Identification and Validation of an Autophagy-Related IncRNA Signature for Patients With Breast Cancer

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Background: Autophagy is a “self-feeding” phenomenon of cells, which is crucial in mammalian development. Long non-coding RNA (IncRNA) is a new regulatory factor for cell autophagy, which can regulate the process of autophagy to affect tumor progression. However, poor attention has been paid to the roles of autophagy-related IncRNAs in breast cancer.

Objective: This study aimed to construct an autophagy-related IncRNA signature that can effectively predict the prognosis of breast cancer patients and explore the potential functions of these IncRNAs.

Methods: The RNA sequencing (RNA-Seq) data of breast cancer patients was collected from The Cancer Genome Atlas (TCGA) database and the GSE20685 database. Multivariate Cox analysis was implemented to produce an autophagy-related IncRNA signature in the TCGA cohort. The signature was then validated in the GSE20685 cohort. The receiver operator characteristic (ROC) curve was performed to evaluate the predictive ability of the signature. Gene set enrichment analysis (GSEA) was used to explore the potential functions based on the signature. Finally, the study developed a nomogram and internal verification based on the autophagy-related IncRNAs.

Results: A signature composed of 9 autophagy-related IncRNAs was determined as a prognostic model, and 1,109 breast cancer patients were divided into high-risk group and low-risk group based on median risk score of the signature. Further analysis demonstrated that the over survival (OS) of breast cancer patients in the high-risk group was poorer than that in the low-risk group based on the prognostic signature. The area under the curve (AUC) of ROC curve verified the sensitivity and specificity of this signature. Additionally, we confirmed the signature is an independent factor and found it may be correlated to the progression of breast cancer. GSEA showed gene sets were...
notably enriched in carcinogenic activation pathways and autophagy-related pathways. The qRT-PCR identified 5 lncRNAs with significantly differential expression in breast cancer cells based on the 9 lncRNAs of the prognostic model, and the results were consistent with the tissues.

Conclusion: In summary, our signature has potential predictive value in the prognosis of breast cancer and these autophagy-related lncRNAs may play significant roles in the diagnosis and treatment of breast cancer.

Keywords: long non-coding RNA, autophagy, breast cancer, The Cancer Genome Atlas, prognostic signature

INTRODUCTION

Breast cancer is the most common malignant tumor and the leading cause of cancer-related deaths in women worldwide, with an incidence and mortality of 24.2% and 15.0%, respectively (1, 2). Current treatment options for breast cancer usually combine surgery with a variety of adjuvant therapies, such as chemotherapy, radiotherapy, endocrine therapy, targeted therapy, and immunotherapy (3–8). Most patients respond to initial treatment within a certain period of time, but there are still some breast cancers, especially triple negative breast cancer, that will develop into a more invasive tumor form, resulting in a poor prognosis (9–11). Because of the strong heterogeneity of breast cancer, multi-parameter signals are more valuable than single biomarkers in predicting the prognosis of breast cancer.

Autophagy is a physiological process that guides the degradation of damaged, denatured, or senescent proteins and damaged organelles in lysosomes. There are two states of autophagy activation: normal state and pathological state. Autophagy responds to various stresses by providing circulating metabolic substrates needed for survival under normal physiological conditions (12). Recent studies further indicate that abnormal autophagy is considered to be a potential cause of many diseases, including cardiovascular and cerebrovascular diseases, liver diseases, and cancer (13, 14). Autophagy is closely related to the occurrence, development, and metastasis of tumor. Multiple studies have reported that autophagy may play especially important roles in supporting cell growth and drug resistance to targeted therapy in BRAF-mutant melanoma (15–17). Some researchers found that MIR106A-5p upregulation suppressed autophagy and accelerated malignant phenotype in nasopharyngeal carcinoma (18). Furthermore, recent findings have revealed the autophagy drugs such as sirolimus and arsenic trioxide can induce autophagy of tumor cells and improve the outcome of certain cancer patients. However, only a limited proportion of patients benefited from autophagy-based treatment. Therefore, establishing predictive biomarkers for autophagic drug therapy response is required to identify patients who can benefit from autophagy-based treatment.

lncRNA is a small RNA with a length of more than 200bp and no protein coding function (19). Although they cannot be translated into proteins, they are involved in the process of protein translation (20). LncRNA plays a critical role in complex autophagy regulatory networks by regulating the biological effects of a variety of autophagy-related DNA, RNA, or proteins (21). Some studies have reported that lncRNA-HOTAIRM1 regulates autophagy and degrades tumor protein PML-RARA, during differentiation arrest of bone marrow cells, suggesting that lncRNA is a potential therapeutic target for leukemia (22). Other researchers have found the expression of lncRNA MEG3 is significantly down-regulated in glioma tissues and cells, and its overexpression can significantly inhibit cell proliferation and promote apoptosis and autophagy of glioma cells (23).

Considering the significance of lncRNA and autophagy in breast cancer biology, we determined an autophagy-related lncRNA signature to predict the prognosis of breast cancer patients and provide a theoretical basis for the diagnosis and treatment of breast cancer.

MATERIALS AND METHODS

Datasets Preparation

The workflow of our analysis is illustrated in Figure 1. TCGA (https://cancergenome.nih.gov) RNA-Seq data of breast cancer patients was used as a training set to construct a signature composed of autophagy-related lncRNAs. The training cohort included TCGA mRNA expression (FPKM) in 1,109 breast cancer patients and related clinical information. We collected an independent dataset GSE20685 (series matrix files), which was performed using the platform GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20685). In order to validate the predictive capability and the universal applicability of the signature, GSE20685 dataset including 327 breast cancer patients was employed as a testing set in our present study.

LncRNAs and Autophagy Genes Mining

LncRNAs and mRNAs were extracted based on the annotations provided by GENCODE (https://www.gencodegenes.org). Obtain the list of autophagy gene from the human autophagy data (HADb, http://autophagy.lu/clustering/index.html) and extract the mRNA related to autophagy gene. Analyze the correlation between lncRNAs and autophagy-related genes in...
breast cancer by using Pearson method. LncRNAs with correlation coefficient $R^2 > 0.3$ and $P < 0.001$ are considered to be autophagy-related lncRNAs. Extract the shared lncRNAs from autophagy-related lncRNAs and GSE20685 for the following analysis. Then, the shared lncRNAs in TCGA were used as a training dataset ($n=1,109$), and the shared lncRNAs in GSE20658 were used as a testing dataset ($n=327$). All RNA-seq sequencing data were normalized by log2 conversion.

### Signature Development

Univariate Cox regression analysis was executed to obtain the prognosis-related lncRNAs from the training dataset. Then the lncRNAs with $P < 0.05$ were included in the multivariate Cox hazard model analysis to produce an autophagy-related lncRNA signature model. In order to avoid over-fitting, Akaike Information Criterion (AIC) was conducted to select the stepwise signature, and the signature with the lowest AIC value was considered to be the optimal signature. The risk score of each patient with breast cancer was calculated according to the following formula: Risk score = $b_{\text{lncRNA}1} \times \text{expr}_{\text{lncRNA}1} + b_{\text{lncRNA}2} \times \text{expr}_{\text{lncRNA}2} + \ldots + b_{\text{lncRNA}_n} \times \text{expr}_{\text{lncRNA}_n}$. $b$ is the regression coefficient of the corresponding lncRNA, expr is the expression level of lncRNA, and its unit is FPKM. Based on the median risk score, 1,109 patients with breast cancer in the training dataset were divided into two groups: high-risk group and low-risk group. In order to compare the difference of OS between high-risk group and low-risk group, Kaplan Meier-plotter was implemented. In addition, ROC curve was performed to evaluate the predictive ability of the signature by using the SurvivalROC package. Finally, 327 breast cancer patients with credible prognostic data from the GSE20685 set were regarded as a testing set to evaluate the predictive capability of the signature. All $P<0.05$ were regarded as a significant difference.

### Gene Set Enrichment Analysis

GSEA was executed to discover changes in the expression of predefined gene sets rather than individual gene, so it can be performed to identify whether gene sets show statistically remarkable differences between the two biological states of samples. In our analysis, GSEA version 4.0.3 was used to verify whether the differentially expressed gene sets between the low-risk group and high-risk group were enriched in cancer-related and autophagy-related processes.

### Cell Culture

Human normal breast cells (HBL-100) and breast cancer cells (MCF-7 and T-47D) were purchased from the Cell Bank of the...
Chinese Academy of Sciences, Shanghai, China. All cells were incubated in RPMI-1640 medium (Solarbio, Beijing, China) supplemented with 10% fetal bovine serum (Biological Industries, Israel, USA), 100 units/ml penicillin G, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a damp incubator, which was supplemented with 5% CO2.

LncRNAs Validation Using qRT-PCR

After being isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantified by NanoDrop 2000 (Thermo Fisher Scientific Inc, Rockford, IL, USA), 1 μg of total RNA underwent reverse transcription by UEIris II RT-PCR System for First-Strand cDNA Synthesis (US EVERBRIGHT INC, Suzhou, China) according to the manufacturer’s instruction. The product was then used for real-time PCR by Universal SYBR Green qPCR Supermix (US EVERBRIGHT INC, Suzhou, China). PCR was conducted as follows: pre-degeneration at 95°C for 5 min; denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, repeating the previous process 40 times. GRAPDH was used as internal references, and experiment of each group was repeated three times. Quantification of the relative expression levels of lncRNA by 2^ΔΔCt method. Primers sequences are presented in Table 1.

Statistical Analysis

All statistical analysis and drawings were carried out using R software (version 3.6.3) and Bioconductor. The co-expression

### TABLE 1 | Primer sequences for RT-PCR analysis.

| LncRNA   | Forward Primer   | Reverse Primer   |
|----------|-----------------|-----------------|
| USP30-AS1-F | 5'-TGAAACCCTCCTCCGCTACC-3' | 5'-TTGAATCCCGATCTCTT-3' |
| USP30-AS1-R | 5'-CTTCTTGGGCTGCGCACAC-3' | 5'-AACATGCACGTCGACGTC-3' |
| TFAP2A-AS1-F | 5'-GCCCAACTCCATCCCTCCTC-3' | 5'-GCCCACTCCATCCCTCCTC-3' |
| MAPT-AS1-F | 5'-GAGGAGGGATGGTGACTGAT-3' | 5'-GAGGAGGGATGGTGACTGAT-3' |
| MAPT-AS1-R | 5'-GAGGAGGGATGGTGACTGAT-3' | 5'-GAGGAGGGATGGTGACTGAT-3' |
| LINC01087-F | 5'-GGGATCCATCGCTCGCTGCTGCT-3' | 5'-GGGATCCATCGCTCGCTGCTGCT-3' |
| LINC01087-R | 5'-GGGATCCATCGCTCGCTGCTGCT-3' | 5'-GGGATCCATCGCTCGCTGCTGCT-3' |
| GAPDH-F | 5'-CGAGGCTGGGCTCATTT-3' | 5'-CGAGGCTGGGCTCATTT-3' |
| GAPDH-R | 5'-CGAGGCTGGGCTCATTT-3' | 5'-CGAGGCTGGGCTCATTT-3' |

### FIGURE 2 | Identification of an autophagy-related lncRNA signature in breast cancer. (A) Venn diagram describes 180 shared lncRNAs from 1,270 autophagy-related lncRNAs and 1,146 lncRNAs of GSE20685. (B) The network of autophagy-genes and lncRNAs. The blue nodes indicate autophagy genes and the pink nodes indicate lncRNA. The co-expression network is performed by CYTOSCAPE 3.7.2. (C) Univariate Cox regression analysis was used to confirm the lncRNAs associated with autophagy were strongly with patients’ OS in training dataset. (D) Multivariate Cox regression analysis to construct the prognostic signature.
network was constructed by Cytoscape to visualize candidate lncRNAs related to prognosis. The autophagy-related lncRNAs in patients with breast cancer were analyzed with R software package “ggalluvial,” “ggplot2,” and “pheatmap.” Then Sankey diagram, risk score, survival status, and lncRNA heat map were drawn. Kaplan Meier-plotter was performed to evaluate the OS differences between high-risk group and low-risk group of the signature. Univariate and multivariate Cox regression were constructed to assess whether the prognostic signature was independent of clinical characteristics such as age, gender, clinical stage, and TNM stage. And the Cox regression also was used to confirm the clinical value of the signature in age, gender, clinical stage, and TNM stage. The above operations are implemented using the R software package “Survival.” The nomogram was implemented using rms R package to predict the OS. All P<0.05 was regarded as a significant difference. The Limma package was used to identify the differentially expressed lncRNAs in view of |log2 fold change (FC)|≥1 and false discovery rate (FDR)<0.05 based on 9 lncRNAs. All P<0.05 were considered statistically significant.

RESULTS

Construction of Autophagy-Associated lncRNA Co-expression Network
We identified a total of 19,658 mRNAs and 14,142 lncRNAs, which were screened from the TCGA dataset. According to 222 autophagy genes in Human Autophagy Database, we developed a co-expression network to determine autophagy-related lncRNAs. The Pearson correlation analysis showed that 1,270 lncRNAs were associated with autophagy-related genes in breast cancer (R² > 0.3, P < 0.001). Finally, we obtained 180 shared lncRNAs from 1,270 autophagy-related lncRNAs and 1,146 lncRNAs of GSE20685 (Figures 2A, B).

Identification of an Autophagy-Related lncRNA Signature in Breast Cancer
By using univariate cox regression analysis, we identified 25 lncRNAs that were strongly with patients’ OS from 180 autophagy-related lncRNAs in training dataset (Figure 2C) (P<0.05). Stepwise multivariable cox proportional hazards regression analysis were carried out to identify the optimal prognostic lncRNAs among the 25 candidate lncRNAs. Subsequently, 9 candidate lncRNAs were identified to be independent prognostic factors for breast cancer patients (Figure 2D). We then developed autophagy-related lncRNAs co-expression networks based on the 9 genes (Table 2, Figure 3A).

| Table 2 | Correlation between autophagy genes and lncRNAs in breast cancer. |
|---------------------------------|-----------------|-------|
| Autophagy genes | lncRNAs | Correlation | P value |
| ARSA | KMT2E-AS1 | 0.38 | 7.24E-40 |
| ARSB | KMT2E-AS1 | -0.33 | 2.06E-30 |
| ATF6 | KMT2E-AS1 | -0.31 | 2.37E-26 |
| ATG18L2 | KMT2E-AS1 | 0.30 | 9.31E-25 |
| ATG4B | KMT2E-AS1 | 0.50 | 2.12E-72 |
| ATG4D | KMT2E-AS1 | 0.39 | 4.97E-25 |
| BAX | KMT2E-AS1 | 0.46 | 2.74E-60 |
| BNIP1 | KMT2E-AS1 | 0.31 | 3.85E-26 |
| CANX | KMT2E-AS1 | -0.32 | 1.44E-27 |
| CAPN10 | KMT2E-AS1 | 0.51 | 2.75E-75 |
| CLN3 | KMT2E-AS1 | 0.35 | 5.52E-33 |
| DDIT3 | KMT2E-AS1 | 0.30 | 1.24E-24 |
| HSPA8 | KMT2E-AS1 | -0.30 | 3.95E-25 |
| ITGB1 | KMT2E-AS1 | -0.43 | 1.63E-51 |
| MAP2K7 | KMT2E-AS1 | 0.32 | 2.21E-27 |
| MAPK1 | KMT2E-AS1 | -0.53 | 6.64E-66 |
| MLST8 | KMT2E-AS1 | 0.36 | 2.58E-35 |
| NCKAP1 | KMT2E-AS1 | -0.40 | 1.48E-43 |
| PELP1 | KMT2E-AS1 | 0.37 | 2.15E-36 |
| PEX14 | KMT2E-AS1 | 0.35 | 8.71E-33 |
| PKIR4 | KMT2E-AS1 | -0.33 | 4.89E-30 |
| RAB24 | KMT2E-AS1 | 0.50 | 6.73E-72 |
| RB1 | KMT2E-AS1 | -0.37 | 1.37E-37 |
| SH3GLB1 | KMT2E-AS1 | -0.33 | 8.19E-29 |
| STK11 | KMT2E-AS1 | 0.38 | 6.93E-39 |
| WDR45 | KMT2E-AS1 | 0.42 | 6.85E-49 |
| APOL1 | USP30-AS1 | 0.65 | 2.41E-136 |
| BAK1 | USP30-AS1 | 0.36 | 3.88E-35 |
| BAX | USP30-AS1 | 0.30 | 5.64E-25 |
| CASP1 | USP30-AS1 | 0.63 | 4.65E-122 |
| CASP4 | USP30-AS1 | 0.51 | 7.21E-74 |
| CCR2 | USP30-AS1 | 0.51 | 4.73E-74 |
| CFLAR | USP30-AS1 | 0.37 | 2.25E-36 |
| FAS | USP30-AS1 | 0.42 | 4.41E-48 |
| IKKBE | USP30-AS1 | 0.44 | 7.15E-55 |
| MLN | USP30-AS1 | 0.31 | 8.82E-26 |
| PDCD5 | USP30-AS1 | 0.48 | 7.33E-46 |
| RGS19 | USP30-AS1 | 0.41 | 7.33E-46 |
| EGFR | ST7-AS1 | 0.38 | 4.60E-39 |
| ATF4 | VPS9D1-AS1 | 0.33 | 8.33E-29 |
| ATG12 | VPS9D1-AS1 | -0.33 | 1.00E-28 |
| BID | VPS9D1-AS1 | 0.37 | 1.14E-37 |
| BIRC5 | VPS9D1-AS1 | 0.32 | 1.29E-29 |
| CD46 | VPS9D1-AS1 | -0.33 | 5.29E-30 |
| CDKN2A | VPS9D1-AS1 | 0.36 | 7.50E-36 |
| GAPDH | VPS9D1-AS1 | 0.36 | 4.73E-36 |
| CCL5 | VPS9D1-AS1 | 0.51 | 1.57E-56 |
| CASP1 | VPS9D1-AS1 | -0.33 | 4.45E-35 |
| TFAP2A-AS1 | VPS9D1-AS1 | 0.34 | 4.47E-31 |
| BIRC6 | VPS9D1-AS1 | 0.36 | 2.32E-35 |
| TSC1 | VPS9D1-AS1 | 0.40 | 5.96E-45 |
| WDFY3 | VPS9D1-AS1 | 0.35 | 1.37E-34 |
| KIF5B | OTUB6B-AS1 | 0.31 | 1.60E-26 |
| MAPK1 | OTUB6B-AS1 | 0.33 | 5.57E-30 |
| RB1CC1 | OTUB6B-AS1 | 0.59 | 1.86E-104 |
| CAPN2 | HOXB-AS1 | 0.32 | 1.41E-28 |
| ATG2B | LINC01087 | 0.31 | 2.50E-25 |
| BIRC6 | LINC01087 | 0.34 | 4.44E-32 |
| WDFY3 | LINC01087 | 0.40 | 6.66E-44 |
| STK11 | MAPT-AS1 | 0.32 | 4.11E-27 |

LncRNAs, long noncoding RNAs; P<0.05 was regarded as a significant difference.
risk score, 1,109 patients with breast cancer in training dataset were divided into two groups: high-risk group and low-risk group. In Figure 3B, we can see 7 lncRNAs are favorable prognostic factors (USP30-AS1, ST7-AS1, VPS9D1-AS1, TFAP2A-AS1, HOXB-AS1, LINC01087, and MAPT-AS1) and 2 lncRNAs are unfavorable prognostic factors (KMT2E-AS1, OTUB6DB-AS1) for breast cancer patients.

Validation of Prognostic Performance of the Autophagy-Related lncRNA Signature in Breast Cancer

To further assess the capability of the signature on the prognosis of breast cancer, Kaplan-Meier survival analysis and ROC curve analysis were operated in the training cohort from the TCGA dataset. The results demonstrated that the OS of breast cancer...
patients in the high-risk group was poorer than the low-risk group based on the prognostic signature (HR: 1.794, 95% CI: 1.556–2.069; \( P < 0.001 \)) (Figure 4A). Additionally, Figure 4B illustrated that risk score of the signature had an AUC of 0.806 under the ROC curve, indicating a credible diagnostic value. The risk score of the prognostic signature in the low-risk group and high-risk group, survival status of breast cancer patients, and the expression heat map are displayed in Figure 4C. To assess the robustness of the signature in OS prediction of breast cancer patients, we further examined it in the testing cohort from the

| LncRNAs     | \( \beta \) | HR    | Lower 95%CI | Upper 95%CI | \( P \)-value |
|-------------|-------------|-------|-------------|-------------|---------------|
| KMT2E-AS1   | 0.2520      | 1.2866| 0.9278      | 1.7840      | 0.1308        |
| USP30-AS1   | -0.4604     | 0.6310| 0.4794      | 0.8305      | 0.0010        |
| ST7-AS1     | -0.5304     | 0.5884| 0.3985      | 0.8687      | 0.0076        |
| VPS9D1-AS1  | -0.2506     | 0.7784| 0.6457      | 0.9383      | 0.0086        |
| TFAP2A-AS1  | -0.5438     | 0.5805| 0.3770      | 0.8940      | 0.0136        |
| OTUD6B-AS1  | 0.2797      | 1.3227| 0.9840      | 1.7779      | 0.0638        |
| HOXB-AS1    | -0.1847     | 0.8314| 0.6605      | 1.0464      | 0.1157        |
| LINC01087   | -0.0873     | 0.9164| 0.8188      | 1.0257      | 0.1290        |
| MAPT-AS1    | -0.4423     | 0.6426| 0.4955      | 0.8334      | 0.0009        |

LncRNAs, long noncoding RNAs; \( \beta \) is the regression coefficient of the corresponding lncRNA; HR, Hazard ratio; \( P < 0.05 \) was regarded as a significant difference.

![Figure 4](image-url)
FIGURE 5 | Risk score analysis of the prognostic signature in the testing dataset. (A) Kaplan-Meier survival for low-risk group and high-risk group. (B) ROC curves for predicting OS based on risk score. (C) Risk score distribution, survival status, and expression heat map.

TABLE 4 | Predictive values of related clinical features and risk score in the training dataset and the testing dataset.

| Variables | Univariate analysis | Multivariate analysis |
|-----------|---------------------|----------------------|
|           | HR                  | 95% CI               | P-value   | HR                  | 95% CI               | P-value   |
| Training dataset |                    |                      |          |                     |                      |          |
| age       | 1.035               | 1.022–1.049          | <0.001   | 1.035               | 1.020–1.050          | <0.001   |
| gender    | 0.830               | 0.116–5.943          | 0.853    | 0.645               | 0.069–4.672          | 0.664    |
| stage     | 2.205               | 1.756–2.768          | <0.001   | 1.461               | 0.880–2.427          | 0.143    |
| T         | 1.461               | 1.196–1.785          | <0.001   | 1.070               | 0.796–1.438          | 0.654    |
| M         | 4.935               | 2.944–8.273          | <0.001   | 1.908               | 0.843–4.318          | 0.121    |
| N         | 1.620               | 1.364–1.923          | <0.001   | 1.294               | 0.971–1.724          | 0.079    |
| risk score| 1.794               | 1.556–2.069          | <0.001   | 1.787               | 1.525–2.093          | <0.001   |
| Testing dataset |                  |                      |          |                     |                      |          |
| age       | 0.992               | 0.971–1.014          | 0.483    | 0.996               | 0.974–1.018          | 0.697    |
| T         | 1.863               | 1.440–2.412          | <0.001   | 1.288               | 0.891–1.860          | 0.178    |
| M         | 5.204               | 2.391–11.326         | <0.001   | 1.202               | 0.421–3.436          | 0.731    |
| N         | 1.757               | 1.448–2.134          | <0.001   | 1.633               | 1.317–2.026          | <0.001   |
| risk score| 1.215               | 1.105–1.336          | <0.001   | 1.210               | 1.083–1.351          | 0.001    |

HR, hazard ratio; P<0.05 was regarded as a significant difference.
GSE20685 dataset using the same formula. The results revealed that the OS of patients with high-risk score was also worse than those with low-risk score in the GSE20685 cohort (HR: 1.215, 95% CI: 1.105–1.336; P<0.001) and the AUC of the ROC curve of the prognostic model was 0.788 (Figures 5A–C). This result is consistent with the training set. Finally, Cox regression analyses were used to confirm the possibility that risk score of the signature could significantly help to forecast the prognosis of breast cancer patients, eliminating the influence of clinical features (gender, age, clinical stage, and TNM stage) (P<0.001) (Table 4, Figures 6A–D).

**Clinical Value of the Prognostic Signature in Patients With Breast Cancer**

We further evaluated the clinical value of the signature in breast cancer patients by determining the correlation between lncRNA and the clinical characteristics (age, gender, clinical stage, and TNM stage) of breast cancer patients. The results showed that

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**TABLE 5 | Clinical impact of risk score signature for the TCGA cohort.**

| Clinical Features | n   | Risk Score | Mean | SD  | t    | P    |
|------------------|-----|------------|------|-----|------|------|
| Age              |     |            |      |     |      |      |
| <=65             | 665 | 1.270      | 0.857| -2.673| 0.008|
| >65              | 243 | 1.452      | 0.932|      |      |
| Gender           |     |            |      |     |      |      |
| Female           | 897 | 1.319      | 0.882| 0.314| 0.700|
| Male             | 11  | 1.238      | 0.853|      |      |
| Stage            |     |            |      |     |      |      |
| I-II             | 691 | 1.277      | 0.862| -2.443| 0.000|
| III-IV           | 217 | 1.451      | 0.929|      |      |
| T                |     |            |      |     |      |      |
| T1-2             | 774 | 1.304      | 0.870| -1.169| 0.161|
| T3-4             | 134 | 1.405      | 0.938|      |      |
| M                |     |            |      |     |      |      |
| M0               | 891 | 1.310      | 0.881| -2.325| 0.186|
| M1               | 17  | 1.746      | 0.763|      |      |
| N                |     |            |      |     |      |      |
| N0               | 449 | 1.265      | 0.867| -1.799| 0.013|
| N1-3             | 459 | 1.370      | 0.892|      |      |

P<0.05 was regarded as a significant difference.
the risk score of patients over 65 years old (t = -2.673, P = 0.003), stage III–IV (t = -2.433, P = 0.000) and N1–3 (t = -1.799, P = 0.013) tends to increase significantly, suggesting that older age, advanced stage, and lymphatic metastasis may be correlated with the progression of breast cancer (Table 5).

**Gene Set Enrichment Analysis**

To further explore the potential biological behavior of this signature in breast cancer patients, we determined a total of 25 gene sets using GSEA, which were notably enriched with a nominal P value of < 0.05 and FDR q-value < 0.25 (Table 6). The results revealed that the differentially expressed genes between the high-risk and low-risk groups were enriched in the cancer-related and autophagy-related pathways. As shown in Table 5 and Figures 7A–F, stromal and carcinogenic activation pathways were markedly enriched in high risk group, such as cell adhesion, ECM receptor interaction, TGF beta signaling pathway, Renal cell carcinoma pathway, and Prostate cancer pathway. Surprisingly, we also found that glucose metabolic pathways were significant such as Glycolysis/Gluconeogenesis pathway, TCA cycle pathway, and starch and sucrose metabolism pathway enriched in high risk groups (Figures 7G–I). It is worth noting that TGF-β signaling pathway and glucose metabolism pathway are closely related to autophagy. The results from GSEA have revealed these autophagy-related genes contribute to carcinogenic activation pathways and autophagy-related pathways, which may provide sufficient evidence for targeted therapy of breast cancer.

**The Nomogram Establishing and the Prognostic Value Validating Base on 9 IncRNAs in TCGA Dataset**

Based on these autophagy-related IncRNAs of the signature, a nomogram was constructed. Multivariate Cox analysis was performed to assign the points in the nomogram to each variable. By drawing a vertical line between the total point axis

### Table 6 | Gene set enrichment analysis based on the signature of 9 autophagy-related IncRNAs.

| Name                                               | Size   | ES    | NES   | NOM P-val | FDR q-val | FWER P-val | Rank at max | Leading edge |
|----------------------------------------------------|--------|-------|-------|-----------|-----------|------------|-------------|--------------|
| OOCYTE MEIOSIS                                     | 112.000| 0.536 | 2.039 | 0.000     | 0.047     | 0.066      | 464900.000  | tags=39%, list=8%, signal=43% |
| VIBRIO CHOLEREA_INFECTION                          | 54.000 | 0.570 | 1.956 | 0.002     | 0.087     | 0.150      | 885500.000  | tags=52%, list=16%, signal=62% |
| GAP_JUNCTION                                       | 90.000 | 0.449 | 1.760 | 0.002     | 0.138     | 0.414      | 634400.000  | tags=39%, list=11%, signal=44% |
| CITRAT CYCLE_TCA_CYCLE                             | 31.000 | 0.718 | 2.040 | 0.006     | 0.095     | 0.066      | 459700.000  | tags=55%, list=8%, signal=60% |
| PATHOGENIC_ESCHERICHIA_COLL_INFECTION              | 56.000 | 0.546 | 1.789 | 0.006     | 0.217     | 0.363      | 605300.000  | tags=43%, list=11%, signal=48% |
| O_GLUCAN_BIOSYNTHESIS                              | 30.000 | 0.534 | 1.758 | 0.010     | 0.127     | 0.417      | 728400.000  | tags=50%, list=13%, signal=58% |
| STEROID_BIOSYNTHESIS                               | 17.000 | 0.700 | 1.776 | 0.011     | 0.151     | 0.384      | 299500.000  | tags=53%, list=5%, signal=56% |
| TGF_BETA_SIGNALING_PATHWAY                         | 85.000 | 0.469 | 1.752 | 0.012     | 0.112     | 0.427      | 397300.000  | tags=31%, list=7%, signal=33% |
| GLYOULYSIS_GLUCONEOGENESIS                          | 62.000 | 0.488 | 1.771 | 0.013     | 0.140     | 0.396      | 547100.000  | tags=34%, list=10%, signal=38% |
| TEPENIOD_BACKBONE_BIOSYNTHESIS                     | 15.000 | 0.705 | 1.786 | 0.015     | 0.196     | 0.368      | 503000.000  | tags=40%, list=9%, signal=44% |
| N_GLUCAN_BIOSYNTHESIS                              | 46.000 | 0.548 | 1.783 | 0.016     | 0.163     | 0.371      | 440800.000  | tags=39%, list=8%, signal=42% |
| REGULATION_OF_ACTIN_CYTOSKELETON                   | 213.000| 0.401 | 1.643 | 0.016     | 0.161     | 0.576      | 612700.000  | tags=35%, list=11%, signal=59% |
| ADHERENS_JUNCTION                                  | 73.000 | 0.497 | 1.836 | 0.017     | 0.192     | 0.291      | 939400.000  | tags=52%, list=17%, signal=63% |
| RENAL_CELL_CARCINOMA                               | 70.000 | 0.435 | 1.643 | 0.023     | 0.170     | 0.575      | 585000.000  | tags=37%, list=11%, signal=41% |
| PROGESTERONE_MEDIATED_OOCYTE_MATURATION             | 85.000 | 0.434 | 1.621 | 0.026     | 0.173     | 0.611      | 450500.000  | tags=33%, list=8%, signal=36% |
| STARCH_AND_SUROSE_METABOLISM                        | 52.000 | 0.468 | 1.568 | 0.028     | 0.202     | 0.684      | 954100.000  | tags=44%, list=17%, signal=53% |
| PROTEIN_EXPORT                                      | 24.000 | 0.643 | 1.687 | 0.029     | 0.137     | 0.514      | 437900.000  | tags=50%, list=8%, signal=54% |
| DORSOVENTRAL_AXIS_FORMATION                         | 24.000 | 0.549 | 1.717 | 0.029     | 0.122     | 0.473      | 569800.000  | tags=42%, list=10%, signal=46% |
| EPITHELIAL_CELL_SIGNALING_IN_HELIKOCCUS_PPIORI_INFECTION | 68.000 | 0.416 | 1.538 | 0.035     | 0.209     | 0.728      | 397000.000  | tags=29%, list=7%, signal=32% |
| VIBRIO CHOLEREA_INFECTION                           | 74.000 | 0.450 | 1.596 | 0.038     | 0.190     | 0.621      | 611200.000  | tags=35%, list=11%, signal=39% |
| RENAL_CELL_CARCINOMA                               | 70.000 | 0.387 | 1.557 | 0.040     | 0.197     | 0.697      | 634400.000  | tags=31%, list=11%, signal=35% |
| ECM_RECEPTOR_INTERACTION                            | 84.000 | 0.536 | 1.726 | 0.040     | 0.122     | 0.454      | 466900.000  | tags=36%, list=8%, signal=39% |
| FOCAL_ADHESION                                      | 199.000| 0.433 | 1.610 | 0.046     | 0.175     | 0.628      | 611200.000  | tags=36%, list=11%, signal=41% |
| PROSTATE CANCER                                     | 89.000 | 0.405 | 1.534 | 0.046     | 0.206     | 0.730      | 455800.000  | tags=33%, list=8%, signal=35% |
| AMINO_SUGAR_AND_NUCLEOTIDE_SUGAR_METABOLISM         | 43.000 | 0.476 | 1.568 | 0.047     | 0.194     | 0.684      | 532300.000  | tags=37%, list=10%, signal=41% |

*ES, enrichment score; NOM P-value, nominal P-value; FDR, false discovery rate; FWER, familywise-error rate; NES, normalized enrichment score.*
FIGURE 7 | Gene set enrichment analysis. (A–F) GSEA suggested notably enrichment of cancer-related pathways in the high-risk group based on training set. (G–I) GSEA suggested significant autophagy-related enrichment based on training set.
and each prognostic axis, the estimated survival rate of breast cancer patients at 1, 3, and 5 years can be calculated, which may help professionals make clinical decisions for breast cancer patients (Figure 8A). In order to further explore the prognostic value of the 9 lncRNAs, the Kaplan Meier curve was built to confirm the relationship between these lncRNAs and OS. In our analysis, a total of 6 of the 9 lncRNAs (USP30-AS1, ST7-AS1, VPS9D1-AS1, OTUB6DB-AS1, LINC01087, and MAPT-AS1) were identified. The results indicated that the 6 autophagy-related lncRNAs were correlated to the OS in breast cancer patients (Figure 8B).

Validation the Expression of 9 IncRNAs in Breast Cancer Tissues and Cells

to further validate the expression profiles of the 9 lncRNAs, we explored the expression levels of the lncRNAs in breast cancer tissues based on TCGA database as well as in breast cancer lines by qRT-PCR. The results showed that USP30-AS1, TFAP2A-AS1, MAPT-AS1, and LINC01087 expression were significantly increased and HOXB-AS1 expression was significantly decreased in breast cancer tissues compared with normal breast tissue (Figure 9A, P<0.001). qRT-PCR results showed that USP30-AS1, TFAP2A-AS1, MAPT-AS1, and LINC01087 were overexpressed and HOXB-AS1 was lowexpressed in MCF-7 and T-47D cell lines compared to HBL-100 cell line (Figures 9B–F, P<0.05).

**DISCUSSION**

Breast cancer is characterized by the accumulation of abnormal cells, which may be attributed to the imbalance of cell proliferation, apoptosis, and autophagy regulation disorder. Autophagy plays a dual role in inhibiting and promoting the occurrence and development of cancer at different stages (24). Increasing evidence shows that lncRNAs and autophagy are closely associated with the occurrence, development, and prognosis of many different tumors. Recent evidence suggests that lncRNA NAMPT-AS promoted breast cancer progression and regulated autophagy through the mTOR pathway (25). Existing studies showed that there is a positive relationship between the expression of lncRNA LCPAT1 and LC3β in lung cancer (26).
Other studies showed that lncRNA DICER1-AS1 expression was up-regulated in osteosarcoma cells, while knockdown of DICER1-AS1 could inhibit the proliferation, migration, and invasion of osteosarcoma cells, and reduce the protein expression levels of ATG5, LC3, and Beclin1, indicating that knocking down DICER1-AS1 can inhibit autophagy of osteosarcoma cells (27).

The specific mechanisms of lncRNAs regulating autophagy can be divided into three categories: lncRNAs act as competitive endogenous RNAs (ceRNAs) combined with miRNAs to regulate the expression of miRNAs, thus affecting the process of autophagy (28); lncRNAs can also affect the expression of ATG genes cis or trans (24); lncRNAs promote tumor progression by inhibiting autophagy-mediated apoptosis through AKT/mTOR pathway (29). LncRNAs regulate the occurrence and development of autophagy in both directions, which provides new ideas and ways for further research on the prevention and treatment of autophagy-related diseases, such as cancer. At present, using high-throughput biotechnology to detect genetic changes to predict tumor recurrence and metastasis has become a research hotspot. However, a single lncRNA may not be sufficient to forecast the prognosis of breast cancer patients. Therefore, it is urgent to construct an autophagy-related lncRNA signature to predict the survival of breast cancer patients.

In our analysis, the TCGA dataset and GSE20685 dataset were downloaded to investigate the prognosis signature of autophagy-related lncRNAs for breast cancer patients. Firstly, we identified 1,270 lncRNAs associated with autophagy-related genes in breast cancer (R² > 0.3, P < 0.001). Because the breast cancer samples in TCGA and GEO databases are different, the extracted lncRNAs are not exactly the same. We analyzed the common lncRNAs in the two databases to ensure that these...
IncRNAs are general and universal in breast cancer samples, and narrow the scope of biological marker screening. Then we obtained 180 shared IncRNAs from TCGA dataset and GSE20685 dataset. We further identified a signature composed of 9 autophagy-related IncRNAs that could divide breast cancer patients into high-risk group or low-risk group. Among the 9 autophagy-related IncRNAs, USP30-AS1, ST7-AS1, VPS9D1-AS1, TFAP2A-AS1, HOXB-AS1, LINC01087, and MAPT-AS1 are protective factors, while KMT2E-AS1 and OTUB6DB-AS1 are risk-related factors. In addition, it was also found that the OS of breast cancer patients in the high-risk group was poorer than that in the low-risk group based on the prognostic signature. The validation in training set and testing set revealed that the prognostic signature has better diagnostic capability. Besides, we used Cox regression analysis that concluded that the signature is an independent factor of breast cancer. We also evaluated the clinical value of the signature in age, gender, clinical stage, and TNM stage and found that the signature may be associated with the progression of breast cancer. To further analyze these 9 IncRNAs of the signature, we developed a nomogram and validated their prognostic value and expression. We found USP30-AS1, ST7-AS1, VPS9D1-AS1, OTUB6DB-AS1, LINC01087, and MAPT-AS1 were correlated to the OS in breast cancer patients. And we further verified that USP30-AS1, TFAP2A-AS1, MAPT-AS1, and LINC01087 were significantly increased and HOXB-AS1 was significantly decreased in breast cancer tissues and cells.

Moreover, in our analysis, we found that several pathways have been established in cancer such as ECM receptor interaction, TGF beta signaling pathway, cell adhesion, Renal cell carcinoma pathway, and Prostate cancer pathway, which were markedly enriched in high-risk group. We also detect that the glucose metabolism pathways were enriched in high-risk patients, especially glycolysis, gluconeogenesis, and tricarboxylic acid cycle. Previous studies have shown that abnormal cell metabolism, especially glucose metabolism, is related to the occurrence and progression of tumors (30, 31). Tumor cells absorb a large amount of glucose and tend to carry out glycolysis in the cytoplasm even under the condition of sufficient oxygen supply, thus promoting the rapid growth and proliferation of cells (31, 32). Recent studies have found an unexpected link between glucose metabolism and autophagy. They found that increase of glycolysis in autophagic cells can promote Ras-mediated adhesion-independent transformation, indicating autophagy may promote Ras-driven tumor growth in a specific metabolic environment (33). This is consistent with our present findings.

However, the molecular mechanism of these 9 autophagy-related IncRNAs is still inadequately understood in breast cancer, and further investigation of the underlying mechanisms may be meaningful. So we established a nomogram to more intuitively predict the OS in 1, 3, and 5 years. Subsequently, we discover the prognostic value of these 9 autophagy-related IncRNAs using the Kaplan-Meier survival analysis and the results were basically consistent with the prognostic analysis results of TCGA dataset. Furthermore, targeted analysis on a specific histological group of breast cancer patients will increase the specificity and individuality of the autophagy-related IncRNAs. In our further study, we will analyze and verify these IncRNAs in breast cancer patients with the specific morphological and molecular features.

Taken together, our study defines an innovative autophagy-related IncRNA signature in breast cancer. It is a comprehensive analysis of RNA sequencing data and clinical information available in TCGA database. The autophagy-related IncRNA signature may provide new insights into predicting the prognosis of patients with breast cancer. More importantly, the functions and approaches associated with our signature may contribute to the development of a new therapeutic strategy for breast cancer.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.cancergenome.nih.gov/, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20685, http://www.autophagy.lu/.

AUTHOR CONTRIBUTIONS

This study was designed by YZ, JY, and RZ. Data were analyzed by RZ, QZ, DY, ZY, JG, and JZ. The original manuscript was written by RZ. Supervision and manuscript revision were performed by YZ and JY. Funding was acquired by YZ and JY. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (No.: 81402266, 81572747, 52111005), the key research project of Henan high school (18A320051), the Medical Science and Technology Research Projects of Henan Province (No.: SBG202002082), and Henan Scientific and Technological Research Projects (No.: 202102310044, 162102410057). Support has also been provided by the Outstanding Young Talent project of Scientific and Technological Innovation in Henan Health (No.: YXKC2020032) and the Clinical Pharmacy Branch of Chinese Medical Association-Wu Jieping Medical Foundation (No.: 320.6750.19090-3).

ACKNOWLEDGMENTS

We thank all the participants who were involved in this study and gratefully acknowledge contributions from the TCGA and the GEO Network.
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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