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ORIGINAL RESEARCH

Acyl-CoA Thioesterase 8 (ACOT8) is a Poor Prognostic Biomarker in Breast Cancer

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Purpose: This study aimed to investigate the expression of Acyl-CoA thioesterase 8 (ACOT8) in breast cancer (BC) and its association with clinicopathological characteristics, patient survival, and immune infiltration.

Methods: We conducted a comprehensive analysis of ACOT8 mRNA differential expression across various cancer types, followed by survival analysis. We focused on BC, where ACOT8 expression was evaluated at both the mRNA and protein levels using online databases, qRT-PCR, and immunohistochemistry. Associations between ACOT8 expression and clinicopathological parameters were assessed using different databases. Additionally, we investigated the prognostic significance of ACOT8 in BC patients by analyzing various cohorts and databases. Furthermore, we predicted a potential signaling pathway and identified miR-1-3p as a possible upstream regulator of ACOT8. Finally, the relationship between ACOT8 and immune system infiltration, as well as immune checkpoint molecules, was examined.

Results: Our findings demonstrated upregulated ACOT8 mRNA and protein levels in BC. Elevated ACOT8 expression correlated positively with various clinicopathological characteristics, indicating an unfavorable prognosis for patients. Functional enrichment analysis suggested ACOT8 involvement in lipid metabolism, mitochondrial components, and ribosomal functions. Moreover, we identified connections between ACOT8 and immune system markers, immune cell infiltration, and immune checkpoints.

Conclusion: This study provides compelling evidence for ACOT8 upregulation in BC and its association with clinicopathological features and patient outcomes. Additionally, our findings suggest that targeting ACOT8 and immune checkpoints might enhance the effectiveness of immunotherapy in BC patients.

Keywords: ACOT8, breast cancer, clinicopathological features, immune infiltration, immunotherapy

Introduction

Breast cancer (BC), the leading cancer affecting women and the second most common cancer overall,[1] is classified into four molecular subtypes: basal-like triple-negative, luminal A, luminal B, and human epidermal growth factor receptor 2 (HER2)-positive.[2] Despite significant advancements in treatment modalities, including surgery, radiation, chemotherapy, and multidisciplinary approaches, the prognosis for breast cancer patients remains suboptimal. Immunotherapy and targeted therapies offer benefits to only a limited subset of patients.[3] Therefore, identifying reliable prognostic biomarkers and novel therapeutic targets remains a critical unmet need.

ACOT8, known by several aliases including hACTE-III, hTE, NAP1, PTE-2, and PTE1, belongs to the acyl-CoA thioesterase superfamily. Located at chromosome 20q13.12 in the human genome, this gene encodes a crucial enzyme responsible for the degradation of fatty acyl-CoA esters into CoA and free fatty acids (FFAs).[4] This study underscores the critical role of ACOT8 in regulating lipid metabolism. ACOT8 is localized within cellular vesicles and mitochondria, facilitating the breakdown of acyl-CoA into free fatty acids and Coenzyme A (CoA). This process contributes to maintaining intracellular homeostasis of fatty acid acyl-CoA, free fatty acids, and CoA. Notably, ACOT8 exhibits a broad substrate range, effectively hydrolyzing medium-chain length (C2-C20) linear, saturated, and unsaturated fatty acid acyl-CoAs despite lacking strong selectivity for the carboxylic acid moiety. Beyond its primary function in fatty acid

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metabolism, ACOT8 participates in various biological processes, including host-virus interactions, peroxisome biogenesis, and lipid metabolism in general.[4,5] Interestingly, ACOT8 plays a role in HIV-infected individuals, potentially influencing their ability to evade immune responses.[6] This suggests a potential role for ACOT8 in viral infection and immune regulation. The expression of ACOT8 has been observed to be elevated in various human cancers, including lung adenocarcinoma.[7] Conversely, ACOT8 gene expression was significantly downregulated in clear cell renal cell carcinoma (ccRCC) samples, suggesting a complex role in tumorigenesis and progression.[8] Additionally, studies have shown a decrease in ACOT8 enzyme activity, responsible for fatty acid metabolism, following treatment with orlistat, a medication linked to the induction of apoptosis in liver cancer cells.

This study investigated ACOT8 expression across various cancer types, followed by an analysis of survival rates in breast cancer patients. We further explored the association between BC clinicopathological characteristics and ACOT8 expression at both the mRNA and protein levels (ie transcriptomic and proteomic levels). To elucidate the underlying mechanisms of BC, we analyzed patient outcomes from well-characterized BC cohorts and datasets. Our primary focus was to identify upstream regulators of ACOT8. Additionally, we investigated the connection between ACOT8 and the tumor microenvironment (TME), immune checkpoint inhibitors, immune cells, and the relationship between ACOT8 and immune infiltration within the TME. In conclusion, this research identifies potential therapeutic targets for BC and offers novel insights into BC development.

Methods

Database Analysis for Estimating the Immune Response of Tumors

Utilizing the TIMER algorithm and data from the TIMER database (https://cistrome.shinyapps.io/timer/),[9] we performed tumor immune infiltration analysis. This analysis estimated the abundance of six immune cell populations within the tumor microenvironment. Furthermore, we investigated ACOT8 mRNA expression levels across various human tumors and assessed its correlation with immune checkpoints and immune cell types. The TIMER database was employed to evaluate the relationships between ACOT8 expression and specific immune cell biomarkers.

Gene Expression Profiling Database with Interactive Analysis

This study employed the GEPIA database (http://gepia.cancer-pku.cn/), an online tool that leverages gene expression data from TCGA and Genotype-Tissue Expression (GTEx) projects. GEPIA facilitates user access to gene expression data, survival statistics, and differential expression analyses.[10] By utilizing GEPIA, we were able to investigate the relationship between ACOT8 expression and other immune cell markers.

TNM Plot Database, UALCAN Database, TCGA Database, and KM Plotter Database

To assess the prognostic value of ACOT8 mRNA expression in BC, we obtained gene expression data from the TNMplot and UALCAN databases.[11,12] Additionally, we leveraged the METABRIC and TCGA datasets from the Breast Cancer International Consortium (BCIC) as discovery cohorts to explore the prognostic significance of ACOT8 expression at the genomic level.[13,14] For validation purposes, we employed the KM plotter online database accessible at https://kmplot.com/analysis/[15].

LinkedOmics Database Analysis

The LinkedOmics database was utilized to investigate the role of ACOT8 and its potential interactions with signaling pathways.

StarBase Database Analysis

A significant correlation was observed between the identified mRNA-miRNA pairs using the starBase database.[16,17] The LinkedOmics database was employed to elucidate the role of ACOT8 and its association with specific signaling pathways.

Patients and Tissue Samples

This study enrolled 222 female BC patients who underwent surgical resection between January 2013 and December 2015. All patients were newly diagnosed with histologically confirmed, primary invasive BC with no prior anti-tumor treatment (surgery, chemotherapy, or radiotherapy). Inclusion criteria also encompassed an age range of 35–91 years (median: 52 years) and the absence of significant pre-existing medical conditions. Patient follow-up was conducted from the surgery date until December 2020, encompassing a maximum period of five years or until patient death. The study protocol was approved by the ethical committee of Nantong University's Affiliated Hospital (protocol code 2020-L125), and all patients provided informed consent. Corresponding para-cancerous tissue samples, obtained approximately 5 cm from the tumor margin, served as controls. All tissue samples (n=444, 222 pairs) were immediately frozen in liquid nitrogen and stored at −80°C after surgery. Pathological examination confirmed all specimens originated from single primary BC cases. For biomarker analysis, IHC arrays were constructed from the clinical samples. These were then evaluated for comparison with publicly available databases. Clinicopathological data, including tumor size, lymph node status, histological grade, and the expression status of estrogen receptor (ER), progesterone receptor (PR), and p53, were documented for each patient. The histological grading system classified breast cancer into three grades (I, II, and III), with grade I representing the most well-differentiated and grade III the least differentiated tumors. IHC staining intensity was scored on a scale of 0 (negative) to 3 (strongly positive), while the percentage of positive cells in each of three randomly selected high-power fields was calculated and averaged (scores ranged from 1: 0–25% to 4: 76–100%). A final IHC score was obtained by multiplying the staining intensity score by the percentage of positive cells. Scores ≥6 were designated as high ACOT8 expression.

The Technique of Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from 80 mg of fresh tissue samples using 1 mL of TRIzol reagent (Invitrogen, Life Technologies, Paisley, UK) following the manufacturer's instructions. Complementary DNA (cDNA) was subsequently synthesized from the extracted RNA using the Takara PrimeScript RT kit (Takara, Dalian, Liaoning, China) according to the manufacturer's protocol. qRT-PCR was performed on a 7500 real-time PCR system (Applied Biosystems, USA) to quantify the mRNA expression levels of ACOT8 and miR-1-3p. The reactions were carried out using the SYBR Green I Master Mix kit (Invitrogen, Carlsbad, CA, USA). The thermal cycling program consisted of an initial denaturation step at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 10 seconds each. Following amplification, a melting curve analysis was performed by gradually increasing the temperature from 60°C to 95°C over 20 minutes, with a final hold at 95°C for 15 seconds. U6 served as the endogenous control for miR-1-3p expression. Primer sequences used in this study are listed below: ACOT8, 5′-ACTGTGCCACCACCAGAAGAG-3′ (forward) and 5′-CCTGAGCAGCAATTCGGTTGAG-3′ (reverse); miR-1-3p, 5′-GCCGCGGTGGAATGTAAAGAAG-3′ (forward), 5′-ATCCAGTGCAGGGTCCGAGG-3′ (reverse), and 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATACAT-3′ (RT Primer); β-actin, 5′-AGTTGCGTTACACCTTTTC-3′ (forward) and 5′-GCTGTCACCTTCACCTTC-3′ (reverse); U6, 5′-CTCGCTTCGGCAGCACA-3′ (forward) and 5′-AACGCTTCACGAATTTGCGT-3′ (reverse). All primers were synthesized by Sangon Biotechnology Co., Ltd. The relative expression levels of target genes were calculated using the 2-ΔΔCT method.

Immunohistochemical Staining and Analysis

A tissue microarray (TMA) was constructed using formalin-fixed paraffin-embedded (FFPE) tissue cores from 222 breast cancer cases. The FFPE tissue sections were deparaffinized and rehydrated through a series of graded alcohols followed by rinsing in phosphate-buffered saline (PBS, 0.1 M). Antigen retrieval was performed using Retriever 2100 in pH 9 EDTA buffer. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in methanol for 15 minutes, followed by three washes in PBS. The sections were then incubated overnight at 4°C with a rabbit polyclonal antibody against ACOT8 (dilution 1:200; Abcam, USA). After three washes with PBS, the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (dilution 1:500) for 30 minutes. Following additional PBS washes, the sections were stained with a chromogenic substrate, resulting in a brown precipitate at antibody binding sites. The sections were then dehydrated, cleared, and mounted. Immunohistochemical analysis revealed positive ACOT8 expression localized to the cytoplasm and membrane of tumor cells.

Statistical Analysis

Statistical analyses were performed using SPSS software version 27.0 (IBM Corp., Armonk, NY, USA) and R software (version to be specified) with the ggplot2 package for data visualization. Patients were categorized into high and low ACOT8 expression groups based on a median cut-off value of mRNA expression. Comparisons between the BC and normal groups were conducted using a two-sided parametric *t*-test, assuming the normality of data distribution. The chi-square test was employed to assess the association between ACOT8 expression and other categorical variables at both the transcriptome and proteome levels. Kaplan-Meier survival analysis was used to evaluate patient outcomes, with statistical significance for differences in survival curves assessed using the Log rank test. Multivariate analysis to identify independent prognostic factors was performed with Cox proportional hazards regression models. For analyses involving the online database mentioned in the paper, all experiments were replicated at least three times to ensure data reliability. A *p*-value less than 0.05 was considered statistically significant.

Results

Pan-Cancer Analysis of ACOT8 Expression

To elucidate the potential role of ACOT8 in tumorigenesis, we examined its expression in various cancer cell lines retrieved from the Tumor Immune Estimation Resource (TIMER) database (http://cistrome.org/TIMER/). Our analysis revealed elevated ACOT8 gene expression in several cancer types, including bladder urothelial carcinoma (BLCA), BC, esophageal carcinoma (ESCA), hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), cervical squamous cell carcinoma (CESC), cholangiocarcinoma (CHOL), and head and neck squamous cell carcinoma (HNSC). Conversely, ACOT8 mRNA expression was downregulated in colon adenocarcinoma (COAD), glioblastoma multiforme (GBM), renal cell carcinoma (KIRC), kidney chromophobe carcinoma (KICH), and pheochromocytoma and paraganglioma (PCPG) compared to their corresponding normal tissues. Interestingly, no significant difference in ACOT8 mRNA expression was observed between certain cancer types, such as KICH and sarcoma (SARC). To validate these findings, we employed the TNMplot online database (https://tnmplot.com/analysis/). Consistent with the results obtained from the TIMER database (Figure 1A and B), TNMplot analysis demonstrated a significant increase in ACOT8 mRNA expression in various cancers, including BC, CESC, ESCA, LIHC, LUAD, LUSC, and uterine corpus endometrial carcinoma (UCEC), when compared to their corresponding healthy tissues. In conclusion, our investigation suggests upregulated ACOT8 expression in a broad spectrum of human cancers, potentially implicating its involvement in promoting oncogenesis.

The Predictive Significance of ACOT8 in Human Malignancies

This study explored the impact of ACOT8 expression on mortality rates across 33 human tumor types. Notably, elevated ACOT8 levels were associated with an unfavorable prognosis in patients with LIHC, Uveal Melanoma (UVM), Mesothelioma (MESO), BC, and Prostate Adenocarcinoma (PRAD), as demonstrated in Figures 2A–E. To gain a deeper understanding of the relationship between ACOT8 expression and overall survival (OS), we analyzed its expression variability and its effect on patient outcomes. Our findings revealed a correlation between increased ACOT8 levels and a poorer prognosis in individuals diagnosed with luminal A breast cancer (Figure 2F). Therefore, these results suggest that ACOT8 may serve as a potential adverse prognostic marker for patients with breast cancer.

BC Exhibits Varying Levels of ACOT8 mRNA and Protein Expression

To investigate ACOT8 mRNA expression in breast cancer, we employed the CancerSEA database (http://biocc.hrbmu.edu.cn/CancerSEA/) to retrieve relevant data. Our analysis revealed elevated ACOT8 expression in breast cancer tissues compared to controls (Figure 3). These findings were further validated using qRT-PCR analysis of breast cancer data from The Cancer Genome Atlas (TCGA). As depicted in Figure 3A–C, tumors exhibited significantly higher ACOT8 mRNA expression than normal tissues. To investigate protein expression, we constructed tissue microarrays and performed immunohistochemical staining. Consistent with the mRNA data, our results demonstrated a positive

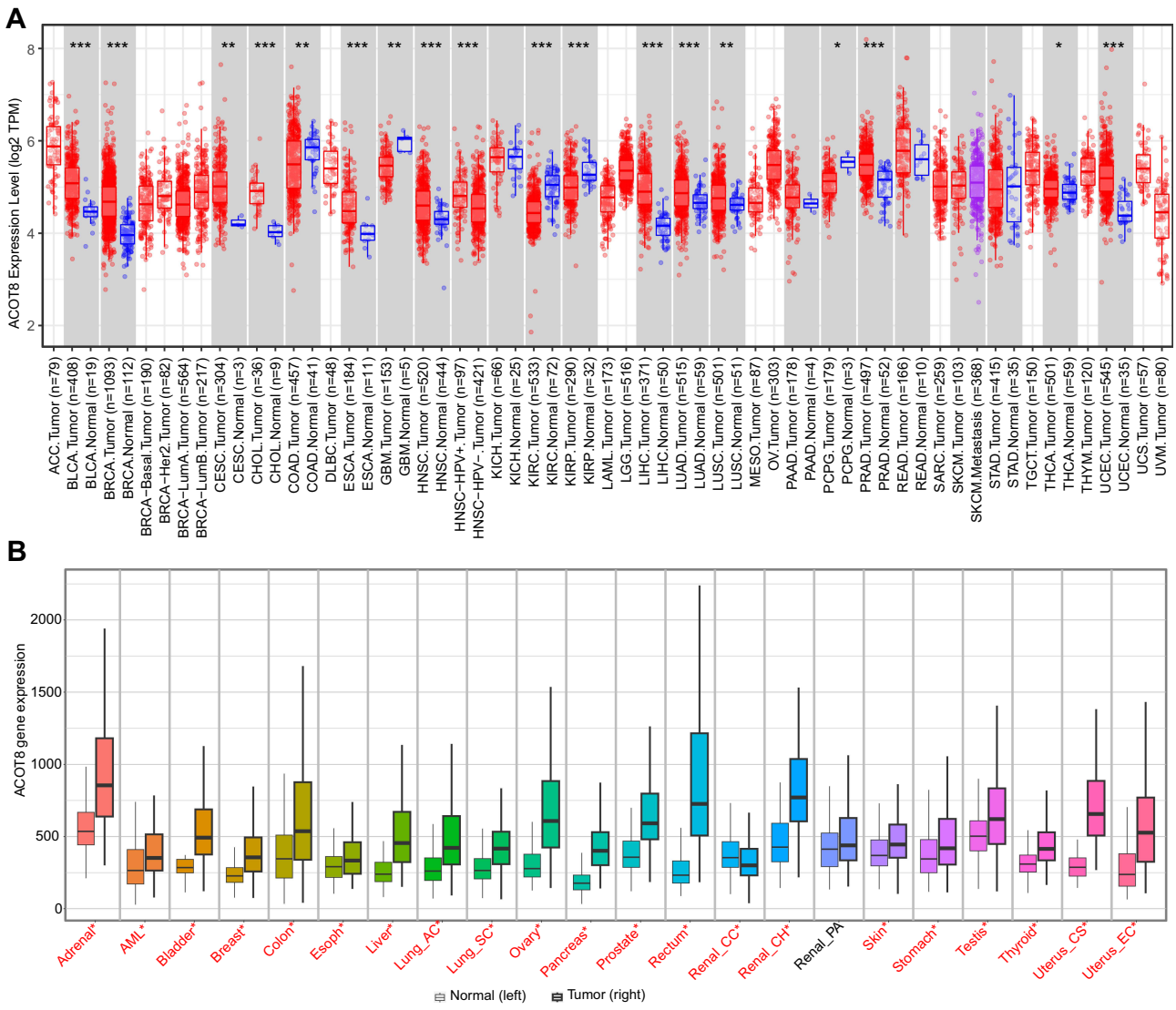

Figure 1 ACOT8 mRNA expression in different human cancers. (**A**) The levels of ACOT8 mRNA in different cancer cell lines were obtained from the TIMER database. (**B**) The TNM plot database was used to analyze the expression of ACOT8 mRNA in various cancers.

correlation between ACOT8 protein (Figure 3E) expression and mRNA levels (Figure 3D). Notably, immunohistochemistry revealed ACOT8 localization within the cytoplasm and membrane of breast cancer cells.

Correlation Between the ACOT8 Expression and Clinicopathological Features

We employed The University of ALabama CANcer (UALCAN) database (http://ualcan.path.uab.edu/) to investigate the association between ACOT8 mRNA expression and clinicopathological parameters in BC patients. Our analysis revealed a correlation between variations in ACOT8 mRNA levels and various patient characteristics, including age, ethnicity, cancer stage, lymph node metastasis status, luminal type, menstrual status, and histological type (Figure 4A–G). Furthermore, significantly higher ACOT8 mRNA levels were associated with larger tumor size (p = 0.010 in METABRIC cohort, p = 0.024 in TCGA cohort), advanced tumor grade (p = 0.019 in METABRIC cohort, p < 0.001 in TCGA cohort), PAM50 subtype (p < 0.001), and high nodal stage in the TCGA cohort (p = 0.044) (Table 1). To validate the association between ACOT8 expression and clinicopathological factors, we constructed tissue microarrays (TMAs) encompassing 222 BC samples. Consistent with Table 2, the data in Figure 3D demonstrates a significant correlation between ACOT8 expression and both tumor size (p = 0.011) and lymph node metastasis (p = 0.006).

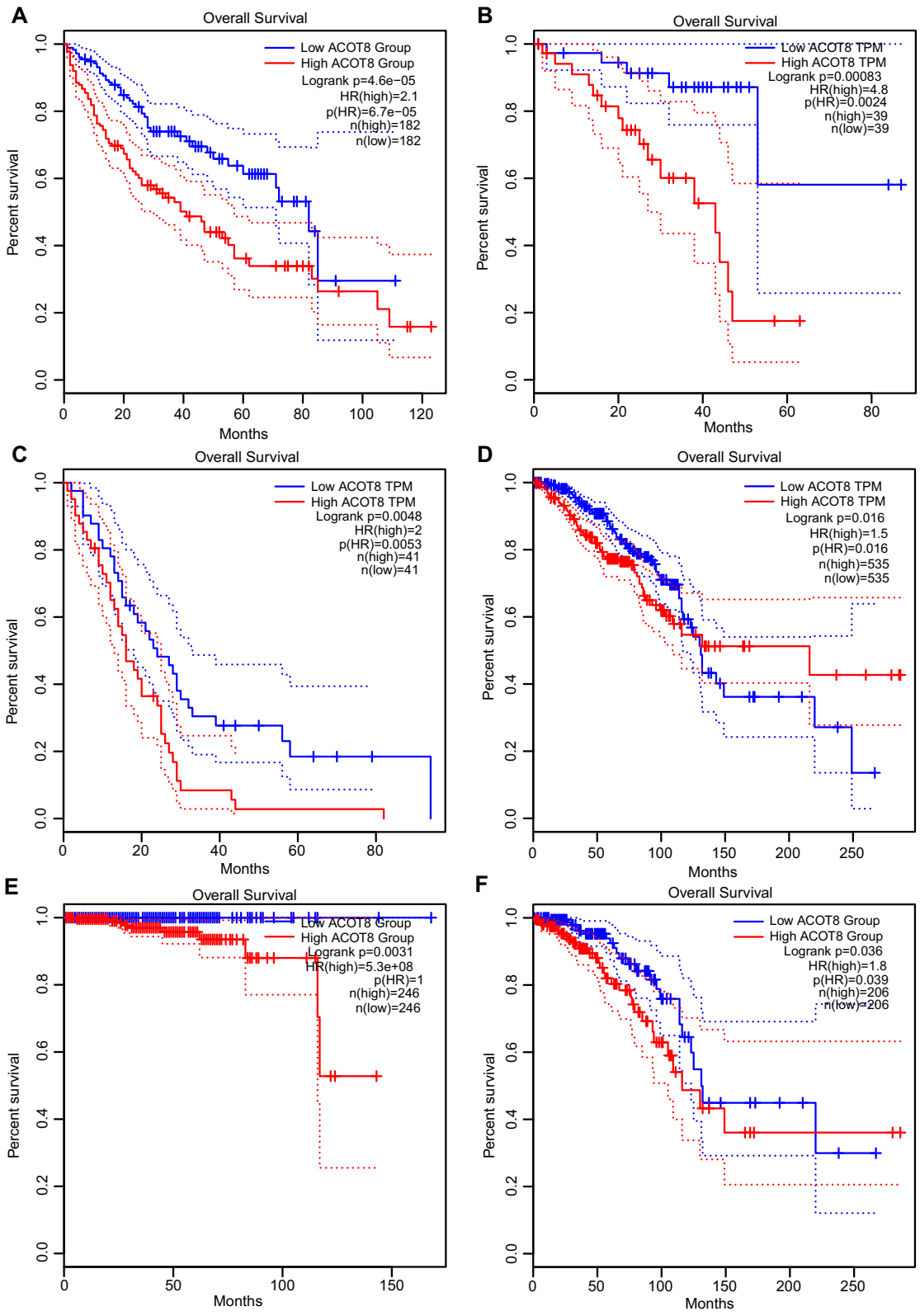

Figure 2 The relationship between ACOT8 expression levels (obtained from GEPIA) and overall survival (OS) in various cancer types.The OS map for ACOT8 is displayed for LIHC (**A**), UVM (**B**), MESO (**C**), BC (**D**), PRAD (**E**), and luminal A breast cancer (**F**).

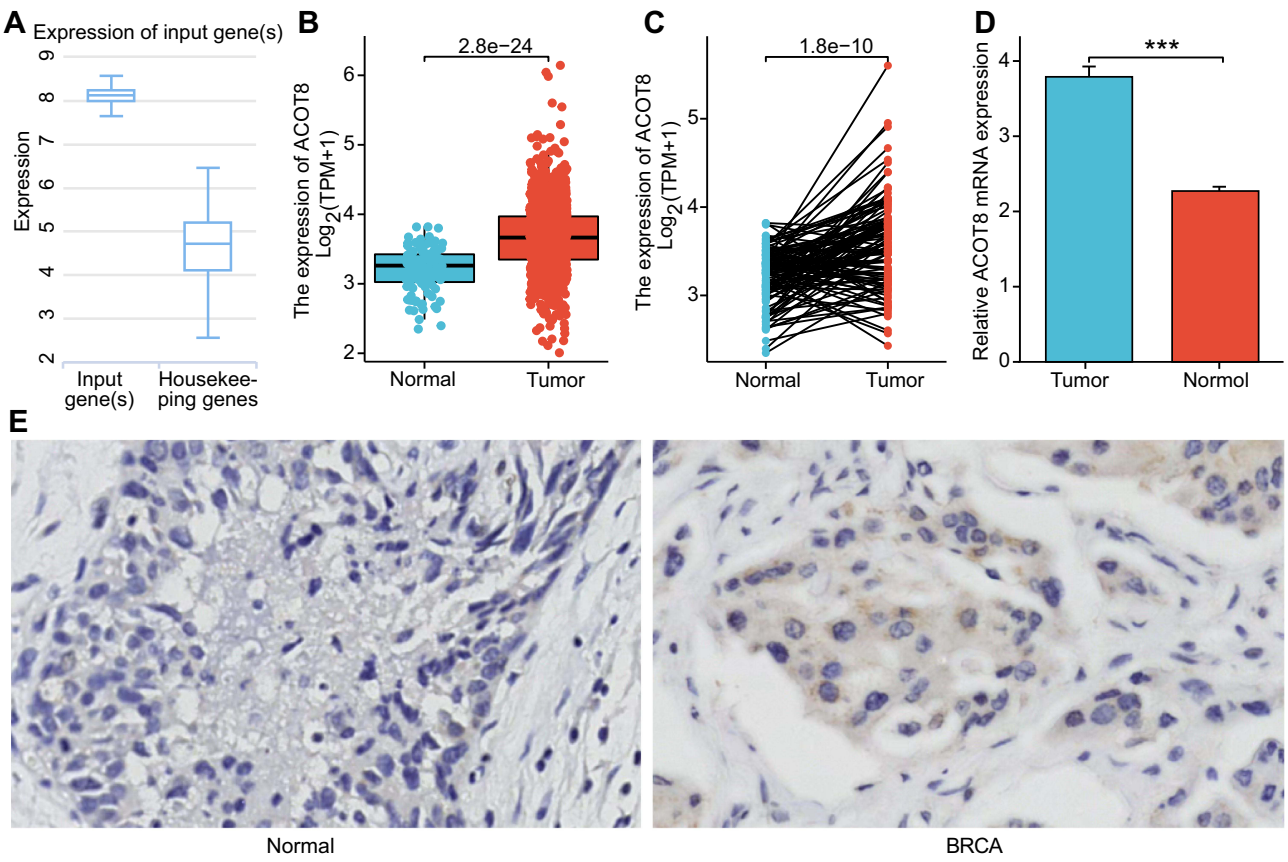

Figure 3 The mRNA and protein expression levels of ACOT8 observed in BC. (**A**) The mRNA levels of ACOT8 in BC were analyzed using the CancerSEA database. (**B**) R software was utilized to examine the mRNA expression. (**C**) Validation of the levels of mRNA of ACOT8 through qRT-PCR analysis. (**D**) Validation of the levels of ACOT8 protein using immunohistochemistry (IHC). (**E**) Comparison of normal tissue and tumor tissue in immunohistochemistry.*P < 0.05, **P < 0.01, ***P < 0.001.

Prognosis Prediction Based on ACOT8 mRNA Expression

Analysis of the METABRIC cohort revealed a significant association between elevated ACOT8 mRNA levels and reduced OS in breast cancer patients (p = 0.005, HR = 1.24, 95% CI 1.07–1.45; Figure 5A). Similar findings were observed in the TCGA cohort (p = 0.024, HR = 1.45, 95% CI 1.05–1.99; Figure 5B) and the IHC cohort (p < 0.001, HR = 3.03, 95% CI 1.73–5.29; Figure 5C). Additionally, the Kaplan-Meier plotter database analysis yielded a significant association (p = 0.0012, HR = 1.39, 95% CI 1.14–1.70; Figure 5D). Furthermore, multivariate analyses conducted on the TCGA, METABRIC, and IHC cohorts identified ACOT8 expression as an independent prognostic indicator for poor overall survival in breast cancer (Table 3 and Table 4). Notably, this association remained significant regardless of pathological stage, tumor size, ER, PR, and HER2 status.

The OS of breast cancer patients was examined in four cohorts at the transcriptome level: METABRIC, TCGA, IHC, and the KM plotter database. Patient OS was categorized based on ACOT8 mRNA expression. Figure 4A depicts patient OS in the METABRIC cohort, while Figures 4B–D display patient OS in the TCGA, IHC, and KM plotter database cohorts, respectively. To identify independent predictors of OS in breast cancer, a multivariate Cox proportional hazards regression analysis was conducted in the METABRIC and TCGA cohorts (Table 3). Similarly, for the IHC cohort, univariate and multivariate Cox proportional hazards regression analyses were employed to assess predictors of OS (Table 4).

ACOT8's Enrichment Analysis

To elucidate the functional role of ACOT8, we explored its interactions with various signaling pathways using the LinkedOmics database (http://www.linkedomics.org/). Our analysis revealed negative correlations between ACOT8 expression and biological

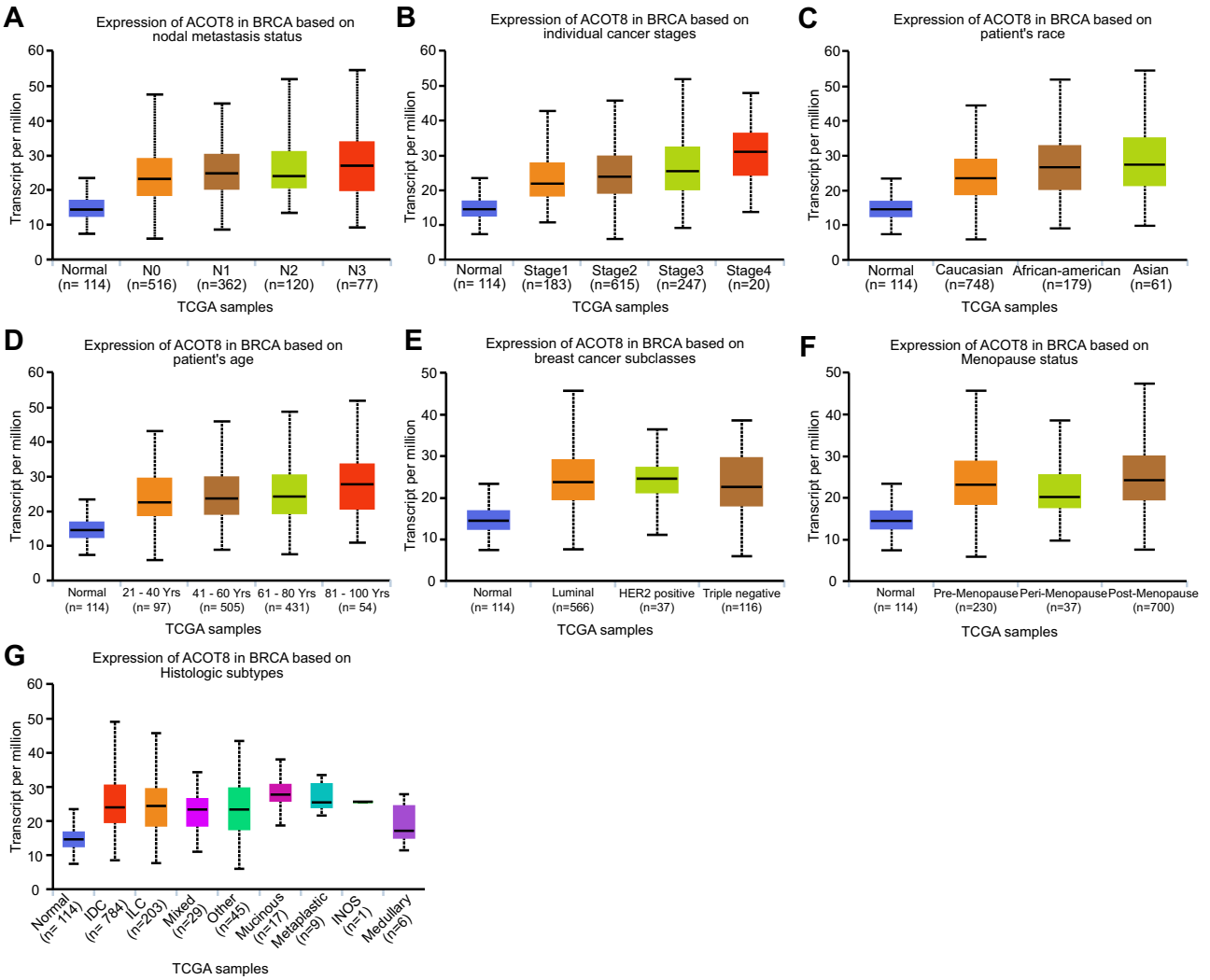

Figure 4 The UALCAN database was used to investigate the correlation between ACOT8 expression and clinicopathological factors. The factors analyzed encompassed lymph node metastasis status (**A**), cancer stage (**B**), race (**C**), age (**D**), luminal stage (**E**), menstrual status (**F**), and pathological type (**G**) of the individuals.

processes such as translational elongation, precursor metabolite and energy generation, and non-coding RNA (ncRNA) processing. Additionally, ACOT8 expression demonstrated negative associations with the regulation of cell shape, peptidyl-serine modification, and cell motility (Figure 6). Conversely, a significant positive correlation was observed between ACOT8 expression and the mitochondrial components, namely the inner membrane and matrix (Figure 6A). Furthermore, our investigation identified positive associations between ACOT8 and the structural component of ribosomes, lyase activity, and cofactor binding (Figure 6B and C). Notably, a negative relationship was found between ACOT8 and the leading edge of the cell (Figure 6C). Figure 6A–C further demonstrates negative associations with protein serine/threonine kinase function, extracellular matrix structural elements, and helicase interaction. The Spearman correlation test was employed to determine the enrichment analysis of ACOT8 in breast cancer. Figure 6 depicts the results for biological processes (BP), cellular components (CC), and molecular functions (MF).

Prediction of ACOT8's Upstream miRNAs

Given the well-established role of ncRNAs in gene expression regulation, we hypothesized that miRNAs interacting with ACOT8 might influence its regulatory mechanisms. We employed correlation analysis, differential expression analysis, and survival analysis of miRNAs to identify potential regulators. Our findings suggested miR-1-3p as the

Table 1 Comparing the Expression ofACOT8 mRNA with the Clinicopathological Traits in the METABRIC and TCGA Breast Cancer Series Breast Cancer Datasets

Characteristics	TCGA Cohort			METABRIC Cohort		
	Low ACOT8 N (%)	High ACOT8 N (%)	*p*-value	Low ACOT8 N (%)	High ACOT8 N (%)	*p*-value
Tumor size			<0.001			0.019
≤ 2.0 cm	165 (15.2)	113 (10.4)		264(46.8)	245 (40)	
> 2.0 cm	377 (34.8)	429 (39.6)		300(53.2)	367 (60)	
Lymph Node status			0.044			0.084
Negative	274 (25.7)	242 (22.7)		315(55.9)	311 (50.8)	
Positive	259 (24.3)	293 (27.4)		249(44.1)	301 (49.2)	
Pathologic stage, n (%)			0.024			0.010
Stage I&Stage II	416 (39.1)	385 (36.2)		528(93.6)	547 (89.4)	
Stage III&Stage IV	115 (10.8)	147 (13.8)		36(6.4)	65 (10.6)	
Age, n (%)			0.978			0.125
≤ 60	301 (27.7)	302 (27.8)		274(48.6)	270 (44.1)	
> 60	242 (22.3)	242 (22.3)		290(51.4)	342 (55.9)	
PR status, n (%)			0.567			0.475
Negative	176 (17)	166 (16.1)		239 (42.4)	272 (44.4)	
Positive	343 (33.2)	349 (33.8)		325 (57.6)	340 (55.6)	
ER status, n (%)			0.062			0.361
Negative	133 (12.8)	107 (10.3)		98 (17.4)	119 (19.4)	
Positive	387 (37.3)	410 (39.5)		466 (82.6)	493 (80.6)	
HER2 status, n (%)			0.152			0.072
Negative	293 (40.9)	267 (37.2)		501 (88.8)	522 (85.3)	
Positive	72 (10)	85 (11.9)		63 (11.2)	90 (14.7)	
PAM50, n (%)			<0.001			<0.001
LumA	307 (28.2)	257 (23.6)		268 (47.5)	226 (36.9)	
Normal	32 (2.9)	8 (0.7)		61 (10.8)	31 (5.1)	
LumB	73 (6.7)	133 (12.2)		114 (20.2)	210 (34.3)	
Her2	35 (3.2)	47 (4.3)		53 (9.4)	82 (13.4)	
Basal	96 (8.8)	99 (9.1)		68 (12.1)	63 (10.3)	

Table 2 Presents the Correlation Between the ACOT8 Protein Expression and the Clinicopathological Features of the IHC Cohort

Clinicopathological Factors	ACOT8 Protein Expression		
	Low N (%)	High N (%)	*p-value*
Tumor size			
≤ 2.0 cm	81 (61.4)	51 (38.6)	**0.011**
> 2.0 cm	34 (37.8)	**56 (62.2)**	
Lymph node status			
Negative	96 (66.2)	49(33.8)	**0.006**
Positive	36 (46.8)	**41 (53.2)**	
Histological grade			
Grade 1 & Grade 2	107 (61.1)	68 (38.9)	**0.403**
Grade 3	25 (53.2)	22 (46.8)	
Age			
≤ 60	63 (66.3)	32 (33.7)	0.075
> 60	69 (54.3)	58 (45.7)	

(*Continued*)

Table 2 (Continued).

Clinicopathological Factors	ACOT8 Protein Expression		
	Low N (%)	High N (%)	*p-value*
Oestrogen receptor (ER)			
Negative	21 (15.9)	**111 (84.1)**	**0.157**
Positive	8 (8.9)	82 (91.1)	
Progesterone receptor (PR)			
Negative	31 (23.5)	**101(76.5)**	**0.745**
Positive	19 (21.1)	71 (78.9)	
P53			
Negative	100 (58.8)	70 (41.2)	**0.75**
Positive	32 (61.5)	**20 (38.5)**	

Table 3 In the METABRIC, TCGA Cohort, a Multivariate Cox Proportional Hazards Regression Analysis Was Conducted to Determine Predictors of Overall Survival (OS) in Breast Cancer

Characteristics	Multivariate Analysis in METABRIC		Multivariate Analysis in TCGA	
	Hazard ratio (95% CI)	*p*-value	Hazard Ratio (95% CI)	*p*-value
ACOT8				
Low	Reference		Reference	
High	1.174 (1.007–1.368)	0.040	1.864 (1.165–2.982)	0.009
Tumor size				
≤ 2.0 cm	Reference		Reference	
> 2.0 cm	1.779 (1.514–2.090)	< 0.001	0.887 (0.509–1.546)	0.672
Pathologic stage				
Stage I & Stage II	Reference		Reference	
Stage III & Stage IV	1.127 (0.951–1.334)	0.168	2.977 (1.802–4.916)	< 0.001
PAM50				
TNBC	Reference		Reference	
NTNBC	0.764 (0.553–1.057)	0.104	0.404 (0.051–3.213)	0.391
PR status				
Negative	Reference		Reference	
Positive	0.813 (0.680–0.972)	0.023	0.866 (0.408–1.841)	0.709
ER status				
Negative	Reference		Reference	
Positive	0.801 (0.601–1.607)	0.130	0.459 (0.181–1.166)	0.102
HER2 status				
Negative	Reference		Reference	
Positive	1.218 (0.946–1.568)	0.127	1.233 (0.658–2.311)	0.514

sole candidate regulator of ACOT8 (Figure 7A–C). To validate this hypothesis, we examined the expression levels of ACOT8 and miR-1-3p in 35 clinical breast cancer samples. Consistent with our previous analysis, the results demonstrated increased ACOT8 expression and decreased miR-1-3p expression in breast cancer tissues (Figure 7D). Furthermore, Figure 7E depicts a negative correlation between ACOT8 and miR-1-3p expression levels.

To elucidate the potential regulatory mechanisms of ACOT8 expression, we focused on miR-1-3p, identified in Figure 7 as a candidate upstream miRNA. The starBase database (https://reporter.nih.gov/project-details/8111303) was

Table 4 In the IHC Cohort of Breast Cancer, the Predictors of Overall Survival (OS) Were Examined Through the Use of Both Univariate and Multivariate Cox Proportional Hazards Regression Analyses

Clinicopathological Parameters	Univariate Analysis (*p*-value)	Multivariate Analysis		
		Hazard Ratio	HR (95% CI)	*p*-value
Age (≤60yr vs >60 yr)	0.696	0.842	0.477~1.488	0.555
Tumor size (≤2cm vs.>2cm)	<0.001	2.921	1.457~5.856	0.003
Pathological grade (G1+G2 vs G3)	0.003	1.003	0.483~2.082	0.994
ER (negative vs positive)	0.838	1.727	0.596~5.000	0.314
PR (negative vs positive)	0.094	0.629	0.262~1.513	0.301
Lymph node invasion (negative vs positive)	<0.001	2.319	1.280~4.202	0.006
P53 (negative vs positive)	0.018	1.503	0.791~2.854	0.214
ACOT8 (negative vs positive)	<0.001	2.041	1.102~3.779	0.023

employed to assess the correlation between miRNA expression and ACOT8 levels in BC tissues. Additionally, we compared miR-1-3p expression levels in BC tissues (n = 70) with those in adjacent normal tissues using the starBase database. Furthermore, Kaplan-Meier plots were utilized to evaluate the prognostic significance of miR-1-3p expression in BC patients. We then performed qRT-PCR analysis to quantify miR-1-3p levels in BC tissues (n = 70). Finally, Pearson's correlation analysis was employed to assess the potential association between ACOT8 and miR-1-3p mRNA levels in these BC tissues.

ACOT8 is Negatively Correlated with Immune Cell Infiltration in BC

This study investigated the interplay between immune cells and ACOT8 expression in BC patients. Utilizing the TIMER database, researchers observed significant variations in the extent of immune cell infiltration across BC patients with different ACOT8 copy number states (Figure 8A). Notably, ACOT8 expression demonstrated a negative correlation with the infiltration levels of three immune cell types. The strongest negative correlation was observed with $CD8^+$ T cells, followed by macrophages (Figure 8B). While a correlation between ACOT8 and neutrophils was also identified, the association was weaker. Overall, these findings suggest a connection between ACOT8 expression and immune cell infiltration in BC, supporting the initial hypothesis.

Correlation Between Expression of ACOT8 and Immune Cell Biomarkers in BC

We employed the Gene Expression Profiling Interactive Analysis (GEPIA) and TIMER databases to investigate the expression correlation between ACOT8 and immune cell biomarkers in BC. Data from these analyses are presented in Tables 5 and 6.

Relationship Between ACOT8 and Immune Checkpoints in BC

To further substantiate the observed association between ACOT8 and the mitochondrial compartment, we performed a correlation analysis using the GEPIA and TIMER databases. The results from these analyses were consistent with our previous findings (Figure 9A and B).

Discussion

Breast cancer remains a significant global health burden. Despite advancements in treatment, the prognosis for breast cancer patients is not always ideal. Identifying reliable prognostic biomarkers and novel therapeutic targets is crucial for improving patient outcomes. This study investigates the role of ACOT8, a lactyl-CoA thioesterase, in breast cancer progression. We explore its potential as both a prognostic indicator and a therapeutic target. ACOT8 belongs to the acyl-CoA thioesterase family and is a key enzyme in mammalian fatty acid metabolism.[18] It is critical in biological processes, including β-oxidation, lipogenesis, signal transduction, and ion channel regulation. Disrupted lipid metabolism is a hallmark of the metabolic reprogramming observed in cancer cells. Consequently, the ACOT gene family is implicated

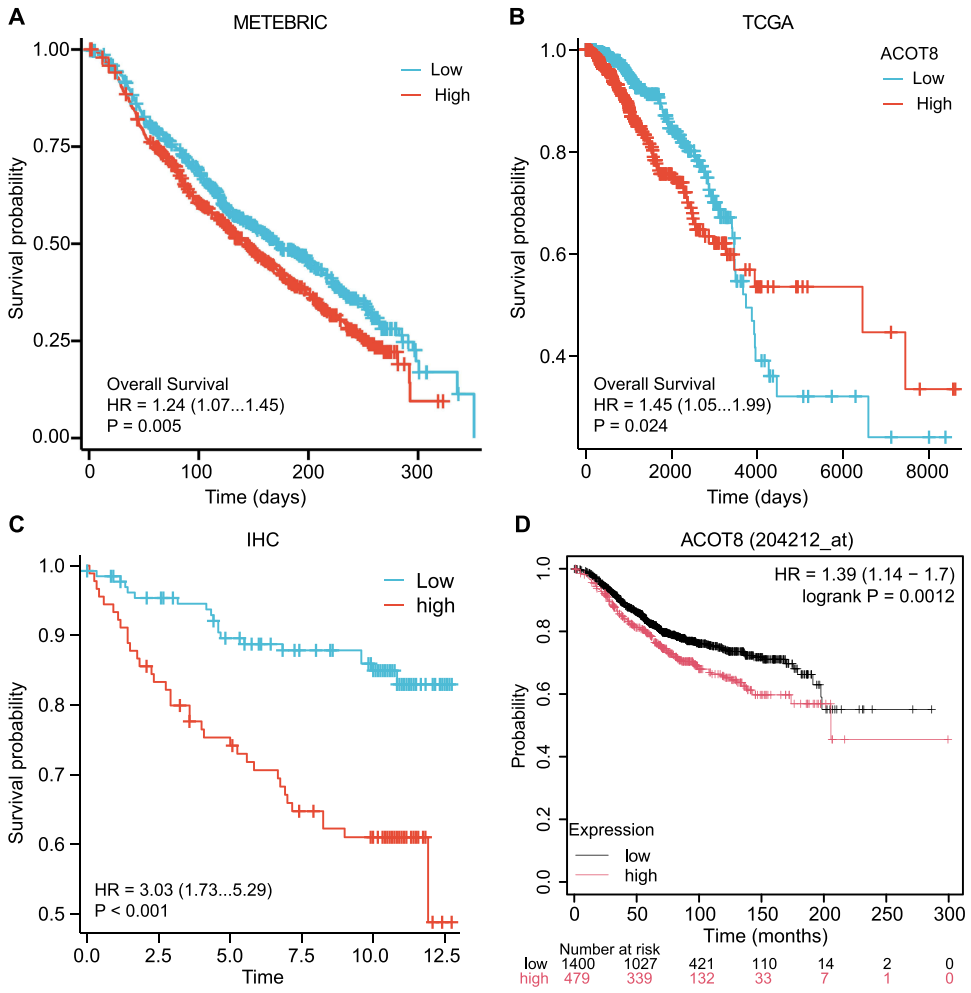

Figure 5 The overall survival (OS) of patients based on ACOT8 mRNA expression in various cohorts: (**A**) METABRIC cohort, (**B**) TCGA cohort, (**C**) IHC cohort, and (**D**) KM plotter database cohort.

in cancer initiation and progression. Previous studies have suggested a role for ACOT8 in the development of several malignancies. However, the precise function of ACOT8 in breast cancer pathogenesis and its underlying mechanisms remain poorly understood.

Our comprehensive analysis revealed a significant upregulation of ACOT8 mRNA expression in multiple human cancers, including vesicourethral carcinoma, breast invasive carcinoma, esophageal carcinoma, hepatocellular carcinoma, and lung adenocarcinoma. This suggests a potential role for ACOT8 in promoting oncogenesis across diverse cancer types. Furthermore, our survival analysis demonstrated a correlation between elevated ACOT8 expression and unfavorable prognosis in patients with liver hepatocellular carcinoma, uveal melanoma, mesothelioma, breast invasive carcinoma, and prostate adenocarcinoma. These findings suggest that ACOT8 expression may hold promise as a prognostic biomarker in these specific cancers.

This study investigated ACOT8 mRNA and protein levels in breast cancer tissues. Consistent with our observations across various cancers, we found significantly elevated ACOT8 expression in breast cancer compared to normal tissues. This suggests a potential role for ACOT8 in breast cancer development and progression. Furthermore, our analysis of clinicopathological data revealed a significant correlation between high ACOT8 levels and factors such as tumor size, grade, PAM50 classification, disease stage, and lymph node involvement. These findings suggest that ACOT8 may contribute to aggressive tumor behavior and could potentially serve as a marker for breast cancer characteristics and dissemination. To gain insights into the molecular mechanisms underlying ACOT8 dysregulation in breast cancer, we

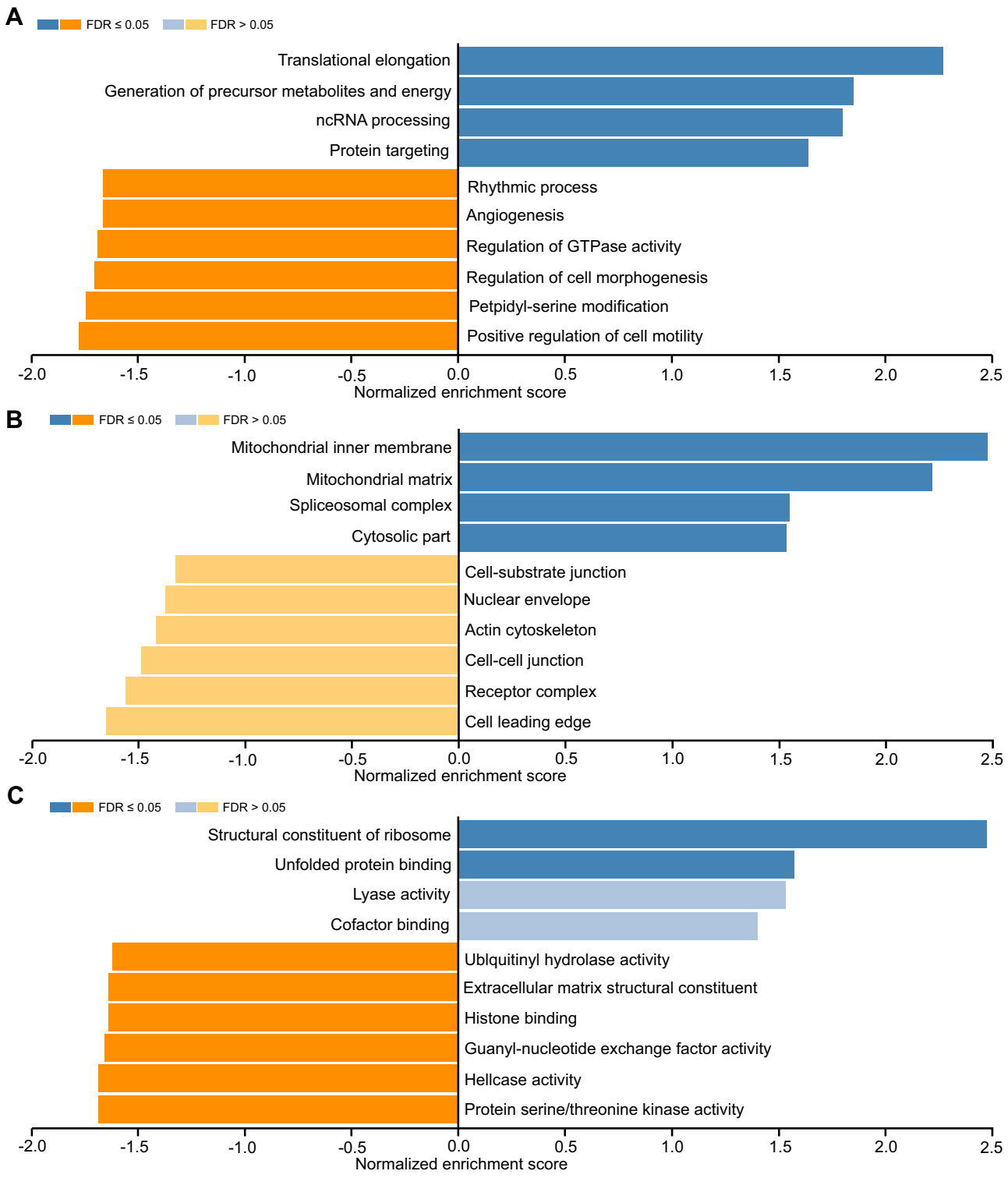

Figure 6 Enrichment Analysis of ACOT8 Expression in Breast Cancer Using Spearman Correlation. (**A**) BP, biological process. (**B**) CC, cellular components. (**C**) MF, molecular function.

explored its associated signaling pathways using the LinkedOmics database. Interestingly, our analysis identified a positive association between ACOT8 expression and ncRNA processing. Since dysregulation of miRNAs is well-established in cancer, targeting specific miRNAs holds therapeutic potential. Following an integrated analysis of various databases, including survival data, we identified miR-1-3p as a potential regulator of ACOT8 in breast cancer. Notably,

A

miRNAname	geneName	PITA	RNA22	miRmap	microT	miRanda	PicTar	TargetScan	TDMDScore	AgoExpNum	CleveExpNum	phyloP	Pan-cancer
hsa-miR-1-3p	ACOT8	1[1,0]	0[0,0]	0[0,0]	0[0,0]	0[0,0]	0[0,0]	0[0,0]	0.4745	1	0	0.381	8
hsa-miR-206	ACOT8	1[1,0]	0[0,0]	0[0,0]	0[0,0]	0[0,0]	0[0,0]	0[0,0]	0.9844	1	0	0.381	5
hsa-miR-613	ACOT8	1[1,0]	0[0,0]	0[0,0]	0[0,0]	0[0,0]	0[0,0]	0[0,0]	0.372	1	0	0.381	0
hsa-miR-873-5p	ACOT8	1[1,0]	0[0,0]	0[0,0]	0[0,0]	0[0,0]	0[0,0]	0[0,0]	0.3543	1	0	0.621	3

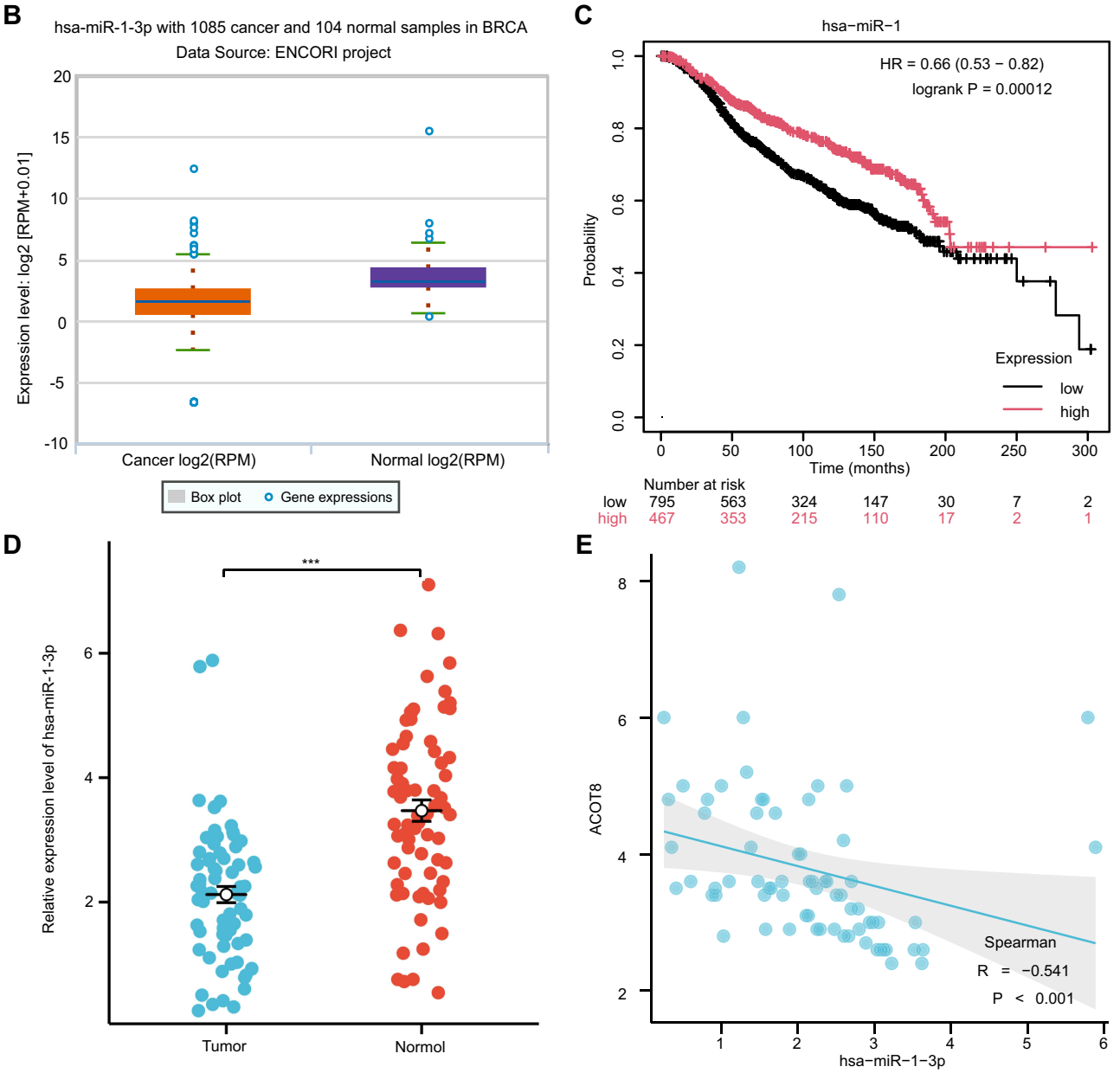

Figure 7 miR-1-3p was discovered as a potential miRNA upstream of ACOT8 in BC. (**A**) Using the starBase database, the correlation between miRNAs and ACOT8 in BC was estimated. (**B**) A comparison of miR-1-3p expression in BC and nearby normal tissues was conducted using the starBase database. (**C**) Kaplan-Meier plots were used to evaluate miR-1-3p's predictive significance in BC. (**D**) miR-1-3p levels in BC tissues (N = 35) were detected using qRT-PCR. (**E**) Pearson's correlation analysis was employed to assess the relationship between ACOT8 and miR-1-3p mRNA levels in BC tissues (N = 35).

previous studies have shown that LINC00242 promotes aerobic glycolysis in gastric cancer cells via the miR-1-3p/G6PD pathway, thereby impacting tumor growth.[19] Further functional investigations are warranted to elucidate the regulatory role of miR-1-3p on ACOT8 and its potential significance in breast cancer.

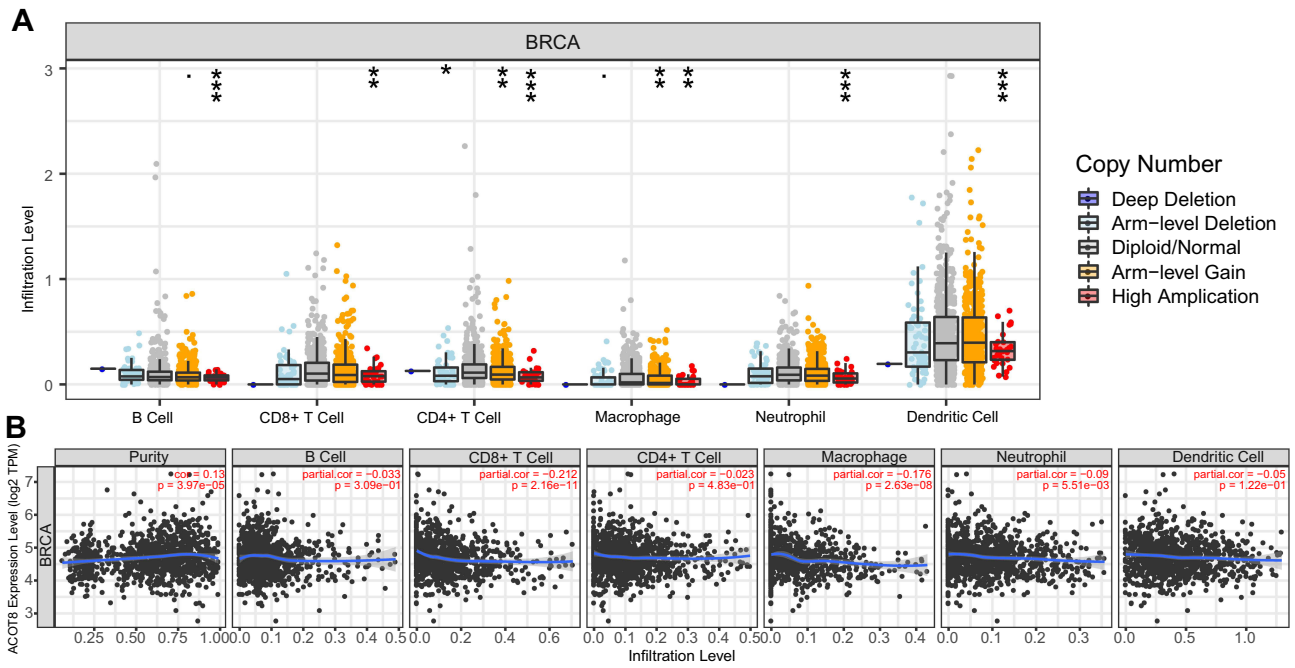

Figure 8 The correlation between the infiltration of immune cells and ACOT8 expression in BC. (**A**) Projected levels of infiltration by different immune cells at varying ACOT8 copy numbers in BC. (**B**) The relationship between the expression of ACOT8 and the levels of infiltration by B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells in BC is being examined.*P < 0.05, **P < 0.01, ***P < 0.001.

This study further explored the relationship between ACOT8 and the tumor immune microenvironment. We identified a correlation between ACOT8 expression and cytokine binding. Interestingly, previous research has shown ACOT8 involvement in HIV's evasion of the host immune response, suggesting a potential link between ACOT8 and immune-related factors.[6] To elucidate the underlying mechanisms of ACOT8's role in breast cancer, we investigated its association with immune cells and immune checkpoint inhibitors using the TIMER database. Our analysis revealed an

Table 5 Correlation Analysis Was Performed by GEPIA to Investigate the Association Between ACOT8 and Producers of Macrophages, B Cells, and T Cells

Description	Marker Genes	Tumor R	*p*-value	Normal R	*p*-value
$CD8^+$ T cell	CD8A	−0.071	0.019	0.28	0.0026
	CD8B	−0.0036	0.91	0.36	0.00011
	CD45	−0.16	***	−0.013	0.89
T cell (overall)	CD3D	−0.029	0.34	0.39	***
	CD3E	−0.059	0.053	0.3	0.0011
	CD2	−0.055	0.068	0.25	0.0075
B cell	CD19	0.0014	0.96	0.29	0.002
	CD79A	−0.088	0.0036	0.28	0.0026
	CD27	−0.054	0.078	0.34	***
	CD20(MS4A1)	−0.098	0.0012	0.21	0.023
TAM	CD68	−0.048	0.11	−0.13	0.17
	CD11B	−0.096	0.0015	0.026	0.78
M1 Macrophage	INOS(NOS2)	−0.076	0.012	0.054	0.57
	IRF5	0.15	***	0.34	***
	COX2(PTGS2)	−0.25	***	−0.14	0.14

(*Continued*)

Table 5 (Continued).

Description	Marker Genes	Tumor R	*p*-value	Normal R	*p*-value
M2 Macrophage	CD163	−0.005	0.87	−0.19	0.045
	CD206	−0.14	***	−0.37	***
Treg	FOXP3	0.024	0.43	0.54	***
	CD25(IL2RA)	−0.031	0.31	−0.17	0.069
	CCR8	−0.034	0.26	−0.015	0.88
	TGFβ(TGFB1)	0.94	0.0021	0.26	0.0048
	STAT5B	−0.13	***	−0.26	0.0057

Notes: *P < 0.05, **P < 0.01, ***P < 0.001.

Table 6 Analysis of ACOT8 Expression and Immune Cell-Related Genes in TIMER Database

Description	Marker Genes	None Cor	*p*-value
$CD8^+$ T cell	CD8A	−0.067	***
	CD8B	−0.004	***
	CD45(PTPRC)	−0.173	***
T cell (overall)	CD3D	−0.022	***
	CD3E	−0.048	0.11
	CD2	−0.048	0.108
B cell	CD19	0.017	0.563
	CD79A	−0.058	0.0536
	CD27	−0.03	0.314
	CD20(KRT20)	−0.125	0.046
Monocyte	CD14	0.021	0.489
	CD86	−0.114	***
	CD115(CSF1R)	−0.13	***
TAM	CD68	−0.042	0.061
	CD11B(ITGAM)	−0.103	***
M1 Macrophage	INOS(NOS2)	−0.112	***
	IRF5	0.099	**
	COX2(PTGS2)	−0.289	***
M2 Macrophage	CD163	−0.081	**
	CD206(MRC1)	−0.159	***
Neutrophil	CD66b(CEACAM8)	−0.012	0.695
	CD11B(ITGAM)	−0.103	***
	CD15(FUT4)	−0.231	***
	CCR7	−0.036	0.229
Natural killer cell	KIR2DL1	−0.055	0.0675
	KIR2DL3	−0.023	0.44
	KIR2DL4	−0.012	0.688
	KIR3DL1	−0.047	0.121
	KIR3DL2	−0.034	0.258
	KIR3DL3	0.054	0.0736
	KIR2DS4	−0.064	0.0345
	CD335(NCR1)	−0.106	***

(*Continued*)

Table 6 (Continued).

Description	Marker Genes	None Cor	*p*-value
Dendritic cell	BDCA-1(CD1C)	−0.143	***
	BDCA-3(CD141)	−0.187	***
	BDCA-4(NRP1)	−0.327	***
	CD11c(ITGAX)	−0.051	0.0883
Th1	T-bet (TBX21)	−0.004	0.896
	STAT4	−0.125	***
	IFN-r(IFNG)	−0.016	0.59
	TNF	−0.044	0.147
	STAT1	−0.036	0.237
Th2	GATA3	0.051	0.0883
	STAT5A	−0.206	***
	STAT6	−0.046	0.126
Tfh	BCL6	−0.152	***
	IL21	−0.05	0.0959
Th17	STAT3	−0.213	***
	IL17A	0.044	***
Treg	FOXP3	−0.018	0.545
	CD25(IL2RA, ISG20)	−0.08	**
	CCR8	−0.064	*
	TGFB1	−0.016	0.586
	STAT5B	−0.18	***
T cell exhaustion	PD-1(PDCD1)	0.046	0.131
	CTLA4	0.001	0.968
	LAG3	0.135	***
	TIM-3(HAVCR2)	−0.117	***
	GZMB	−0.026	0.394

Notes: *P < 0.05, **P < 0.01, ***P < 0.001.

inverse correlation between ACOT8 expression and the presence of $CD8^+$ T cells, macrophages, and neutrophils. Additionally, a negative correlation was observed between ACOT8 expression and several other immune cell markers.

Our findings suggest a potential role for ACOT8 in regulating the tumor microenvironment and immune response in breast cancer. Further investigation into the specific mechanisms by which ACOT8 modulates the tumor microenvironment, particularly its interaction with immune checkpoint molecules, could provide valuable insights for the development of novel immunotherapeutic strategies for breast cancer treatment.

This study investigated the association between ACOT8 expression and the efficacy of immune checkpoint inhibitors (ICIs) in BC patients receiving PD-1 blockade therapy. Specifically, we analyzed the relationship between ACOT8 and the expression of PD-1, SIGLEC15, LAG3, and PDCD1LG2, all immune checkpoint molecules. Utilizing data from the TIMER database, we identified an inverse correlation between ACOT8 expression and PD1, SIGLEC15, and PDCD1LG2. Conversely, a positive correlation was observed between ACOT8 and LAG3. These findings were corroborated by analyzing data from the GEPIA database. Overall, our research highlights the potential of ACOT8 as a target for immunotherapy in breast cancer patients. However, further investigation is necessary to validate these findings and elucidate the underlying mechanisms by which ACOT8 expression may influence the response to PD-1 blockade therapy.

This study has limitations that should be acknowledged. The retrospective nature of the investigation precludes establishing causality. Future studies employing in vivo or in vitro models are necessary to elucidate the mechanistic underpinnings of the observed associations and to determine cause-and-effect relationships.In vivo experiments can simulate the impact of changes in ACOT8 expression levels on the formation and progression of breast cancer by constructing transgenic or gene knockout mouse models. In vitro experiments can utilize breast cancer cell lines to observe biological behaviors such as cell proliferation, migration, invasion, and metabolic changes by overexpressing or knocking down the ACOT8 gene.

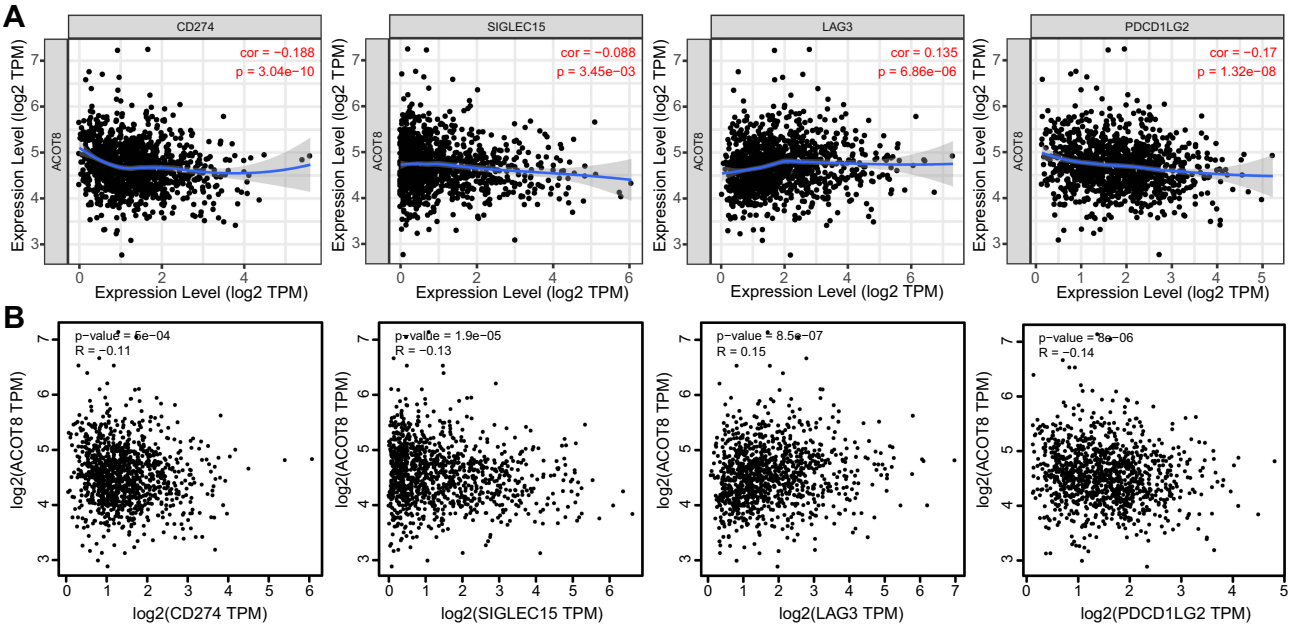

Figure 9 The correlation between the expression of ACOT8 and PD-1, SIGLEC15, LAG3, and PDCD1LG2 was examined in the TIMER (**A**) and GEPIA (**B**) databases.

Conclusion

This study underscores the potential significance of ACOT8 as a dual target in breast cancer: a prognostic biomarker and a therapeutic target. The observed upregulation of ACOT8 in breast cancer tissues, coupled with its correlation with poor prognosis and aggressive clinical features, suggests its involvement in breast cancer development and progression. Furthermore, the link between ACOT8 expression and immune infiltration hints at its potential influence on the tumor microenvironment and response to immunotherapy. Future investigations are necessary to elucidate the underlying molecular mechanisms and functional roles of ACOT8 in breast cancer. Unraveling these mechanisms could pave the way for developing novel targeted therapies and improved patient outcomes.

Ethics Approval

The study was approved by the ethics committee of Nantong University's Affiliated Hospital's ethical committee and all patients provided informed consent to participate in the study. All methods were carried out in accordance with relevant guidelines and regulations. This study was carried out in compliance with the Declaration of Helsinki.

Disclosure

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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