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Appraising histone H4 lysine 5 lactylation as a novel biomarker in breast cancer

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Background Posttranslational modifications of histone lysine (K) have integral connections with cell metabolism, and participate in the carcinogenesis of various cancers. This study focuses on evaluating the expression of histone H4 lys 5 lactylation (H4K5lac) and its clinical role in breast cancer (BC). **Methods** During this research, immunohistochemistry (IHC) and immunoblotting, utilizing a specific primary anti-L-lactyl-histone H4 (Lys 5) rabbit monoclonal antibody, were employed to assess H4K5lac expression in BC tissue chips. H4K5lac expression in the peripheral blood mononuclear cells (PBMCs) of BC patients was investigated through immunoblotting. **Results** IHC revealed upregulation of histone H4K5lac in both triple-negative breast cancer (TNBC) and non-TNBC tissues, with positive rate of 91.40% [170/(150 + 19 + 17)] and 93.64% (103/110) in TNBC and non-TNBC tissues, respectively. The expression of H4K5lac demonstrated positive correlations with lymph nodes (%), and Ki-67 expression. Survival analysis indicated a negative correlation between H4K5lac expression and overall survival (OS) time in both TNBC (HR [hazard ratio] = 2.773, 95%CI [confidence interval]: 1.128–6.851, $P = 0.0384$) and non-TNBC cases (HR = 2.156, 95%CI: 1.011–4.599, $P = 0.0275$). Furthermore, elevated levels of H4K5lac were observed in the PBMCs of BC cases, and H4K5lac expression is positively correlated with serum lactate and carcinoma embryonic antigen (CEA) levels. **Conclusions** Histone H4K5lac exhibits increased levels in both BC tissues and PBMCs, suggesting its potential as a promising biomarker for BC. This study might pave the way toward novel lactylation treatment strategies in BC.

Keywords Breast cancer, Histone H4K5lac, Lactylation, Prognosis, Biomarker

Abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myelocytic leukemia
BC	breast cancer
BRD4	bromodomain containing protein 4
CCM	cerebral cavernous malformation
ER	estrogen receptor
HDACs	histone deacetylases
H4K5lac	H4 lys 5 lactylation
HR	hazard ratio
HG	histologic grading
IHC	immunohistochemistry
K	lysine
OS	overall survival
PBMCs	peripheral blood mononuclear cells
PTM	post-translational modification
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TME	tumor microenvironment

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TNBC	triple-negative breast cancer
VHL	von Hippel-Lindau
YTHDF2	YTH domain family protein 2

Breast cancer (BC) ranks among the most prevalent malignancies affecting women globally, with approximately 2.26 million new cases diagnosed in 2020 according to Global Cancer Statistics, representing the highest incidence rate among all cancers[1]. The intricate pathogenesis of BC involves multifactorial, multistep, and multigene co-regulated processes within tumor microenvironment (TME), encompassing complex molecular biological processes[2,3]. In recent years, advances in molecular biology technologies, such as high-throughput sequencing, have unveiled numerous BC-related genes and proteins, offering valuable insights for the exploration and development of potential biomarkers[4,5].

Post-translational modification of histone or non-histone lysine (K) emerges as a pivotal player in the initiation and progression of malignant tumors[6,7]. A recent breakthrough by Zhao's group in 2019 in protein post-translational modification is the discovery of lysine lactylation, actively regulated by lactic acid, a glycolytic metabolite[8]. In human cancer cells, mass spectrometry revealed a 72.021Da mass shift on lysine residues by mass spectrometry, matching the attachment of a lactyl group to the ε-amino group of a lysine residue, facilitated by glycolytic metabolite—lactate[8]. Lactylation is intricately linked to tumors, influencing metabolic alterations, epigenetic modification, immune escape, and immunosurveillance[9,10]. Histones, fundamental proteins in eukaryotic chromatin, being composed of core histones (H2A, H2B, H3, and H4) and linker histones (H1 and H5)[7,11]. Post-translational modifications of certain histone amino acid residues alter chromatin configuration, modulating transcriptional activation or gene silencing[12,13]. Histone lactylation has been implicated in the regulation of tumor initiation and progression[9,14]. Yu et al. explained that von Hippel-Lindau (VHL) inactivation participates in the pathogenesis of clear cell renal cell carcinoma by triggering histone lactylation, establishing a positive feedback loop with PDGFRβ signaling[15]. Additionally, YTH domain family protein 2 (YTHDF2), an m6A reader protein, is activated by histone lactylation, offering new therapeutic targets for ocular melanoma[15]. However, the role of histone lactylation in BC, especially at the clinical application level, remains underexplored. The status of histone lactylation in BC and its potential as a biomarker for BC remains undisclosed. The development of the Anti-L-Lactyl-Histone H4 (Lys 5) Rabbit Monoclonal Antibody has facilitated our examination of histone H4K5lac expression in BC and its clinical prognostic implications. Our preliminary findings offer new evidence, paving the way for extended research and evaluation of the clinical utility of histone lactylation in BC.

Materials and methods

Clinical samples

This study included 386 tissue microarray samples (HBreD129Su01 [Non-TNBC, $n=110$; TNBC, $n=19$], HBreD180Bc01[TNBC, $n=150$, paired adjacent, $n=30$], HBreD132Su07 [TNBC, $n=17$]; HBre-Duc090Sur-01 [BC adjacent tissue, $n=90$]), and all were sourced from Sanghai Outdo Biotech Co.,Ltd. Additionally, the study involved six samples each of TNBC (fresh tissues, and PBMCs, mean age: 53.6 years), non-TNBC (fresh tissues, and PBMCs; mean age: 50.2 years), and PBMCs from healthy individuals from Henan Provincial People's Hospital for subsequent verification; all blood or tissue samples were collected without any intervention, and the serum/plasma testing parameters as CEA, CA125, CA153, and lactate were obtained from the LIS Diagnostic Database of Henan Provincial People's Hospital as well. Ethical approval was granted by the Ethics Committee of Henan Provincial People's Hospital (approval number: No.2023-Ethics-43), and study was carried out according to the Declaration of Helsinki. Informed consent was obtained from the study subjects before their enrolment. For the included TNBC cases, a total of 36 cases (data from the HBreD129Su01 and HBreD132Su07 tissue chips) retained the survival follow-up information. All tissue samples were untreated, and patient informed consent was obtained prior to sample acquisition. Ethical approval was also obtained for the acquisition of the samples for commercial tissue chips (No.SHYJS-CP-2210008, and No.SHYJS-CP-1804031 by the Ethics Committee of Sanghai Outdo Biotech Co.,Ltd).

Main reagents

Anti-L-Lactyl-Histone H4 (Lys 5) Rabbit Monoclonal Antibody (Cat no. PTM-1407RM, PTM BIO, Hangzhou, China), EpiQuik Total Histone Extraction Kit (Catalog.OP-0006, Epigentek), Histone H4 Rabbit Monoclonal Antibody (Product no. AF2581), SDS-PAGE Gel SuperQuick Preparation Kit (Beyotime, Shanghai, China). EliVisionTM Plus Two-Step Detection Kit (Product No. KIT-9903, Fuzhou Maixin).

Immunohistochemistry (IHC)

The EliVisionTM Plus two-step detection system of Fuzhou Maxim was utilized, following the kit manual's specified procedures. Anti-L-Lactyl-Histone H4 (Lys 5) Rabbit Monoclonal Antibody was diluted at 1: 300, and H4 expression was used as a positive control, while PBS served as negative control. DAB staining was followed by hematoxylin contrast staining. The expression of H4K5lac is currently semi-quantitatively analyzed using IHC, assessed by two diagnostic pathologists, and the evaluation criteria for staining intensity were as follows: 0 for no immunochromogenic, 1 for brown/yellow, 2 for medium brown, and 3 for dark brown. Scores based on the positive staining ratio of slices included 0 for < 5%, 1 for 5–25%, 2 for 25–50% and 3 for > 50%. The product of H4K5lac expression intensity score and expression categorized as 0 = -, 1–2 = +, 3–5 = + +, 6–9 = + + +. Among them, 0–2 (- to +) indicated low expression, while 3 or above (++, +++) indicated relatively high expression.

Extraction of PBMCs

A total of 2 mL blood was mixed with an equal volume of PBS 1: 1. The diluted venous blood then carefully drawn into a sterile straw, and gently introduced onto the liquid surface of human lymphocyte separation liquid along the tube wall. The anticoagulant blood was thoroughly mixed according to a 1: 1 ratio, ensuring a clear interface. The prepared mixture was subjected to horizontal centrifugation at 800 g for 20 min. Post centrifugation, the 15 mL test tube was carefully removed. The tube exhibited three layers, with a distinct narrow band of white cloud primarily consisting of mononuclear cells at the interface between the upper and middle layers, these mononuclear cells were the target. The mononuclear cells within the narrow band of the white cloud layer were aspirated using a sterile straw and transferred to another 15 mL centrifuge tube containing 10 mL PBS . This new mixture was centrifuged at 800 g at room temperature for 5 min. The supernatant was discarded. Subsequently, the precipitate underwent erythrocyte lysis by adding an erythrocyte lysis solution, left at room temperature for 2 min, and then centrifuged at 800 g for 5 min. The supernatant was discarded again. The remaining precipitate was resuspended in 5 mL PBS , subjected to centrifugation at 500 g at room temperature for 10 min, and the supernatant was discarded once more. This final step yielded purified mononuclear cells for further analysis.

Histone protein extraction & Immunoblotting

The tissue samples were weighed, and small pieces (1–2 mm^3) were meticulously cut using a scalpel or scissors. The cut samples were then transferred to a Dounce homogenizer. A 10× Pre-Lysis Buffer was diluted with distilled water at a 1:10 ratio (e.g., 1 ml of 10× Pre-Lysis Buffer + 9 ml of water). Next, the Diluted 1× Pre-Lysis Buffer was added at 1 mL per 200 mg of tissue, and the tissue pieces were disaggregated by 50–60 strokes. The homogenized mixture was subsequently transferred to a 15 mL conical tube and centrifuged at 3000 rpm for 5 min at 4 °C. If the total volume of the mixture was less than 2 mL, the mixture was then transferred to a 2 mL vial and centrifuged at 10,000 rpm for 1 min at 4 °C, followed by the removal of the supernatant.

Total proteins were collected using the EpiQuik Total Histone Extraction Kit and quantified using the BCA Protein Assay Kit (Beyotime, Shanghai, China). Protein isolation was carried out using a 10–15% SDS-PAGE gel, subjected to electrophoresis at 90 V for 120 min. Subsequently, the proteins were transferred onto a PVDF membrane and sealed with 5% fat-free milk at room temperature for 2 h. Antibodies including Anti-L-Lactyl-Histone H4 lys5 (1 : 500), and H4 (1 : 1000) served as positive control. The incubation occurred at room temperature for 2 h, followed by an overnight incubation at 4℃. Secondary antibodies were added and incubated for an additional 3 h. Protein development was performed using a BD protein imaging system (Bio-Rad Laboratories, Inc, USA), and the band's gray value was analyzed using the National Institutes of Health Image J software, allowing for the calculation of the expression of the relevant proteins.

Peripheral blood CEA, CA125, CA153, and lactate assays

On the day after admission for BC patients and on the morning of the same day for health check participants, 3 to 5 mL of venous blood was collected on an empty stomach, and the blood was placed in coagulation-promoting tubes. The samples were centrifuged using a Zhongjia Li KD-1044 centrifuge at 1728 × g for 5 min. The Roche Cobas e8000 Electrochemical Luminescence Instrument was then used to test serum levels of CEA, CA125, CA153 based on electrochemiluminescence. The assay for lactate was measured by Ossendo Dry biochemical analyzer (VITROS 5600) using the lactate oxidase method. All experiments were performed in accordance with relevant guidelines and regulations.

Statistical analysis

Data were presented as mean ± standard deviation (SD), and statistical analyses were conducted using SPSS 16.0 software. Graphpad (version 8.0) was employed for creating statistical charts. A two-sample t-test was utilized for comparing the means of two samples, accompanied by homogeneity analysis of variance. In cases where variance heterogeneity was observed, the corrected t-test, variable transformation, or rank sum test were applied. Additionally, the relationship between histone H4K5lac expression and clinicopathological features of BC patients was explored using χ^2 tests. Survival analysis was performed using the Kaplan-Meier method. The significance level (α) was set at 0.05, with $P < 0.05$ considered statistically significant. These rigorous statistical methods aimed to provide robust and reliable insights into the observed differences and associations in the study.

Results

Demographic characteristics of the tissue chips

The study's flowchart is illustrated in Fig. 1. The BC and para-carcinoma tissue microarray (HBreD129Su01; HBre-Duc090Sur-01) included 110 cases of Non-TNBC, from January 2001 to August 2004. All of which were female with an average age of 54 years. Pathological diagnoses revealed 1 case of intraductal carcinoma, 10 cases of invasive carcinoma (comprising invasive ductal carcinoma or lobular carcinoma), and 99 cases of non-specific invasive carcinoma. All cases were primary, with no definitive distant organ metastasis detected. Lymph node status included 66 cases with metastasis, 40 cases without metastasis, and 4 cases with missing lymph node data. Clinical staging, based on the AJCC sixth edition, comprised 7 cases at stage I, 61 cases at stage II, 39 cases at stage III, and 3 cases with missing stage information of the 110 non-TNBC cases. The HBre-Duc090Sur-01 tissue chip included 90 points of BC adjacent tissues correspondingly from 90 BC cases.

TNBC high-throughput tissue microarray (HBreD180Bc01-1, $n = 150$; HBreD129Su01, $n = 19$; HBreD132Su07, $n = 17$) consisted of 186 cases of TNBC and 30 adjacent tissues. All 186 samples were obtained from female patients with an average age of 52.9 years, diagnosed through histopathology. Histological types included non-specific invasive carcinoma (96 cases), invasive ductal carcinoma (77 cases), medullary carcinoma (4 cases), metaplastic carcinoma (3 cases), mixed metaplastic carcinoma (1 case), basal carcinoma (1 case),

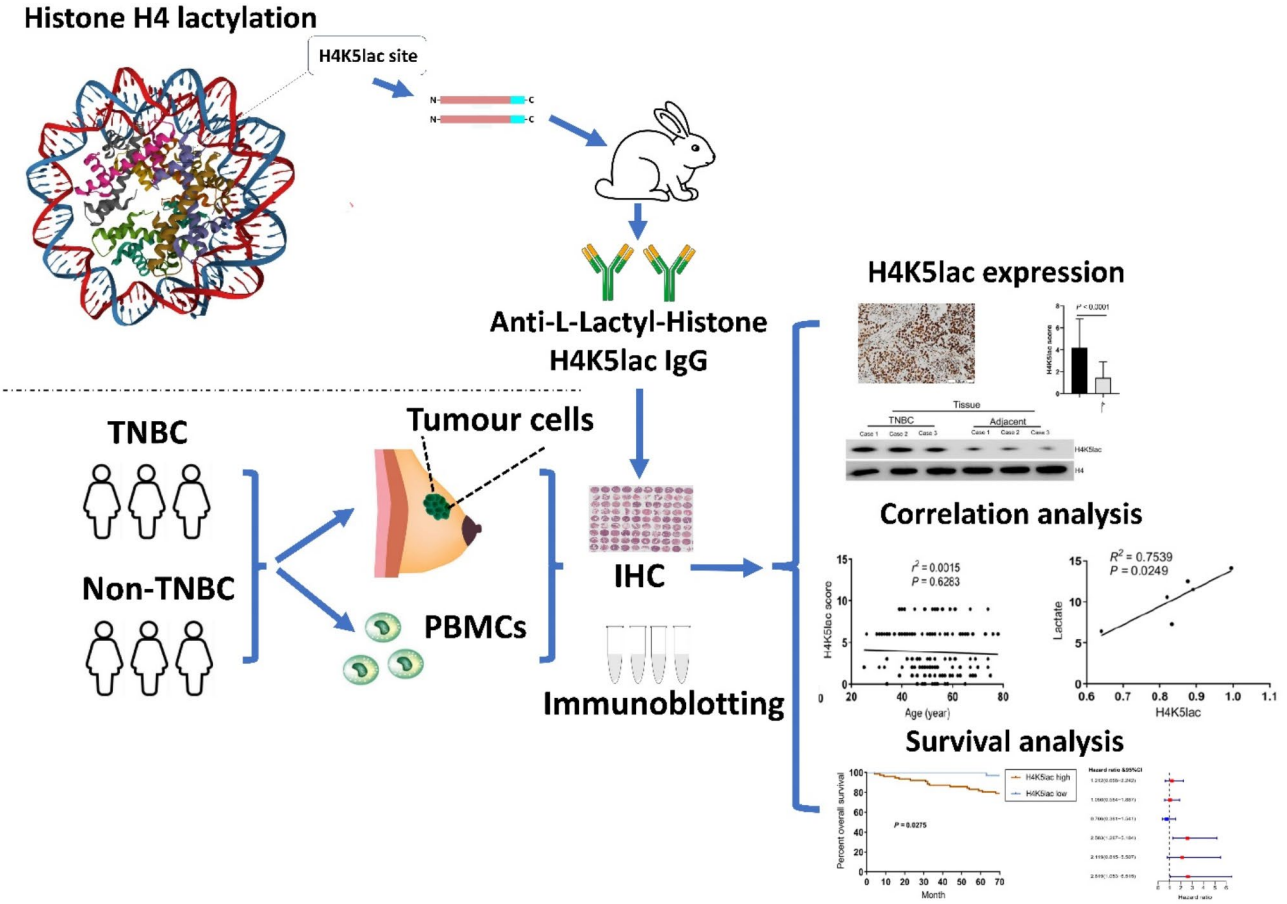

Fig. 1. Schematic illustration of the study design.

squamous cell carcinoma (1 case), adenoid cystic carcinoma (1 case) and mucinous carcinoma (2 case). Clinical characteristics of the included BC cases is summarized in Table 1.

Expression of lactylated histone H4K5lac in non-TNBC tissue and its prognostic significance

The detection of histone H4K5lac expression in BC tissue microarrays (non-TNBC) revealed prominent nuclear expression within tumor cells (Fig. 2A). IHC indicated a positive expression rate of 93.64% (103/110) in non-TNBC cancer tissues, with 76 cases exhibiting high expression, 27 cases displaying low expression, and 7 cases demonstrating negative expression. In adjacent tissues, the positive expression rate of histone H4K5lac was 73.33% (66/90), including 20 cases with high expression, 46 cases with low expression, and 24 cases with negative expression. The expression of H4K5lac (rating scores) in cancer tissues was significantly higher than that in adjacent tissues (Fig. 2B; $P < 0.0001$).

Subgroup analysis showed that H4K5lac expression (rated expression score) was lower in patients with estrogen receptor (ER) positive than in those with ER negative; H4K5lac expression was higher in patients with clinical stage III-IV and BC than those with stage I-II (Fig. 2C). Similarly, it was also found that H4K5lac rated expression score was elevated in patients with histologic grading II-III, but it was not significant when stratified with tumor location (right vs. left) (Fig. 2C). Moreover, H4K5lac expression was positively correlated with positive lymph node ratio, but not related to tumor size, and age (Fig. 2D). The $\chi 2$ analysis also underscored that H4K5lac expression (component ratio) was associated with clinical stage ($P = 0.042$; Table 2).

A cohort of 110 non-TNBC patients underwent a follow-up for a median duration of 110 months (9 to 150 months). The median OS for the high expression group and the low/negative expression group of histone H4K5lac was 123 months and 105 months, respectively. The overall 5-year survival time for the high expression group of histone H4K5lac was notably lower than that of the low/negative expression group (HR = 2.156, 95%CI: 1.011–4.599, $P = 0.0275$) (Fig. 2E). The univariable and multivariable Cox hazards model demonstrated that advanced clinical staging, and high H4K5lac expression were risk factors for the prognosis of TNBC patients (Fig. 2F).

Expression of lactylated histone H4K5 site in TNBC tissue

Histone H4K5lac expression in two high-throughput TNBC tissue microarrays (HBreD180Bc01-1 and HBreD132Su07) were similarly examined. The positive expression rate in cancer tissues was 91.40% [170/(150 + 19 + 17)], comprising 115 cases with high expression, 55 cases with low expression, and 16 cases with

Clinicopathological features	TNBC ($n=150$, $n=19$, $n=17$)**	Non-TNBC ($n=110$)
Age (years)		
≥ 50	120	48
< 50	66	62
Location		
Left	99	43
Right	87	67
Tumor size		
> 2 cm	125	88
≤ 2 cm	44	22
Histologic grading		
I	1	11
I-II	5	17
II	60	76
II-III	10	5
III	110	1
Clinical stage		
I	6	7*
II	12	61*
III	18	39*
Ki-67 expression		
≥ 14%	140	/
< 14%	10	/
Lymph node metastasis		
Yes	20+	66#
No	15+	40#
Pathological type		
Non-specific invasive carcinoma	96	99
Invasive ductal carcinoma	77	10
Intraductal carcinoma	/	1
Medullary carcinoma	4	/
Metaplastic carcinoma	3	/
Mixed metaplastic carcinoma	1	/
Basal carcinoma	1	/
Squamous cell carcinoma	1	/
Adenoid cystic carcinoma	1	/
Mucinous carcinoma	2	/

Table 1. Clinical characteristics of the included BC cases. ** HBreD180Bc01 ($n=150$), HBreD129Su01 ($n=19$), HBreD132Su07 ($n=17$). *Stage information loss ($n=3$); # lymph node information loss ($n=4$); +lymph node information loss ($n=1$).

negative expression. In adjacent tissues, the positive expression rate of histone H4K5lac was 63.33% (19/30), including 7 cases with high expression, 12 cases with low expression, and 11 cases with negative expression. Notably, H4K5lac expression in cancer tissues was significantly higher than that in adjacent tissues ($P<0.0001$) (Fig. 3A and B). It was found that mean H4K5lac levels showed no significance when grouped by histologic grading and tumor location (Fig. 3C). The $\chi 2$ and correlation analyses revealed that histone H4K5lac levels were associated with Ki-67, and histologic grading, but showed no significant association with age, location, and tumor size (Table 3; Fig. 3D). The Kaplan-Meier survival analysis shows that H4K5lac expression is significantly correlated with TNBC prognosis (Fig. 3E; $n=36$; HR = 2.773, 95%CI: 1.128–6.851, $P=0.0384$). The Cox hazards model revealed that H4K5lac expression, clinical stage, histologic grading, tumor size, tumor location, and age were not risk factors for the prognosis of TNBC patients (Fig. 3F).

Histone H4K5 lactylation in BC tissue and PBMCs

Verification of histone H4K5lac expression in tissues and PBMCs were conducted through immunoblotting on 6 BC samples (including 3 TNBC and 3 non-TNBC cases, respectively) and 3 healthy individuals. Immunoblotting demonstrated histone H4K5lac expression in all 6 BC tissue samples (Fig. 4A and C), with an average expression level higher than that of adjacent tissues (Fig. 4B and D). Similarly, histone H4K5lac expression was also found to be elevated in the PBMCs of both 3 TNBC and 3 non-TNBC cases as compared with the healthy controls (Fig. 4E and F). Correlation analysis revealed that histone H4K5lac expression was positively correlated to serum

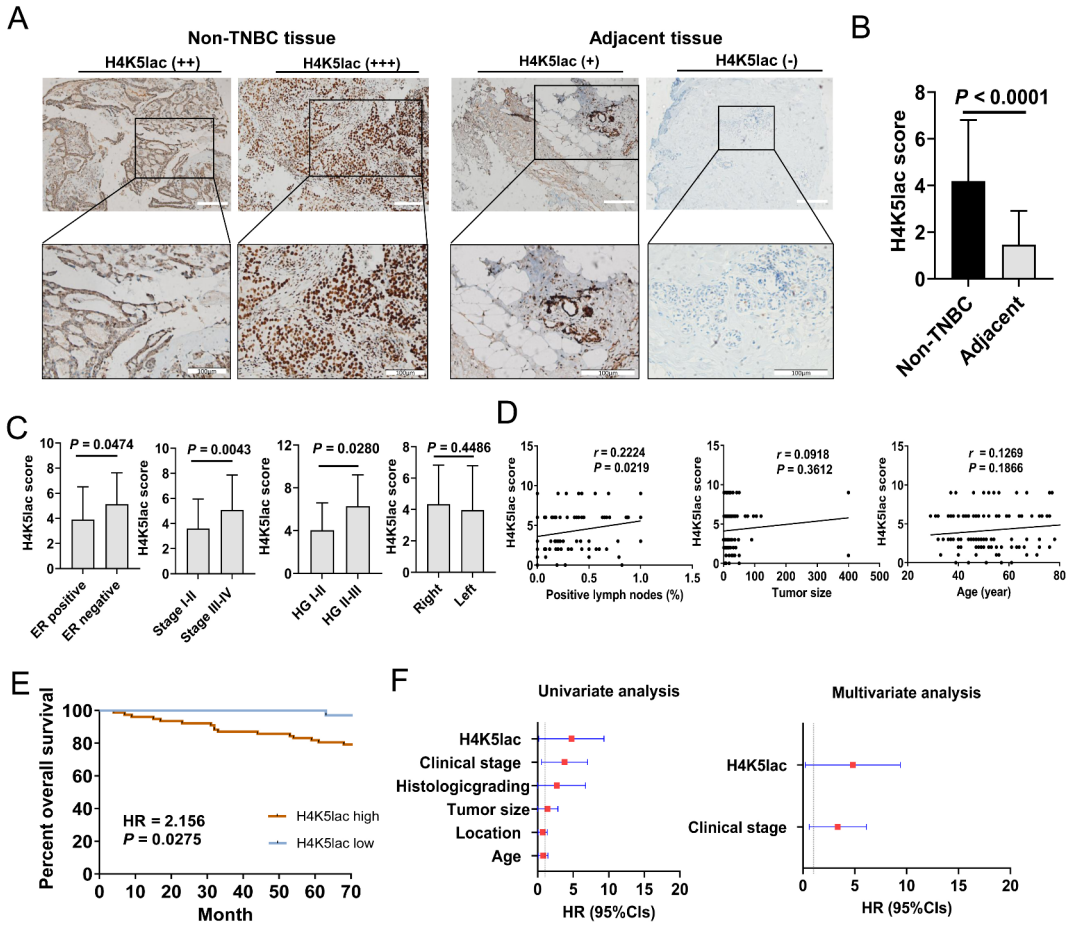

Fig. 2. Expression of H4K5lac in non-TNBC and its prognostic value. (**A**) IHC reveals the expression and subcellular localization of lactylated H4K5lac in non-TNBC cases. (**B**) Quantification of lactylated H4K5lac expression in the non-TNBC cases was based on IHC rating scores. (**C**) Subgroup analyses of H4K5lac expression based on ER status, clinical stage, histological grading, and location. (**D**) Correlation analysis demonstrates that H4K5lac expression is associated to positive lymph nodes (%); (**E**) Survival curve plotted based on H4K5lac expression (high vs. low) in non-TNBC cases. (**F**) Forest plot of the univariate and multivariate Cox hazards model in predicting the prognosis of non-TNBC cases. All data are presented as means ± standard deviations [SDs]. Statistical analysis was performed by Student's *t*-test or Kruskal-Wallis test in B, and C, or by Pearson's correlation coefficient analysis in D, or by Log-rank test in E, or by Cox hazards analysis in F. HG: histologic grading, ER: estrogen receptor.

lactate and CEA levels (Fig. 4G). This finding suggests that while H4K5lac is expressed at a baseline level in both BC and healthy individuals, but its expression is elevated in BC cases.

Discussion

Lactate, a significant carbon-containing metabolite in the cellular glycolysis pathway, plays a pivotal role in histone lysine lactation during cell metabolism[16]. In 2019, Zhao's team demonstrated that lactate, an accumulation product in metabolism, acts as a precursor, inducing lactation modification of histone lysine[8]. This modification regulates gene expression and contributes to the steady-state regulation of M1 macrophages during bacterial infection[8]. Moreover, lactate modification was confirmed to regulate the immunosuppressive function of tumor-infiltrated myeloid cells, mediating tumor immune escape[17,18]. The study also highlighted lactate-induced upregulation of METTL3 via H3K18 lactylation, underscoring the importance of lactylation in diverse cellular processes[19]. Despite these findings, research on histone lactylation in BC and its clinical application is limited. This study aims to explore the expression of H4K5lac in TNBC, Non-TNBC tissues, and PBMCs and its potential clinical value.

Post-translational modifications (PTMs) of H4 lys5, such as acetylation (H4K5ac) and butyrylation (H4K5bu), play a crucial role in nervous system disease and tumors[20–23]. In cerebral cavernous malformation (CCM), the loss of NGBR impairs the binding of HBO1 and acetylated histone H4K5 and H4K12 on the promotors of the CCM1 and CCM2 genes, leading to the pathogenesis and progression of CCMs[21]. Low levels of H4K5ac were notably prominent in patients with complex karyotype acute myelocytic leukemia (AML) and were associated with inferior overall survival; furthermore, ChIP-seq experiments in primary AML patient

Clinicopathological features	Total cases ($n=110$)	H4K5lac low (Negative ~ ±)	H4K5lac high (+ ~ +++)	χ2 value	*P* value
Age				0.584	0.445
≥ 50	48	13	35		
< 50	62	21	41		
Location				1.312	0.252
Left	43	16	27		
Right	67	18	49		
Tumor size				0.170	0.680
> 2 cm	88	28	60		
<=2 cm	22	6	16		
Histologic grading				0.614	0.433
I-II	28	7	21		
II, II-III	82	27	55		
Clinical stage	107*				
I + II	68	29	39	4.145	0.042
III	39	9	30		

Table 2. Correlations between H4K5lac expressions and clinical characteristics in non-TNBC patients. *Stage information loss ($n=3$).

blasts revealed widespread deregulation of H4K5Ac, particularly at gene promoters[22]. Gao et al. reported that the ratio of histone H4K5 acylation/acetylation drives and regulates the genomic distribution of bromodomain-containing protein 4 (BRD4) in acute lymphoblastic leukemia (ALL) malignancies[20]. Another type of PTM, H4K5bu, was evaluated in patients affected by testicular cancers, such as seminoma and teratoma[23]. Recently, it was reported that the lactylation level of H4K5lac can be regulated by "erasers": histone deacetylases (HDACs) 1 and 3[24]. However, reports of H4K5lac in tumors are scarce.

Based on tissue microarray, we detected the expression of H4K5lac in TNBC and Non-TNBC tissues by IHC, and found that H4K5lac was predominantly localized in the nucleus, positive rate and average expression level of H4K5lac in TNBC and Non-TNBC tissues surpassed those in adjacent tissues. H4K5lac expressions (rated IHC expression score) were also found to be higher in patients with clinical stage III-IV or histologic grading II-III. Moreover, a correlation was observed between histone H4K5lac levels and positive lymph nodes (%) as well as clinical stage and Ki-67 expression, indicating potential involvement in the malignant pathophysiological processes of BC patients. Upon examining the follow-up data from the tissue microarray, our study revealed a higher 5-year survival rate in the group exhibiting high histone H4K5lac expression compared to the low/negative expression group. Intriguingly, both TNBC and non-TNBC patients with elevated histone H4K5lac expression exhibited a worse prognosis. Importantly, H4K5lac emerged as an independent risk factor for OS in non-TNBC patients. In HCC, an integrative analysis of the lactylome and proteome in tumors and adjacent livers unveiled 9,275 Kla sites, with 9,256 located on non-histone proteins[25]. This underscores the prevalence of Kla as a modification extending beyond histone proteins, suggesting involvement in various cellular processes beyond transcriptional regulation[25]. Additionally, histone lactylation was found to be elevated in tumors, correlating with the poor prognosis of ocular melanoma[15]; it was also identified as contributing to tumorigenesis by facilitating YTHDF2 expression[15]. However, we found that the mean H4K5lac levels (IHC rating scores) were elevated in the non-TNBC patients with histologic grading II-III than patients with histologic grading I, but no significant were found in TNBC cases; intriguingly, the χ2 test revealed that histone H4K5lac levels were associated with histologic grading in TNBC. This inconsistency may be caused by the following reasons: (1) Different statistical methods: the former is the evaluation of means, whereas the latter describes the composition ratio; and (2) Different BC subtypes may lead to altered H4K5lac levels among them.

We further validated the expression of histone H4K5lac in PBMCs and observed its up-regulation in PBMCs of BC patients. In contrast, the expression of histone H4K5lac in healthy subjects was found to be very low. This suggests that histone H4K5lac in PBMCs holds promise as a noninvasive biomarker, warranting further investigation for its potential clinical translational value. In a parallel context, a study by Chu et al. identified upregulated expression of lactylated histone H3K18lac in the PBMCs of septic shock patients[26]. Notably, H3K18lac protein expression positively correlated with APACHE II scores, SOFA scores on day 1, ICU stay, mechanical ventilation time, and serum lactate levels in non-tumor diseases[26]. Cui et al. recently plotted the whole protein lactylation signature in TNBC, and also found that H4K12lac were elevated in TNBC tissues, and H4K12lac expression was negatively correlate to the OS of TNBC cases[27]. These studies suggested that histone lactylation may be developed as novel biomarkers, and also provide new clues for further in-depth research into lactylation treatment strategies and targeted therapies in BC.

Our study still retains several limitations. First, the sample size for TNBC was limited, which may contribute to the lack of significant findings in the univariate and multivariate Cox analyses. Second, since the data for TNBC and non-TNBC were derived from commercial tissue arrays, we lacked data on Ki-67 in non-TNBC, resulting in unmatched clinicopathological outcomes between TNBC and non-TNBC. Third, the number of samples included in the correlation study was small, potentially leading to biased results. Lastly, the detection

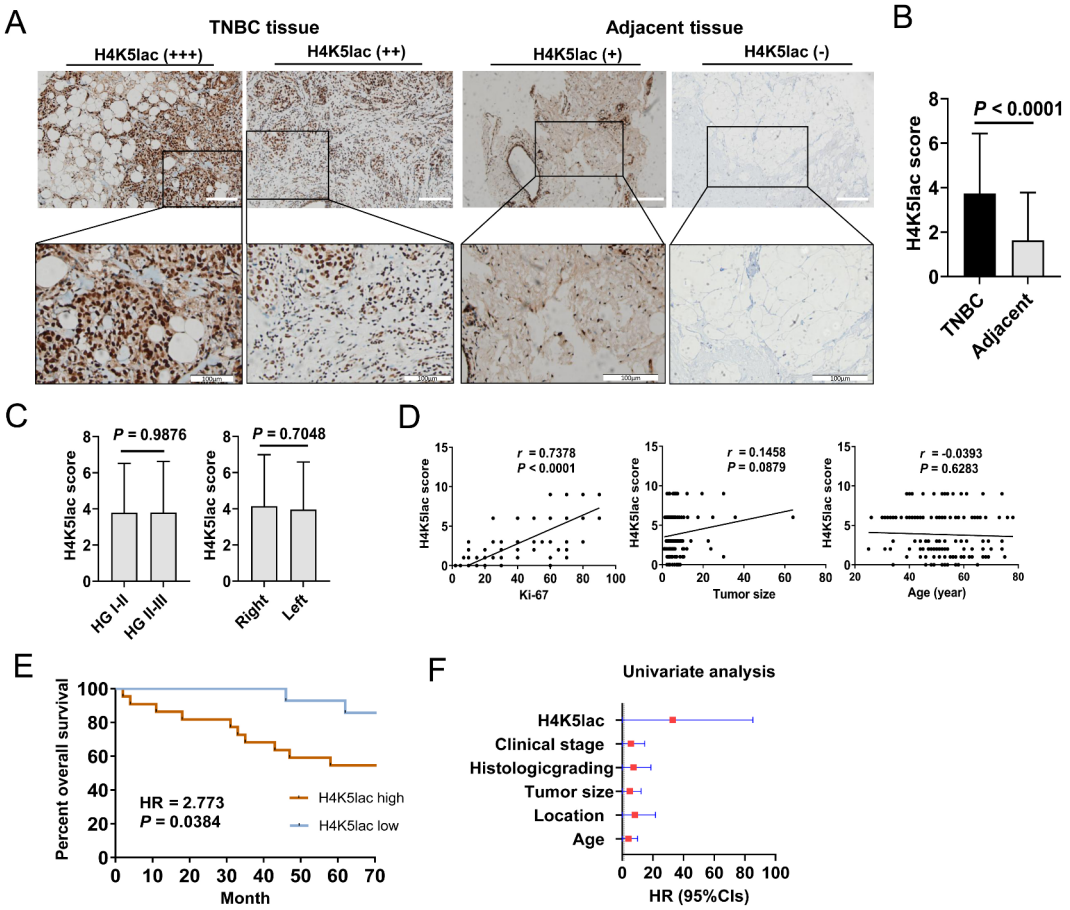

Fig. 3. Expression of H4K5lac in TNBC tissue. (**A**) Expression and subcellular localization of lactylated H4K5lac in TNBC tissues by IHC. (**B**) Quantification analysis reveals higher lactylated H4K5lac expression in TNBC compared to paired adjacent tissues. (**C**) Subgroup analyses of H4K5lac expression according to ER expression, histological grading (I-II vs. II-III), and location (right vs. left). (**D**) Lactylated H4K5lac expression positively associated with Ki-67 expression in TNBC. (**E**) Survival curve of H4K5lac expression (high vs. low) in predicting the overall survival of TNBC cases ($n = 36$). (**F**) Forest plot of the Cox hazards model utilized for prognosis prediction of TNBC cases. All data are shown as the means ± SDs. Statistical analysis was conducted utilizing Student's *t*-test or Kruskal-Wallis test in B, and C, or Pearson's correlation coefficient analysis in D, or Log-rank test in E, or Cox hazards analysis in F. HG: histologic grading; HR: hazard ratio.

methods for H4K5lac protein were limited to IHC and immunoblotting, highlighting the need for developing novel methods for detecting H4K5lac and other lactylated histone proteins in the future. There is a pressing need to develop novel methods for detecting protein lactylation in subsequent stages of research. The biological function of histone H4K5lac in BC is a facet that merits further exploration.

In summary, the identification of lactic acid modification opens up a novel avenue for exploring the involvement of the metabolite lactic acid in tumor and immune-related fields. This discovery implies that the elevated expression of histone H4K5lac could potentially serve as a marker for BC. However, to validate the findings of this study, a comprehensive large-scale investigation is imperative in the subsequent stages of research.

Clinicopathological features	Total cases ($n = 150$, $n = 36$)	H4K5lac low (Negative ~ ±)	H4K5lac high (+ ~ +++)	χ2 value	*P* value
Age				0.376	0.540
≥ 50	120	51	69		
< 50	66	25	41		
Location				0.027	0.870
Left	99	41	58		
Right	87	35	52		
Tumor size				0.560	0.454
> 2 cm	125	46	79		
≤ 2 cm	44	19	25		
Histologic grading				7.378	0.007
I-II	43	25	18		
II, II-III	143	50	93		
Ki-67 expression*				4.436#	0.046
≥ 14%	140	51	89		
< 14%	10	7	3		

Table 3. Correlations between H4K5lac expressions and clinical features in TNBC patients. *The cohort of 19 TNBC cases from HBreD129Su01 chip was not included. # Continuity Correction χ2 test was used.

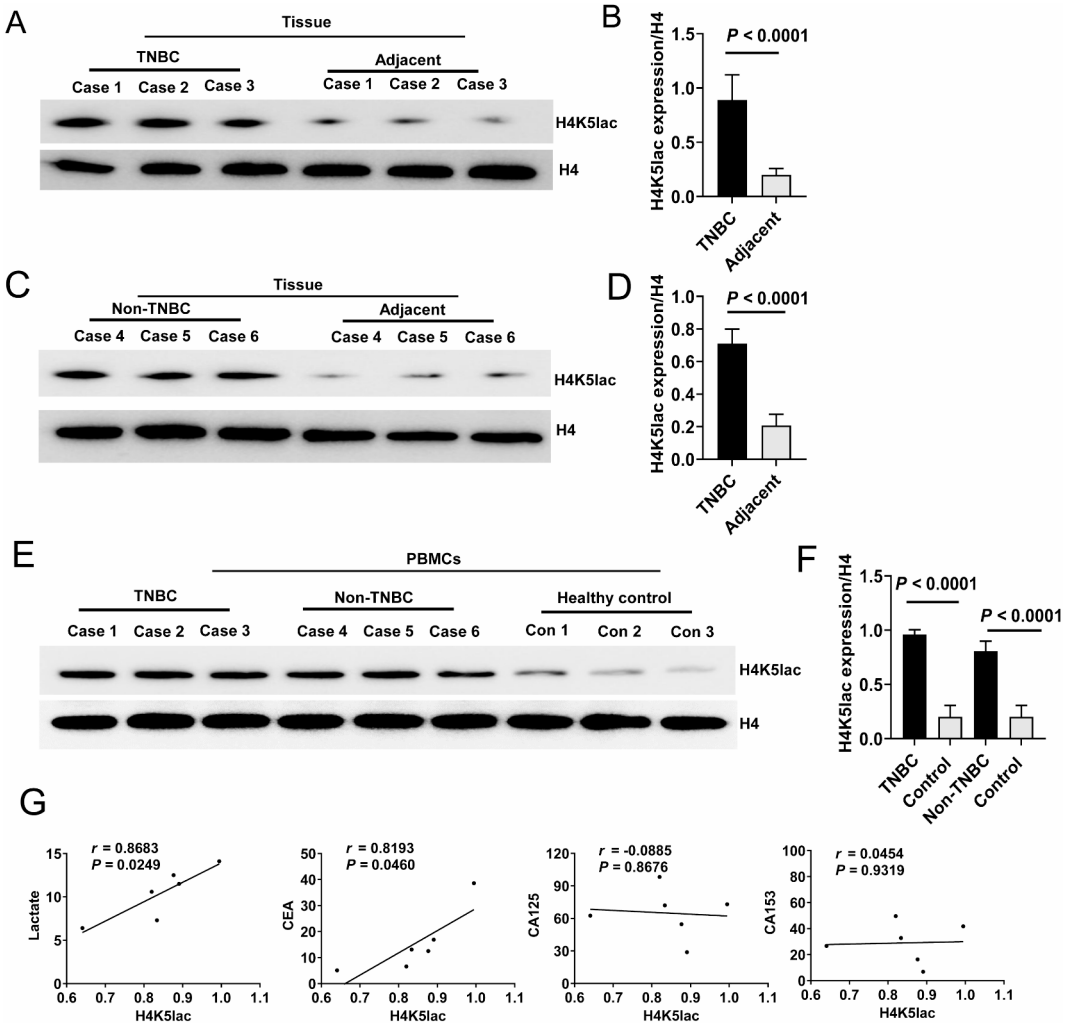

Fig. 4. Expression of H4K5lac in tissues and PMBCs of BC cases by immunoblotting. (**A, B**) TNBC tissue, and paired adjacent tissue ($n = 6$). (**C, D**) Non-TNBC tissue, and paired adjacent tissue ($n = 6$). (**E**, **F**) Expression of H4K5lac in PBMCs ($n = 6$) and healthy subjects ($n = 3$). (**G**) Correlation analyses between H4K5lac expression and serum BC related indicators. H4 utilized as a reference protein in nucleus. All data are shown as the means ± SDs. Student's *t*-test or Kruskal-Wallis test was performed in B, D, and F, and Pearson's correlation coefficient analysis were used in G. BC: breast cancer, TNBC: triple-negative breast cancer, Con: control, H4: histone 4, PMBCs: peripheral blood mononuclear cells.

Data availability

The data that support the results of this study are available from the corresponding author upon reasonable request.

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Author contributions
Ruili Yu and Ya Zhu designed the study; Yuping Fu, Fengzhen Liu, and Sha Yan collected the data, performed the experiments, and conducted analyses of available data; Ya Zhu drafted the manuscript; Ruili Yu and Ya Zhu proofread or revised critically for important intellectual content; all authors have reviewed the paper and approved the final manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Conflict of interest
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