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# Pan-cancer analysis of DNA epigenetic modifications by hydrophilic interaction liquid chromatography-tandem mass spectrometry

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## ABSTRACT

Accumulating evidence in recent years indicates that DNA methylation (5-methyl-2′-deoxycytidine, 5-mdC) and hydroxymethylation (5-hydroxymethyl-2′-deoxycytidine, 5-hmdC) have been implicated in various biological processes, and the aberrations of these DNA cytosine modifications is tightly associated with cancer. *N*$^6$-methyl-2′-deoxyadenosine (m$^6$dA), as a newly discovered epigenetic modification in genome of mammals, has been demonstrated to play vital regulatory roles in tumorigenesis. However, the content information of m$^6$dA in human tumor tissues is still limited and pan-cancer analysis of these DNA epigenetic modifications is lacked. Herein, we developed a sensitive and robust stable isotope-diluted hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS) method for accurate quantification of m$^6$dA, 5-mdC and 5-hmdC in genomic DNA from 82 pairs of human tumor tissues and matched tumor-adjacent normal tissues. The types of tumors included esophagus cancer, lung cancer, breast cancer, liver cancer, pancreatic cancer, gastric cancer, stromal tumor and colorectal cancer. Compared to the normal tissues, we revealed the level of m$^6$dA was increased in tumor tissues of esophagus cancer, lung cancer and liver cancer, whereas the level of m$^6$dA was diminished in tumor tissues of pancreatic cancer and gastric cancer; while the contents of 5-mdC and 5-hmdC exhibited significant decrease in tumor tissues of most types of cancer. It is worth noting that we revealed, for the first time, the content of genomic m$^6$dA in pancreatic cancer, stromal tumor and colorectal cancer. The significant changes of these DNA epigenetic modifications indicate they may serve as indicators of cancers. In addition, this study will benefit for better understanding of the regulatory roles of these DNA epigenetic modifications in cancers.

In the past few decades, DNA methylation has drawn great attention and tremendous efforts have been devoted into this exciting field. DNA cytosine methylation (5-methyl-2′-deoxycytidine, 5-mdC) is a widespread form of methylation in genome of mammals and has been involved in numerous crucial biological processes, such as the maintenance of advanced chromosome structure, genomic imprinting, transposon suppression and X chromosome inactivation [1,2]. And aberrant alteration of 5-mdC level contributes to inappropriate expression of tumor suppressor genes and oncogenes, leading to tumorigenesis [3,4].

Under the catalysis of ten-eleven translocation (TET) proteins, 5-mdC can be oxidized to 5-hydroxymethyl-2′-deoxycytidine (5-hmdC), 5-formyl-2′-deoxycytidine (5-fodC), and 5-carboxyl-2′-deoxycytidine (5-cadC) [5–7]. 5-hmdC which is considered as the sixth nucleoside in genome is especially enriched in the gene bodies and enhancers, and plays important roles on cellular differentiation and epigenetic regulation [8,9]. It has been demonstrated that compared with adjacent normal tissues, the level of 5-hmdC in tumor tissue is significantly lower, and the degree of reduction is proportional to tumor stage [10–14].

Apart from DNA cytosine methylation, methylation of DNA at the *N*$^6$ position of adenine gives rise to *N*$^6$-methyl-2′-deoxyadenosine (m$^6$dA), which is recently revealed to be an important epigenetic mark in eukaryotes [15]. It was found in the genomes of *Chlamydomona reinhardtii* [16], *Drosophila melanogaster* [17], *Caenorhabditis elegans* [18], *Xenopus laevis* [19], zebrafish, pigs [20] and mice [21]. In 2018, m$^6$dA is reported to be extensively present in the human genome [22]. Although some controversy

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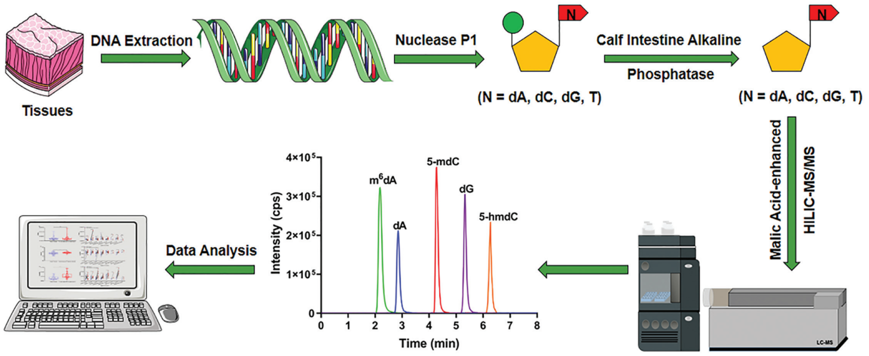

**Fig. 1.** Schematic diagram to illustrate the analytical procedures for the analysis of m$^6$dA, 5-mdC and 5-hmdC in human tissues. Genomic DNA was isolated from tumor and matched tumor-adjacent normal tissues from various types of cancer patients, followed by enzymatic digestion and malic acid-enhanced HILIC-MS/MS measurement.

exists in the realm about the mammalian origin of m$^6$dA (*e.g.*, the contamination of bacterial DNA), previous studies revealed that m$^6$dA participated in many life activities, such as transposon expression, embryonic development, intergenerational inheritance and tumorigenesis [18,20-22]. The level of m$^6$dA was dynamically regulated by methyltransferases and demethylases [22,23], and the aberrant level of m$^6$dA was correlated to human cancers including liver cancer [22], gastric cancer [22], glioblastoma [24], lung cancer [25], esophagus cancer [26] and breast cancer [27]. Recently, we confirmed the presence of m$^6$dA in human urine and revealed that the level of urinary m$^6$dA was diminished in gastric as well as colorectal cancer patients compared with healthy controls [28].

The abnormal DNA epigenetic modification is a characteristic hallmark of cancer, and deciphering the alteration of the contents of DNA epigenetic modifications will offer valuable information for better understanding of tumorigenesis and the underlying regulatory roles of these epigenetic modifications. From this point of view, analysis of these DNA epigenetic modifications in various types of cancer is desirable.

Compared with other analytical techniques, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been considered a powerful quantitative analytical platform for analysis of DNA/RNA epigenetic modifications due to its great advantages in selectivity, sensitivity and accuracy [28–37]. In our previous work, we found hydrophilic-interaction liquid chromatography (HILIC) coupled with MS/MS method has excellent detection sensitivity for analysis of modified nucleosides in human urine, especially when malic acid was used as a mobile phase additive [28,37]. Herein we systematically performed the evaluation of the alteration of genomic m$^6$dA, 5-mdC, and 5-hmdC in tumor tissues and matched tumor-adjacent normal tissues from various types of cancer patients by malic acid-enhanced HILIC-MS/MS analysis (Fig. 1). Moreover, stable isotope dilution method was used to realize the accurate quantification of these DNA epigenetic modifications.

A HILIC-MS/MS method to detect genomic m$^6$dA, 5-mdC and 5-hmdC was first established. The chemical structures of these DNA modifications and their stable isotope-labeled internal standards were illustrated in Fig. 2a. In order to avoid interference from canonical nucleosides on the multiple reaction monitoring (MRM) detection of modified nucleosides, effective separation of m$^6$dA, 5-mdC, 5-hmdC and eight canonical nucleosides (*i.e.*, rA, rC, rG, rU, dA, dC, dG, T) is needed. As shown in Fig. 2b, excellent separation was obtained by using a BEH Amide column (2.1 mm × 100 mm, 1.7 µm) under optimized chromatographic separation conditions. Besides, these eleven nucleosides could be rapidly separated within 8 min, which indicates the analytical method is capable for measurement of a large number of clinical samples. The optimized MRM parameters can be found in Table S1 (Supporting information).

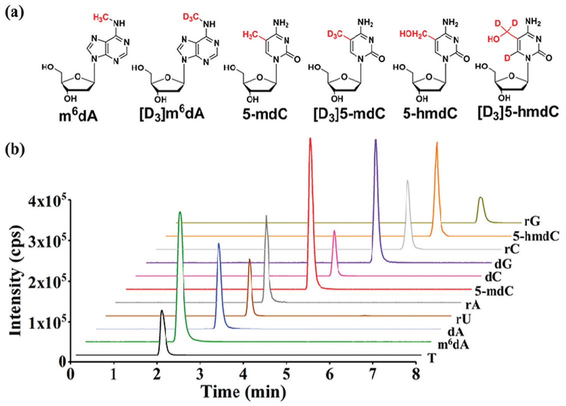

**Fig. 2.** (a) The chemical structures of m$^6$dA, 5-mdC, 5-hmdC and their stable isotope-labeled internal standards. (b) The MRM chromatograms of rA, rC, rG, rU, dA, dC, dG, T, m$^6$dA, 5-mdC and 5-hmdC standards. The concentration of rU was 300 nmol/L. For other nucleosides, the concentration was 100 nmol/L each.

We next evaluated the feasibility of the developed method. Parameters including linearity, limits of detection (LODs), limits of quantification (LOQs), intra- and inter-day precision and accuracy were evaluated. As shown in Table S2 (Supporting information), the calibration curve of each analyte showed excellent linearity with a coefficient value ($R^2 > 0.999$). The LODs of m$^6$dA, 5-mdC and 5-hmdC were 0.005 nmol/L, 0.01 nmol/L and 0.025 nmol/L, respectively, which were better than those previously reported [38,39]. The intra- and inter-day precision values were within 3.54%. The accuracy of the intra- and inter-day analysis was in the range of 94.79% to 104.84%, indicating that outstanding reproducibility and accuracy were achieved (Table S3 in Supporting information). These results declared that the sensitivity, precision and accuracy of established HILIC-MS/MS method could be guaranteed during measurement.

The validated HILIC-MS/MS method was then applied to measure genomic m$^6$dA, 5-mdC and 5-hmdC from 82 pairs of tumor tissues and matched tumor-adjacent normal tissues from various types of cancer patients, including esophagus cancer, lung cancer, breast cancer, liver cancer, pancreatic cancer, gastric cancer, stromal tumor and colorectal cancer. The information of subjects was listed in Table S4 (Supporting information). For the measurement of 5-mdC and 5-hmdC, 60 ng of DNA was injected, while 300 ng of DNA was injected for the analysis of m$^6$dA due to its extremely low abundance. The retention times of m$^6$dA, 5-mdC and 5-hmdC were identical to those of their corresponding isotope-labeled internal standards, while these modifications were not detectable

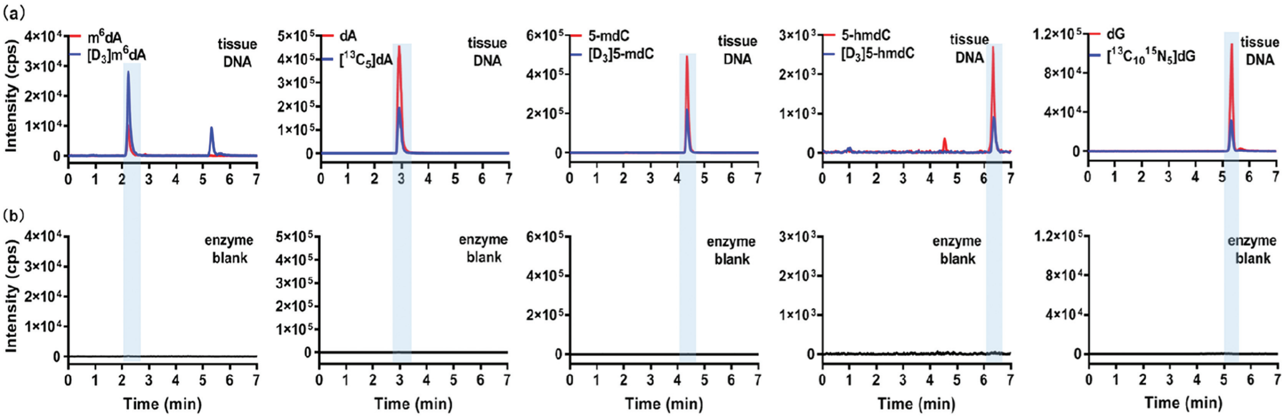

**Fig. 3.** Identification of m$^6$dA, dA, 5-mdC, 5-hmdC and dG in human tissues. (a) MRM chromatograms of m$^6$dA, dA, 5-mdC, 5-hmdC, dG and their corresponding stable isotope-labeled internal standards in a human tissue sample. (b) MRM chromatograms of m$^6$dA, dA, 5-mdC, 5-hmdC and dG in enzyme blank samples, which only contain enzymes used for digesting DNA.

in the enzyme control samples, further confirming these detected DNA epigenetic modifications were from DNA samples (Fig. 3).

We next quantified genomic m$^6$dA, 5-mdC and 5-hmdC in tumor tissues and matched tumor-adjacent normal tissues. The results showed that the contents of m$^6$dA, 5-mdC and 5-hmdC in all tumor tissues ranged from 0.000066% to 0.001% (m$^6$dA/dA), 1.58% to 3.16% (5-mdC/dG), and 0.007% to 0.080% (5-hmdC/dG), respectively. In all paracancerous tissues, the contents of m$^6$dA, 5-mdC and 5-hmdC ranged from 0.000056% to 0.0014% (m$^6$dA/dA), 2.31% to 3.15% (5-mdC/dG), and 0.011% to 0.138% (5-hmdC/dG), respectively (Table S5 in Supporting information).

The measured levels of these DNA epigenetic modifications were comparable to the previously reported levels in human genomic DNA [22,25,40]. Moreover, in order to exclude the contribution from bacterial DNA which carries abundant m$^6$dA, Dpn I digestion combined with size-exclusion ultrafiltration was performed [25]. As shown in Fig. S1 (Supporting information), the content of m$^6$dA in DNA treated with Dpn I was consistent with that in DNA without treatment of Dpn I, indicating there was no contamination of bacterial DNA in the genomic DNA extracted from tissue samples. It is worth noting that, to the best of our knowledge, this is the first time to achieve quantitative analysis of genomic m$^6$dA in tissues from pancreatic cancer, stromal tumor and colorectal cancer patients.

Then, we compared the levels of these DNA epigenetic modifications in tumor tissues and matched tumor-adjacent normal tissues. The mean contents of m$^6$dA in genomic DNA from tumor tissues and matched tumor-adjacent normal tissues ($n = 82$) were 0.00028% and 0.00031% (m$^6$dA/dA), respectively (Table S6 in Supporting information). The results showed that there was no significant difference in levels of m$^6$dA between tumor tissues and matched tumor-adjacent normal tissues (Fig. 4a). This could be attributed to that the level of m$^6$dA was elevated or diminished in different types of cancer. We found the level of m$^6$dA was significantly higher in tumor tissues of esophagus cancer ($P < 0.05$), lung cancer ($P < 0.01$) and liver cancer ($P < 0.05$) than matched tumor-adjacent normal tissues, whereas the level of m$^6$dA was significantly lower in tumor tissues of pancreatic cancer ($P < 0.01$) and gastric cancer ($P < 0.05$) than matched tumor-adjacent normal tissues (Fig. 4a). Previous studies also demonstrated the diminished level of m$^6$dA in gastric cancer and elevated level of m$^6$dA in glioblastoma [22,24]. However, there was no significant difference in the level of m$^6$dA between tumor tissues and normal tissues in breast cancer, stromal tumor and colorectal cancer ($P > 0.05$, Fig. 4a). These results suggest that m$^6$dA may play different roles in the initiation and development of different cancers.

As for 5-mdC, its mean content in tumor tissues and matched tumor-adjacent normal tissues ($n = 82$) was 2.65% and 2.77% (5-mdC/dG), respectively (Table S6 in Supporting information), and the content of 5-mdC was lower in tumor tissues than matched tumor-adjacent normal tissues ($P < 0.001$, Fig. 4b). We found that the level of 5-mdC was obviously lower in tumor tissues of esophagus cancer ($P < 0.01$), breast cancer ($P < 0.05$), liver cancer ($P < 0.05$) and gastric cancer ($P < 0.05$), compared to matched tumor-adjacent normal tissues; whereas there was no significant difference in level of 5-mdC between tumor tissues and normal tissues in lung cancer, pancreatic cancer, stromal tumor and colorectal cancer ($P > 0.05$, Fig. 4b).

The mean content of 5-hmdC in tumor tissues and matched tumor-adjacent normal tissues ($n = 82$) was 0.030% and 0.056% (5-hmdC/dG), respectively (Table S6 in Supporting information), and the content of 5-hmdC was also significantly lower in tumor tissues than matched tumor-adjacent normal tissues ($P < 0.0001$, Fig. 4c). We found that the level of 5-hmdC was obviously lower in tumor tissues of esophagus cancer ($P < 0.0001$), breast cancer ($P < 0.0001$), liver cancer ($P < 0.0001$), pancreatic cancer ($P < 0.05$) and gastric cancer ($P < 0.05$), compared to matched tumor-adjacent normal tissues; whereas there was no significant difference in the level of 5-hmdC between tumor tissues and normal tissues in lung cancer, stromal tumor and colorectal cancer ($P > 0.05$, Fig. 4c). In previous studies, the level of 5-hmdC was found to be dramatically diminished in several types of cancer, such as liver cancer, brain tumor, lung cancer, breast cancer, pancreatic cancer and colorectal cancer [13,40-42]. Our results, together with previous studies, revealed that the aberrant levels of DNA epigenetic modifications were tightly associated with cancer.

We further evaluated the potential of m$^6$dA, 5-mdC and 5-hmdC as biomarkers for the early detection and prognosis of human cancers by performing receiver operating characteristic (ROC) curve analysis. As shown in Fig. S2 (Supporting information), m$^6$dA was highly effective in the detection of lung cancer and pancreatic cancer, with the area under the curve (AUC) being 0.80 and 0.81, respectively. As for 5-mdC, it was an effective indicator of esophagus cancer (AUC = 0.83). The AUCs of 5-hmdC for liver cancer, esophagus cancer and breast cancer were 0.93, 0.92, and 0.98, respectively, indicating that 5-hmdC was a more effective indicator of these three types of cancer.

Currently, several tumor biomarkers were used in clinical practice for cancer screening. For instance, carcino-embryonic anti-

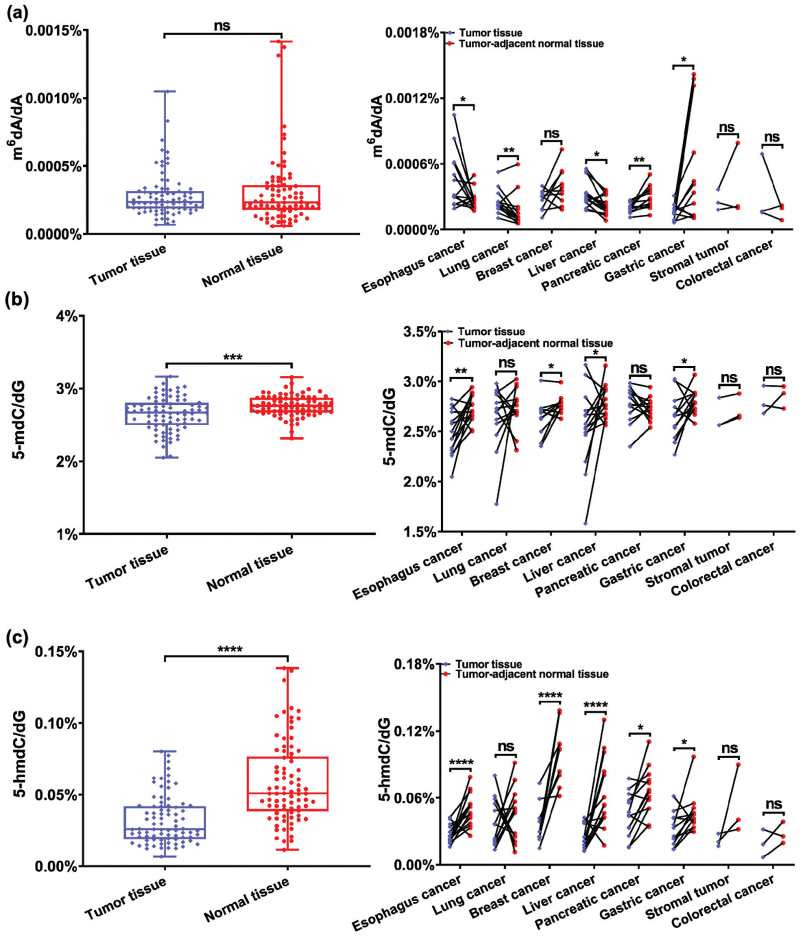

**Fig. 4.** Quantification results of (a) m$^6$dA, (b) 5-mdC, and (c) 5-hmdC in 82 pairs of tumor tissues and matched tumor-adjacent normal tissues. ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

gen (CEA) was commonly used for detection of multiple cancers. Herein, we also estimated Pearson correlation coefficients to evaluate the correlation between the levels of these DNA epigenetic modifications and the levels of several tumor biomarkers. As shown in Fig. S3 (Supporting information), the level of m$^6$dA was positively correlated with the level of CEA in lung cancer ($r = 0.8391$, $P = 0.0003$), whereas negatively correlated with carbohydrate antigen 242 (CA242) in gastric cancer ($r = -0.6084$, $P = 0.0358$). The level of 5-mdC was negatively correlated with the level of CEA ($r = -0.5888$, $P = 0.0267$) and squamous cell carcinoma associated antigen (SCCA) ($r = -0.9066$, $P < 0.0001$) in esophagus cancer. However, there was no significant correlation between the level of 5-hmdC and CEA, CA242 or SCCA.

DNA cytosine methylation (5-mdC) plays crucial roles in tumor pathogenesis, and hypermethylation of promoter CpG island in tumor-suppressor genes is a general characteristic of cancer. TET proteins could catalyze the oxidation of 5-mdC to form 5-hmdC which is recognized as an intermediate in DNA demethylation. It is common that a lower level of 5-hmdC is present in cancer [38–42]. Interestingly, we demonstrated that the global levels of 5-mdC and 5-hmdC were both diminished in tumor tissues of esophagus cancer, breast cancer, liver cancer and gastric cancer.

As a newly discovered DNA modification in eukaryotes, m$^6$dA has attracted great interest in the past few years. However, the content of genomic m$^6$dA in human tissues was seldom measured previously, which may be ascribed to the extremely low abundance of m$^6$dA. By using the developed sensitive malic acid-enhanced HILIC-MS/MS method, we found that m$^6$dA could be determined with a DNA sample of only 150 ng, and we accurately quantified, for the first time, the content of genomic m$^6$dA in tissues of pancreatic cancer, stromal tumor and colorectal cancer. We demonstrated the higher levels of m$^6$dA in tumor tissues of esophagus cancer, lung cancer and liver cancer, while the lower level of m$^6$dA in tumor tissues of pancreatic cancer and gastric cancer, compared to their matched normal tissues. This also indicates that m$^6$dA plays disparate roles in different types of cancer. Although the underlying mechanisms to elucidate the link between m$^6$dA and tumorigenesis remain unclear, the alteration of m$^6$dA may be attributed to the aberrant expression of upstream enzymes and the contributions of the methyltransferases, demethylases, reader and anti-reader proteins in initiation and development of cancer need to be further investigated.

In summary, we established a HILIC-MS/MS method for the accurate quantitation of m$^6$dA, 5-mdC and 5-hmdC in genomic DNA. With the developed method, we evaluated the alterations of these DNA epigenetic modifications in 82 pairs of tumor tissues and matched tumor-adjacent tissues from various types of cancer patients. It is worth mentioning that we revealed, for the first time, the content of genomic m$^6$dA in tissue samples from pancreatic cancer, stromal tumor and colorectal cancer patients. Compared with normal tissues, the level of m$^6$dA in tumor tissues was increased or decreased in different types of cancer, indicating the disparate roles of m$^6$dA. As for 5-mdC and 5-hmdC, their contents in tumor tissues were both diminished in most types of cancer studied, compared to normal tissues. Deciphering the alteration of these DNA epigenetic modifications in tumor tissues and normal tissues would provide valuable information for better understanding of carcinogenesis. Future investigation on the underlying mechanisms of the changes of these DNA epigenetic modifications may provide a new strategy for the treatment of cancer by targeting these epigenetic modifications and their regulating enzymes.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2022.108023.

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