mL min^{-1}) was pumped with a Waters 515 isocratic pump and mixed with the first-dimension column effluent by using a UPLC low-dead-volume tee. The following columns were used: Phenomenex Kinetex F5 column (150 mm × 2.1 mm, 1.7 μm) for the first dimension, Waters X-select C18 CSH (30 mm × 2.1 mm, 1.7 μm) for the second dimension, and Waters X-select C18 CSH (30 mm × 2.1 mm, 1.7 μm) as the trap/transfer column. The chromatographic system operated in a heart-cutting mode, which means that selected portions of effluent from the first dimension were directed to the trap/transfer column via the 6-port valve switching, which served as an "injector" for the second-dimension chromatography system. The flow rate at the first dimension was 0.25 mL min^{-1} and the injection volume amounted to 0.3 and 2 μL. The separation was performed with a gradient elution for 10 min using a mobile phase of 0.1% acetate (A) and acetonitrile (B) (1–5% B for 5 min, column washing with 30% acetonitrile, and re-equilibration with 99% A for 3.6 min). The flow rate at the second dimension was 0.35 mL min^{-1}. The separation was performed with a gradient elution for 10 min by using a mobile phase of 0.01% acetate (A) and methanol (B) (4–50% B for 4 min, isocratic flow of 50% B for 1.5 min, and re-equilibration with 96% A up to next injection). Mass spectrometric detection was conducted with a Waters Xevo TQ-S tandem quadrupole mass spectrometer equipped with an electrospray ionization source. The following common detector parameters were used: source temperature 150 °C, nitrogen desolvatation gas flow 1000 L h^{-1}, nitrogen cone-gas flow 200 L h^{-1}, desolvatation temperature 500 °C, and nebulizer gas pressure 7 bar. Collision-induced dissociation was obtained with argon (6.0 at 3×10^{-6} bar pressure) as a collision gas. The instrument's response to all compounds was optimized by the

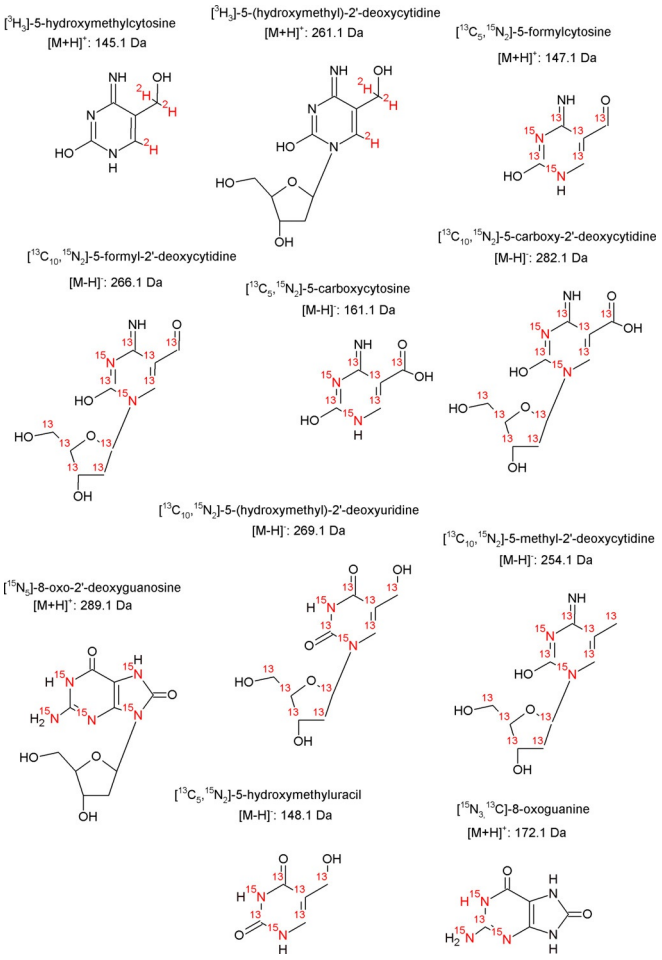

Figure 2. Structures of the internal standards.

Table 1. Compound-specific validation parameters.

Parameter[a]	5-hmCyt	5-hmdC	5-fCyt	5-fdC	5-caCyt	5-cadC	5-hmdU	8-oxodG	5-mdC
recovery [%] (25 fmols per injection)	111	111	123	66	101	107	110	93	87
recovery [%] (250 fmols per injection)	97	116	124	100	96	108	102	95	83
day-to-day RSD $n=3$ [%]	12.5	14.5	12.4	14.4	15.3	5.9	15.2	7.7	13.8
within sample RSD $n=5$ [%]	3.3	5.5	6.2	5.8	7.7	5.3	4.7	4.1	2.5
LOD [fmols]	0.3	3	0.5	0.3	2	0.05	2	0.2	0.2
LOQ [fmols]	0.6	8	1.7	0.8	5.2	0.13	9	0.52	0.55

[a] RSD: relative standard deviation, LOD: limit of detection, LOQ:limit of quantification.

infusion of 10 μM genuine compounds dissolved in water (10 μL min^{-1}), in mobile phase A stream, through the mass spectrometer fluidics system operating in the "mixed" mode, using MassLynx 4.1 Intelli-Start feature. Quantitative and qualitative transition patterns, as well as specific settings of the detector, are summarized in Table S1. The chromatographic system was operated with MassLynx 4.1 Software from Waters. Quantitative analyses were performed by using the Target Lynx application. All samples were analyzed in three to six technical replicates. The analyzed urine samples contained all modified bases and nucleosides, except 5-fdC and 5-cadC. The results of the quantification are summarized in Figure 1.

The recovery, inter- and intra-sample reproducibility results are presented in Table 1. To assess the recovery, the samples of urine were spiked with two amounts of unlabeled compounds (three replicates per amount) and analyzed according to the standard procedure along with three unspiked DNA samples. The recovery was determined as a percentage ratio of the difference between the absolute concentration of the compound in the spiked and unspiked sample to the effective concentration of the spiked compound in the injected sample. The results of this experiment were also used to determine the inter- and intra-sample reproducibility.

Acknowledgements

This work was supported by the National Science Centre grant number: DEC-2013/09/B/NZ5/00767.

Keywords: 2D UPLC–MS/MS · DNA damage · DNA methylation · epigenetics · urine

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Received: September 8, 2016
Published online on November 15, 2016

