Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Plasma Exosome miR-203a-3p is a Potential Liquid Biopsy Marker for Assessing Tumor Progression in Breast Cancer Patients

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Background: Timely detection of tumor progression in breast cancer (BC) patients is critical for therapeutic management and prognosis. Plasma exosomal miRNAs are potential liquid biopsy markers for monitoring tumor progression, but their roles in BC remain unclear.

Methods: In the TCGA database, we first screened for miRNAs significantly associated with BC progression by comparing miRNA expression in para-carcinoma tissues, stage I BC tissues, and stage II–III BC tissues (n = 1026). Cox regression analyses and survival analyses were performed on candidate miRNAs to explore their prognostic value (n = 848). KEGG, GO, and PPI analyses were used to identify enriched pathways associated with cancer. Finally, the potential of candidate miRNAs as liquid biopsy markers was evaluated by sequencing and analyzing plasma exosomal miRNAs from our collection of 45 BC patients (14 in stage I, 31 in stage II–III) and 5 healthy controls, combined with qRT-PCR analysis to assess the correlation of candidate gene expression in plasma exosomes and BC tissues.

Results: We found that only miR-203a-3p was progressively elevated with BC progression and was associated with poor prognosis in the TCGA dataset. Its potential target genes were enriched in pathways related to tumor progression, and the downregulation of 48 of these genes was associated with poor prognosis. More importantly, plasma exosomal miR-203a-3p was also found to gradually increase with BC progression, and its expression was positively correlated with miR-203a-3p in BC tissues. This result suggests that plasma exosomal miR-203a-3p may reflect the expression of miR-203a-3p in tumor tissues and serve as a potential liquid biopsy marker for monitoring BC progressions.

Conclusion: We found for the first time that elevated miR-203a-3p was associated with BC progression and poor prognosis. Our findings suggested that plasma exosomal miR-203a-3p could hold potential as a liquid biopsy marker for evaluating BC progression in patients.

Keywords: plasma exosomal miR-203a-3p, breast cancer, tumor progression, liquid biopsy marker

Introduction

Breast cancer (BC) is the most prevalent malignant tumor in females, with over 2.3 million women globally diagnosed in 2023.[1] Although the 5-year survival rate for patients with stage I BC is 98%, the survival rate of BC patients gradually decreases as the tumor stage advances.[2–4] Additionally, the choice of treatment regimen is closely related to the stage of BC progression.[5,6] Therefore, selecting a treatment regimen inconsistent with the patient's current stage may adversely affect their prognosis. Existing studies have shown that the mortality rate of BC has continued to increase in recent years, suggesting that current monitoring and treatment of tumor staging and progression in BC patients are still inadequate. Currently, mammography, ultrasound, and MRI are the principal techniques used to assess BC progression.[7,8] Yet, these methods present challenges including radiation exposure, invasiveness, potential inaccuracies, and inconvenience.[9]

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Therefore, there is an urgent need for safer, simpler, and more accurate methods for timely assessment of tumor progression in BC patients.

Exosomes, abundant in the circulation of human body fluids and tissue cells, play a vital role in intercellular communication. These extracellular vesicles have a diameter ranging from approximately 30 to 120 nm and comprise lipid bilayers. They transport a variety of nucleic acids, including different small RNAs such as microRNAs (miRNAs), along with proteins and other molecules.[10–12] MiRNAs, specifically, have attracted considerable attention due to their functional importance in regulating gene expression.[13,14] Plasma exosomal miRNAs have emerged as liquid biopsy marker for tumor diagnosis and prognostic prediction, offering several advantages over free miRNAs in body fluid circulation.[15,16] The protective lipid bilayer of exosomes ensures miRNAs stability, making them less susceptible to degradation compared to their free counterparts. This characteristic, coupled with their non-invasive accessibility via liquid biopsy, positions plasma exosomal miRNAs as valuable diagnostic tools.[17–20] Current research suggests that dysregulated miRNAs in plasma exosomes can act as liquid biopsy markers for monitoring the progression of some cancers. Yet, studies focusing on plasma exosomal miRNA in relation to BC progression are still in their infancy.[21]

In this study, we utilized the TCGA database and plasma exosomal miRNA sequencing (miRNA-seq) from our BC patients. We found that the expression level of miR-203a-3p in tissues gradually increases with the progression of BC and is positively correlated with poor patient prognosis. Moreover, the expression level of miR-203a-3p in plasma exosomes can reflect its expression status in BC tissues and also increases as the BC in patients progresses. In conclusion, our research suggests that plasma exosomal miR-203a-3p has potential as a liquid biopsy marker for assessing the tumor progression of BC patients.

Materials and Methods

Screening Candidate miRNAs in TCGA Dataset

We downloaded miRNA-seq data from the TCGA database (TCGA provisional dataset, 2021) for 1,026 tissue samples from BC patients, including all para-carcinoma tissue samples (n=104) as well as BC tissue samples with clinical stage information (322 stage I BC samples and 600 stage II–III BC samples). We screened for differentially expressed genes (DEGs) between para-carcinoma tissues and stage I BC tissues, as well as between stage I BC tissues and stage II–III BC tissues, respectively. Subsequently, selected DEGs that consistently increased or decreased with tumor progression as the candidate miRNAs. The Count data of miRNA-seq were corrected and normalized using the "limma" R package,[22] and DEGs analysis was conducted using the "edgeR" R package.[23] The filtering condition for all differentially expressed miRNAs (DEMs) was median of normalized count data ≥ 10, P-value < 0.05, and $|\log_2 \text{fold change (FC)}| > 0.5$.

Cox Regression Analysis and Disease-Free Survival (DFS) Analysis

The miRNA-seq data and clinical data from BC tissues of patients meeting the following criteria in the TCGA dataset of 1,026 samples, as described above, were used in univariate and multivariate Cox regression analyses, as well as DFS analyses, to further screen for candidate genes with clinical value (n = 848):

1. Female patients who were diagnosed with BC between the ages of 20–85 years;
2. Those at stages I–III;
3. Those without distant metastasis (M0);
4. Those who have completed therapeutic interventions;
5. By the end of the follow-up, patients with distant metastasis or censored data.

Candidate miRNAs that were statistically significant in univariate Cox regression analyses, along with patient clinical factors, were included in multivariate Cox regression analyses. Univariate and multivariate Cox regression with forward stepwise regression based on likelihood ratio test (forward LR model) were performed to investigate the impact of independent factors on DFS time, with the hazard ratio (HR), 95% confidence intervals (CI), and P-value being reported. Further, DFS analysis was used to verify the relationship between the candidate miRNA and BC patient prognosis. The "survival" and "survminer" R packages were utilized to draw Kaplan–Meier survival curves and compare the high and

low expression groups based on the HR, and Log-Rank P-value < 0.05. The high and low expression of candidate miRNA is divided based on its median value.

Clinical Sample Collection and Processing

Our blood samples were collected from 45 BC patients (stage I, n = 14; stage II–III, n = 31) and 5 healthy controls using 5mL EDTA-anticoagulated vacutainer tubes between April 2021 and May 2022 at Beijing Hospital. Following collection, samples were centrifuged at 1500 × g for 10 minutes at 4 °C, and subsequently at 3000 × g for 15 minutes at 4 °C. From this, 2mL of plasma was extracted and stored at −80 °C, awaiting further isolation and sequencing of exosomal miRNA. In addition to these, 12 BC tissue samples were sourced from the aforementioned BC patients and stored in liquid nitrogen.

All participants written informed consent for the collection of plasma and tissue samples, and for their pathological data to be utilized in this study. The study protocol was approved by the Ethics Committee of Beijing Hospital, based on the Declaration of Helsinki, and written informed consent was obtained from the patients.

Isolation and Identification of Plasma Exosomes

Plasma exosomes were isolated using size exclusion chromatography. One millilitre of blood plasma, filtered through a 0.8 μm filter, was diluted 1.5 times with PBS and further purified using Exosupur columns (Echobiotech, China). The samples were then eluted with PBS, and 2 mL eluate fractions were collected. These fractions were then concentrated down to 200 μL using 100 kDa molecular weight cut-off Amicon Ultra spin filters (Millipore, Germany).

The exosomes were identified using three methods: nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and Western blot (WB). Initially, vesicle suspensions at concentrations of about 1×10^8/mL were inspected using a ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) outfitted with a 405 nm laser. The size and quantity of the isolated particles were then determined. A total of 20 μL of exosomes was analysed, and particle movement was assessed using NTA software (ZetaView 8.02.28). Subsequently, the exosomes were resuspended in PBS and placed on an electron microscope's copper mesh. After a 10-minute incubation at room temperature, 1% uranyl acetate was used for negative staining for 10 minutes. Observations and images were made with a TEM (H-7650, Hitachi Ltd., Tokyo, Japan). Finally, exosome-enriched supernatant was denatured in 5 × sodium dodecyl sulfonate (SDS) buffer and subjected to WB using TSG101 (sc-13611, Santa Cruz), CD9 (60232-I-Ig, Proteintech, Rosemont, IL), Alix (ab275377, Abcam), and calnexin (10,427–2-AP, Promega, Madison, WI).[24] The X-ray exposure system used for developing the PVDF membrane. The original Ponceau stained PVDF membrane and the film exposure results can be seen in Supplementary Figure 1.

Plasma Exosomal miRNA-Seq and Analysis

From each of the 50 plasma exosome samples, we extracted approximately 10 ng of RNA using the miRNeasy Kit (Qiagen, Germany). MiRNA-seq was subsequently executed on the Illumina HiSeq platform (Echobiotech, China). The miRNA-seq data, denoted as Unique Molecular Identifiers (UMIs), underwent correction and normalization via the "limma" R package. We employed the "edgeR" R package for the differentially expressed plasma exosomal miRNAs analysis between normal controls and stage I BC patients, as well as between stage I BC patients and stage II–III BC patients. The filtering criteria for identifying differentially expressed exosomal miRNAs were set as follows: transcripts per million (TPM) ≥ 30, P-value < 0.05, and $|\log_2 FC| > 0.5$.

Quantitative Analysis of Candidate miRNA in BC Tissues

Total RNA was extracted from 12 BC tissues using RNAiso Plus (TaKaRa). To detect U6 and miR-203a-3p, real-time polymerase chain reaction (qRT–PCR) analyses were performed using the PrimeScript RT reagent Kit and SYBR Premix Ex Taq (TaKaRa), with U6 serving as the control. The relative expression levels of miR were calculated and quantified using the ΔCT method. The qRT–PCR primers were as follows: miR-203a-3p stem–loop RT primer: 5'- GTCGTAT CCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTAGTGGT-3'; miR-203a-3p: Forward 5'-GCCGCTCCCCC

AGGTGTGATT-3' and reverse 5'-GTGCAGGGTCCGAGGT-3'; U6: Forward 5'-GGCAGGAAGAGGGCCTA-3' and reverse 5'-GTGCAGGGTCCGAGGT-3'.

Prediction of miRNA Target Genes

Initially, we identified potential target genes that were inversely related to the expression of candidate miRNAs through simple regression analysis in the TCGA dataset (n = 848). Subsequently, potential target genes that overlapped between the TCGA dataset and online miRNA target analysis algorithms (The miRWalk database[25]) were defined as candidate target genes. Venn analysis was applied to identify intersections in the data obtained from the two databases.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analysis

Candidate target genes were subject to GO[26] and KEGG[27] analysis. GO analysis was utilized to investigate the crucial functions of the target genes according to Gene Ontology. Likewise, pathway analysis was carried out to scrutinize the significant pathways of the targeted genes based on KEGG. Fisher's exact test and the χ^2 test were executed to select the significant GO terms and pathways. The threshold of significance was determined by a $P < 0.05$. The validated miRNAs in enriched biological pathways were selected to construct miRNA-target-pathway networks using Cytoscape software (Version: 3.5.1).

Protein–Protein Interaction (PPI) Network Construction

To predict the interaction pattern of prognostic-related target genes in BC, we employed the Retrieval of Interacting Genes/Proteins (STRING[28]) database (https://string-db.org/) to construct a PPI network of prognosis-related gene expression, specifying the retrieval conditions as Organism: Homo sapiens, and requiring a minimum interaction score of low confidence (0.15). Following this, the simple table text exported from STRING was imported into Cytoscape v3.10.0. The top 10 nodes were then identified using the "betweenness" ranking method in cyto-Hubba, and these were defined as key pivotal genes.

Statistical Analysis

Statistical analysis of the data was conducted using SPSS version 25 (IBM Corp., Armonk, NY, USA) and R software version 4.2.3 (R core Team, Vienna, Austria). The Mann–Whitney *U*-test was used to process continuous variables. Linear regression models were deployed to detect the linear trend test. *P*-value of less than 0.05 was considered statistically significant.

Results

Identification of Candidate miRNA

Utilizing the TCGA database, we screened for differential expression miRNAs between 104 para-carcinoma tissues and 322 stage I BC tissues, as well as between the 322 stage I BC tissues and 600 stage II–III BC tissues (Figure 1A). We eventually identified 3 candidate miRNAs related to tumor progression (Figure 1B). Among them, the expression levels of miR-203a-3p and miR-9-5p in tissues gradually increased with BC progression (Figure 1C and D), while the expression of miR-144-5p gradually decreased with BC progression (Figure 1E).

MiR-203a-3p is a Poor Prognostic Factor Positively Correlated with BC Progression

Initially, to explore the roles of the three candidate miRNAs in BC disease progression and prognosis, we included miR-203a-3p, miR-9-5p, miR-144-5p, and clinical factors related to prognosis into univariate and multivariate Cox regression analyses using the TCGA dataset (n = 848) (Figure 2A and B). Detailed clinical data for the 848 BC patients was shown in Table 1. We found that among the three candidate genes, only miR-203a-3p is an independent risk factor related to DFS in BC patients. Its high expression can lead to poor prognosis for BC patients (univariate Cox regression: HR=1.691 [1.049–2.725], $P = 0.031$; multivariate Cox regression: HR = 1.66 [1.027–2.681], $P = 0.038$). To further confirm the

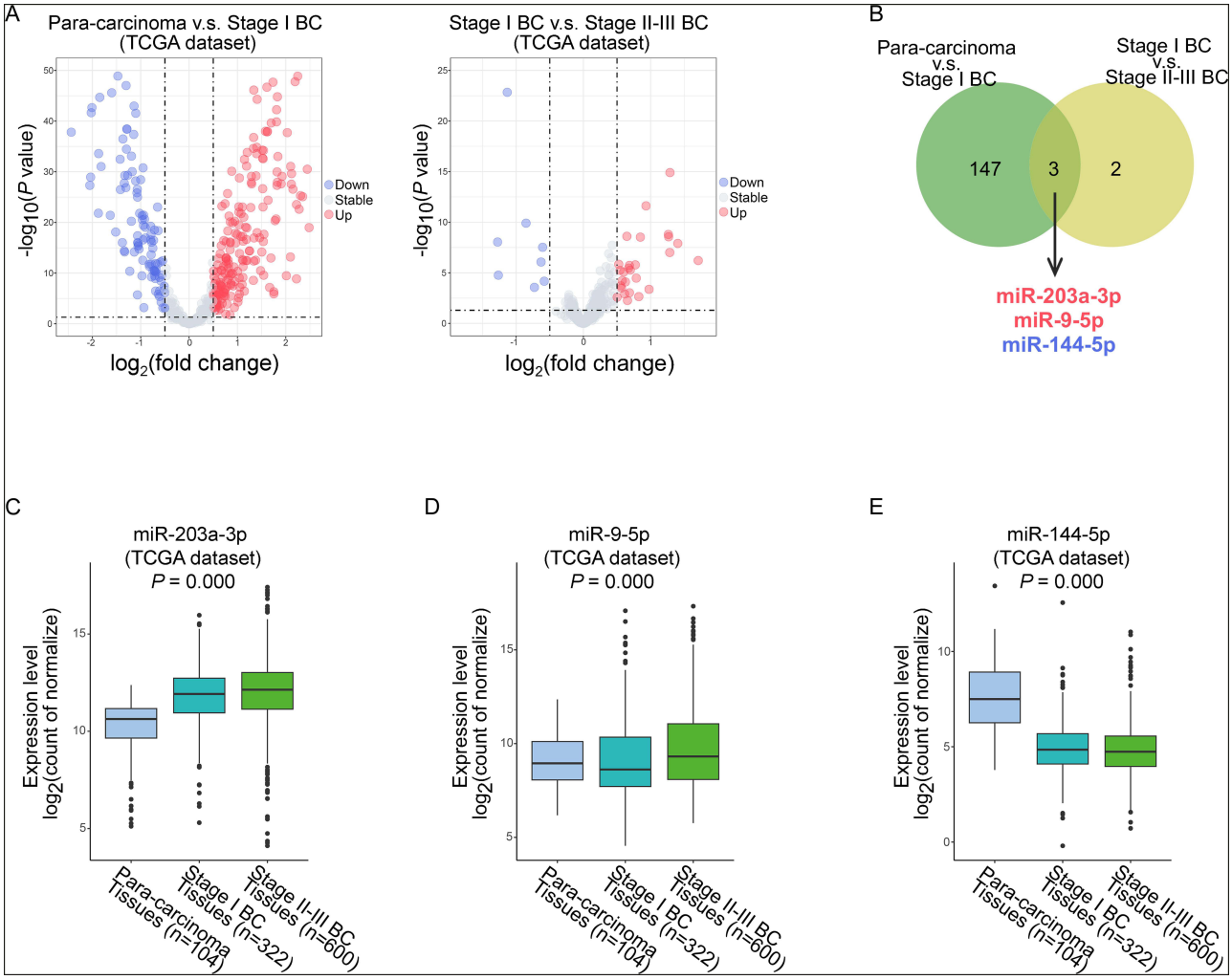

Figure 1 Screening candidate miRNAs associated with BC progression in TCGA dataset. (**A**) Screening the differential miRNAs between 104 para-cancer tissues and 322 stage I BC tissues, as well as between 322 stage I BC tissues and 600 stage II–III BC tissues, presented as a volcano plot. The Venn diagram indicates that miR-203a-3p, miR-9-5p and miR-144-5p were candidate genes. (**B**) The box plot shows the expression trends of the three candidate miRNAs among para-cancer tissues, stage I BC tissues, and stage II–III BC tissues. (**C** and **D**) The expression levels of miR-203a-3p and miR-9-5p in BC tissues gradually increased with BC progression. (**E**) The expression of miR-144-5p in BC tissues gradually decreased with BC progression.

prognostic value of miR-203a-3p in BC, we carried out DFS analysis. Our results showed that patients with high expression of miR-203a-3p had a significantly shorter DFS time (Figure 2C: Log-Rank $P = 0.029$). These findings underscore the potential of miR-203a-3p as a prognostic marker in BC. Based on the above results suggested that the expression level of miR-203a-3p in tissues gradually increases with the progression of BC. Furthermore, its high expression leads to a shortened DFS time for patients, indicating a poor prognosis for BC patient.

Target Gene Prediction and Its Functional Enrichment Analysis

To further understand the biological functions and mechanisms of miR-203a-3p in BC, we performed prediction and functional enrichment analysis of its potential target genes. First, we identified genes negatively associated with miR-203a-3p expression levels in the TCGA dataset and merged these results with target gene prediction outcomes from the miRWalk public database. As a result, we identified 234 potential target genes showed (Figure 3A).

Subsequently, we conducted KEGG pathway and GO enrichment analyses to uncover the signaling pathways and functions of potential target genes. As shown in Figure 3B and C, the potential target genes were significantly enriched in crucial tumor proliferation and invasion-related pathways, including the MAPK signaling pathway, PI3K-Akt signaling

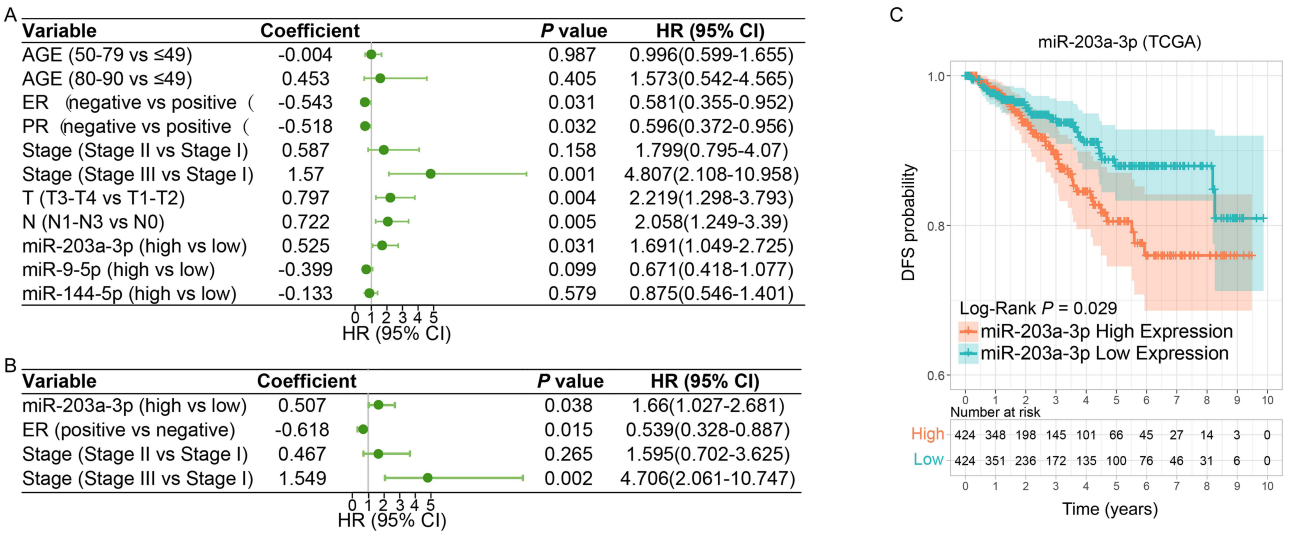

Figure 2 miR-203a-3p is positively correlated with poor prognosis in BC patients. (**A**) Using the TCGA dataset, a univariate Cox regression analysis indicated that among the three candidate genes, only miR-203a-3p is a factor related to the DFS in BC patients. (**B**) Multivariate Cox regression analysis showed that high expression of miR-203a-3p is an independent risk factor for poor prognosis in BC patients. (**C**) Kaplan-Meier analysis showed that high expression of miR-203a-3p leads to a shortened DFS time.

pathway, and the Ras signaling pathway. These results suggest that miR-203a-3p might promote the progression of BC through these pathways.

Further Screening for Prognosis-Related Key Target Genes and PPI Network

In this study, we found that high expression of miR-203a-3p was positively associated with shorter DFS time in BC patients. Therefore, we further screened for potential target genes that might be associated with prognosis in BC patients. Through survival analysis, we discovered that 48 of the 234 potential target genes were associated with shorter DFS in BC patients when these genes were downregulated. The specific results of the survival analysis are shown in Table 2. Consequently, we hypothesize that the role of miR-203a-3p in regulating the aforementioned 48 genes may account for its prognostic impact among BC patients (Figure 3D). Next, we mapped these 48 genes to the PPI network, revealing 48 nodes and 78 edges (Supplementary Figure 2). Based on the node degree calculated by Betweenness in Cytoscape, we

Table 1 The Clinical Characteristics of Female 848 BC Patients Enrolled for Prognostic Analysis in the TCGA Dataset

Variables		N (%)
Age	< 50	231 (27.2)
	≥ 50	617 (72.8)
Stage*	I	150 (17.7)
	II–III	698 (82.3)
Tumor size†	≤ 20mm	725 (85.5)
	> 20mm	123 (14.5)
Lymph node Metastasis	Negative	410 (48.3)
	Positive	438 (51.7)
Estrogen Receptor	Negative	194 (22.9)
	Positive	654 (77.1)
Progesterone Receptor	Negative	275 (32.4)
	Positive	573 (67.6)

Notes: *Staging was based on the tumor-node-metastasis (TNM) classification of American. †The greatest dimension of the tumor measured.

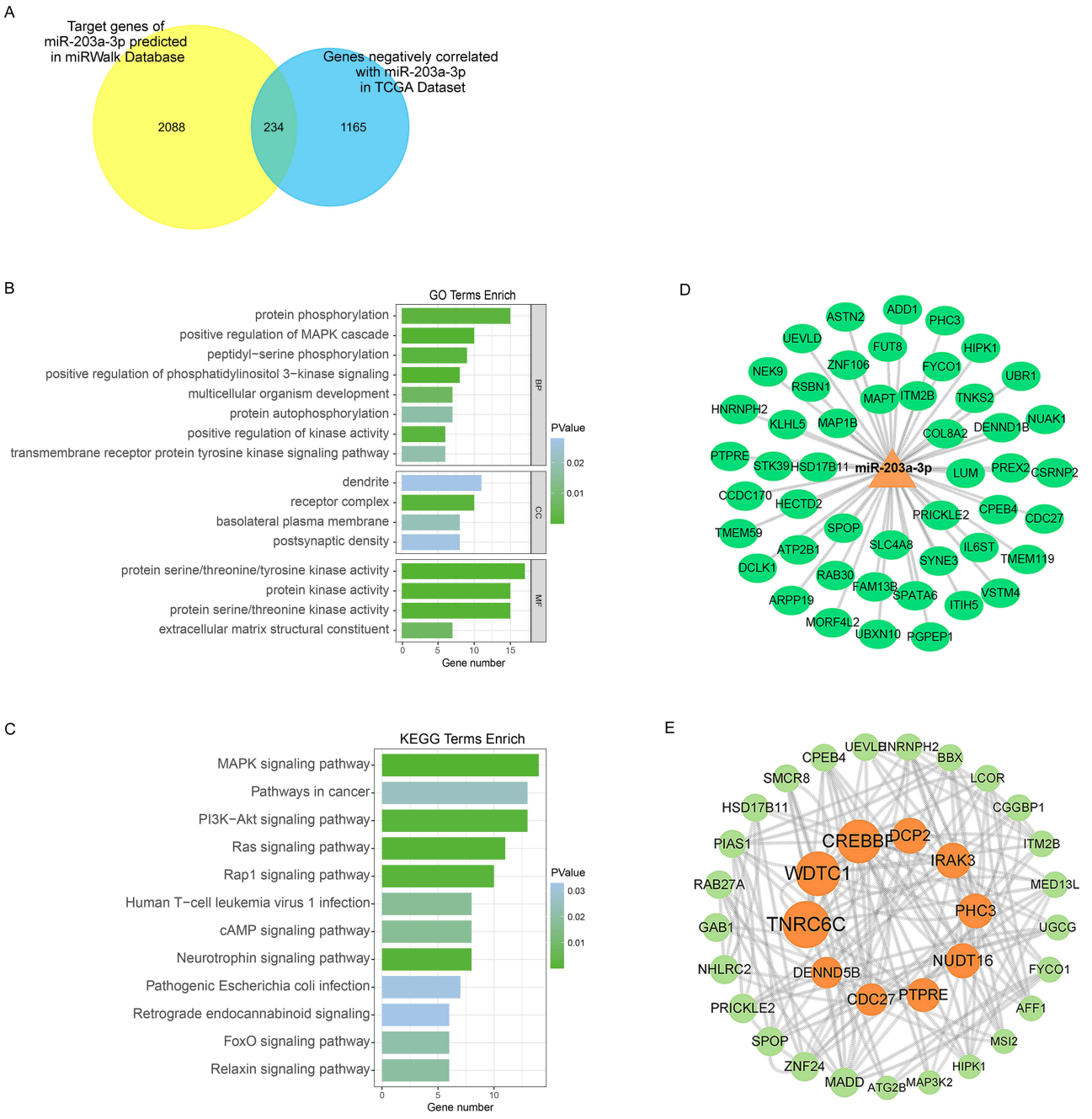

Figure 3 Potential pathway analysis of miR-203a-3p in BC. (**A**) Venn diagram showing 234 potential target genes. (**B** and **C**) KEGG pathway and GO enrichment analyses to uncover the signaling pathways and functions of the potential target genes. (**D**) miRNA–mRNA network showing the 48 prognosis-related key target genes of miR-203a-3p. (**E**) Further screening of 35 target genes, among which the top 10 genes emerged as key target genes in the miR-203a-3p regulatory network.

further screened 35 target genes, among which the top 10 genes emerged as key target genes in the miR-203a-3p regulatory network (Figure 3E).

Extraction and Characterization of Plasma Exosomes in BC Patients and Healthy Controls

To assess whether miR-203a-3p can serve as a liquid biopsy marker for evaluating BC progression, we procured plasma samples from 45 BC patients and 5 healthy controls, followed by conducting exosomal miRNA-seq on these samples. Detailed clinical data for the 45 BC patients was shown in Table 3. Before sequencing, we first identified the extracted

Table 2 There are 48 Potential Target Genes with Prognostic Value, and Their Expression Levels are Negatively Correlated with miR-203a-3p Expression Level in TCGA Dataset

Gene	Correlation analysis		Kaplan-Meier		Gene	Correlation analysis		Kaplan-Meier	
	P-value	β*	*P*-value	HR†		*P* value	β*	*P*-value	HR†
IL6ST	0.000	−0.33	0.006	1.94	*SPOP*	0.038	−0.01	0.015	1.81
LUM	0.023	−0.21	0.022	1.75	*UBXN10*	0.000	−0.01	0.024	1.73
ITM2B	0.008	−0.07	0.012	1.87	*UBR1*	0.000	−0.01	0.038	1.65
TMEM59	0.004	−0.06	0.021	1.75	*FAM13B*	0.000	−0.01	0.042	1.63
MAPT	0.005	−0.06	0.047	1.61	*PHC3*	0.016	−0.01	0.041	1.63
MORF4L2	0.041	−0.05	0.015	1.81	*ITIH5*	0.002	−0.01	0.017	1.78
RAB30	0.001	−0.05	0.036	1.67	*ZNF106*	0.035	−0.01	0.005	1.99
DCLK1	0.011	−0.04	0.040	1.64	*TNKS2*	0.022	−0.01	0.002	2.12
ARPP19	0.049	−0.03	0.042	1.64	*ASTN2*	0.011	−0.01	0.004	2.03
DENND1B	0.023	−0.03	0.008	1.91	*FYCO1*	0.030	−0.01	0.039	1.64
MAP1B	0.006	−0.03	0.014	1.81	*ATP2B1*	0.017	−0.01	0.044	1.62
ADD1	0.007	−0.03	0.026	1.71	*CDC27*	0.044	−0.01	0.007	1.91
COL8A2	0.026	−0.02	0.030	1.68	*HSD17B11*	0.000	−0.01	0.046	1.61
HNRNPH2	0.001	−0.02	0.025	1.70	*CSRNP2*	0.024	−0.01	0.030	1.69
HIPK1	0.005	−0.02	0.036	1.66	*HECTD2*	0.000	−0.01	0.046	1.62
NEK9	0.000	−0.02	0.021	1.74	*SPATA6*	0.000	−0.01	0.001	2.37
CPEB4	0.001	−0.02	0.036	1.66	*UEVLD*	0.022	−0.01	0.006	1.96
FUT8	0.048	−0.02	0.034	1.67	*NUAK1*	0.015	−0.01	0.001	2.32
TMEM119	0.002	−0.02	0.033	1.67	*RSBN1*	0.011	−0.01	0.036	1.66
STK39	0.002	−0.02	0.016	1.79	*PRICKLE2*	0.004	−0.01	0.001	2.20
SLC4A8	0.006	−0.02	0.002	2.17	*PTPRE*	0.007	−0.01	0.024	1.72
KLHL5	0.000	−0.02	0.026	1.71	*VSTM4*	0.005	−0.01	0.028	1.69
CCDC170	0.000	−0.01	0.003	2.07	*PREX2*	0.000	−0.01	0.045	1.62
PGPEP1	0.000	−0.01	0.004	2.02	*SYNE3*	0.002	−0.01	0.007	1.92

Notes: *Pearson correlation coefficient. †Hazard Ratio.

exosomes. The hydrodynamic diameter distribution of the exosomes was then measured, with the particle diameter peaking close to 100nm (Figure 4A). The morphology of the extracted exosomes was examined by TEM (Figure 4B). Lastly, we extracted the total protein from the exosomes and assessed the expression levels of exosome-positive markers TSG101, CD9, and Alix, as well as the negative marker Calnexin, through Western blotting (Figure 4C). These results demonstrated that the particle diameter distribution, morphological characteristics, and marker expression levels were consistent with previously reported findings, thereby indicating the successful extraction of exosomes.

Plasma Exosomal miR-203a-3p Can Reflect the Expression Level of miR-203a-3p in BC Tissue and Increases Gradually with the BC Progression in Patient

Firstly, we utilized miRNA-seq to determine miRNA expression levels in plasma exosomes. Upon performing differential gene screening, we found that 22 plasma exosomal miRNAs were differentially expressed in five healthy controls compared to 14 stage I BC patients, and 6 plasma exosomal miRNAs were differentially expressed in 14 stage I BC patients compared to 31 stage II–III BC patients (Figure 5A). In above results, we were delighted to find that only plasma exosomal miR-203a-3p was the common DEG, and it also increased gradually with the progression of the patient's BC (Figure 5B).

To further examine the association between plasma exosomal miR-203a-3p and its expression in BC tissues, we performed qRT–PCR analysis on 12 BC tissues. Through correlation analysis, we noted a positive correlation between the expression levels of plasma exosomal miR-203a-3p and that in BC tissues (Figure 5C, linear regression $P = 0.045$, $R^2 = 0.344$). Such results suggest that plasma exosomal miR-203a-3p could potentially serve as a liquid biopsy marker to indicate the expression status of miR-203a-3p in BC tissue. Furthermore, by comparing the expression levels of miR-

Table 3 Clinical Characteristics of Our 45 Female BC Patients in Plasma Exosomal miRNA-Seq Analysis

Variables		N (%)
Age	< 50	21 (46.7)
	≥ 50	24 (53.3)
Stage*	I	14 (31.1)
	II–III	31 (68.9)
Tumor size†	≤ 20mm	22 (48.9)
	> 20mm	23 (51.1)
Lymph node Metastasis	Negative	20 (44.4)
	Positive	25 (55.6)
Grade#	I	3 (6.7)
	2	30 (66.7)
	3	12 (26.7)
Molecular subtyping‡	Luminal A	10 (22.2)
	Luminal B	14 (31.1)
	HER2+	9 (20.0)
	TNBC	12 (26.7)

Notes: *Staging was based on the tumor-node-metastasis (TNM) classification of American Joint on Cancer. †The greatest dimension of the tumor measured by ultrasound. #The grade according to the American Joint on Cancer is based on how much the cancer cells look like normal cells. ‡The molecular subtype is based on the classification system established by the American Joint Committee on Cancer, which takes into account the expression levels of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki-67.

203a-3p in 4 stage I BC tissues and 8 stage II–III BC tissues among these 12 BC specimens, we further verified that the expression of miR-203a-3p in BC tissue gradually increases with the progression of BC (Figure 5D).

Finally, to evaluate the potential of plasma exosomal miR-203a-3p to distinguish between BC patients, particularly those in the early stage, and healthy women, we employed receiver operating characteristic (ROC) curve analysis to calculate the sensitivity and specificity of plasma exosomal miR-203a-3p in detecting BC patients. The result shown that the potential of plasma exosomal miR-203a-3p as a discriminatory marker for stage II–III BC with a sensitivity of 80.00% and specificity of 91.10% (Figure 5E: AUC = 0.849, 95% CI: 0.640–1.000, $P = 0.011$), and for stage I BC with sensitivity of 80.00% and specificity of 84.60% (Figure 5F: AUC = 0.831, 95% CI: 0.605–1.000, $P = 0.034$).

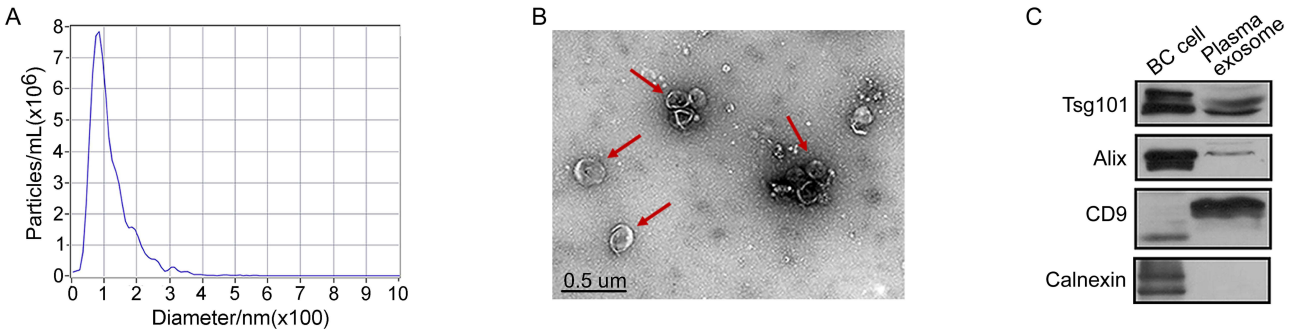

Figure 4 Identification of plasma exosomes. (**A**) Results from NTA analysis show the diameters of the exosomes. (**B**) A graphical illustration of TEM images depicting plasma-derived exosomes (the red arrows indicate the exosomes; magnification, × 40,000). (**C**) WB was utilized to determine the expression levels of Tsg101, Alix, CD9, and calnexin in exosomes isolated from plasma samples collected from BC patients and the BC cell line MCF-7.

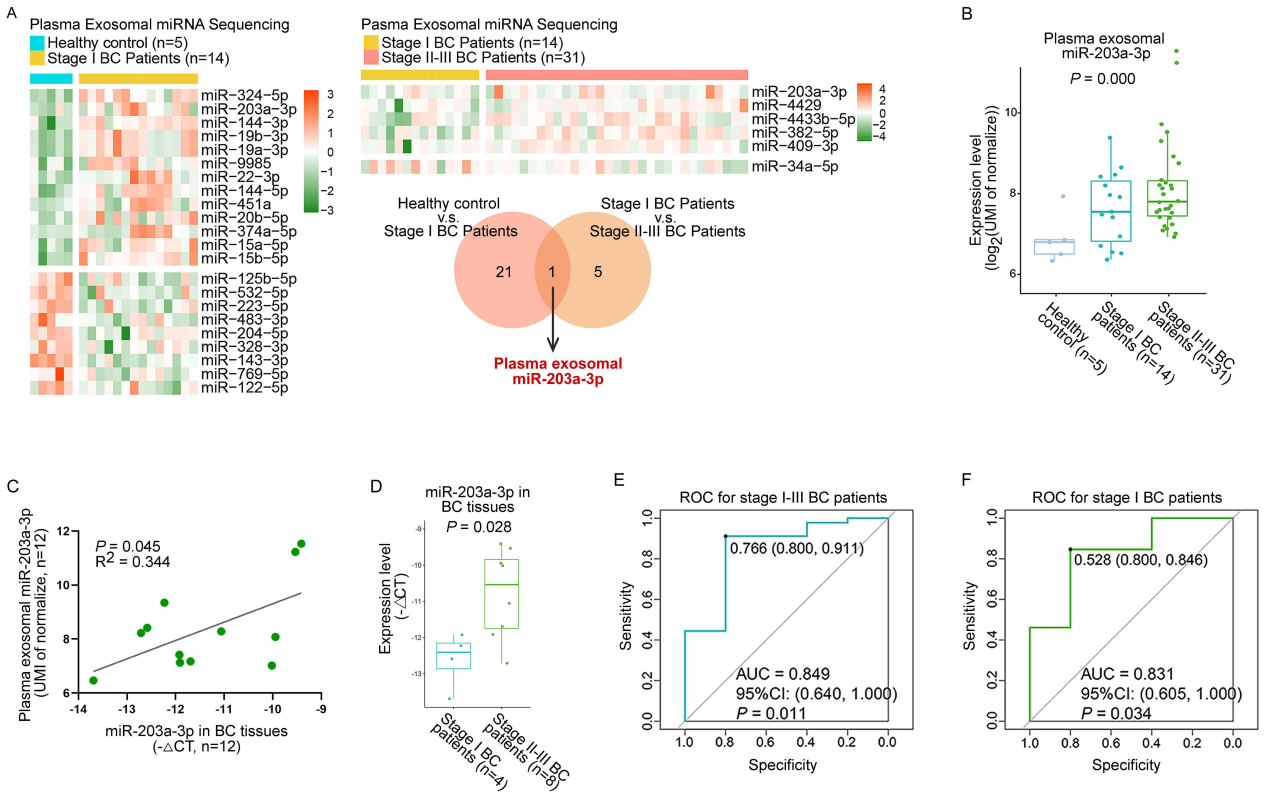

Figure 5 Plasma exosomal miR-203a-3p is a potential liquid biopsy marker for assessing BC progression. (**A**) The heatmap shows significant differences in the 22 plasma exosomal miRNAs between 5 healthy women and 14 stage I BC patients, and in the 6 plasma exosomal miRNAs that were differentially expressed between 14 stage I BC patients and 31 stage II–III BC patients. Venn diagram showing one common differentially expressed plasma exosomal miRNA, miR-203a-3p. (**B**) The box plot indicates that plasma exosomal miR-203a-3p also gradually increases with the BC progression in patients. (**C**) The expression level of plasma exosomal miR-203a-3p is positively correlated with its expression level in BC tissues. (**D**) The box plot shows that miR-203a-3p also gradually increases with the tumor progression in our BC tissues. (**E** and **F**) ROC analysis showed the potential of plasma exosomal miR-203a-3p as a discriminatory marker for BC and for stage I BC.

Discussion

In this study, we found for the first time that miR-203a-3p in both tumor tissues and plasma exosomes of BC patients gradually increased with tumor progression, and their expression levels were positively correlated. Our results suggest that plasma exosomal miR-203a-3p has the potential to be used as a liquid biopsy marker for real-time monitoring of tumor progression in BC patients.

Previous studies have demonstrated that miR-203a-3p plays a key regulatory role in multiple cancer types. In some cancers, miR-203a-3p behaves as an oncogene. For example, miR-203a-3p promotes tumor metastasis and proliferation by targeting PDE4D in colon[29] cancer and promotes the progression of lung cancer by targeting DNMT3B.[30] Additionally, miR-203a-3p has been shown to promote the proliferation and metastasis of BC cells by targeting and regulating SOCS3.[31] Conversely, in other cancers, such as gastric cancer,[32] non-small-cell lung cancer,[33] and ovarian cancer,[34] miR-203a-3p acts as a tumor suppressor, inhibiting tumor proliferation and metastasis or promoting tumor apoptosis. These differing findings may be attributed to the complex roles of miR-203a-3p in tumor growth and progression, which can vary depending on the specific cellular environment, tumor heterogeneity, and the different stages of cancer development. Nevertheless, these findings underscore the significant regulatory role of miR-203a-3p in tumor cells and highlight its potentially wide-ranging impact on tumor initiation and progression. In this study, we enriched potential miR-203a-3p target genes in BC tissue samples using KEGG and GO analyses and found that it may promote BC progression by regulating key signaling pathways. These pathways include MAPK, PI3K-AKT, and Ras, which play critical roles in tumor proliferation and invasion.[35–37] Furthermore, among the 48 potential target genes associated with shorter DFS in BC, MAPT was found to be enriched in the MAPK pathway according to KEGG analysis. Additionally, aberrant downregulation of eight genes, including CPEB4, DCLK1, IL6ST, ITM2B, KLHL5, PREX2, SPOP, and TMEM59, has been reported to modulate the activation of the MAPK, RAS, or PI3K-

AKT pathways, thereby enhancing tumor cell viability.[38–45]Additionally, in the study by Cai Kaiteng et al,[46] they demonstrated through the analysis of 11 datasets from the TCGA, GEO, and UCSC databases that the expression level of miR-203a-3p in tumor tissues of BC patients is significantly higher than in adjacent non-cancerous tissues. ROC curve analysis further indicated that this gene has the capability to distinguish between BC tissues and adjacent normal tissues. Our findings are consistent with theirs, and through further analysis, we revealed that miR-203a-3p levels gradually increase with tumor progression in BC patients, underscoring its important role in tumor progression. Moreover, in our study, we were the first to discover that miR-203a-3p in plasma exosomes of BC patients also increases with tumor progression and is positively correlated with the expression levels of miR-203a-3p in BC tissues. Through ROC curve analysis, we found that miR-203a-3p in plasma exosomes has the potential to distinguish between healthy controls and (early-stage) BC patients.

Recent studies have shown that highly enriched nucleic acid substances (including miRNAs) in plasma exosomes of cancer patients may originate from the tumor or result from tumor-induced changes in the body's microenvironment. Some studies suggest that the elevation of miR-203a-3p in plasma exosomes of colon cancer patients may originate from highly metastatic colon cancer cells and further induce liver metastasis of colon cancer.[47] Other research has proposed that exosomal miR-203a-3p secreted by hepatocytes can promote liver metastasis of colon cancer.[48] In addition, the results of related studies in lung and pancreatic cancers suggested that miR-203a-3p in plasma exosomes may serve as a fluid marker.[49,50] These findings imply that exosomal miR-203a-3p in body fluids or microenvironments may indicate tumor invasiveness or reflect tumor progression. In our study, we were the first to propose that plasma exosomal miR-203a-3p may reflect the expression levels of miR-203a-3p in tumor tissues and suggest tumor progression in BC patients. Although our study provides valuable insights, it has some limitations. A larger sample size is needed to validate the role of plasma exosomal miR-203a-3p as an indicator of tumor progression in breast cancer (BC) patients. Additionally, our study did not elucidate the specific molecular mechanisms by which exosomal miR-203a-3p affects BC cell viability through in vitro or in vivo experiments. Nonetheless, our results reveal a correlation between the expression levels of miR-203a-3p in plasma exosomes and tumor tissues of BC patients and BC progression, suggesting that plasma exosomal miR-203a-3p may have the potential to be used as a liquid biopsy marker for monitoring BC disease progression.

Conclusion

In conclusion, our research indicates that the expression of miR-203a-3p in tissues gradually increases with the tumor progression in BC patients and is a factor for poor prognosis. Most importantly, miR-203a-3p in the plasma exosomes of BC patients also increased gradually with BC progression, and its expression level was positively correlated with that in BC tissues, suggesting that plasma exosomal miR-203a-3p may reflect the expression of miR-203a-3p in tumor tissues. These observations point towards the potential of plasma exosomal miR-203a-3p as a liquid biopsy marker to gauge disease progression in BC patients.

Abbreviation

BC, breast cancer; miRNA, microRNA; miRNA-seq, miRNA sequencing; DFS, disease-free survival; DEMs, differentially expressed miRNAs; DEGs, differentially expressed genes; TCGA, The Cancer Genome Atlas; qRT–PCR, quantitative real-time polymerase chain reaction; NTA, nanoparticle tracking analysis; TEM, transmission electron microscopy; WB, Western blot; UMIs, Unique Molecular Identifiers; TPM, transcripts per million; HR, hazard ratio; CI, confidence intervals; FC, fold change; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, Protein-Protein Interaction; ROC, receiver operating characteristic.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

Ethics Approval and Consent to Participate

The clinical breast tumor specimens were collected from the BC Biobank of the Breast Centre at Beijing Hospital. The study protocol was approved by the Ethics Committee of Beijing Hospital on the basis of the Declaration of Helsinki (IRB Number in Ethical approval: 2022BJYYEC-114-01), and written informed consent was obtained from the patients.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests in this work.

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