Abstract. DNA methylation at the 5 position of cytosine (5-mC) is an epigenetic hallmark that is critical in various biological and pathological processes such as DNA methylation regulation, and initiation and development of cancers. 5-mC can be oxidized to 5-hydroxymethylcytosine (5-hmC) by the ten-eleven translocation family of DNA hydroxylases. Accumulating evidence has reported that loss of 5-hmC is associated with cancer development. However, its level in papillary thyroid carcinoma (PTC) remains unclear. The present study reports that the loss of 5-hmC is an epigenetic mark of PTCs, associated with their malignant biological behavior, providing diagnostic and predictive advantages over DNA hypomethylation (5-mC), an acknowledged epigenetic alteration in cancer. In addition, the 5-hmC staining levels were decreased in cases of micro-carcinoma with lymph node metastasis, which suggests that 5-hmC expression levels could be used as valuable biomarkers for predicting malignant potential and assist in the selection of therapeutic strategies in PTC; therefore, 5-hmC has the potential to provide a more precise direction for PTC therapy.

Introduction

Thyroid cancer is a class of heterogeneous diseases that have various subtypes with different biological behaviors. Papillary thyroid carcinoma (PTC) is the most common subtype of thyroid cancer, and its incidence has been increasing considerably in recent years (1). A majority of PTCs are considered to have a good prognosis, whereas others may behave more aggressively (2). The risk stratification in patients with PTC has important clinical implications for the selection of therapeutic strategies. Therefore, novel biomarkers are urgently required for predicting the malignant potential of primary lesions and clinical outcome. To the best of our knowledge, the present study is the first to provide an epigenetic biomarker to help identify the differences between benign and malignant lesions and to assist in predicting their biological behaviors.

Epigenetic modifications serve a key role in numerous biological and pathological processes. DNA methylation at the 5 position of cytosine (5-mC) acts as a key epigenetic marker that serves essential roles in genomic imprinting and regulation of gene expression (3). Aberrant DNA methylation is frequently observed in cancer. Hypermethylation of tumor suppressors and hypomethylation of oncogenes triggers tumorigenesis and tumor development (4). Disrupted DNA methylation patterns at the 5 position of cytosine have been substantiated as mechanism-based evidence in cancer (5). 5-mC can become oxidized to 5-hydroxymethylcytosine (5-hmC) by the ten-eleven translocation (TET) family of 5-mC hydroxylases, including TET1, 2 and 3 (6,7). Accumulating evidence has suggested that loss of 5-hmC is an epigenetic hallmark in various types of cancer, with diagnostic and prognostic implications (5,8-10). Aberrant methylation of various candidate genes have been reported potentially associated with thyroid carcinogenesis (11). However, the role of 5-hmC with regard to thyroid cancer remains largely unknown. In the present study, it was observed that the global level of 5-hmC is significantly decreased in PTC compared with benign thyroid disease. Furthermore, 5-hmC reduction is associated with potential malignant biological behavior, contributing to our present understanding of thyroid cancer epigenetics.

Materials and methods

Ethics statement. Thyroid cancer and nodular goiter samples were obtained from the archives of the Pathology Department of the First Affiliated Hospital of Dalian Medical University (Dalian, China), following the approval of the institutional review board. The Committee of Research Ethics waived the requirement of informed consent for the tissue sections used in this study, as patient data were anonymized.
Patient cohorts and tissue samples. Cancer tissue and adjacent matching healthy tissue samples from 88 patients with PTC and 20 patients with nodular goiter (NG) treated at the First Affiliated Hospital of Dalian Medical University (Dalian, China) between January 2015 and September 2017 were included in the present study. The cohort had an age range of 23-75 years with 26 males and 82 females. All tissue sections were reviewed by an expert pathologist for verification of the clinical diagnosis. Each tissue sample had at least three independent tissue sections for the following analyses.

Immunohistochemistry (IHC) staining. IHC was employed to analyze 5 mm tissue sections, which had been fixed in 10% formalin for 24 h at room temperature and paraffin-embedded. Sections were dewaxed and rehydrated following standard protocols (12). Subsequently, antigen retrieval was performed by boiling the sections for 5 min in citric acid. The sections were dipped in the endogenous peroxidase blocker (cat. no. PV-6000D; OriGene Technologies, Inc., Beijing, China) for 10 min at room temperature. For immunolabeling of 5-hmC and 5-mC, a rabbit monoclonal 5-hydroxymethylcytosine antibody (cat. no. ab214728; Abcam, Cambridge, UK) and a mouse monoclonal 5-methylcytosine antibody (cat. no. ab10805; Abcam) were applied at 1:200 and 1:100 dilution, respectively, for 1 h at room temperature. After the sections were washed with PBS, they were incubated with peroxidase-conjugated anti-rabbit/mouse immunoglobulin G (cat. no. PV-6000D; OriGene Technologies, Inc.; undiluted) at room temperature for 20 min, followed by DAB chromogenic reaction performed according to the protocol of the DAB chromogenic reagent kit (cat. no. ZLI-9019; OriGene Technologies, Inc.). The sections were then counterstained with hematoxylin at room temperature for 1 min, dehydrated with graded alcohol and xylene, and mounted onto coverslips. The stained cell images were captured under the light microscope (Olympus, Tokyo, Japan), and cells were counted and assessed at a magnification of x100.

Scoring system. Immunoreactivity of the nucleus was evaluated for each tissue section. In the case of NG, sections that indicated strong immunostaining in follicular cell nuclei were further evaluated. In the case of PTC, adjacent non-neoplastic cells were used as internal controls to evaluate the immunoreactivity. Thereafter, the H-score system was applied to evaluate positive immunoreactivity (9). Briefly, nuclei staining intensity (0, 1, 2 or 3) was first determined for each cell in a fixed field corresponding to the presence of negative, weak, intermediate and strong staining, respectively. The percentage of cells at each staining intensity level was subsequently calculated, and an H-score (0-300) was assigned using the following formula: H-score=0 x (% of cells staining 0) + 1 x (% of cells staining 1) + 2 x (% of cells staining 2) + 3 x (% of cells staining 3). H-scores for each case were calculated as the mean score of at least three individual section scores for each case, from which the mean score of all the individual field scores of each section was derived. In total 330 sections across 108 samples were analyzed.

Statistical analysis. The data are presented as either median ± interquartile range or the mean ± standard deviation and were analyzed using GraphPad Prism 7.00 software (GraphPad Software, Inc., La Jolla, CA, USA). The χ² test was used to analyze differences of clinical parameters between two groups of patients. A Student's t-test was used to analyze differences in 5-hmC level (H-scores). Pearson or Spearman’s rank correlation analysis was used to evaluate the correlation between 5-hmC level and pathological parameters. P<0.05 was considered to indicate a statistically significant difference.

Results

5-hmC expression level is high in NG and lost in PTC. To evaluate the global 5-hmC landscape, 5-hmC levels in the thyroid tissues were examined by IHC staining with a specific anti-5-hmC antibody in two representative cohorts (Table I), including PTC (n=88) and NG (n=20). IHC analysis of NG tissues indicated robust 5-hmC staining of the nucleus, whereas the cancer cells in PTC tissues exhibited reduced 5-hmC staining by comparison (Fig. 1A). The H-scores indicated that the 5-hmC level was significantly decreased in PTC tissues compared with NG tissues (P<0.0001; Fig. 1B). Low nuclear 5-hmC staining was also observed in thyroid cancer tissues compared with the adjacent non-neoplastic tissues (Fig. 1C and D). However, there was no difference in 5-mC levels between NG and PTC, as well as cancer and adjacent normal tissue (Fig. 2). These data indicate that a high level of 5-hmC is a distinct epigenetic signature in benign thyroid disease, and a significant loss of 5-hmC is a distinctive hallmark of thyroid cancer. In addition, the 5-hmC level has a more sensitive diagnostic value compared with 5-mC in PTC.

5-hmC is a useful molecular hallmark of potential PTC. 5-hmC levels between PTCs with lymph node metastases (N+)) and PTCs without lymph node metastases (N-) were analyzed by IHC in order to evaluate if 5-hmC level correlates with malignancy of PTC. H-score indicated that the 5-hmC level was significantly lower in PTCs with N+ (n=45) compared with

Table I. Clinical characteristics of NG and PTC cohort.

| Parameter          | NG (%) | PTC (%) | P-value (χ² test) |
|--------------------|--------|---------|------------------|
| Age at surgery (years) |        |         | 0.354            |
| <50                | 8 (40) | 48 (54.5) |                   |
| ≥50                | 12 (60)| 40 (45.5) |                   |
| Sex (%)            |        |         | 0.329            |
| Male               | 7 (35) | 19 (21.6)|                   |
| Female             | 13 (65)| 69 (78.4)|                   |
| Nodule size, cm    |        |         | 0.068            |
| ≤1                 | 6 (30) | 49 (55.7)|                   |
| >1                 | 14 (70)| 39 (44.3)|                   |
| Location           |        |         | 0.183            |
| Left lobe          | 7 (35) | 48 (54.5)|                   |
| Right lobe         | 13 (65)| 40 (45.5)|                   |

PTC, papillary thyroid carcinoma; NG, nodular goiter.
PTCs with N- (n=43; P=0.0039; Fig. 3A and B). Consistently, there was a negative correlation between 5-hmC staining and the number of metastatic lymph nodes, a predictor of malignant potential (Fig. 3A; r²=0.2104; P<0.0001). A correlation analysis was subsequently conducted comparing the 5-hmC level to tumor size (the largest diameter the tumor), with no correlation indicated, while the 5-hmC levels of micro-carcinomas with N+ (n=22) were lower compared than those of macro-carcinomas with N (n=21; P=0.0086; Fig. 3C). In addition, in the limited number of patients that exhibited tumor development during the follow-up duration (12-42 months), no significant correlation between cancer 5-hmC levels and cancer recurrence was indicated (P=0.3679; data not shown). Therefore, these results support the conclusion that a reduction in 5-hmC serves as a distinctive epigenetic event in the initiation and progression of PTC, suggesting it may represent a novel epigenetic hallmark for PTC recognition and prediction of malignancy.

**Discussion**

DNA methylation is an essential epigenetic modification that is often altered in cancer (13). In general, global 5-mC reduction at specific sites of the genome is associated with cancer progression (14,15). Current evidence suggests that the oxidation of 5-mC to 5-hmC serves a critical role in epigenetic plasticity (16-18). The family of TET enzymes are 5-methylcytosine oxidases that transform 5-mC to 5-hmC (19). 5-hmC is a key intermediate of DNA demethylation that can affect global gene expression in mammals (20). A number of previous studies have reported that the amount of 5-hmC is substantially decreased in various types of cancer, including in brain, lung, breast, liver, pancreatic, colon and prostate.
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5-hmC is an epigenetic biomarker in papillary thyroid carcinoma (PTC). However, the 5-hmC level in PTC remains unknown. In the present study, it was indicated that reduced 5-hmC is a characteristic epigenetic event in PTCs that is associated with malignant biological behavior. Given that 5-hmC is oxidized from 5-mC, and hypomethylation is an acknowledged epigenetic alteration in cancer (27), it was hypothesized that the underlying mechanism for the global decrease in 5-hmC levels in PTC is due to a reduction in 5-mC. However, it was indicated that 5-mC levels were similar between PTC and normal glands (NG), therefore the mechanism is derived from the oxidation process rather than 5-mC loss. As TET enzymes mediate the oxidation process of 5-mC to 5-hmC, and partial or complete loss of 5-hmC is frequently associated with significantly decreased expression of the three TET genes, suggesting a potential mechanism to explain the loss of 5-hmC in cancer cells (21). In addition, the catalytic oxidation reaction through TET enzyme requires the cofactor α-ketoglutarate (6,7), which is mainly controlled by isocitrate dehydrogenase (IDH). Therefore, understanding the role of 5-hmC, TET and IDH involved in PTC initiation and progression is crucial. Further examination of the underlying mechanism of this epigenetic marker is required, in addition to the role of the associated enzymes involved in PTC.

PTC is a unique cancer, and the prognosis varies widely. PTCs measuring <10 mm at the largest diameter are termed micro-carcinomas (28). Due to the advent of high-resolution ultrasound detection and increasing regular fine-needle aspiration biopsies, the incidence of micro-carcinomas has increased worldwide (29). Micro-carcinomas are often considered as indolent tumors and have a good prognosis; however, a small proportion of these tumors are capable of metastasizing when the volume of the original tumor is estimated to be as little as 1 mm³ (29). Accumulating evidence indicates that surgical excision is not recommended for micro-carcinomas, particularly indolent micro-carcinomas (30,31). Radiofrequency ablation can effectively eliminate low-risk micro-carcinomas with a low complication rate (32,33). Therefore, distinguishing aggressive from indolent micro-carcinomas has important clinical implications for the selection of therapeutic strategies. Current routine characteristics, based on clinical assessment and pathology, cannot explicitly distinguish aggressive from indolent PTCs at an early phase (34), and, therefore, uncovering novel prognostic biomarkers is required. In the present study, it was indicated that 5-hmC expression levels in micro-carcinomas with metastasis (high risk) is lower compared with macro-carcinoma without metastasis (low risk), which suggests that the level of 5-hmC may serve as a valuable biomarker for predicting the malignant potential of PTC and assist in the selection of therapeutic strategies.

This study also endeavors to evaluate the patients’ survival data by directly associating the low 5-hmC levels with PTC prognosis. However, recurrence of PTC following optimized surgery is uncommon, and the latent durations prior to detection of distant and lethal metastases may vary from years to decades (35). The follow-up duration of the current study is not long enough to observe the prognostic value of 5-hmC in PTC. Studies of hallmarks used for predicting clinical outcome of thyroid cancer also have this limitation, as investigation is hindered by the barriers of the follow-up interviews. Consequently, clinically annotated biospecimen archives can serve as valuable substitutes for theoretical and impractical prospective strategies.
The present study demonstrates that global 5-hmC levels are greatly diminished in the majority of PTCs. Ongoing mechanistic investigations and identification of target genes through comprehensive genome-wide mapping will shed further light on cancer epigenetics (5,36-38). In the present study, 5-hmC loss in PTC was a basic epigenetic event that suggested that fundamental molecules in the 5-hmC biological pathway can be therapeutically targeted to re-establish the level of 5-hmC, and therefore reveal novel approaches in the therapy of aggressive PTC. With the significance of clinical therapeutics, the current study provides a novel direction for cancer prevention and treatment, by targeting the cellular molecules and biochemical pathways that restore the landscape of 5-hmC in PTC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the author on reasonable request.

Authors' contributions

MT, SG and WQ performed experiments, analyzed the data and wrote the paper. MQ, ZS, CS, ZW, FY, HL and SB carried out additional analyses and supported the study. YC and LW jointly designed, oversaw and directed the study. All authors read and approved the final version of this manuscript.

Ethics approval and consent to participate

Thyroid cancer and nodular goiter samples were obtained from the archives of the Pathology Department of the First Affiliated Hospital of Dalian Medical University (Dalian, China) following the approval of the committee of research ethics. The committee of research ethics of the First Affiliated Hospital of Dalian Medical University (Dalian, China) waived the requirement of informed consent for the tissue sections used in this study as patient data were anonymized.

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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