Breast Cancer: Targets and Therapy Dovepress

Open Access Full Text Article ORIGINAL RESEARCH

Identification and Validation of circDOCK1/miR-138-5p/GRB7 Axis for Promoting Breast Cancer Progression

Yan Zhang[1,*], Mei Yang[1,2,*], Yiping Wang[1], Junhao Zhao[1], Pei Yao Lee[1], Yuhua Ma[1], Shaohua Qu[1]

[1]Department of Breast Surgery, The First Affiliated Hospital of Jinan University, Jinan University, Guangzhou, 510630, People's Republic of China; [2]Department of Breast Surgery, JiangMen Maternity and Child Health Care Hospital, Jiangmen, 529000, People's Republic of China

*These authors contributed equally to this work

Correspondence: Yuhua Ma; Shaohua Qu, Email mayuhua2110@126.com; qushaohua2009@163.com

Background: Non-coding RNAs have received increasing attention in human tumors, with RNA interaction networks playing important roles in breast cancer. This study aims to explore novel circular RNAs and their mechanisms of biological function in breast cancer.

Methods: Six HER2-positive breast cancer tissues and paired normal tissues were obtained for the whole transcriptome RNA sequencing. Differentially expressed (DE) circRNAs, miRNAs and mRNAs were identified and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of DERNAs were performed. DECircRNAs- DEmiRNAs-DEmRNAs networks were constructed and further verified by bioinformatics database analyses, luciferase assays and RIP assays. The expression level of circDOCK1 in breast cancer specimens was measured using qRT-PCR. Functional rescue experiments were conducted to explore the role of circDOCK1/miR-138-5p/GRB7 axis in breast cancer cells. The correlation of circDOCK1 expression and clinicopathologic features of 102 HER2 positive breast cancer patients was analyzed.

Results: A total of 6960 DEmRNAs, 133 DE miRNAs and 1691 DE circRNAs were identified from HER2-positive breast cancer tissues and paracancerous tissues. Enrichment Analysis showed that the differential mRNAs were associated with cell division in biological processes and cell cycle and signaling pathways. GO and KEGG analysis demonstrated that DE circRNAs were mainly enriched in double-strand break repair, positive regulation of transcription by RNA polymerase II, nucleoplasma, nucleus, chromatin binding and protein binding. Forty networks of competing endogenous RNAs (ceRNAs) were constructed and circDOCK1/miR-138-5p/GRB7 axis was verified. Functional experiments revealed that the axis promotes migration and invasion of breast cancer cells. CircDOCK1 expression was elevated in breast cancer patients and correlated with adverse clinicopathologic parameters. Patients with high circDOCK1 level had poor outcomes.

Conclusion: A novel circDOCK1/miR-138-5p/GRB7 axis promotes HER2 positive breast cancer metastasis and progression, providing a potential therapeutic target in the treatment of breast cancer.

Keywords: circular DOCK1, ceRNA, breast cancer, progression, patient survival

Introduction

Human epidermal growth factor receptor 2 (HER2) positive breast cancer accounts for 20–25% of all the breast cancer patients.[1] With the development of targeted treatment strategies, the outcome of patients with HER-2 positive breast cancer has been markedly improved.[2] However, advanced HER-2 positive breast cancer seriously jeopardized women's health due to metastasis and treatment insensitivity.[3] Thus, the molecular regulatory networks involved in breast cancer metastasis and treatment resistance remain to be further investigated.

The competitive endogenous RNA (ceRNA) hypothesis, which suggests that coding and non-coding RNAs sharing miRNA response elements (MREs) can regulate each other by competing for a limited pool of miRNAs, was a novel intrinsic molecular mechanism to regulate essential biological processes in cancer. Circular RNAs (circRNAs) are

Received: 15 October 2024
Accepted: 13 November 2024
Published: 26 November 2024

generated by the back splicing of pre-mRNAs to form a structurally stable loop without a 5'-cap or a 3'-poly (A) tail, which are less exposed to degradation driven by exonucleases.[4] They can be served as potential diagnostic and prognostic predictors for cancers because of their widespread expression, relatively high stability, and abundant presence in body fluid and exosomes.[5,6] Moreover, circRNAs are particularly effective as sponges because they harbor a high number of MREs.[7–9] Accumulating evidence demonstrated that circRNA-miRNA-mRNA regulatory axes are involved in cell cycle, apoptosis, proliferation, invasion, and migration in HER-2 positive breast cancer. For instance, circGFRA1 promotes malignant progression in HER-2 positive breast cancer and enhances AIFM2 expression by acting as a sponge for miR-1228;[10] circ-ERBB2 promotes cancer progression through the circ-ERBB2/miR-136-5p/miR-198/TFAP2C axis;[11] hsa-circ-001783 promotes breast cancer cell progression by sponging miR-200c-3p;[12] circBCBM1 acts as an endogenous miR-125a sponge to upregulate BRD4 and is involved in breast cancer brain metastasis.[13] Therefore, it is of great significance to explore ceRNA networks in HER2 positive breast cancer.

In this study, differentially expressed circRNAs, miRNAs and mRNAs were identified through RNA sequencing in six cancer tissues and paired normal tissues from patients with HER2-positive breast cancer in our hospital. Subsequently, bioinformatic analyses such as functional enrichment analyses of DEmRNAs were performed. The ceRNA networks were constructed for DEcircRNA targeting DEmiRNA and DEmRNA. We found a novel circDOCK1/miR-138-5p/GRB7 ceRNA network presenting in breast cancer and promoting breast cancer progression. Our findings revealed that circDOCK1/miR-138-5p/GRB7 axis is a potential therapeutic target for breast cancer.

Materials and Methods

Patients and Tissue Collection

Tissue samples were collected from 108 female HER2 positive breast cancer patients who underwent mastectomy without preoperative chemotherapy at the First Affiliated Hospital of Jinan University, Guangzhou, China, from 2019 to 2022. Among these samples, 6 cancer tissues and paired normal tissues were collected for whole transcripts RNA sequencing and 102 cancer tissues were stored for real time PCR. All experimental procedures were approved by the Ethics Committee of the First Affiliated Hospital of Jinan University. The samples were frozen in liquid nitrogen immediately after resection and stored at −80°C before use. All patients were free of any other forms of cancer, and all specimens were examined by two pathologists before storage in liquid nitrogen. Detailed patient information is given in Table S1.

Cell Culture and Transfection

SKBR3 cells were purchased from the American Type Culture Collection (ATCC, USA). Cells were cultured in DMEM media containing 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C with 5% CO_2. All si-RNAs and plasmids used in this study were obtained from Guangzhou IGE Biotechnology Co, Ltd. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used for transfection assays in SKBR3 cells. Forty-eight hours after the transfection, cells were collected for further experiments.

RNA Extraction and Sequencing

Total RNA was extracted from frozen tumor tissues and their paired normal tissues with TRIzol according to the manufacturer's protocol. Initial RNA concentration and purity were assessed with a NanoDrop 2000 spectrophotometer (Thermo Scientific), and precise quantification was performed with a Qubit (ABI). Purified RNA was stored at −80◦C. RNA-seq libraries were generated using the V AHTS Universal V8 RNA-seq Library Prep Kit for Illumina according to the manufacturer's protocol. The quality of each library was controlled using an Agilent 2100 TapeStation (Agilent). Following the manufacturer's protocol, libraries were pooled and sequenced in the Illumina PE150, resulting in 150bp paired-end reads.

Quality Control and Comparison of RNA Sequencing Reads

Raw RNA sequencing reads were filtered using fastp to remove r-RNA for clean reads. The Q20, Q30, and GC contents of the clean reads were recalculated to assess the quality of the sequencing. Clean reads were aligned to the reference genome using Hisat2. To define novel RNAs with or without coding potential, transcripts from each sample were assembled by StringTie from mapped reads. To estimate mRNA and circRNA expression levels, TPM (Transcripts Per Million reads) was calculated by Salmon (https://combine-lab.github.io/salmon/): ie, the number of reads per million reads from a given transcript and the TMM (Trimmed mean of M-values). Trimmed mean of M-values): weighted truncated mean of M-values.

Identification of DE Transcripts

DESeq2 was analyzed for DE in cancer and paracancer. P value < 0.05, |log2FoldChange| >1 could be used as a threshold for significant identification of DE mRNAs, DE miRNAs and DE circRNAs. Volcano maps and heat maps were plotted on DE mRNAs, DEmiRNAs and DE circRNAs using the R package.

Analysis of Functional Enrichment Pathways

GO and KEGG enrichment pathways of DE mRNAs and DE circRNAs were analyzed using the clusterProfiler package in R. GO terms and KEGG pathways enriched in ≥2 genes and with a significance threshold of $p \leq 0.05$ were considered significant.

Construction of Protein-Protein Interaction Networks

The STRING database (version 12.0) was used to predict potential interactions between proteins translated by DE mRNAs. Upregulated mRNAs in cancer tissues were screened with Fold Change > 2, p-value < 0.05 and expression > 50 in at least one cancer sample and visualized with Cytoscape (version 3.10). Protein networks with Hub gene association scores >0.4 (no more than 20) were predicted using the STRING database (version 12.0).

CircRNAs-miRNAs-mRNAs Network Construction

circRNA-miRNA-mRNA interactions were predicted using the following online bioinformatics tools: STARBASE (http://starbase.sysu.edu.cn/), miRanda (http://www.microrna.org/) and TargetScan (http://www.targetscan.org/vert_72/). The igraph package in R was used to construct the ceRNA networks.

RNA Extraction and qRT–PCR

Total RNAs were isolated from cell lines or tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and transcribed into cDNA using a PrimeScript RT Reagent Kit (TaKaRa Bio, Inc., China). The expression of circDOCK1, miR-138-5p and GRB7 was normalized to the GAPDH level and the U6 level. The primers were shown in Table S2. The fold change in RNA expression was assessed by the $2^{-\Delta\Delta Ct}$ method.

Transwell Assays

Migration and invasion assays were conducted using transwell chambers with or without matrigel (100μL). Breast cancer cells (10^5 cells per well) were placed on the inserts in the upper chambers in 200μL serum-free DMEM. A 600μL of DMEM supplemented with 10% FBS was added into the lower chambers. Cells were cultured for 12h (migration) or 24h (invasion) in at 37°C in 5% CO_2. And then, cells on the upper surface of the membrane filter were removed. The migrated and invaded cells that crossed the inserts to the lower surface were fixed with 4% formaldehyde stained with 0.1% crystal violet solution, and counted as cells per field of view under an inverted fluorescence microscope.

Luciferase Reporter Assay

The circDOCK or GRB7 fragments with the mutant (MUT) or wild-type (WT) miR-138-5p binding sites were subcloned downstream of the Renilla gene in the dual-luciferase reporter vectors (Promega). SKBR3 cells were cotransfected with

the reporter vectors and the miR-138-5p mimic or NC mimic with the Lipofectamine 2000 reagent (Invitrogen). After incubation for 48h, a Dual-Luciferase reporter Assay System (Promega) was utilized to measure luciferase activity.

RNA Immunoprecipitation Assay

The Magna RIP binding protein immunoprecipitation kit (Millipore) was used to perform the RIP analysis. According to the instructions, after the initial lysis of the SKBR3 cells, the lysate was incubated overnight with anti-Ago2 or anti-IgG antibody-coated beads. Enrichment of circDOCK1 and miR-138-5p was measured by qRT-PCR.

Statistical Analysis

All experiments were performed with three technical replicates. Student's *t*-test or one-way ANOVA were used to analyze differences between groups. Kaplan-Meier method and the Log rank test were used to analyze survival. Pearson correlation analysis were used to correlate among the levels of circRNA, miRNA and mRNAs. $P < 0.05$ was considered as a significant difference. Data were presented as means±standard deviations. All analyses were performed using SPSS 20.0 software (IBM, USA).

Results

Differential Expression Analysis

Volcano plots showed that the differentially expressed mRNAs, miRNAs and circRNAs were clearly delimited in cancer tissues and paired normal tissues (Figure 1A-C). A total of 6960 DE mRNAs were determined according to the screening criteria, of which 3303 mRNAs were up-regulated and 3657 mRNAs were down-regulated. A total of 133 DE miRNAs were identified in breast cancer samples compared with normal control samples. Of these, 83 miRNAs were up-regulated and 50 miRNAs were down-regulated. Among 1691 DE circRNAs, 106 circRNAs were observed up-regulated and 1585 circRNAs were down-regulated. In addition, hierarchical clustering analyses of DE mRNAs, DE miRNAs and DE circRNAs further explained the reliability of differential expression analysis (Figure 1D-F).

Functional Enrichment Analysis and PPI Network for mRNAs

GO and KEGG enrichment analyses were performed to predict the potential biological functions of DE mRNAs. The top 20 enriched terms by dysregulated DE mRNAs were shown in Figure 2. The results indicated that most of the up-regulated DE mRNAs were associated with nucleosome, immune response, extracellular matrix organization as well as cell division in GO enrichment analyses. Up-DEmRNAs were highly clustered in several signaling pathways such as systemic lupus erythematosus, neutrophil extracellular trap formation, cell cycle et al (Figure 2A and B). Most of the down-regulated DE mRNAs were involved in ion channel activity, ion transmembrane transport and chemical synaptic transmission (Figure 2C). Additionally, KEGG pathway analyses suggested that down-regulated DE mRNAs were mainly associated with neuroactive ligand-receptor interaction, cAMP signaling pathway and calcium signaling pathway et al (Figure 2D).

To better investigate the impact of protein interactions in breast cancer, a PPI network was constructed using STRING based on up-regulated DE mRNAs with expression >50 in at least one cancer sample, indicating 114 nodes and 761 interaction pairs (Figure S1). Nodes with high degree of expression were considered as key proteins, including MMP9, FN1, COL1A1 and ERBB2. These proteins were involved in several important signaling pathways in cancer cells, suggesting their vital roles in breast cancer progression.

Functional Enrichment Analysis of miRNAs and circRNAs

Enrichment Analysis of GO and KEGG for DE miRNAs showed that target genes were highly enriched in intracellular signal transduction (Figure 3A). Bubble map results showed that the majority of target genes were involved in proteoglycans in cancer (Figure 3B). GO and KEGG enrichment analyses of DE circRNAs revealed that DE circRNA were related to lamellipodium, chromatin organization, positive regulation of GTPase activity, GTPase activator activity and DNA repair et al (Figure 3C). The most relevant pathways of DE circRNA were regulation of actin cytoskeleton,

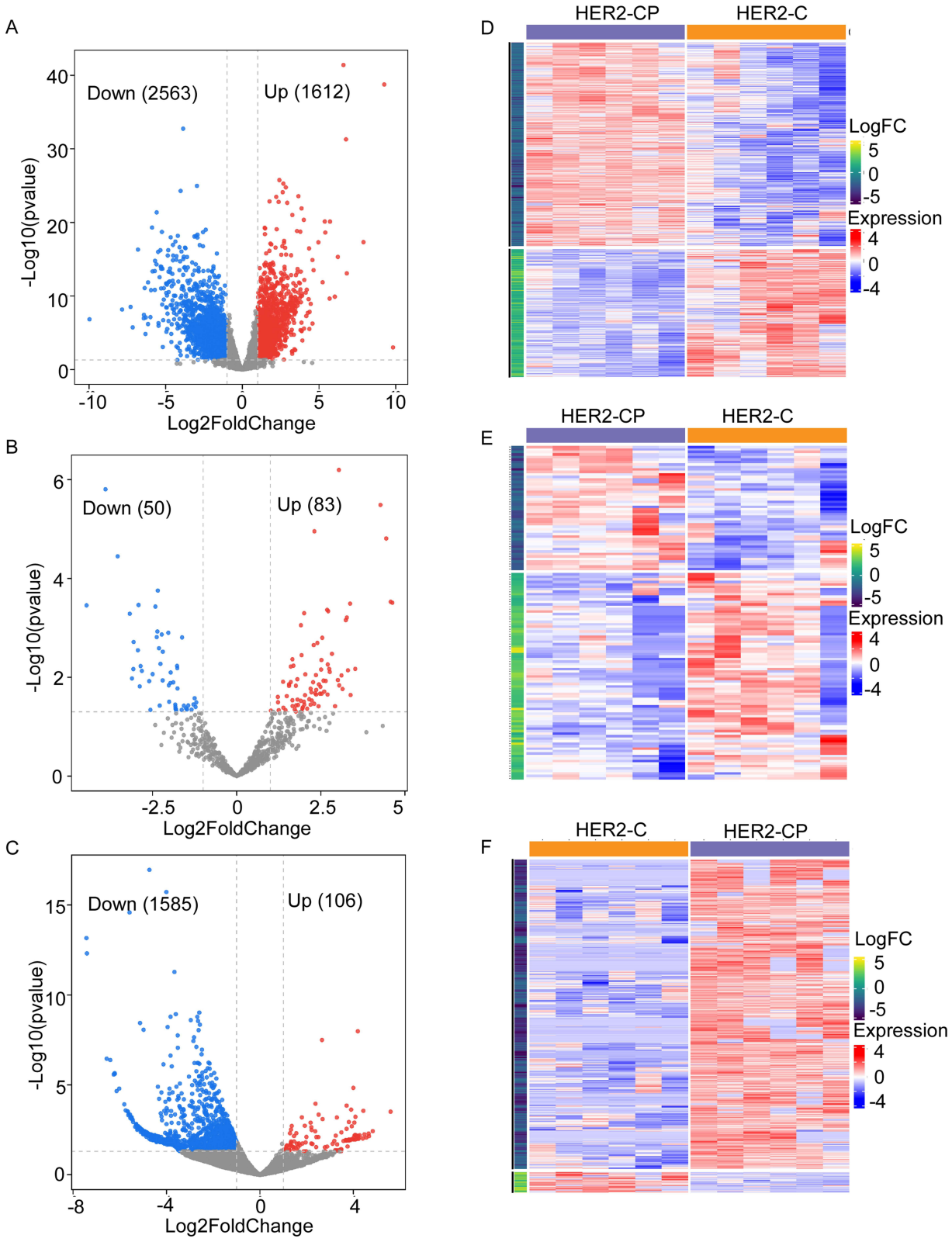

Figure 1 Overview of DE mRNAs, miRNAs, and circRNAs. (**A–C**) Volcano plots of DE mRNAs (**A**), DE miRNAs (**B**), and DE circRNAs (**C**). (**D–F**) Heatmaps of DE mRNAs (**D**), DE miRNAs (**E**), and DE circRNAs (**F**). Red indicates up-regulation and blue indicates down-regulation.

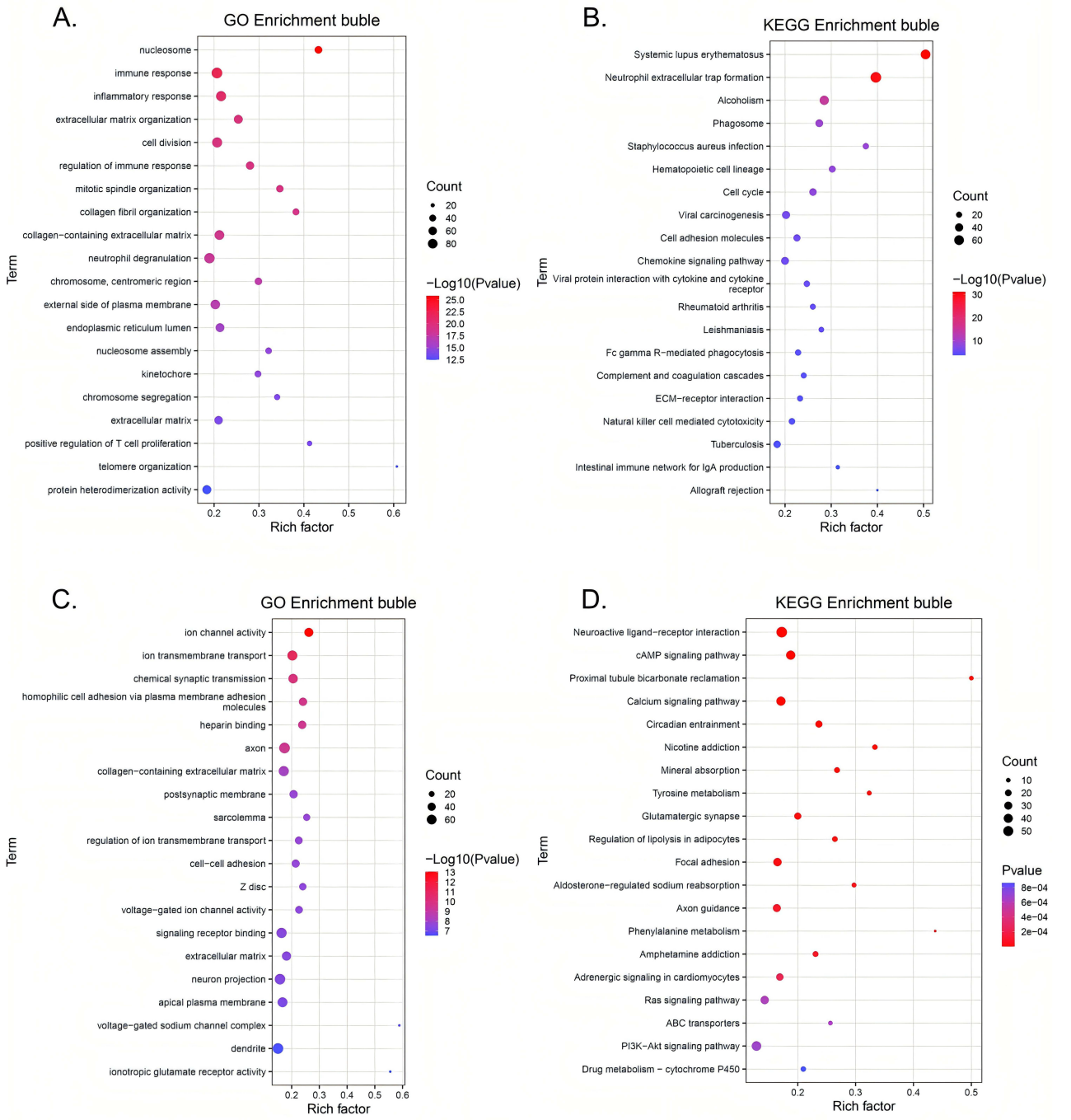

Figure 2 The GO and KEGG enrichment analysis of DE mRNAs. (**A**) Top 20 GO terms enriched by up-regulated DE mRNAs. (**B**) Top 20 pathways enriched by up-regulated DE mRNAs. (**C**) Top 20 GO terms enriched by down-regulated DE mRNAs. (**D**) Top 20 pathways enriched by down-regulated DE mRNAs.

lysine degradation, adherens junction and EGFR tyrosine kinase inhibitor resistance according to the KEGG pathway analyses (Figure 3D).

CeRNA Networks Construction and circDOCK1/miR-138-5p/GRB7 Axis Identification

CircRNAs can competitively sponge miRNAs, indirectly regulating mRNAs expression. To construct circRNAs-miRNAs-mRNAs regulatory networks, we predicted DE mRNAs targeting miRNAs and DE circRNAs targeting miRNAs by starBase,

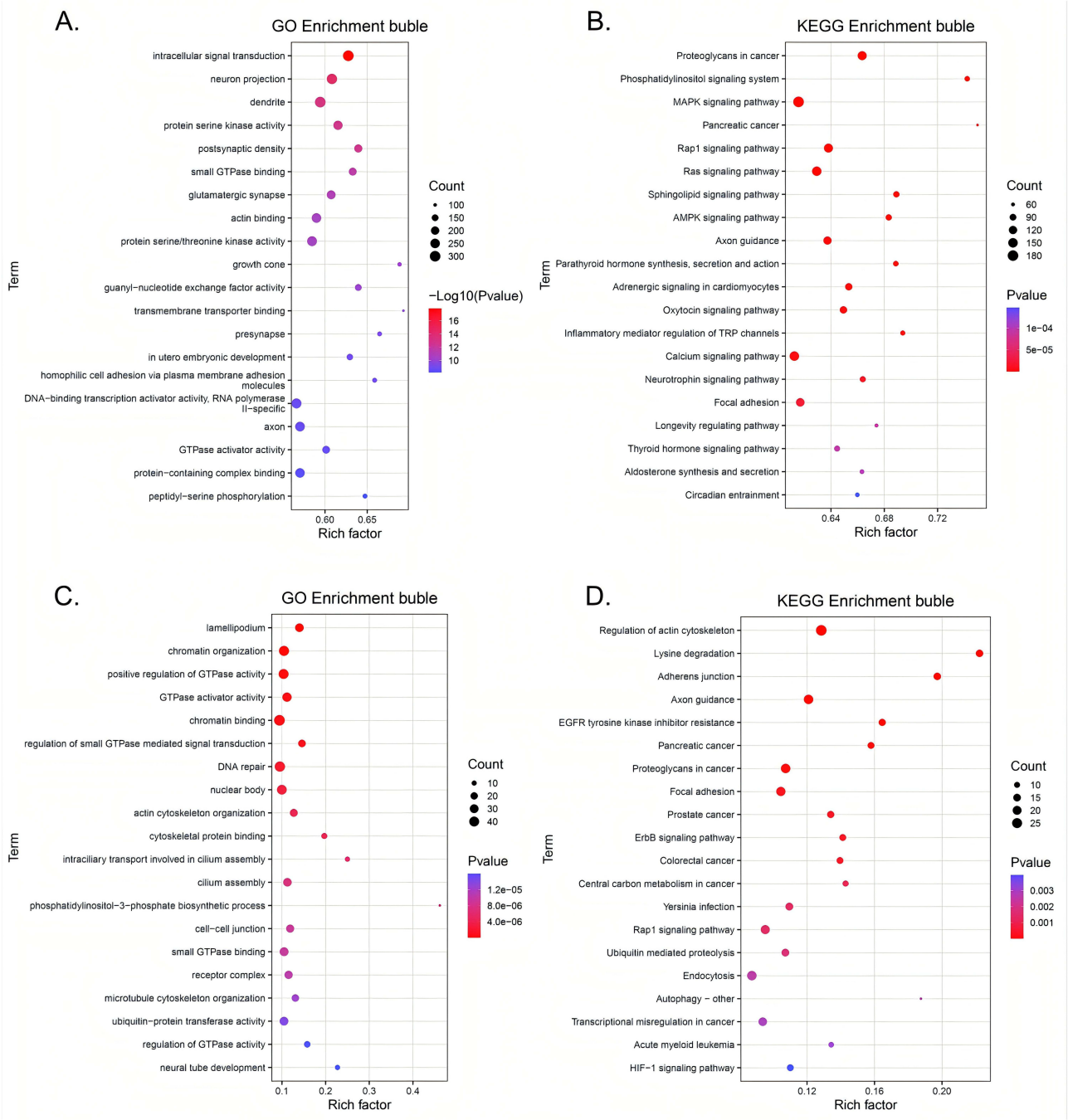

Figure 3 Bubble maps of enrichment analysis of miRNAs and circRNAs. (**A**) GO analysis of DE miRNAs. (**B**) Pathways analysis of DE miRNAs. (**C**) GO analysis of DE circRNAs. (**D**) Pathways analysis of DE circRNAs.

Miranda and TargetScan based on DE circRNAs, DE miRNAs and DE mRNAs. Finally, forty ceRNA networks were constructed including 15 circRNAs, 9 miRNAs, and 17 up-regulated mRNAs (Table S3).

Among these 40 ceRNA networks, we chose circDOCK1/miR-138-5p/GRB7 axis for further analyses (Figure 4A and B). GO annotation analyses showed that circDOCK1 was mainly enriched in double-strand break repair, positive regulation of transcription by RNA polymerase II (BP), nucleoplasma, nucleus (CC), chromatin binding, protein binding (MF), suggesting that circDOCK1 may have vital biological functions (Figure 4C-E). KEGG annotation analysis showed that circDOCK1 was predominantly enriched for Human T-cell leukemia virus 1 infection (Figure S2).

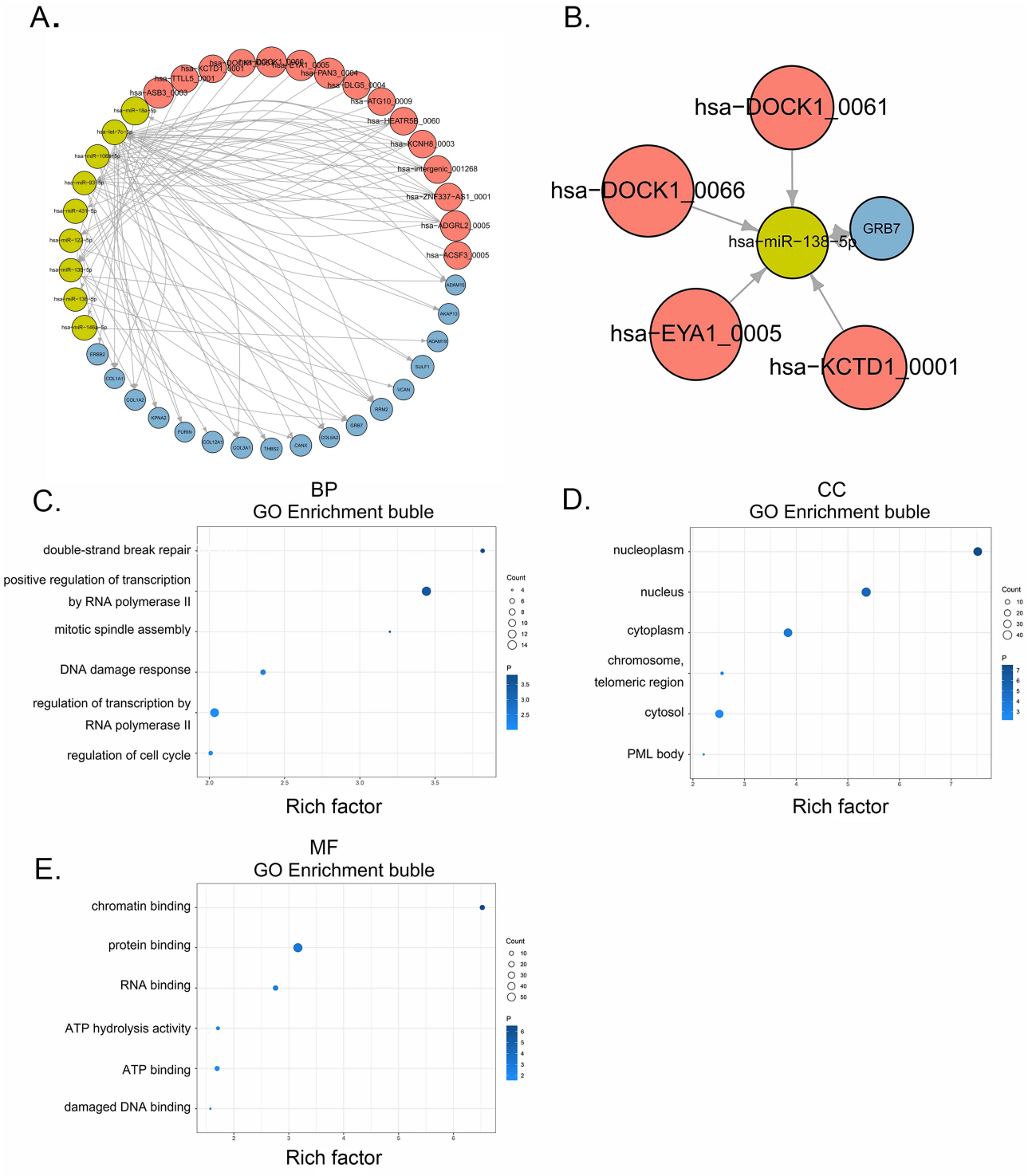

Figure 4 circRNA-miRNA-mRNAs interaction network. (**A**) Interaction network between DE circRNA and its target miRNA and target mRNA. Red color represents DE circRNA, yellow color represents DE miRNA, and blue color represents DE mRNA. (**B**) CircDOCK1-miR-138-50-GRB7 crosstalk. (**C**) Top 6 GO BP (biological process) enriched by DOCK1. (**D**) Top 6 GO CC (cellular component) enriched by DOCK1. (**E**) Top 6 GO (molecular function) enriched by DOCK1.

GO Analyses for GRB7 indicated that it was mainly enriched in transmembrane transport, Golgi membrane, and ATPase activity. KEGG pathways analyses identified that GRB7 was closely related to ABC transporters, cell adhesion and tight junction, which were associated with breast cancer progression (Figure 5A-D). In addition, we found that GRB7 expression is closely correlated with ERBB2, GRB2, SHC1, EGFR, SRC and other proteins based on String website prediction with GRB7

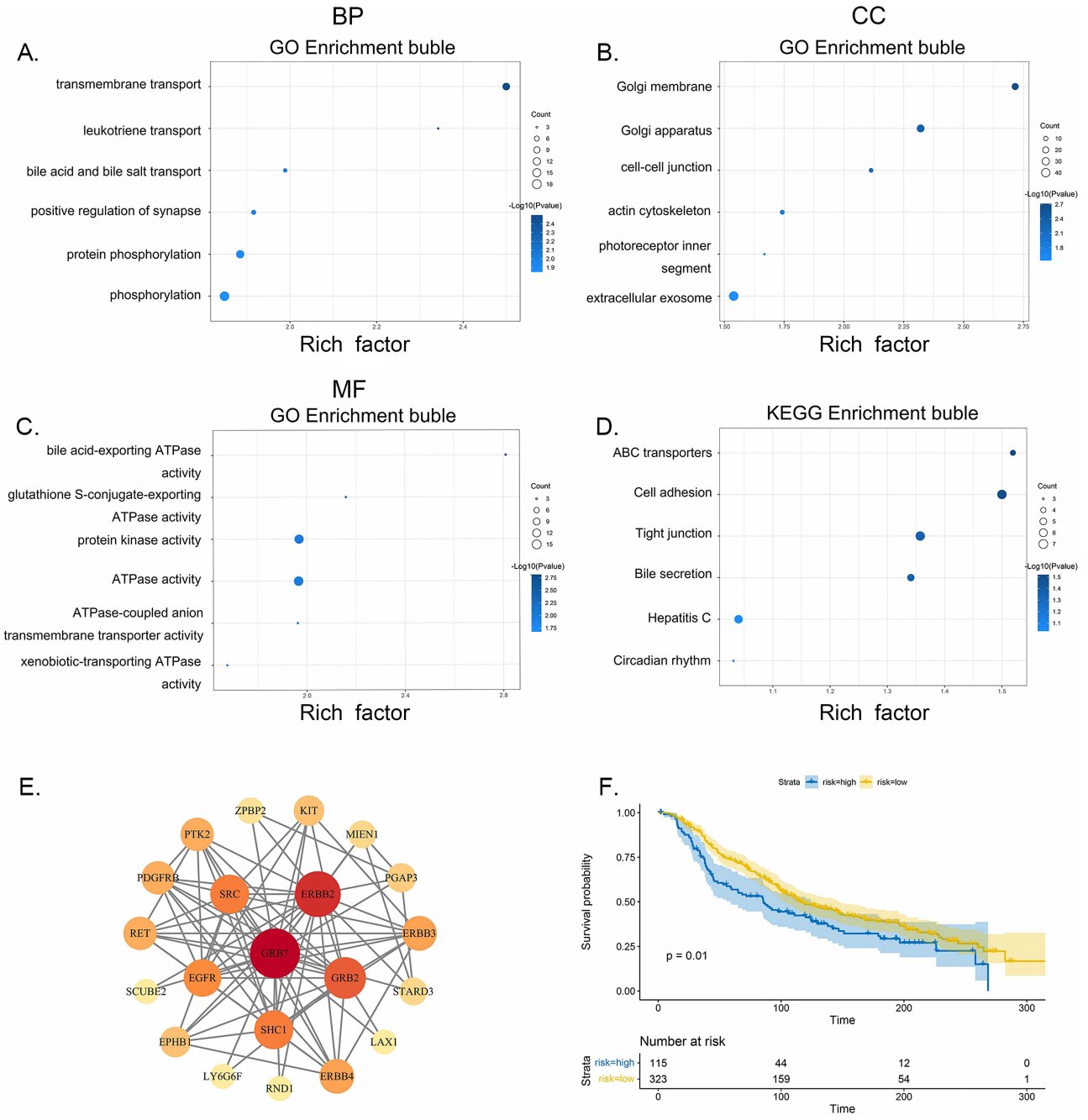

Figure 5 Functional enrichment analysis of GRB7 and its prognostic predictive value. (**A**) Top 6 GO BP (biological process) enriched by GRB7. (**B**) Top 6 GO CC (cellular component) enriched by GRB7. (**C**) Top 6 GO (molecular function) enriched by GRB7. (**D**) Top 6 pathways enriched by GRB7. (**E**) Cytoscape v3.10.0 A network of proteins related to GRB7 protein. Darker colors mean more relevant. (**F**) Kaplan-Meier analysis of the correlation between GRB7 expression and overall survival (OS) in METABRIC dataset.

association score above 0.4 (Figure 5E). To explore whether GRB7 could be served as a prognostic predictor, we validated the survival data of GRB7 in HER2-positive breast cancer in the METABRIC dataset. The results demonstrated that patients with low GRB7 expression had a better prognosis than those with high expression ($p<0.01$, Figure 5F). Collectively, these data suggests that circDOCK1/miR-138-5p/GRB7 may exhibit potential biological roles in HER2 positive breast cancer.

Verification of circDOCK1/miR-138-5p/GRB7 ceRNA Network

To further investigate roles of circDOCK1/miR-138-5p/GRB7 axis, we first examined the circDOCK1, miR-138-5p and GRB7 expression in HER2 positive breast cancer tissues. The results showed that the expression level of circDOCK1 and GRB7 were elevated in breast cancer tissues compared with paired normal tissues. On the contrary, the expression level of miR-138-5p was downregulated in cancer tissues compared with paired normal tissues (Figure 6A-C, n=20). Pearson correlation analyses revealed that circDOCK1 expression level was positively correlated with GRB7 level and negatively correlated with miR-138-5p expression level (Figure 6D and E). In addition, circDOCK1 level was higher in HER2$^+$

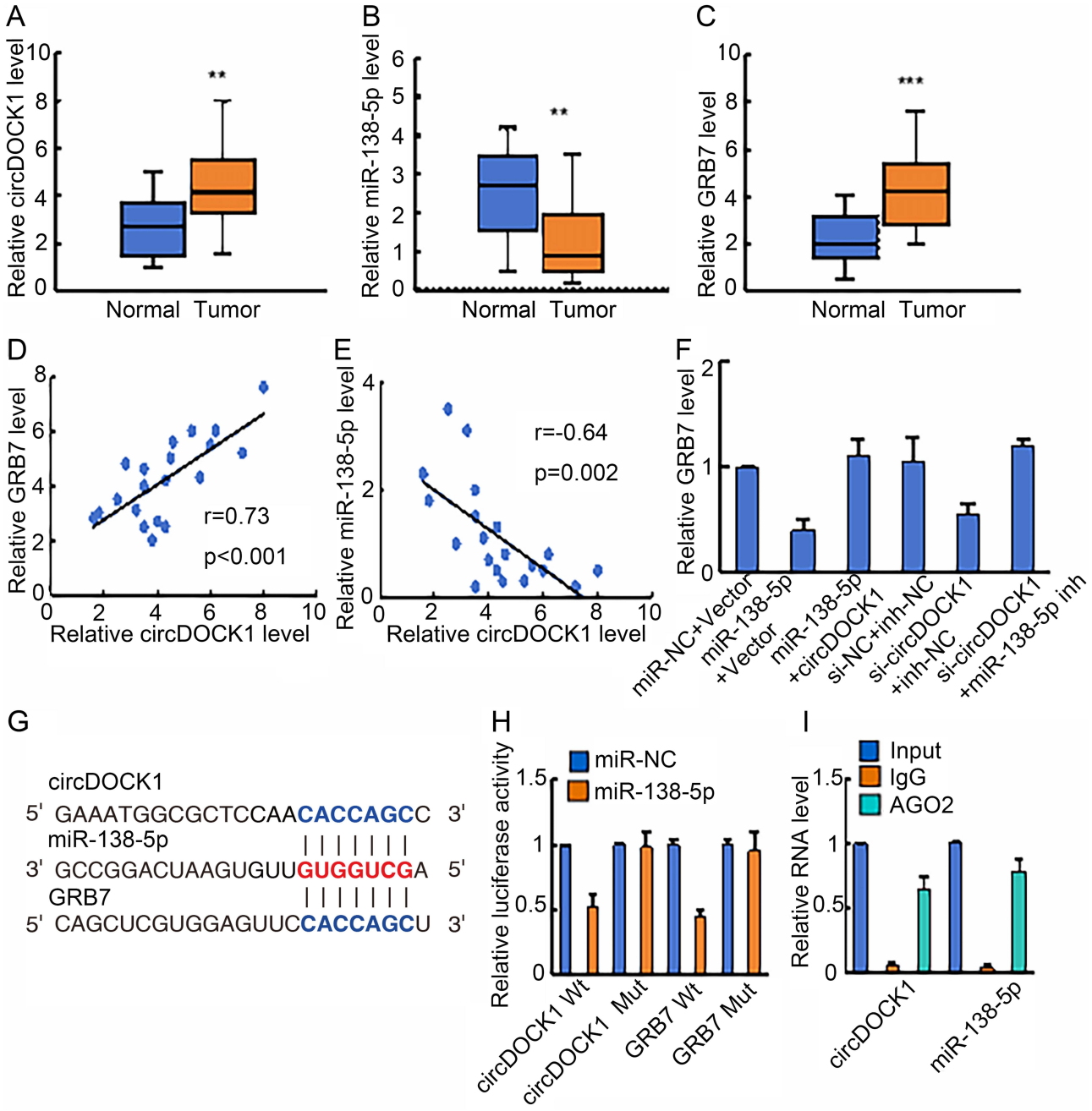

Figure 6 Verification of circDOCK1/miR-138-5p/GRB7 ceRNA Network. (**A-C**) The relative expression of circDOCK1 (**A**), miR-138-5p (**B**) and GRB7 (**C**) was determined by qRT-PCR in 20 paired HER2$^+$ breast tissues and paired noncancerous tissues. (**D-E**) Pearson correlation analysis of circdock1 and GRB7 (**D**) and miR-138-5p (**E**) expression in HER2$^+$ breast cancer. (**F**) Effects of miR-138-5p and circDOCK1 on the expression of GRB7. (**G**) The putative binding sites among circDOCK1, miR-138-5p and GRB7. (**H**) Luciferase reporter assay detecting the binding of miR-138-5p to circDOCK1 and GRB7 in SK-BR3 cells (normalized to miR-NC). (**I**) RIP with an anti-AGO2 antibody in the SK-BR3 cells evaluating the transcript levels of circDOCK1 and miR-138-5p. (**, p<0.01, ***, p<0.001).

BT474 and SKBR3 cells than that in HER2$^-$ cells (Figure S3). Moreover, miR-138-5p mimics significantly decreased GRB7 expression, whereas circDOCK1 upregulated GRB7 expression and weakened the inhibitory effect of miR-138-5p mimics on GRB7 expression. The decrease of GRB7 caused by circDOCK1 siRNA transfection was reversed by transfection with miR-138-5p inhibitor in SKBR3 cells (Figure 6F).

The putative binding sites of miR-138-5p to circDOCK1 and GRB7 were identified through bioinformatics database analysis (Figure 6G). Next, dual-luciferase reporter assays were performed to verify the existence of the circRNA-miRNA-mRNA regulatory axis. We found that miR-138-5p directly binds to a site in circDOCK1 and a site in the 3'-UTR of GRB7 (Figure 6H). RIP was then performed using an anti-AGO2 antibody. We found that the amounts of circDOCK1 and miR-138-5p were higher in the anti-AGO2 precipitate than in the IgG precipitate (Figure 6I). Taken together, these data suggests that circDOCK1 may regulate GRB7 expression by sponging miR-138-5p.

circDOCK1/miR-138-5p/GRB7 Axis Promotes Breast Cancer Progression

We performed rescue experiments to investigate whether circDOCK1 promotes breast cancer progression by competitively interacting with miR-138-5p and subsequently elevating the expression of GRB7. Briefly, cells with stable circDOCK1 silencing were transfected with the miR-138-5p inhibitor alone or in combination with sh-GRB7. The transwell assay results demonstrated that the inhibitory effects of circDOCK1 depletion on cells migration and invasion capacities were dramatically attenuated through miR-138-5p inhibitor. However, in cells with stable knockdown of circDOCK1, the suppressive effects of sh-circDOCK1 on cells migration and invasion capacities were restored after cotransfection with the miR-195-5p inhibitor and sh-GRB7 (Figure 7A). The results reveled the modulation effect of circDOCK1/miR-138-5p/GRB7 axis on the migration and invasion abilities of breast cancer cells.

Next, we evaluated the correlation of the circDOCK1 expression with the clinicopathologic parameters of breast cancer patients. As shown in Table 1, overexpression of circDOCK1 was correlated with adverse parameters in HER2$^+$ breast cancer, including advanced grading, positive lymph nodes, large tumor size as well as distant metastasis. No significant relationship was found for other parameters such as age, HR status (Figure 7B-D). Kaplan-Meier survival analyses with the Log rank test were performed in breast cancer patients with high or low circDOCK1 expression (based on the median level) and revealed that patients with higher circDOCK1 expression levels exhibited worse disease-free survival and overall survival rates (Figure 7E and F). These data further revealed that circDOCK1/miR-138-5p/GRB7 axis promotes breast cancer progression.

Discussion

HER2 positive breast cancer is highly metastatic accounting for 20–25% of all breast cancer patients.[1] More than 30% HER2 positive patients may suffer relapse and metastasis even under anti- HER2 treatment, making it necessary to explore novel mechanisms underlying metastasis.[14,15] Accumulating evidence have demonstrated that circRNA-miRNA-mRNA regulatory pattern plays crucial roles in malignancies including breast cancer.[16–18] Herein, we constructed novel circRNA-miRNA-mRNA regulatory networks in HER2 positive breast cancer and circDOCK1/miR-138-5p/GRB7 axis was identified and confirmed. Functional analyses further validated the role of circDOCK1/miR-138-5p/GRB7 axis in promoting metastasis. Analyses of the clinicopathological characteristics of breast cancer patients revealed that circDOCK1 expression was positively correlated with lymph nodes involvement, HER2 status, distant metastasis as well as worse outcome. Therefore, our findings provided further evidence that circRNA-miRNA-mRNA axes played vital roles for breast cancer progression.

Advances in high-throughput transcriptome sequencing technology have facilitated the discovery of various non-coding RNAs (circRNAs and lncRNAs) and its ceRNAs networks.[19–21] In our study, we chose circDOCK1/miR-138-5p/GRB7 axis for further study for several reasons. First, the expression of cirDOCK1 and GRB7 were elevated, whereas the level was decreased in breast cancer tissues, which is consistent with the ceRNA theory.[22–24] Second, the biological function of circDOCK1 in breast cancer is unknown. CircDOCK1 is located on chromosome 10:128,594,022–-128,926,028 and is 2848 nucleotides in length. Here, GO annotation analysis displayed that circDOCK1 were primarily enriched in "positive regulation of transcription by RNA polymerase II", "nucleoplasm" and "protein binding". We found

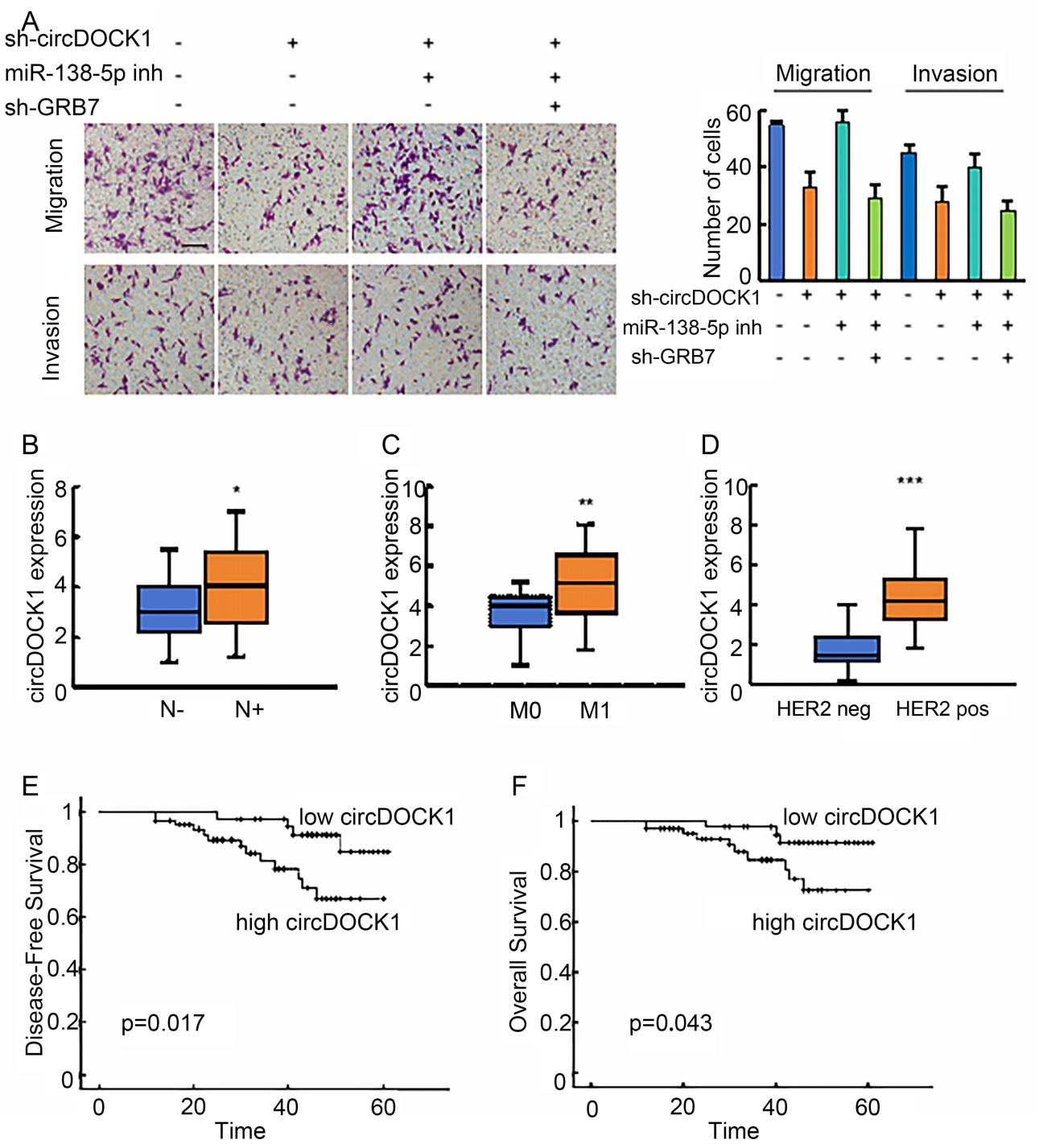

Figure 7 The validation of the involvement of the circDOCK1/miR-138-5p/GRB7 axis in breast cancer progression and metastasis. (**A**) Transwell assays were performed to evaluate the effect of circDOCK1/miR-138-5p/GRB7 axis on the migration and invasion abilities of BT-474 cells. (**B-D**) The correlation of cirDOCK1 expression with lymph node involvement (**B**), metastasis (**C**) and HER2 status (**D**). (**E**) Kaplan-Meier analysis of the correlation between circDOCK1 expression and disease-free survival (DFS). (**F**) Kaplan-Meier analysis of the correlation between circDOCK1 expression and overall survival (OS). (*, p<0.05, **, p<0.01, ***, p<0.001).

that circDOCK1 was upregulated in breast cancer and correlated with adverse clinicopathologic parameters as well as worse patient outcomes.

CircRNAs have emerged as reliable and promising ceRNAs owing to their stable structures. Previous evidence demonstrated that circDOCK1 is mainly a cytoplasmic RNA and functions as ceRNA regulating progression of various malignancies. For instance, circDOCK1 has been shown to promote tumor proliferation, invasion and induce cisplatin

Table 1 Relationship Between circDOCK1 Expression and Clinicopathologic Features in Patients with HER2$^+$ Breast Cancer No. (%)

Item	Total No.	circDOCK1		χ^2	*p*
		Low	High		
Age(years)				0.048	0.84
≤50	42 (41.2)	17 (16.7)	25 (24.5)		
>50	60 (58.8)	23 (22.5)	37 (36.1)		
Tumor size				0.919	0.631
≤2cm	22 (21.6)	10 (9.8)	12 (11.8)		
2–5cm	55 (53.9)	22 (21.6)	33 (32.4)		
>5cm	25 (24.5)	8 (7.8)	17 (16.7)		
Lymph node status				12.199	0.007
N0	20 (19.6)	14 (13.7)	6 (5.9)		
N1	32 (31.4)	13 (12.7)	19 (18.6)		
N2	28 (27.5)	6 (5.9)	22 (21.6)		
N3	22 (21.6)	7 (6.9)	15 (14.7)		
Grade				3.58	0.167
I	30 (29.4)	16 (15.7)	14 (13.7)		
II	41 (40.2)	14 (13.7)	27 (26.5)		
III	31 (30.4)	10 (9.8)	21 (20.6)		
ER status				1.148	0.284
Positive	70 (68.6)	25 (24.5)	45 (44.1)		
Negative	32 (31.4)	15 (14.7)	17 (16.7)		
PR status				1.316	0.251
Positive	68 (66.7)	24 (23.5)	44 (43.1)		
Negative	34 (33.3)	16 (15.7)	18 (17.6)		
Ki67 status				1.689	0.194
low	38 (37.3)	18 (17.6)	20 (19.6)		
high	64 (62.7)	22 (21.6)	42 (41.2)		
Relapse				4.942	0.026
no	87 (85.3)	38 (37.3)	49 (48.0)		
yes	15 (14.7)	2 (2.0)	13 (12.7)		

resistance through the miR-339-3p/IGF1R axis in osteosarcoma;[25] In oral squamous cell carcinoma, circDOCK1 regulates BIRC3 expression by acting as a ceRNA and is involved in the process of apoptosis;[26] CircDOCK1 affects bladder cancer progression through circDOCK1/hsa-miR-132-3p/Sox5 axis.[27] Our bioinformatics analyses and experimental verification for the first time showed the regulatory role of circDOCK1/miR-138-5p/GRB7 axis in breast cancer progression. These findings indicated the potential application of molecular therapy targeting circDOCK1/miR-138-5p/GRB7 axis in breast cancer.

MiR-138-5p, a tumor suppressor, has been reported to suppress metastasis and progression of various malignancies including breast cancer.[28] Shadbad reported that ectopic expression of miR-138-5p inhibited the development of non-small cell lung cancer;[29] Jing Xun reported that tumor cell-derived exosome miR-138-5p promotes breast cancer progression by inhibiting KDM6B expression in macrophages.[30] Qi Zhang found that aerosolized miR-138-5p targeting PD-L1 can highly effective in preventing lung cancer development and progression, making it a potential treatment strategy for cancer.[31] Furthermore, reports on the interactions between miR-138-5p and circRNAs in cancer are not a new phenomenon. For instance, circ-002136 functionally targeted miR-138-5p in an RNA-induced silencing complex (RISC), thereby regulating SPON2 expression and angiogenesis in Glioma;[32] CircC16orf62 functions as an oncogene and promotes hepatocellular carcinoma progression through the miR-138-5p/PTK2/AKT axis;[33] Circ-ERBIN promotes growth and metastasis of colorectal cancer through miR-138-5p/4EBP-1 axis.[34] However, whether miR-138-5p interacts with circRNAs in breast cancer is unknown. In our study, miR-138-5p was for the first time identified as the potential

target of circDOCK1 in breast cancer. The binding relationship was further verified through bioinformatics analyses, pulldown assays and luciferase reporter assays.

Moreover, we found that GRB7 is a downstream target of miR-138-5p in breast cancer cells. Our study revealed a positive correlation of GRB7 expression with circDOCK1 expression and a negative correlation of GRB7 expression with miR-138-5p expression in breast cancer tissues, which was consistent with the competing endogenous RNA theory. GRB7 (Growth factor receptor bound protein 7), a gene commonly co-expressed with HER2 due to their proximity on chromosome 17 position q12, binds to phosphorylated tyrosine kinases to promote metastatic proliferative pathway such as PI3K/AKT/mTOR pathway through its C-terminal SH2 domain.[35,36] Yun Ling reported that GRB7 mediated circCDYL2 induced trastuzumab resistance in HER2 positive breast cancer;[37] Chune Yu found that GRB7 was a driver for MEK inhibitor resistance in KRAS mutant colon cancer;[38] Pei YY reported that up-regulated GRB7 correlated with increased stem cell properties in gastric cancer.[39] Thus, peptide inhibitors targeting GRB7 SH2 domain may be promising molecular therapeutic in cancer. In our study, we found that high GRB7 expression was associated with poor patient survival in METABRIC dataset. In addition, functional rescue experiments revealed that circDOCK1 promoted breast cancer cells migration and invasion through circDOCK1/miR-138-5p/GRB7 axis, emphasizing the value of this axis as a prognostic marker and a therapeutic target.

Conclusion

In summary, our finding elucidate a novel circDOCK1/miR-138-5p/GRB7 regulatory network that promotes metastasis and progression of patients with HER2 positive breast cancer. This axis may be a therapeutic target for breast cancer. However, further research on the role of circDOCK1/miR-138-5p/GRB7 in breast cancer is warranted.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

All experimental protocols were approved by the Ethics Committee of the First Affiliated Hospital of Jinan University and conducted in accordance with the principles of the World Medical Association Declaration of Helsinki. Written informed consent was obtained from all participants prior to sample collection.

Acknowledgments

Breast cancer samples were obtained from Biobank of The first affiliated hospital of Jinan University. All patients had signed informed consent for donating their samples to Biobank of The first affiliated hospital of Jinan University.

Funding

This work was supported by The Guangdong Basic and Applied Research Foundation (2023A1515010553); Science and Technology Projects in Guangzhou (2023A03J1005); Youth Science Foundation of National Natural Science Foundation of China (81702598); The Science Foundation of Guangdong Province (2017A030313803); The Science and Technology Program of Guangzhou (201804010011) and the Flagship specialty construction project-General surgery (711003).

Disclosure

The authors declare that they have no competing interests.

References

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. 1987;235:177–182. doi:10.1126/science.3798106
2. Ross JS, Fletcher JA. The HER-2/neu oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy. *Oncologist*. 1998;3:237–252. doi:10.1634/theoncologist.3-4-237

3. Harbeck N, Penault-Llorca F, Cortes J, et al. Breast cancer. *Nat Rev Dis Primers*. 2019;5:66. doi:10.1038/s41572-019-0111-2
4. Memczak S, Jens M, Elefsinioti A, et al. Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature*. 2013;495:333–338. doi:10.1038/nature11928
5. Liu J, Li D, Luo H, Zhu X. Circular RNAs: the star molecules in cancer. *Mol Aspects Med*. 2019;70:141–152. doi:10.1016/j.mam.2019.10.006
6. Beilerli A, Gareev I, Beylerli O, et al. Circular RNAs as biomarkers and therapeutic targets in cancer. *Semin Cancer Biol*. 2022;83:242–252. doi:10.1016/j.semcancer.2020.12.026
7. Hansen TB, Jensen TI, Clausen BH, et al. Natural RNA circles function as efficient microRNA sponges. *Nature*. 2013;495:384–388. doi:10.1038/nature11993
8. Cheng Z, Yu C, Cui S, et al. circTP63 functions as a ceRNA to promote lung squamous cell carcinoma progression by upregulating FOXM1. *Nat Commun*. 2019;10:3200. doi:10.1038/s41467-019-11162-4
9. Xia S, Feng J, Chen K, et al. CSCD: a database for cancer-specific circular RNAs. *Nucleic Acids Res*. 2018;46:D925–d929. doi:10.1093/nar/gkx863
10. Bazhabayi M, Qiu X, Li X, et al. CircGFRA1 facilitates the malignant progression of HER-2-positive breast cancer via acting as a sponge of miR-1228 and enhancing AIFM2 expression. *J Cell Mol Med*. 2021;25:10248–10256. doi:10.1111/jcmm.16963
11. Zhong JX, Kong YY, Luo RG, et al. Circular RNA circ-ERBB2 promotes HER2-positive breast cancer progression and metastasis via sponging miR-136-5p and miR-198. *J Transl Med*. 2021;19:455. doi:10.1186/s12967-021-03114-8
12. Liu Z, Zhou Y, Liang G, et al. Circular RNA hsa_circ_001783 regulates breast cancer progression via sponging miR-200c-3p. *Cell Death Dis*. 2019;10:55. doi:10.1038/s41419-018-1287-1
13. Fu B, Liu W, Zhu C, et al. Circular RNA circBCBM1 promotes breast cancer brain metastasis by modulating miR-125a/BRD4 axis. *Int J Biol Sci*. 2021;17:3104–3117. doi:10.7150/ijbs.58916
14. Loibl S, Gianni L. HER2-positive breast cancer. *Lancet*. 2017;389(10087):2415–2429. doi:10.1016/S0140-6736(16)32417-5
15. Tarantino P, Viale G, Press MF, et al. ESMO expert consensus statements (ECS) on the definition, diagnosis, and management of HER2-low breast cancer. *Ann Oncol*. 2023;34:645–659. doi:10.1016/j.annonc.2023.05.008
16. Luo J, Cao D, Hu C, Liang Z, Zhang Y, Lai J. Lymphatic metastasis-associated circRNA–miRNA–mRNA network for exploring the pathogenesis and therapeutic target of triple negative breast cancer based on whole-transcriptome sequencing analysis: an experimental verification study. *J Transl Med*. 2022;20:508. doi:10.1186/s12967-022-03728-6
17. Zhang M, Bai X, Zeng X, Liu J, Liu F, Zhang Z. circRNA-miRNA-mRNA in breast cancer. *Clin Chim Acta*. 2021;523:120–130. doi:10.1016/j.cca.2021.09.013
18. Khan S, Jha A, Panda AC, Dixit A. Cancer-Associated circRNA-miRNA-mRNA regulatory networks: a meta-analysis. *Front Mol Biosci*. 2021;8:671309. doi:10.3389/fmolb.2021.671309
19. Fan X, Zhang X, Wu X, et al. Single-cell RNA-seq transcriptome analysis of linear and circular RNAs in mouse preimplantation embryos. *Genome Biol*. 2015;16:148. doi:10.1186/s13059-015-0706-1
20. Lu T, Cui L, Zhou Y, et al. Transcriptome-wide investigation of circular RNAs in rice. *RNA*. 2015;21:2076–2087. doi:10.1261/rna.052282.115
21. Qu S, Zhong Y, Shang R, et al. The emerging landscape of circular RNA in life processes. *RNA Biol*. 2017;14:992–999. doi:10.1080/15476286.2016.1220473
22. Ni Z, Liu W, Pan G, et al. Circular forms of dedicator of cytokinesis 1 promotes breast cancer progression by derepressing never in mitosis related kinase 2 via sponging miR-128-3p. *Environ Toxicol*. 2023;38:1712–1722. doi:10.1002/tox.23799
23. Wang G, Wu Y, Su Y, et al. TCF12-regulated GRB7 facilitates the HER2+ breast cancer progression by activating Notch1 signaling pathway. *J Transl Med*. 2024;22:745. doi:10.1186/s12967-024-05536-6
24. Jian FX, Bao PX, Li WF, Cui YH, Hong HG. Negative regulation of CD44st by miR-138-5p affects the invasive ability of breast cancer cells and patient prognosis after breast cancer surgery. *BMC Cancer*. 2023;23:269. doi:10.1186/s12885-023-10738-0
25. Li S, Liu F, Zheng K, et al. CircDOCK1 promotes the tumorigenesis and cisplatin resistance of osteogenic sarcoma via the miR-339-3p/IGF1R axis. *Mol Cancer*. 2021;20:161. doi:10.1186/s12943-021-01453-0
26. Wang L, Wei Y, Yan Y, et al. CircDOCK1 suppresses cell apoptosis via inhibition of miR-196a-5p by targeting BIRC3 in OSCC. *Oncol Rep*. 2018;39:951–966. doi:10.3892/or.2017.6174
27. Liu P, Li X, Guo X, et al. Circular RNA DOCK1 promotes bladder carcinoma progression via modulating circDOCK1/hsa-miR-132-3p/Sox5 signalling pathway. *Cell Prolif*. 2019;52:e12614. doi:10.1111/cpr.12614
28. Yeh M, Oh CS, Yoo JY, Kaur B, Lee TJ. Pivotal role of microRNA-138 in human cancers. *Am J Cancer Res*. 2019;9:1118–1126.
29. Shadbad MA, Ghorbaninezhad F, Hassanian H, et al. A scoping review on the significance of programmed death-ligand 1-inhibiting microRNAs in non-small cell lung treatment: a single-cell RNA sequencing-based study. *Front Med Lausanne*. 2022;9:1027758. doi:10.3389/fmed.2022.1027758
30. Xun J, Du L, Gao R, et al. Cancer-derived exosomal miR-138-5p modulates polarization of tumor-associated macrophages through inhibition of KDM6B. *Theranostics*. 2021;11:6847–6859. doi:10.7150/thno.51864
31. Zhang Q, Pan J, Xiong D, et al. Aerosolized miR-138-5p and miR-200c targets PD-L1 for lung cancer prevention. *Front Immunol*. 2023;14:1166951. doi:10.3389/fimmu.2023.1166951
32. He Z, Ruan X, Liu X, et al. FUS/circ_002136/miR-138-5p/SOX13 feedback loop regulates angiogenesis in Glioma. *J Exp Clin Cancer Res*. 2019;38:65. doi:10.1186/s13046-019-1065-7
33. Zhang S, Lu Y, Jiang HY, et al. CircC16orf62 promotes hepatocellular carcinoma progression through the miR-138-5p/PTK2/AKT axis. *Cell Death Dis*. 2021;12:597. doi:10.1038/s41419-021-03866-7
34. Chen LY, Wang L, Ren YX, et al. The circular RNA circ-ERBIN promotes growth and metastasis of colorectal cancer by miR-125a-5p and miR-138-5p/4EBP-1 mediated cap-independent HIF-1α translation. *Mol Cancer*. 2020;19:164. doi:10.1186/s12943-020-01272-9
35. Nadler Y, González AM, Camp RL, Rimm DL, Kluger HM, Kluger Y. Growth factor receptor-bound protein-7 (Grb7) as a prognostic marker and therapeutic target in breast cancer. *Ann Oncol*. 2010;21(3):466–473. doi:10.1093/annonc/mdp346
36. Zheng Y, Pei Y, Yang L, et al. Upregulated GRB7 promotes proliferation and tumorigenesis of bladder cancer via Phospho-AKT pathway. *Int J Biol Sci*. 2020;16:3221–3230. doi:10.7150/ijbs.49410
37. Ling Y, Liang G, Lin Q, et al. circCDYL2 promotes trastuzumab resistance via sustaining HER2 downstream signaling in breast cancer. *Mol Cancer*. 2022;21:8. doi:10.1186/s12943-021-01476-7

38. Yu C, Luo D, Yu J, et al. Genome-wide CRISPR-cas9 knockout screening identifies GRB7 as a driver for MEK inhibitor resistance in KRAS mutant colon cancer. *Oncogene*. 2022;41:191–203. doi:10.1038/s41388-021-02077-w
39. Pei YY, Ran J, Wen L, et al. Up-regulated GRB7 protein in gastric cancer cells correlates with clinical properties and increases proliferation and stem cell properties. *Front Oncol*. 2022;12:1054976. doi:10.3389/fonc.2022.1054976