Hypermethylated KLF9 Is An Independent Prognostic Factor For Favorable Outcome In Breast Cancer

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Background and objective: Breast cancer (BC) is the most lethal human malignancy and is the leading cause of cancer-associated death in women worldwide. Krüppel-like factor 9 (KLF9) belongs to a family of transcriptional regulators and its role in BC has not been fully investigated.

Method: Data mining was used to analyze BC data from The Cancer Genome Atlas (TCGA) database, which was downloaded using the UCSC Xena browser. The differential expression and methylation level of KLF9 was analyzed in patients with BC and corresponding normal controls enrolled from our hospital. Besides, the correlation of KLF9 methylation and prognosis was explored, and gene set enrichment analysis (GSEA) was conducted to identify the potential signaling pathway of KLF9 involved.

Results: Both TCGA and BC tissues indicated hypermethylation of the KLF9 promoter region in patients with BC compared with normal controls, which might account for the dysregulation of KLF9 in patients with BC. Besides, hypermethylation of KLF9 was detected in patients with estrogen or progesterone receptor-positive and non-triple-negative disease. Further, hypermethylation of KLF9 was demonstrated to be a potential independent biomarker in obtaining favorable outcomes in BC. By GSEA, tumor-associated biological processes and signaling pathway were identified, which indicated that KLF9 might play a vital role in the carcinogenesis of BC.

Conclusion: KLF9 plays an important role in the carcinogenesis of BC through the multiple tumor-associated signaling pathway. The hypermethylation of KLF9 resulted in its reduced expression in BC, while the hypermethylation of KLF9 has potential in the prediction of favorable outcomes in BC.

Keywords: Krüppel-like factor 9, methylation, breast cancer, prognostic biomarker, GSEA

Introduction

Breast cancer (BC) is the most common cause of cancer-related death in women worldwide.1 Incidence rates are high in well-developed countries, whereas those in developing nations such as Africa and Asia, incidence rates have historically been relatively low, but have increased in recent decades.2 According to the report on the global burden of cancer by the International Agency for Research on Cancer, almost half of the cases and over half of the cancer deaths worldwide occurred in Asia in 2018.3 In 2018, there were approximately 2.1 million new cases of BC, comprising 25% of cancer cases among women, and almost 700,000 deaths, accounting for 16% of cancer-associated deaths among women.

Many established risk factors for BC are related to estrogen.4,5 Risk is increased by early age at menarche,6 later age at menopause,7 and obesity in postmenopausal...
women. Prospective studies have shown that high concentrations of endogenous estradiol are associated with increased risk of BC development. Childbearing reduces risk, with greater protection for early first birth and a larger number of births; breastfeeding also likely has a protective effect. Alcohol consumption increases BC risk, whereas physical activity is probably protective; however, these risk factors account for a minority of cases.

Transcription factors can contribute to carcinogenesis and the progression of various cancers. Transcriptionomics-based screening of molecular signatures has identified multiple differentially expressed genes that are associated with decreasing in the overall survival (OS) of BC patients. The human positive cofactor 4 (PC4), initially identified as a transcriptional cofactor, exerted its oncogenic functions by directly binding to c-Myc promoters and inducing Warburg effect. The expression of PC4 has effects on sensitivity to paclitaxel of cancer cells and is associated with poor survival in patients with BC. Krüppel-like factors (KLFs) are DNA-binding transcription regulators that control essential cellular processes, such as proliferation, differentiation, migration, and maintenance of pluripotency. Previous studies showed that Krüppel-like factor 6 (KLF6) functions as a tumor suppressor in BC by reducing cell proliferation rate through increased c-Jun degradation and proliferating cell signaling. Krüppel-like factor 11 (KLF11) was hypermethylated in BC; this hypermethylation may be associated with low expression and cancer metastases. Krüppel-like factor 8 (KLF8) acts as an oncogene in lung adenocarcinoma, and Kaplan–Meier curves revealed that high expression of KLF8 was related to poor prognosis in patients with lung adenocarcinoma. Expression of Krüppel-like factor 15 (KLF15) was also found to be abnormally high in lung adenocarcinoma tissues and was correlated with tumor TNM stage, and has potential as a cancer prognostic marker. Krüppel-like factor 9 (KLF9) was previously reported as a basic transcription element-binding protein, due to its specific binding to the transcription element GC box in a gene promoter region. Further, KLF9 is implicated in the pathogenesis of several cancers, including endometrial cancer and other endocrine-responsive cancers of female reproductive tissues; however, the role of KLF9 in BC remains largely unknown. Here, we took advantage of public databases to explore the expression and methylation status of KLF9, and to identify the biological processes involving KLF9 and its co-expressed genes in the context of BC. We also validated our findings using laboratory experiments.

Materials And Methods

Tissue Collection
A total of 144 BC and adjacent normal tissue samples were collected and stored in liquid nitrogen after surgery. All patients were enrolled in the thyroid and breast surgery department of Li Huili Affiliated Hospital of Ningbo University and they signed the informed consent forms before surgery and sample collection. All patients were pathologically diagnosed with BC by at least two experienced pathologists. The study was approved by the Human Research Ethical Committee of Ningbo Medical Center Li Huili Hospital.

DNA Extraction And Bisulfite Modification
Genomic DNA was extracted from patients with BC and normal tissue controls (n = 144) using DNA Mini Kit (Qiagen, Germany), according to the manufacturer’s protocol. The quality and concentration of DNA were measured. Extracted DNA was bisulfite-treated using the ZYMO EZ DNA Methylation-Gold Kit, as recommended by the manufacturer (Zymo Research, USA).

Quantitative Methylation-Specific Polymerase Chain Reaction (qMSP)
The promoter region of KLF9 was amplified by fluorescence-based qMSP, using SYBR Green Master Mix (Promega, USA). The qMSP primers for KLF9 were described in a previous study. The primer sequences of KLF9, along with those of primers for amplification of the control gene, ACTB are listed in Table 1.

Bioinformatic Analysis Using The UCSC Xena Browser
All microarray data (including methylation and expression information) from patients with BC and normal tissue controls, along with the clinical characteristics, were from the Cancer Genome Atlas (https://www.cancer.gov/TCGA), which was downloaded using the UCSC Xena browser (https://xenabrowser.net/).

Table 1 Sequences Of KLF9 And ACTB Primers For qMSP

| Characteristics | Sequence |
|-----------------|----------|
| qMSP KLF9-Forward | 5'-TGAGTTAGGAGGTTCCGATC-3' |
| KLF9-Reverse | 5'-TTGCTACTGCGCTCCACC-3' |
| ACTB-Forward | 5'-TGCGTTGAGGAGAGTTGAGT-3' |
| ACTB-Reverse | 5'-AACCAATAAAACCTACTGCTCCCTTA-3' |
Bioinformatic Analysis Using cBioPortal For Cancer Genomics And ClueGo

Genes co-expressed with KLF9 were identified using cBioPortal for Cancer Genomics (www.cbioportal.org, |Pearson’s r| ≥ 0.4; P < 0.01). Then, the genes were loaded into ClueGo in Cytoscape version 3.71 for analysis of KEGG pathways.

Gene Set Enrichment Analysis (GSEA) And Single-Sample GSEA (ssGSEA)

TCGA BC patients were classified into two groups, high expression and low expression, according to the median expression of KLF9. GSEA of KLF9 was performed using GSEA 3.0 software. An enrichment score (ES) >0.4 was obtained and false discovery rate (FDR) value <0.05 was regarded as statistically significant. To compare the activation degree of enriched pathways from GSEA, we used ssGSEA to generate activation pathway score. Using GSVA package and its ssGSEA method (http://www.bioconductor.org), the enrichment pathway score in each sample was calculated. The scatter plot of activated pathway score and KLF9 expression was generated by ggplots package on the R platform.

Statistical Analysis

Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Differences in KLF9 methylation and expression between BC and healthy control tissues, and associations between KLF9 methylation and clinicopathological features were evaluated by independent t-test or one-way ANOVA. Multivariate Cox regression models were constructed to analyze the prognostic power of clinical variables, using the "survival" package on the R platform (https://cran.r-project.org/web/packages/survival/survival.pdf). A heatmap of KLF9 methylation, clustered by sample type, was generated using the "pheatmap" package on the R platform (https://cran.r-project.org/web/packages/pheatmap/index.html). The ssGSEA analysis was performed using GSVA package, and the scatter plots were generated using ggplots package on the R platform. Other data were analyzed using GraphPad Prism 6 software (GraphPad, San Diego, CA). Two-tailed P values <0.05 were deemed statistically significant.

Results

KLF9 Is Significantly Downregulated In BC Tissues Compared With Normal Control Tissues

In the initial data mining, we downloaded KLF9 expression data of 1108 BC and 139 normal control tissue samples using the UCSC Xena browser. After filtering out the samples with null values for KLF9 expression, a total of 1104 BC and 114 normal control tissue samples were included for further analysis. As shown in Figure 1, our results indicated that KLF9 expression was approximately 1.2-fold reduced in BC (cases vs. controls: 9.76 ± 0.87 vs. 11.56 ± 0.77, P = 4.96E-86).

Biological Processes Regulated By KLF9

By data mining using cBioPortal for Cancer Genomics, we identified genes co-expressed with KLF9 in BC (|Pearson’s r|≥ 0.4, P < 0.01; Table S1). A total of 13,010 genes were identified as co-expressed with KLF9 in BC. To further explore possible signaling pathways in which KLF9 may be involved, KLF9 co-expressed genes were subjected to pathway analysis. As shown in Figure 2, the co-expressed genes were enriched in multiple biological processes, including negative regulation of cellular processes, regulation of multicellular organization, cell migration, and cellular responses to growth factor stimuli.

A GSEA was conducted between low and high expression of KLF9 to identify some associated KEGG signaling pathways. The most significant pathways were identified according to the ES and FDR q value. As shown in Table 2, the results showed 50 significantly enriched pathways (P value < 0.05). Figure 3 exhibited top 10 pathways

![Figure 1](https://example.com/figure1.png) Downregulated expression of KLF9 in breast cancer. ***P < 0.001.
correlated with cancer, such as MAPK signaling pathway, pathway in cancer, renal cell carcinoma and prostate cancer (ES > 0.45 and FDR < 0.05).

Single-sample Gene Set Enrichment Analysis (ssGSEA) calculates separate ESs for each sample and pathway. Each ssGSEA ES represents the degree to which the genes in a particular pathway are coordinately up- or downregulated within a sample. Therefore, in order to validate the differentially enriched pathways associated with KLF9 expression from GSEA, we used the top 10 pathways shown in Figure 3 for ssGSEA. Then, the scatter plots about ssGSEA activation score of pathways and KLF9 levels were exhibited in Figure 4, and results showed most activated pathways score (especially MAPK signaling pathway) were significantly associated with KLF9 expression in BC.

Negative Correlation Of KLF9 Expression And Promoter Methylation
To determine the mechanism of downregulation of KLF9 in BC, we simultaneously obtained KLF9 methylation data from the UCSC Xena browser. First, the location of the KLF9 gene in the genome and the distribution of CpG sites at the locus were extracted from UCSC Genome Browser (http://genome.ucsc.edu/) (Figure 5). Subsequently, a heatmap of all KLF9 CpG methylation sites was constructed (Figure 6), which demonstrated that the methylation level of site cg00049440, located in the KLF9 promoter region was significantly higher in BC than normal control tissues (Figure 6). Finally, Pearson’s correlation analysis demonstrated a negative correlation between KLF9 expression

Figure 2 Biological processes involving genes co-expressed with KLF9.
| Name                                                                 | Size | Enrichment Score | P     | FDR q-value |
|----------------------------------------------------------------------|------|------------------|-------|-------------|
| KEGG_FOCAL_ADHESION                                                  | 197  | 0.6090904        | 0     | 0.014636766|
| KEGG_ECM_RECEPTOR_INTERACTION                                        | 84   | 0.713751         | 0     | 0.01304298 |
| KEGG_SMALL_CELL_LUNG_CANCER                                          | 84   | 0.54016006       | 0     | 0.02931023 |
| KEGG_PATHWAYS_IN_CANCER                                              | 321  | 0.4768017        | 0     | 0.025051337|
| KEGG_MELANOMA                                                        | 71   | 0.5331018        | 0     | 0.020671003|
| KEGG_REGULATION_OF_ACTIN_CYTOSKELETON                               | 211  | 0.4975616        | 0     | 0.018928043|
| KEGG_LEUKOCYTE_TRANSITIONENDOTHELIAL_MIGRATION                      | 115  | 0.56087464       | 0     | 0.01696653 |
| KEGG_ADHÉRENS_JUNCTION                                              | 68   | 0.5595902        | 0.9789946 | 0.016274992|
| KEGG_RENAL_CELL_CARCINOMA                                            | 66   | 0.5432381        | 0     | 0.019406032|
| KEGG_PROSTATE_CANCER                                                 | 89   | 0.50076175       | 0     | 0.01972579 |
| KEGG_MAPK_SIGNALING_PATHWAY                                          | 265  | 0.45462683       | 0.01897533 | 0.023419893|
| KEGG_B_CELL_RECEPTOR_SIGNALING_PATHWAY                               | 74   | 0.4663734        | 0.005928854 | 0.021981618|
| KEGG_PANCREATIC_CANCER                                               | 69   | 0.51115805       | 0.003724395 | 0.028878667|
| KEGG_TGF_BETA_SIGNALING_PATHWAY                                      | 85   | 0.5057866        | 0.005976096 | 0.026816 |
| KEGG_CELL_ADHÉRENS_MOLECULES_CAMS                                    | 128  | 0.5760402        | 0.015625 | 0.038524617|
| KEGG_TOLLLIKE_RECEPTOR_SIGNALING_PATHWAY                             | 101  | 0.5005208        | 0.005964215 | 0.038436893|
| KEGG_GLIOMA                                                          | 65   | 0.48526835       | 0.005791506 | 0.0396818|
| KEGG_NODLIKE_RECEPTOR_SIGNALING_PATHWAY                              | 60   | 0.5195665        | 0.015533981 | 0.042915743|
| KEGG_JAK_STAT_SIGNALING_PATHWAY                                      | 151  | 0.47148353       | 0.004065041 | 0.043287273|
| KEGG_FC_GAMMA_R_MEDIATED_PHAGOCYTOSIS                                | 93   | 0.4892478        | 0.00990099 | 0.042204544|
| KEGG_FC_EPSILON_RI_SIGNALING_PATHWAY                                 | 79   | 0.46109742       | 0.039620369 | 0.04183705 |
| KEGG_Axon_guidance                                                   | 127  | 0.4707542        | 0.01183432 | 0.04734439 |
| KEGG_CALCIUM_SIGNALING_PATHWAY                                       | 176  | 0.4929449        | 0.06109996 | 0.047208063|
| KEGG_DORSO_VENTRAL_AXIS_FORMATION                                    | 24   | 0.56431544       | 0.008163262 | 0.05408871 |
| KEGG_NEUROTOPHIN_SIGNALING_PATHWAY                                   | 125  | 0.4376075        | 0.00704563 | 0.05410812 |
| KEGG_ARRHYTHMOGENIC_RIGHT_VENTRICULAR_CARDIOMYOPATHY_ARVC           | 74   | 0.5250181        | 0.014285714 | 0.054433808|
| KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY                               | 106  | 0.47410268       | 0.021526419 | 0.055294024|
| KEGG_CHAT_MOMINE_SIGNALING_PATHWAY                                   | 184  | 0.47442147       | 0.025590552 | 0.053740975|
| KEGG_ALDOSTERONE_REGULATED_SODIUM_REABSORPTION                      | 42   | 0.49038163       | 0.00897166 | 0.05194523 |
| KEGG_HYPERTROPHIC_CARDIOMYOPATHY_HCM                                | 83   | 0.50052696       | 0.010603663 | 0.05285185 |
| KEGG_VASCULAR_SMOOTH_MUSCLE_CONTRACTION                              | 113  | 0.4594074        | 0.022357723 | 0.051408213|
| KEGG_INOSITOL_PHOSPHATE_METABOLISM                                   | 54   | 0.483866         | 0.016427105 | 0.05364621 |
| KEGG_LONG_TERM_POTENTIATION                                          | 69   | 0.42732894       | 0.00813159 | 0.054988716|
| KEGG_ERBB_SIGNALING_PATHWAY                                          | 86   | 0.4261888        | 0.018218623 | 0.059245516|
| KEGG_CHRONIC_MYELOID_LEUKEMIA                                       | 73   | 0.4550269        | 0.01594542 | 0.058499124|
| KEGG_COLORECTAL_CANCER                                               | 62   | 0.43846455       | 0.017475728 | 0.059406566|
| KEGG_GNRH_SIGNALING_PATHWAY                                          | 101  | 0.4099261        | 0.007662835 | 0.063104525|
| KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION                          | 257  | 0.4562621        | 0.03505296 | 0.062216133|
| KEGG_APOPTOSIS                                                       | 86   | 0.43924192       | 0.023255814 | 0.061754137|
| KEGG_DILATED_CARDIOMYOPATHY                                         | 90   | 0.48362222       | 0.028513283 | 0.07178575 |
| KEGG_MTOR_SIGNALING_PATHWAY                                          | 51   | 0.41760606       | 0.014256619 | 0.070806555|
| KEGG_HEMATOPOIETIC_CELL_LINEAGE                                     | 85   | 0.5320989        | 0.044354837 | 0.071416594|
| KEGG_WNT_SIGNALING_PATHWAY                                           | 149  | 0.38095233       | 0.01682243 | 0.07165872 |
| KEGG_NON_SMALL_CELL_LUNG_CANCER                                     | 54   | 0.43223512       | 0.022222223 | 0.07510562 |
| KEGG_HEDGEHOG_SIGNALING_PATHWAY                                     | 56   | 0.44952872       | 0.02504817 | 0.07498143 |
| KEGG_PHOSPHATIDYLINOSITOL_SIGNALING_SYSTEM                           | 76   | 0.42925084       | 0.037030737 | 0.07830575 |
| KEGG_LONG_TERM_DEPRESSION                                           | 68   | 0.40578875       | 0.02173913 | 0.08100538 |
| KEGG_GAP_JUNCTION                                                    | 88   | 0.40297355       | 0.021696253 | 0.0919032334|
| KEGG_TYPE_II DIABETES_MELLITUS                                      | 46   | 0.43765506       | 0.03909465 | 0.09847454 |
| KEGG_TIGHT_JUNCTION                                                 | 128  | 0.3705701        | 0.029354207 | 0.10748016 |

(Continued)
and cg00049440 methylation, indicating that promoter methylation could be the mechanism underlying KLF9 dysregulation in BC \((r = -0.34, P = 5.08E-23; \text{Figure 7})\).

**Associations Between KLF9 Methylation And Clinical Characteristics**

Clinical features of BC were also downloaded using the UCSC Xena browser. There were 788 cases with both KLF9 methylation data and clinical features. Associations between KLF9 methylation and demographic and clinicopathological parameters in patients with BC are summarized in Table 3. The results showed that KLF9 methylation levels were significantly elevated in elderly patients \((\geq 55 \text{ years})\), and patients with estrogen receptor-positive (ER+), progesterone receptor-positive (PR+), and non-triple-negative tumors.

**Prognostic Value Of KLF9 Expression And Methylation In BC**

By performing multivariate analysis, we identified three clinical parameters associated with poor OS: age, lymph node metastasis, and distant organ metastasis \((\text{age, HR} = 1.04, P = 3.3E-07; \text{lymph node metastasis, HR} = 1.79, P = 0.02; \text{distant metastasis, HR} = 4.42, P = 5.6E-05)\). Distant metastasis was associated with poor recurrence-free survival (RFS) \((\text{M stage: HR} = 5.12, P = 3.66E-03)\). Moreover, methylation of KLF9 was an independent prognostic factor for superior OS \((HR = 0.35, P = 0.015)\) and RFS \((HR = 0.31, P = 0.035)\) of patients with BC (Table 4).

**Hypermethylation Of KLF9 In A Validation Dataset**

To verify the methylation levels of KLF9, a fragment of the KLF9 promoter region was amplified using qMSP. A total of 144 bisulfite-converted DNA residues from BC and adjacent normal control tissue samples were analyzed. The results demonstrated elevated KLF9 methylation levels in BC relative to controls \((0.322 \pm 0.193 \text{ vs. } 0.113 \pm 0.059, P = 3.89E-25; \text{Figure 8})\). Next, subgroup analyses were conducted according to clinical features. The results were in accordance with our findings from analysis of TCGA dataset, showing elevated methylation level of KLF9 in patients with ER+, PR+, and non-triple-negative tumors (Table 5).

**Table 2 (Continued).**

| Name                        | Size | Enrichment Score | P         | FDR q-value |
|-----------------------------|------|------------------|-----------|-------------|
| KEGG_ENDOCYTOSIS            | 176  | 0.36516306       | 0.035643563 | 0.12364727  |
| KEGG_VEGF_SIGNALING_PATHWAY | 75   | 0.37441227       | 0.048449613 | 0.13148727  |
Discussion

BC is clinically heterogenous disease. Approximately 10–15% of patients with BC have aggressive disease and develop distant metastasis within 3 years after the initial detection of the primary tumor. Further, patients with BC are at risk of experiencing metastasis for their entire lifetime. The heterogenous nature of BC metastases hinders both the definition of cure for this disease and assessment of risk factors for metastasis.

Biomarkers that can predict the metastasis or prognosis of BC are also scarce. Although the risk of distant metastases increases with the presence of lymph node metastasis, larger-size primary tumor, and poor histopathological grade, which are established prognostic markers in BC,
approximately one-third of women with BC that has not spread to the lymph nodes develop distant metastasis, and around one-third of those with BC that has spread to the lymph node remain free of distant metastasis 10 years after local therapy.\textsuperscript{35,36}

KLFs are a family of transcriptional regulators characterized by a triple zinc finger DNA-binding domain with highly conserved C-terminal binding to GC-rich sequences.\textsuperscript{37–39} By influencing the expression of major regulatory factors, KLFs contribute to virtually all facets of cellular function, including cell proliferation, apoptosis, differentiation, and neoplastic transformation.\textsuperscript{37} An emerging body of evidence indicates that KLFs are associated with various types of cancers, and abnormal expression of KLF genes has been detected in multiple tumor types.\textsuperscript{40–42} The expression profile and functions of some KLFs are overlapping, while others are widely divergent. Reduction or loss of KLF6 has been reported in colorectal cancer.\textsuperscript{42} In BC, KLF5 expression is significantly reduced compared with that in matched normal tissues;\textsuperscript{40} however, KLF4 mRNA expression is increased in early and invasive BC,\textsuperscript{43} and KLF4 has attracted considerable attention for its opposing effects in carcinogenesis as tumor suppressor in gastrointestinal cancer\textsuperscript{44} or an oncoprotein in BC.\textsuperscript{45} As an important member of the KLF family, the role of KLF9 in BC remains largely unknown.

Table 3 Associations Of KLF9 Methylation With Clinical Characteristics Of BC In TCGA Dataset

| Parameters                      | n  | KLF9 Methylation | P value |
|---------------------------------|----|------------------|---------|
| Age (years)                     |    |                  |         |
| ≤55                             | 455| 0.363±0.231      | 0.0062  |
| >55                             | 333| 0.312±0.221      |         |
| Gender                          |    |                  |         |
| Female                          | 779| 0.342±0.228      | 0.075   |
| Male                            | 9  | 0.478±0.233      |         |
| ER status                       |    |                  |         |
| Positive                        | 574| 0.369±0.227      | 6.04E-11|
| Negative                        | 170| 0.246±0.201      |         |
| Null                            | 44 |                  |         |
| PR status                       |    |                  |         |
| Positive                        | 503| 0.359±0.221      | 0.0014  |
| Negative                        | 240| 0.302±0.234      |         |
| Null                            | 45 |                  |         |
| HER2 status                     |    |                  |         |
| Positive                        | 141| 0.309±0.221      | 0.094   |
| Negative                        | 555| 0.348±0.227      |         |
| Null                            | 92 |                  |         |
| Triple-negative breast cancer   |    |                  |         |
| Non-triple                      | 628| 0.360±0.227      | 9.71E-09|
| Negative                        | 117| 0.223±0.194      |         |
| Null                            | 43 |                  |         |
| T                               |    |                  |         |
| T1                              | 200| 0.335±0.225      | 0.32    |
| T2                              | 452| 0.340±0.230      |         |
| T3                              | 109| 0.369±0.226      |         |
| T4                              | 24 | 0.323±0.237      |         |
| Null                            | 3  |                  |         |
| N                               |    |                  |         |
| N0                              | 350| 0.326±0.229      | 0.22    |
| N1                              | 273| 0.351±0.227      |         |
| N2                              | 95 | 0.366±0.226      |         |
| N3                              | 58 | 0.337±0.216      |         |
| Null                            | 12 |                  |         |
| M                               |    |                  |         |
| M0                              | 619| 0.339±0.228      | 0.674   |
| M1                              | 13 | 0.312±0.261      |         |
| Null                            | 156|                  |         |
| Stage                           |    |                  |         |
| Stage I                         | 127| 0.332±0.227      | 0.19    |
| Stage II                        | 443| 0.338±0.233      |         |
| Stage III                       | 200| 0.364±0.218      |         |
| Stage IV                        | 13 | 0.239±0.216      |         |

(Continued)
KLF9 was previously named basic transcription element-binding protein 1 (BTEB) and first identified as a trans-repressor of the CYP1A1 gene and then reported to induce CYP7A. KLF9 mRNA is most strongly expressed in the brain, kidney, lung, and testis. Previous studies have reported regulatory activity of KLF9 in the uterus during the development of BC and pregnancy, with KLF9 knockout mice exhibiting uterine hypoplasia, smaller litter size, reduced numbers of implantation sites, partial progesterone resistance in the uterus, and delayed parturition. In addition to the roles of KLF9 in normal cells and tissues, it has important tumor suppressive and oncogenic functions in cancer. Well-characterized biological effects of KLF9 include a role in endometrial carcinogenesis. In the current study, our results also confirmed that KLF9 is mainly enriched in signaling pathway associated with carcinogenesis by GSEA analysis, including small cell lung cancer, prostate cancer, pancreatic cancer, pathway in cancer, and melanoma. In uterine endometrial cells, progesterone opposes the pro-proliferative effects of estrogen, and the absence of progesterone receptor (PR) signaling can promote cell proliferation. KLF9 can facilitate progesterone-induced effects on uterine gene expression by its co-recruitment to the PR and trans-inhibition of estrogen receptor α activity by promoting its estrogen-induced downregulation, suggesting that KLF9 inhibits proliferation in hormonally responsive cancers. Further, endometrial cancer cells with loss of KLF9 fail to be activated by estrogen, demonstrating that alteration of KLF9 expression may lead to escape from estrogen-mediated growth regulation. These findings suggest the intriguing likelihood that KLF9, regulated in concert with PR and ER, may serve as a prognostic predictor for hormonally responsive diseases.

In our study, we found that KLF9 mRNA expression was significantly reduced in patients with BC relative to normal controls, partly due to hypermethylation of the

| Parameters      | n | KLF9 Methylation | P value |
|-----------------|---|------------------|---------|
| Null            | 5 |                  |         |
| Menopause status|   |                  |         |
| Pre-menopause   | 278 501 9 |                  |         |
| Post-menopause  | 0.34±0.229 0.342±0.228 | 0.932   |

Table 4 Multivariate Analysis Of KLF9 Methylation In BC

| Parameters | OS                        |            | P value | RFS                        |            | P value |
|------------|---------------------------|------------|---------|----------------------------|------------|---------|
|            | HR                        | 95% CI (Lower/Upper) | P value | HR                        | 95% CI (Lower/Upper) | P value |
| Age        | 1.04                      | 0.15 0.82 | 3.30E-07 | 1.02                      | 1.00 1.04 | 0.03    |
| Gender     |                           |            |         |                           |            |         |
| Female (Ref) |                |            |         | Male                       | 0.62 0.09 4.55 | 0.64 | 1.11E-07 0.00 Inf | 0.99 |
| Stage      |                           |            |         |                           |            |         |
| Stage I/II (Ref) |            |            |         | Stage III/IV               | 1.69 0.96 2.98 | 0.069 | 2.16 0.89 5.25 | 0.088 |
| T          |                           |            |         |                           |            |         |
| T1/2 (Ref) |                |            |         | T3/T4                      | 0.96 0.54 1.70 | 0.88 | 1.39 0.70 2.76 | 0.34 |
| N          |                           |            |         |                           |            |         |
| N0 (Ref)   |                |            |         | N1                         | 1.79 0.54 2.94 | 0.02 | 1.13 0.49 2.56 | 0.77 |
| M          |                           |            |         |                           |            |         |
| M0 (Ref)   |                |            |         | M1                         | 4.42 2.15 9.11 | 5.60E-05 | 5.12 1.70 15.41 | 3.66E-03 |
| KLF9 methylation | 0.35 0.15 0.82 | 0.015 | 0.31 0.10 0.92 | 0.035 |
KLF9 gene promoter region. No significant difference in methylation was observed among different TNM stage tumors; however, an upward trend in methylation was detected in advanced TNM stage disease (T3–4 stage, N2 stage, stage III). The reverse phenomenon (lower KLF9 methylation) was exhibited in patients with T4, N3, M1, and clinical stage IV tumors, which may be attributable to the relatively small number of samples from these tumor stages (24 patients with T4, 58 patients with N3, 13 patients with M1, and 13 patients with stage IV). The results of subgroup analysis according to other clinical characteristics demonstrated higher methylation of KLF9 in patients with ER+, PR+, or non-triple-negative BC, which may be accounted for by the relationship of KLF9 expression with hormone levels in BC. These data suggest the possibility of acquired resistance to endocrine therapy following prolonged exposure to tamoxifen; that is, KLF9 expression may be re-induced in breast cells responsive to tamoxifen. This hypothesis requires confirmation by more rigorous and comprehensive prospective studies.

Additionally, we performed multivariate analysis demonstrating that age (HR for OS = 1.04, HR for RFS = 1.00), node metastasis status (HR for OS = 1.79, HR for RFS = 1.13), and distant metastasis (HR for OS = 4.42, HR for RFS = 5.12) are useful prognostic biomarkers of poor outcome in BC. Notably, hypermethylation of KLF9 exhibited the potential to act as a prognostic marker of favorable outcome in BC (HR for OS = 0.35, HR for RFS = 0.31).

Table 5 Associations of KLF9 Methylation With Clinical Characteristics of BC in Our Validation Dataset

| Parameters                  | n  | KLF9 Methylation | P value |
|-----------------------------|----|------------------|---------|
| Age                         |    |                  |         |
| ≥55                         | 78 | 0.323±0.208      | 0.97    |
| <55                         | 66 | 0.322±0.175      |         |
| ER status                   |    |                  |         |
| Positive                    | 94 | 0.368±0.198      | 1.85E-05|
| Negative                    | 50 | 0.336±0.151      |         |
| PR status                   |    |                  |         |
| Positive                    | 93 | 0.3616±0.200     | 3.68E-04|
| Negative                    | 51 | 0.250±0.159      |         |
| HER2 status                 |    |                  |         |
| Positive                    | 40 | 0.244±0.114      | 1.12E-04|
| Negative                    | 104| 0.352±0.209      |         |
| Triple-negative breast cancer |  |                  |         |
| Non-triple negative         | 123| 0.348±0.197      | 2.18E-12|
| Negative                    | 21 | 0.173±0.059      |         |
| T                           |    |                  |         |
| T1/T2                       | 114| 0.320±0.191      | 0.807   |
| T3/T4                       | 30 | 0.330±0.203      |         |
| N                           |    |                  |         |
| N0                          | 64 | 0.302±0.184      | 0.252   |
| N+                          | 80 | 0.339±0.200      |         |
| M                           |    |                  |         |
| M0                          | 139| 0.320±0.195      | 0.361   |
| M+                          | 5  | 0.375±0.115      |         |
| Stage                       |    |                  |         |
| Stage I/II                  | 90 | 0.310±0.199      | 0.317   |
| Stage III/IV                | 54 | 0.343±0.183      |         |
| Menopause status            |    |                  |         |
| Pre-menopause               | 53 | 0.314±0.189      | 0.711   |
| Post-menopause              | 91 | 0.327±0.196      |         |

**Figure 8** KLF9 methylation levels in our validation dataset.

**Conclusion**

In summary, our data demonstrate that hypermethylation of the KLF9 promoter region results in its downregulation in BC.
Multivariate analysis showed that KLF9 methylation was associated with favorable prognosis in patients with BC. Further, the construction of an interaction network of genes co-expressed with KLF9 indicated that this factor may participate in breast carcinogenesis by contributing to cell migration and multiple growth regulation pathways. Moreover, we found that methylation of KLF9 is an independent prognostic factor for superior OS in patients with BC. These findings may inspire new clinical practices for patients with BC, including for diagnosis, treatment, and prognosis.

Disclosure

The authors declare that there are no conflicts of interest.

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