Abstract

**Background:** This study aimed to evaluate the correlations of 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), and ten-eleven translocation enzyme 2 (TET2) expressions in lesion tissue with histological classification of breast precancerous lesion.

**Methods:** Eighty-three patients with breast ductal intraepithelial neoplasia (DIN), 20 patients with breast ductal carcinoma in situ with microinvasion (DCIS-MI), and 10 patients with invasive breast cancer were included. Histological classification of the DIN patients was classified as DIN1A, DIN1B, DIN1C, DIN2, and DIN3. 5mC, 5hmC, and TET2 expressions in lesion tissues from biopsy were assessed by immunohistochemistry (IHC) assay.

**Results:** 5hmC and TET2 were negatively associated with histological classification as validated by both IHC score and IHC semi-quantification expression grades in total patients (all \( P < .05 \)); however, no correlation of 5mC with histological classification was found (all \( P > .05 \)). 5mC (\( P = .004 \)) was negatively but 5hmC (\( P < .001 \)) was positively correlated with TET2, while no association of 5mC with 5hmC was discovered in total patients (\( P = .078 \)). In addition, 5mC was positively associated with ER expression in total patients (\( P = .040 \)). In subgroups, 5mC was negatively correlated with 5hmC in DIN1C patients (\( P = .023 \)) and invasive cancer patients (\( P = .044 \)), and 5mC was negatively associated with TET2 in DIN1B patients (\( P = .004 \)) as well as DCIS-MI patients (\( P = .003 \)).

**Conclusion:** 5hmC and TET2 have the potentials to serve as biomarkers that could assist in the identification of presence and progression of breast precancerous lesion.

**KEYWORDS**
5-methylcytosine, 5-hydroxymethylcytosine, biomarker, breast precancerous lesion, ten-eleven translocation enzyme 2

1 INTRODUCTION

Breast cancer, the most frequent cancer in women worldwide, attacks approximately 2 088 849 patients and results in roughly 626 679 related deaths in 2018.1 Breast cancer is featured by the heterogeneous molecular subtypes, such as luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) enriched type, and basal-like breast cancer, which is closely related to the therapeutic decision-making and prognosis in clinical setting.2 In Asia, an elevation of breast cancer incidence could be seen as a
result of the change of lifestyle and progress of screening program, and mortality is also rising due to lacking timely intervention and leading-edge treatment strategy. Thus, early diagnosis and timely intervention are of note in optimizing the prognosis of patients with breast cancer. In this respect, the identification of breast precancerous lesion is a critical and effective method in enhancing early diagnosis and timely intervention in patients with breast cancer.

Five-methylcytosine (5mC) is a regulator of DNA methylation, which is a process crucial to tumorigenesis; in addition, 5mC can be switched into 5-hydroxymethylcytosine (5hmC) by the activations of ten-eleven translocation (TET) family, including TET2. Previous studies have revealed the roles of 5mC, 5hmC, and TET2 in multiple cancers, including regulating cancer progression and serving as diagnostic or prognostic biomarkers in clinical practice. More importantly, there are reports illuminating that 5mC, 5hmC, and TET2 also participate in breast cancer etiology, including the regulation of tumor suppressor genes and DNA methylation. Since that 5mC and 5hmC have been reported in the modulation of precancerous pathogenesis, such as, 5mC is involved in the stemness in cervical precancerous lesion and 5mC is one of the crucial regulators of DNA methylation dysregulation in colorectal precancerous lesion, and TET2 can catalyze 5mC to 5hmC, we speculated that 5mC, 5hmC, and TET2 may be correlated with the existence or progression of breast precancerous lesion.

Thus, this study aimed to evaluate the correlations of 5mC, 5hmC, and TET2 expressions in lesion tissue with histological classification of breast precancerous lesion.

2 | MATERIALS AND METHODS

2.1 | Patients and specimens

A total of 83 patients with breast ductal intraepithelial neoplasia (DIN), 20 patients with breast ductal carcinoma in situ with microinvasion (DCIS-MI), and 10 patients with invasive breast cancer were retrospectively screened from Huashan Hospital, Fudan University between January 2015 and December 2017 and analyzed in this study. All patients’ diagnosis was confirmed by excisional biopsy and pathological examination, and the formalin-fixed paraffin-embedded (FFPE) pathological sections were collected form Pathology Department of Huashan Hospital, Fudan University. This study was approved by the Institutional Review Board of Huashan Hospital, Fudan University, and all patients or their guardians provided the written informed consent.

2.2 | Data collection

Patients’ age, histological classification, estrogen receptor (ER) status, progesterone receptor (PR) status, human epidermal growth factor receptor-2 (HER2) status, and Ki-67 level were collected from the Case Database of Huashan Hospital, Fudan University. For the histological classification, DIN was classified as DIN1A (corresponding to flat epithelial atypia), DIN1B (corresponding to atypical ductal hyperplasia), DIN1C (corresponding to DCIS grade 1), DIN2 (corresponding to DCIS grade 2), and DIN3 (corresponding to DCIS grade 3), which was referring to World Health Organization: Classification of Tumours: Pathology and Genetics. Tumours of the Breast and Female Genital Organs (Lyon: IARC Press, 2003). Besides, the ER, PR, and HER2 status and the Ki-67 level were determined by immunohistochemical (IHC) assay. A cutoff value of ≥1% of the positively stained nuclei was used to define ER and PR positivity, and HER2 status was identified according to the ASCO/CAP Guideline. In addition, as recommended by the St Gallen International Expert Consensus, a cut-point of ≥14% of Ki-67-positive cells was used to define Ki-67 high expression.

2.3 | IHC assay for 5mC, 5hmC, and TET2

Four-micron FFPE sections were baked in the oven, deparaffinized in xylene, and rehydrated in graded ethanol; then, heat-induced epitope retrieval was carried out in citrate buffer with microwave heating. Next, the sections were treated with H2O2 to block endogenous peroxidase, followed by normal goat serum for blocking of nonspecific binding. After that, the sections were incubated with primary antibody overnight at 4°C. For 5mC, the mouse monoclonal antibody to 5mC (1:100 dilution, Active Motif) was used as primary antibody; for 5hmC, the rabbit polyclonal antibody to 5hmC (1:1000 dilution, Active Motif) was used as primary antibody; and as for TET2, rabbit polyclonal antibody to TET2 (1:200 dilution, ABclonal) was used as primary antibody. Next day, the sections were incubated with horseradish peroxidase-conjugated goat-anti-mouse (for 5mC) immunoglobulin G antibody (1:2000 dilution, Abcam) or goat-anti-rabbit (for 5hmC and TET2) immunoglobulin G antibody (1:2000 dilution, Abcam). Subsequently, the sections were stained with DAB kit (Dako), followed by counterstaining with hematoxylin (Sigma-Aldrich). Finally, the sections were viewed and photographed on a microscope (Nikon Instruments).

2.4 | IHC staining evaluation of 5mC, 5hmC, and TET2

IHC staining assessment of 5mC, 5hmC, and TET2 was based on the density and intensity of positively stained cells. The staining density was evaluated according to the percentage of positively stained tumor cells, and 100 cells in 5 high-power fields were counted for evaluating the percentage of positive cells, which were scored as: 0 (0%), 1(<25%), 2 (26%-50%), 3 (51%-75%), and 4 (>75%). The staining intensity was scored as: 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The IHC score was calculated trough multiplying the density score by the intensity score, which ranged from 0 to 12. IHC semi-quantification: based on the IHC score, the 5mC/5hmC/TET2 expression was classified into 4 grades: IHC score
0-3, negative (−) expression; IHC score 4-6, positive (+) expression; IHC score 7-9, positive (++) expression; and IHC score 10-12, positive (+++) expression.

### 2.5 Statistical analysis

Data were presented as mean and standard deviation (SD) or number (percentage). Correlation was analyzed by Spearman's rank test or linear-by-linear association. SPSS 21.0 statistical software (IBM) was used for statistical data processing, and the GraphPad Prism 7.02 (GraphPad Software Inc) was used to plot graphs. P value < .05 was considered statistically significant.

### 3 RESULTS

#### 3.1 Patients' characteristics

The total of 113 patients had a mean age of 54.1 ± 11.5 years, and the numbers of patients with DIN1A, DIN1B, DIN1C, DIN2, DIN3, DCIS-MI, and invasive cancer were 3 (2.7%), 20 (17.7%), 20 (17.7%), 20 (17.7%), 20 (17.7%), 20 (17.7%), and 10 (8.8%), respectively (Table 1).

In addition, the percentages of patients with ER positive, PR positive, and HER2 positive were 76 (67.3%), 69 (61.1%), and 30 (26.5%), respectively. The numbers of patients who had Ki-67 expression <14%, ≥14%, and unknown expression were 68 (60.2%), 44 (38.9%), and 1 (0.9%), respectively. The other detailed information was listed in Table 1.

#### 3.2 Associations of 5mC, 5hmC, and TET2 with histological classification

Examples of 5mC, 5hmC, and TET2 expressions in patients with different histological classification were presented in Figure 1, and further analyses disclosed that 5mC was not correlated with histological classification as demonstrated by IHC score (P = .150) (Figure 2A) and IHC semi-quantification expression grades (P = .300) (Figure 2B). However, 5hmC expression was negatively correlated with histological classification, which was validated by both IHC score (P < .001) (Figure 2C) and IHC semi-quantification

| TABLE 1 Characteristics of patients |
|-------------------------------------|
| Characteristics | Patients (N = 113) |
| Age (years), mean ± SD | 54.1 ± 11.5 |
| Histological classification, No. (%) |
| DIN1A | 3 (2.7) |
| DIN1B | 20 (17.7) |
| DIN1C | 20 (17.7) |
| DIN2 | 20 (17.7) |
| DIN3 | 20 (17.7) |
| DCIS-MI | 20 (17.7) |
| Invasive cancer | 10 (8.8) |
| ER status, No. (%) |
| Negative | 37 (32.7) |
| Positive | 76 (67.3) |
| PR status, No. (%) |
| Negative | 44 (38.9) |
| Positive | 69 (61.1) |
| HER2 status, No. (%) |
| Negative | 76 (67.3) |
| Positive | 30 (26.5) |
| Unknown | 7 (6.2) |
| Ki-67 expression, No. (%) |
| <14% | 68 (60.2) |
| ≥14% | 44 (38.9) |
| Unknown | 1 (0.9) |

Abbreviations: DCIS-MI, ductal carcinoma in situ with microinvasion; DIN, ductal intraepithelial neoplasia; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor; SD, standard deviation.

**FIGURE 1** Examples of 5mC, 5hmC, and TET2 expressions. The 5mC, 5hmC, and TET2 expressions shown by IHC staining in patients with DIN1A, DIN1B, DIN1C, DIN2, DIN3, DCIS-MI, and invasive cancer. 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; TET2, ten-eleven translocation enzyme 2; DIN, ductal intraepithelial neoplasia; DCIS-MI, ductal carcinoma in situ with microinvasion.
FIGURE 2  Correlations of 5mC, 5hmC, and TET2 with histological classification. Correlations of 5mC IHC score (A), percentage of patients with different 5mC expression categorized by IHC semi-quantification (B), 5hmC IHC score (C), proportion of patients with different 5hmC expression categorized by IHC semi-quantification (D), TET2 IHC score (E), and percentage of patients with different TET2 expression categorized by IHC semi-quantification (F) with histological classification. Comparison among groups was determined by Spearman's rank test. P value < .05 was considered statistically significant. 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; TET2, ten-eleven translocation enzyme 2
expression grades \((P = .001)\) (Figure 2D). As to TET2, it was also negatively correlated with the histopathological classification as illustrated by IHC score \((P < .001)\) (Figure 2E) and IHC semi-quantification expression grades \((P < .001)\) (Figure 2F). These results indicated that 5mC expression was not correlated with histopathological classification, while the 5hmC and TET2 expressions were negatively associated with histological classification of the breast precancerous lesion.

### 3.3 Associations among 5mC, 5hmC, and TET2

In total patients, 5mC expression was negatively correlated with TET2 expression \((r = -.270, P = .004)\), and 5hmC expression was positively associated with TET2 expression \((r = .374, P < .001)\); however, no correlation of 5mC with 5hmC was found \((r = -.167, P = .078)\) (Table 2). As for the correlations among 5mC, 5hmC, and TET2 in subgroups, 5mC was negatively associated with 5hmC in DIN1C patients \((r = -.507, P = .023)\) and invasive cancer patients \((r = -.646, P = .044)\), and 5mC was also negatively correlated with TET2 in DIN1B patients \((r = -.619, P = .004)\) and DCIS-MI patients \((r = -.627, P = .003)\) (Table 3). Additionally, no correlation of 5hmC with TET2 was found in any subgroups \((all \ P > .05)\).

### 3.4 Associations of 5mC, 5hmC, and TET2 with ER/PR/HER2/Ki-67 expressions

In total patients, 5mC expression was positively correlated with ER expression \((r = .194, P = .040)\) but not PR expression \((P = .247)\), HER2 expression \((P = .104)\), or Ki-67 expression \((P = .179)\) (Table 4). In addition, no correlation of 5hmC with ER \((P = .344)\), PR \((P = .272)\), HER2 \((P = .388)\), or Ki-67 \((P = .453)\), PR \((P = .470)\), HER2 \((P = .752)\), or Ki-67 \((P = .297)\) expression, either.

### 4 DISCUSSION

In this study, the correlations of 5mC, 5hmC, and TET2 expressions with histopathological classification of breast precancerous lesion and the associations among 5mC, 5hmC, and TET2 expressions in total patients as well as in subgroups were evaluated, and the results illustrated that (a) 5hmC and TET2 expressions were negatively associated with histopathological grades of breast precancerous lesion; (b) negative correlation between 5mC and TET2, and positive association between 5hmC and TET2 expressions were found; and (c) in subgroups, negative correlations of 5mC with 5hmC in DIN1C and invasive cancer patients and negative associations of 5mC with TET2 in DIN1B and DCIS-MI patients were discovered.

As predominant regulators of DNA methylation, 5mC and 5hmC are abundantly investigated in malignancies, and as one of the most important enzymes that regulate their transversions, TET2 is also increasingly investigated in multiple carcinomas. Regarding 5mC in oncology, a previous study reveals that the decrease in 5mC level in tumor tissue predicts progression (invasion to the muscle) of patients with urothelial carcinoma.22 However, another former study elucidates that 5mC enhances progression of bladder cancer by activating the oncogenes.23 These findings indicate a dual role of 5mC in tumorigenesis. As for 5hmC, a previous study reveals that epigenetically reduction of

### TABLE 2 Correlation among 5mC/5hmC/TET2 in total patients

| Status     | 5hmC | TET2 |
|------------|------|------|
| 5mC        |      |      |
| Negative (-) |     |      |
| Positive (+) |     |      |
| Positive (+++) |   |      |
| Positive (++++) | |     |
| r          | -.167 | -.270 |
| P value    | .078  | .004  |

| Status     | 5hmC | TET2 |
|------------|------|------|
| 5hmC        |      |      |
| Negative (-) |     |      |
| Positive (+) |     |      |
| Positive (+++) |   |      |
| Positive (++++) | |     |

Note: Correlation was determined by Spearman's rank correlation test. Abbreviations: 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; TET2, ten-eleven translocation enzyme 2.
TET3 expression decreases the 5hmC expression in both tumor tissue and cancer cells, and this process subsequently advocates the tumor progression of glioblastoma in vitro. 23 And another study illustrates that restoring the 5hmC using ascorbate represses the tumor growth of clear-cell renal cell carcinoma in vitro. 24 And a previous study elucidates that decline of 5hmC level in tumor tissue correlates with the existence of papillary thyroid carcinoma (PTC) and malignant behavior of PTC in vitro. 25 These results suggest that 5hmC functions as a regulator repressing tumorigenesis. As to TET2, it has been reported to enhance the immunity and treatment efficacy of anti-PD-L1 agents in human colon cancer by modulating the interferon-γ (IFNγ)-JAK-STAT signaling pathway. 26 And a lack of TET2 in B cells contributes

| Items | 5mC and 5hmC | 5mC and TET2 | 5hmC and TET2 |
|-------|--------------|--------------|---------------|
| DIN1A* (n = 3) | - | - | - |
| DIN1B (n = 20) | −.250 .287 | −.619 .004 | .246 .296 |
| DIN1C (n = 20) | −.507 .023 | −.061 .798 | .390 .089 |
| DIN2 (n = 20) | −.237 .315 | −.363 .116 | .296 .206 |
| DIN3 (n = 20) | −.045 .852 | .023 .924 | .136 .567 |
| DCIS-MI (n = 20) | .048 .839 | −.627 .003 | .343 .138 |
| Invasive cancer (n = 10) | −.646 .044 | −.361 .305 | .373 .289 |

Note: Correlation was determined by Spearman’s rank correlation test. Abbreviations: 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; DCIS-MI, ductal carcinoma in situ with microinvasion; DIN, ductal intraepithelial neoplasia; TET2, ten-eleven translocation enzyme 2.

*Correlation was unable to assess due to only 3 patients in the subgroup.

TABLE 4 | Correlation of 5mC/5hmC/TET2 with ER/PR/HER2/Ki-67 in total patients |
|-------------------|-------------------|-------------------|-------------------|-------------------|
| Status            | ER Negative       | ER Positive       | PR Negative       | PR Positive       |
|                   |                   |                   |                   |                   |
| 5mC               |                   |                   |                   |                   |
| Negative (-)      | 8                 | 12                | 8                 | 12                |
| Positive (+)      | 23                | 35                | 26                | 32                |
| Positive (+++)    | 6                 | 28                | 10                | 24                |
| Positive (+++)    | 0                 | 1                 | 0                 | 1                 |
| Pearson's r       | .194              | .109              | −.159             | −.128             |
| P value           | .040              | .247              | .104              | .179              |
| 5hmC              |                   |                   |                   |                   |
| Negative (-)      | 6                 | 18                | 7                 | 17                |
| Positive (+)      | 20                | 42                | 24                | 38                |
| Positive (+++)    | 11                | 14                | 13                | 12                |
| Positive (+++)    | 0                 | 2                 | 0                 | 2                 |
| Pearson's r       | −.098             | −.104             | .084              | .089              |
| P value           | .344              | .272              | .388              | .350              |
| TET2              |                   |                   |                   |                   |
| Negative (-)      | 11                | 22                | 13                | 20                |
| Positive (+)      | 18                | 32                | 21                | 29                |
| Positive (+++)    | 8                 | 19                | 10                | 17                |
| Positive (+++)    | 0                 | 3                 | 0                 | 3                 |
| Pearson's r       | .071              | .068              | −.031             | −.099             |
| P value           | .453              | .470              | .752              | .297              |

Note: Correlation was determined by the linear-by-linear association. Abbreviations: 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; ER, estrogen receptor; HER2, human epidermal growth factor receptor-2; PR, progesterone receptor; TET2, ten-eleven translocation enzyme 2.
to germinal center hyperplasia, damage in class switch recombination, blocking of plasma cell differentiation, and a B cell lymphomagenesis.\textsuperscript{27} In addition, as the oxygenase of 5mC, TET2 has been reported to catalyze the conversion of 5mC to 5hmC progressively in an iterative manner in multiple cell lines.\textsuperscript{28} These studies indicated that TET2 acts as a factor inhibiting tumorigenesis. As for the functions of 5mC, 5hmC, and TET2 in precancerous pathogenesis, it has been reported that TET1 advocates 5hmC-dependent stemness of the cervical precancerous lesion in vitro.\textsuperscript{16} However, to our best knowledge, no direct effect of 5mC or TET2 on precancerous pathogenesis has been reported. Considering that 5mC and TET2 are both closely related to the regulation of DNA methylation, it might be reasonable to hypothesize that 5mC and TET2 also participate in the etiology of the existence or progression of precancerous lesion. In this study, we found that 5hmC and TET2 expressions were both negatively correlated with the histological classification, and here were several possible explanations to the results in our study: (a) 5hmC and TET2 might prevent the formation or repress the progression of breast precancerous lesion via modulating stemness or other related processes, which still need more in vivo and in vitro experiments to validate; (b) 5mC and TET2 might also inhibit precancerous lesion development and progression via similar mechanism by which they suppress the cancer progression, for instance, repressing malignant behaviors of cancer cells by mediating multiple oncogenic signaling pathways.\textsuperscript{9,16,22,29} However, we did not find a statistically significant correlation between 5mC and histopathological classification, which may be due to the dual role of 5mC in cancer pathogenesis found in other cancers, or a relatively small sample size.\textsuperscript{9,22}

In addition, we also found in our study that 5mC was negatively whereas 5hmC was positively correlated with TET2, while in subgroups, 5mC was negatively correlated with 5hmC in DIN1C and invasive cancer patients, and 5mC was negatively correlated with TET2 in DIN1B and DCIS-MI patients. The interactions among 5mC, 5hmC, and TET2 have already been demonstrated in other cancers previously. For instance, a previous study elucidates that in HCC cells, 5-azacytidine starts a TET2-dependent demethylation activity which increases 5hmC expressions.\textsuperscript{29} Another study reveals that the reduction of 5hmC in human blood cells is partially dependent on the mutation of TET2 gene in normal individuals.\textsuperscript{30} And a priori study reveals that idarubicin provokes the 5hmC formation in four cancer cell lines in a TET2 dependent mechanism.\textsuperscript{30} In addition, a study reports that methyl-CpG-binding domain protein 3-like 2 is able to enhance the enzymatic activity of TET2 to regulate the 5mC oxidation in human embryonic kidney cells (293T) and human acute lymphoblastic leukemia Jurkat cells.\textsuperscript{31} As for the association between 5mC and 5hmC, it has been reported for almost a decade that 5mC could be converted to 5hmC by the TET family through the utilization of oxygen, Fe, and u-ketoglutarate.\textsuperscript{32,33} These previous findings could provide some information on the results in our study that 5mC was negatively while 5hmC was positively associated with TET2 in total patients. However, the molecular mechanisms of the interactions among 5mC, 5hmC, and TET2 breast precancerous lesion still need more investigations in the future.

Furthermore, several limitations should be noticed in this study: (a) the sample size was relatively small, which might slightly reduce the statistical power; (b) the correlations of 5mC, 5hmC, and TET2 expressions with survival profiles of patients with breast precancerous lesion and patients with invasive cancer in our study were not evaluated; and (c) the molecular interactions among 5mC, 5hmC, and TET2 in breast precancerous lesion were not assessed in this study. Thus, cohort studies with larger sample size and prognostic analyses and experiments investigating the molecular activities among 5mC, 5hmC, and TET2 in patients with breast precancerous lesion should be conducted in the future.

In conclusion, 5mC and TET2 have the potentials to serve as biomarkers that could assist in the identification of breast precancerous lesion.

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CONFLICT OF INTEREST
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