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CircRNA_100395 protects breast carcinoma deterioration by targeting MAPK6

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Abstract. – OBJECTIVE: This study aims to uncover the differential expression of circRNA_100395 in breast carcinoma specimens, and its regulatory effect on cancer cell phenotypes. The role of circRNA_100395 in affecting breast carcinoma progression and the molecular mechanism are explored as well.

PATIENTS AND METHODS: CircRNA_100395 expressions in breast carcinoma and paracancerous tissues were detected. The influence of circRNA_100395 level on clinical indicators of breast carcinoma patients was analyzed. In vitro regulations of circRNA_100395 on phenotypes of breast carcinoma cells were examined by CCK-8, colony formation, and transwell assay. The interaction between circRNA_100395 and MAPK6 was confirmed by Dual-Luciferase reporter assay and rescue assays.

RESULTS: CircRNA_100395 was downregulated in breast carcinoma tissues and cell lines. Its level was negatively correlated to tumor staging and tumor size of breast carcinoma. Overexpression of circRNA_100395 in SKBR3 and MDA-MB-231 cells weakened proliferative and migratory abilities. MAPK6 was the target gene of circRNA_100395. Overexpression of MAPK6 reversed the anti-cancer effect of circRNA_100395 on breast carcinoma.

CONCLUSIONS: CircRNA_100395 serves as an anti-cancer gene in breast carcinoma progression by targeting MAPK6, and its level is negatively correlated to tumor staging and tumor size of breast carcinoma. CircRNA_100395 can be utilized as a potential biomarker and therapeutic target of breast carcinoma.

Key Words:
CircRNA_100395, MAPK6, Breast carcinoma, Malignant progression.

Introduction

Breast carcinoma (BC) is a commonly detected cancer that severely endangers health of women, and globally 1.5 million patients were newly diagnosed cases of breast carcinoma[1-3]. In China, BC is highly prevalent in women, and it is reported that in 2015, 272,400 people were diagnosed as breast carcinoma, including 70,700 deaths[1,4,5]. Classical epidemiological studies have shown that with the changes in people's fertility and lifestyle, the incidence of this tumor is on the rise, and the disease burden it caused is serious[5]. The occurrence of breast carcinoma can be affected by the age of menarche and menopause, family history of cancer, obesity, and alcohol consumption. Notably, genetic factors are of significance in the tumorigenesis[6]. With the advancement of molecular biology technology and the implementation of the Human Genome Project, identifying high-risk population and developing individualized health management are research focuses[7,8]. Recently, epigenetic changes have been well concerned for the regulation of biological activities[9,10]. In clinical practice, drug resistance of BC largely limits the therapeutic efficacy and clinical outcomes, leading to a poor prognosis of affected people[11]. It is urgent to seek for effective biomarkers for clarifying the molecular mechanism of breast carcinoma[12].

CircRNAs are a novel type of noncoding RNAs responsible for transcriptional or post-transcriptional regulation[13,14]. It used to be neglected as splicing byproducts although the history of circRNAs could be traced back to 30 years ago[15]. Unlike traditional linear RNAs, circRNAs are formed by a covalent closed loop without 5' cap and 3' poly(A) tail[16]. Because of the unique structure, circRNAs are resistant to RNase R[17]. Dynamically expressed circRNAs have been identified to have a relation to the progression of cancer, and the expression levels are correlated to tumor size, differentiation and metastasis rate[14].

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CircRNA_100395 is differentially expressed in the profiles of ovarian cancer and hepatocellular carcinoma[18,19].

Based on the research background, we speculated that circRNA_100395 might be involved in the regulation of breast carcinoma *via* targeting a certain factor. Through bioinformatic prediction, MAPK6 was the target gene of circRNA_100395. In the following experiments, we would elucidate the co-regulation of circRNA_100395 and MAPK6 on the proliferative and metastatic abilities of BC.

Patients and Methods

Breast Carcinoma Specimens

Breast carcinoma and paracancerous ones were collected during biopsy or surgery from 52 patients, which were stored at -80°C. Patients were diagnosed by two experienced doctors independently. Tumor node metastasis (TNM) staging and histological classification of breast carcinoma were defined according to the criteria proposed by UICC/AJCC (the 8th edition). Inclusion criteria: none of patients had the severe diseases from other organs, and preoperative chemotherapy/radiotherapy, endocrine or molecular targeted therapy. Exclusion criteria: patients had distant metastasis, those complicated with other malignancies, those with mental disease, those complicated with myocardial infarction, heart failure or other chronic diseases, or those previously exposed to radioactive rays.

This investigation was approved by the Research Ethics Committee of Shengjing Hospital of China Medical University and complied with the Helsinki Declaration. Informed consent from patients was obtained before collecting samples and clinical data. Follow-up was conducted after discharge by telephone or outpatient review, including general conditions, clinical signs and symptoms, and imaging examinations.

Cell Lines and Reagents

Breast carcinoma cell lines (MCF-7, SKBR3, MDA-MB-231 and MDA-MB-435S) and the mammary epithelial cell line (MCF-10A) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) at 37°C with 5% CO_2. 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin were added in culture medium. Cell passage was conducted every 2-3 days.

Transfection

Eight hours prior to transfection, cells in a good condition were seeded and cultivated to 60% density. Transfection plasmid and Lipofectamine 2000 solution (Invitrogen, Carlsbad, CA, USA) was respectively diluted in medium, and gently mixed together. Cells were cultured in the mixture for 4-6 h, followed by replacement of fresh medium.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 5×10^3 cells/well. At 24, 48, 72 and 96 h, absorbance value at 450 nm of each sample was recorded using the cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Colony Formation Assay

Cells were inoculated in the 6-well plate with 200 cells/well. Medium was replaced once a week in the first week, and twice in the second week. Visible colonies were washed by phosphate-buffered saline (PBS), fixed in methanol for 20 min and dyed with 0.1% crystal violet for 20 min. Colonies were captured for counting.

Transwell Migration Assay

Transfected cells were prepared to serum-free suspension at 3×10^4/mL. In the transwell chamber, 200 μL of suspension and 500 μL of complete medium were added on the upper and bottom chamber, respectively. After 24 h of incubation, the cells in the bottom were fixed in methanol for 15 min, dyed with crystal violet for 30 min and counted using a microscope.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cellular or tissue RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 500 μL of chloroform was added in 1 mL of TRIzol mixed with cell or tissue lysate. After gentle mixture for 30 s and centrifugation at 4°C, the aqua phase was mixed with the same volume of pre-cold isopropanol. The centrifuged precipitant was washed in 75% ethanol, air dried and diluted in 30 μL of diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). Qualified RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using AMV reverse

transcription kit (TaKaRa, Otsu, Shiga, Japan). SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) was used for qRT-PCR with cDNA as the template. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. CircRNA_100395: forward: 5'-GAGCCGTGTCCCAGATTGT-3', reverse: 5'-TGCCGCTGATCCAACATTTC-3'. MAPK6: forward: 5'-GAAGTCCCGTTGTATCAGAGTA-3', reverse: 5'-CCTGGCGGCTGTTGGTTA-3'. GAPDH: forward: 5'-TGTGGGCATCAATGGATTTGG-3', reverse: 5'-ACACCATGTATTCCGGGTCAAT-3'.

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) on ice for 30 min, and centrifuged at 4°C, 14000×g for 15 min. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). The membrane was cut into small pieces according to the molecular size and blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure and grey value analyses using alpha SP.

Dual-Luciferase Reporter Assay

Cells were seeded in a 24-well plate and cultured in Opti-MEN. They were co-transfected with pcDNA3.1-NC/pcDNA3.1-MAPK6 (200 ng) and circRNA_100395-WT/circRNA_100395-MUT (10 ng), respectively. After 24 h co-transfection, cells were lysed and centrifuged at 12,000×g for 3 min. Luciferase intensity of Firefly and Renilla was determined (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used to statistical analysis. The Student's *t*-test was applied to compare differences between two groups. Each experiment was repeated in triplicate and data were expressed as mean±SD (standard deviation). Chi-square test was applied for analyzing the relationship between circRNA_100395 and clinical indicators of breast carcinoma. Pearson correlation test was conducted to assess the correlation between circRNA_100395 and MAPK6 levels in breast carcinoma specimens. $p<0.05$ was considered as statistically significant.

Results

CircRNA_100395 Was Downregulated in Breast Carcinoma Specimens

We collected 53 pairs of breast carcinoma and paracancerous tissues. QRT-PCR data showed that circRNA_100395 was downregulated in breast carcinoma specimens (Figure 1A). Similarly, it was lowly expressed in breast carcinoma cell lines as well (Figure 1B). Of the four tested cancer cell lines, circRNA_100395 level remained the lowest in SKBR3 and MDA-MB-231 cells, which were used in the following experiments.

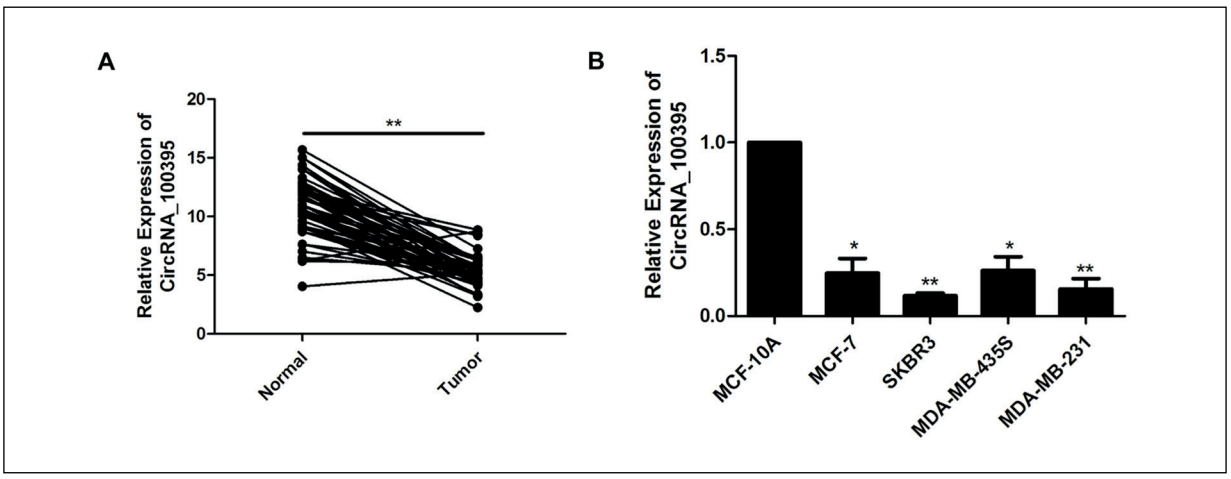

Figure 1. CircRNA_100395 was downregulated in breast carcinoma specimens. **A,** CircRNA_100395 levels in breast carcinoma and paracancerous tissues. **B,** CircRNA_100395 levels in breast carcinoma cell lines. *$p < 0.05$, **$p < 0.01$.

CircRNA_100395 Was Correlated to Tumor Size and T Stage in Breast Carcinoma

Chi-square test was conducted to analyze the clinical significance of circRNA_100395 in breast carcinoma. It is indicated that circRNA_100395 level was negatively linked to tumor size and T stage, while it was unrelated to sex, age, lymphatic metastasis and distant metastasis of breast carcinoma (Table I).

Overexpression of CircRNA_100395 Weakened Proliferative and Migratory Abilities of Breast Carcinoma

By transfection of pcDNA3.1-circRNA_100395, we constructed circRNA_100395 overexpression models in SKBR3 and MDA-MB-231 cells successfully (Figure 2A). CCK-8 assay revealed a lower viability in cells overexpressing circRNA_100395 than controls (Figure 2B). In addition, both wound healing and transwell assay demonstrated the attenuated migratory ability in SKBR3 and MDA-MB-231 cells overexpressing circRNA_100395 (Figure 2C, D).

CircRNA_100395 Directly Targeted MAPK6

Using online bioinformatic databases, we searched three candidates of circRNA_100395 targets, MAPK6, c-myc and SIRT1. Notably, mRNA level of MAPK6 was the most affected by overexpression of circRNA_100395 (Figure 3A). Compared with normal tissues, MAPK6 was upregulated in breast carcinoma tissues (Figure 3B). According to the predicted binding sites in the seed sequences of circRNA_100395 and MAPK6, Luciferase vectors were generated for further verifying their relationship. Overexpression of MAPK6 only declined Luciferase activity in wild-type circRNA_100395 vector, confirming that MAPK6 could be targeted by circRNA_100395 (Figure 3C). Protein level of MAPK6 was found to be markedly downregulated by overexpression of circRNA_100395 in SKBR3 and MDA-MB-231 cells (Figure 3D). Consistently, a negative correlation was identified between circRNA_100395 and MAPK6 levels in breast carcinoma specimens (Figure 3E).

CircRNA_100395 Protected Breast Carcinoma Deterioration by Targeting MAPK6

We thereafter explored the biological function of MAPK6 in breast carcinoma. First of all, transfection efficacy of pcDNA-MAPK6 was tested in SKBR3 and MDA-MB-231 cells overexpressing circRNA_100395 by Western blot (Figure 4A). Compared with cells solely overexpressing circRNA_100395, those co-overexpressing circRNA_100395 and MAPK6 had a higher viability (Figure 4B). Furthermore, we found higher clone number and migratory cell number after overexpression of circRNA_100395 in breast car-

Table I. Association of CircRNA_100395 expression with clinicopathologic characteristics of breast cancer.

Parameters	No. of cases	CircRNA_100395 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.548
< 60	24	14	10	
≥ 60	28	14	14	
Tumor size (cm)				0.012
< 3	25	18	7	
≥ 3	27	10	17	
T stage				0.005
T1-T2	26	19	7	
T3-T4	26	9	17	
Lymph node metastasis				0.299
No	22	10	12	
Yes	30	18	12	
Distance metastasis				0.113
No	32	20	12	
Yes	20	8	12	

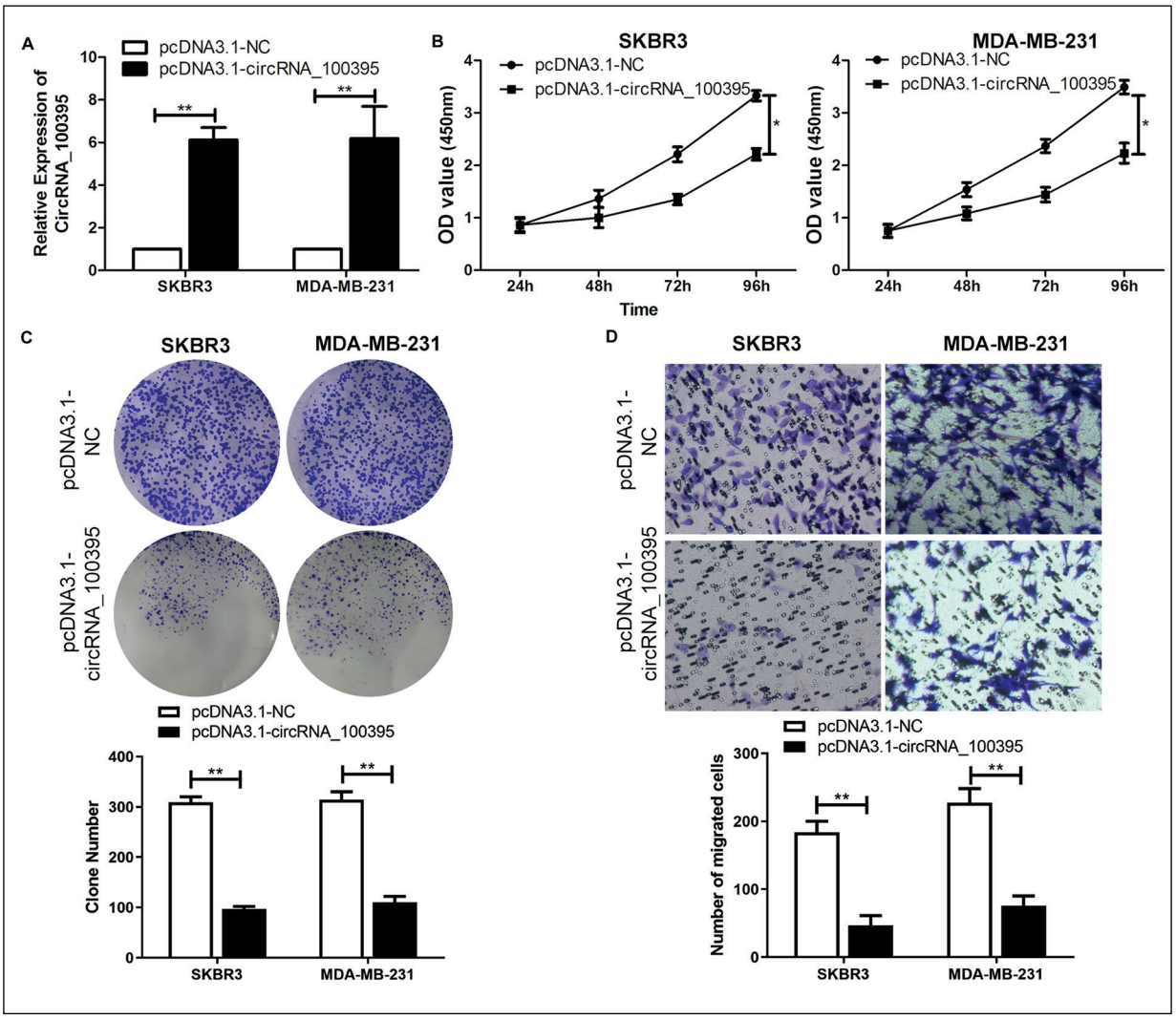

Figure 2. Overexpression of circRNA_100395 weakened proliferative and migratory abilities of breast carcinoma. **A,** Transfection efficacy of pcDNA3.1- circRNA_100395. **B,** Viability in SKBR3 and MDA-MB-231 cells overexpressing circRNA_100395. **C,** Clone number in SKBR3 and MDA-MB-231 cells overexpressing circRNA_100395 (magnification: 10×). **D,** Migration in SKBR3 and MDA-MB-231 cells overexpressing circRNA_100395 (magnification: 40×). *$p < 0.05$.

cinoma cells than those of controls (Figure 4C, 4D). It is suggested that circRNA_100395 alleviated the malignant progression of breast carcinoma by regulating MAPK6.

Discussion

The differential level of circRNA_100395 in breast carcinoma specimens was analyzed and predicted its potential target gene, in this study. CircRNAs are now promising molecular markers and therapeutic targets of human cancers[15,16]. Epigenetic modification is a heritable genetic change (i.e., DNA methylation, histone modification, noncoding RNA regulation) that affects DNA expressions without changing the nucleotide sequences[9,10]. MiRNAs and lncRNAs have been well explored for their functions in the progression of cancer[13]. As a novel type of noncoding RNAs, people are interested in the biological functions of circRNAs[18,19]. CircRNA_100395 is a newly discovered circRNA involved in the maturation, differentiation and growth of cancer. However, the association about circRNA_100395 and breast carcinoma is unclear. Our findings uncovered that circRNA_100395 was downregulated in both breast carcinoma tissues and cell lines. Lat-

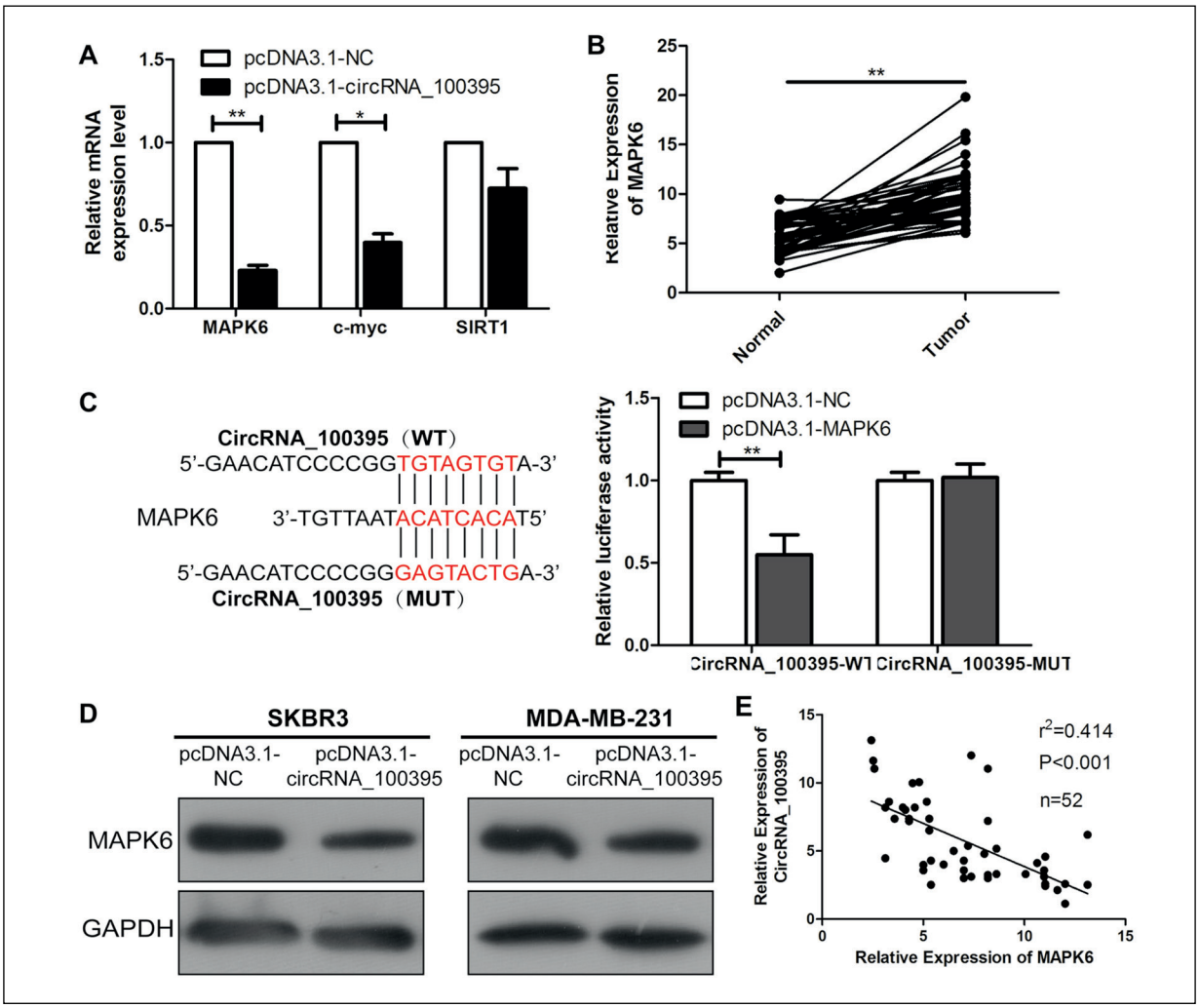

Figure 3. CircRNA_100395 directly targeted MAPK6. **A,** Relative levels of MAPK6, c-myc and SIRT1 in cells overexpressing circRNA_100395. **B,** MAPK6 levels in breast carcinoma and paracancerous tissues. **C,** Luciferase activity in cells co-transfected with pcDNA3.1-NC/pcDNA3.1-MAPK6 and circRNA_100395-WT/ circRNA_100395MUT, respectively. **D,** Protein level of MAPK6 in SKBR3 and MDA-MB-231 cells overexpressing circRNA_100395. **E,** A negative correlation between circRNA_100395 and MAPK in breast carcinoma tissues. *$p < 0.05$, **$p < 0.01$.

er, we constructed circRNA_100395 overexpression models in SKBR3 and MDA-MB-231 cells by transfection of pcDNA3.1-circRNA_100395. Viability, clone number and migratory cell number all decreased in cancer cells overexpressing circRNA_100395, suggesting the anti-cancer effect of circRNA_100395 on BC.

By competitively binding the downstream targets, circRNAs are capable of mediating cancer progression[13,14,16]. Here, we have proven that MAPK6 was the target gene binding circRNA_100395 by Bioinformatics analysis and Luciferase reporter gene experiment. MAPK6 was negatively regulated by circRNA_100395 in SKBR3 and MDA-MB-231 cells. Interestingly, overexpression of MAPK6 reversed the role of circRNA_100395 in regulating proliferative and migratory abilities of breast carcinoma, indicating an oncogenic effect of MAPK6. These findings pointed out a novel biomarker, circRNA_100395, for the diagnosis and treatment of breast carcinoma, which could be utilized in the targeted therapy of BC.

Conclusions

The above data demonstrated that circRNA_100395 serves as an anti-cancer gene involved in breast carcinoma progression by tar-

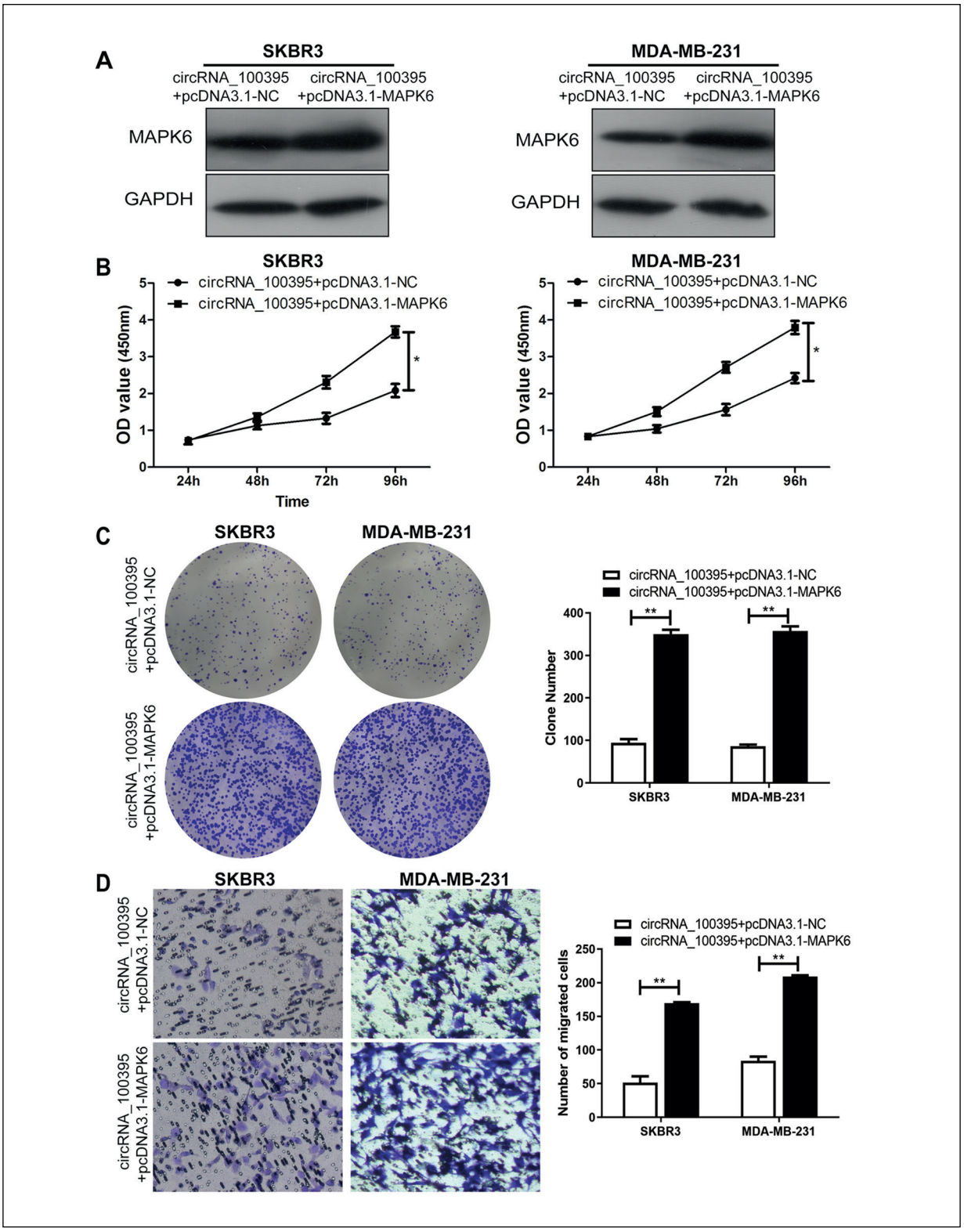

Figure 4. CircRNA_100395 protected breast carcinoma deterioration by targeting MAPK6. **A,** Protein level of MAPK6 in SKBR3 and MDA-MB-231 cells co-overexpressing circRNA_100395 and MAPK6. **B,** Viability in SKBR3 and MDA-MB-231 cells co-overexpressing circRNA_100395 and MAPK6. **C,** Clone number in SKBR3 and MDA-MB-231 cells co-overexpressing circRNA_100395 and MAPK6 (magnification: 10×). **D,** Migration in SKBR3 and MDA-MB-231 cells co-overexpressing circRNA_100395 and MAPK6 (magnification: 40×). *$p < 0.05$, **$p < 0.01$.

geting MAPK6, and its level is negatively correlated to tumor staging and tumor size of breast carcinoma. CircRNA_100395 can be utilized as a potential biomarker and therapeutic target of breast carcinoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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References

1) Ito H, Matsuo K. Molecular epidemiology, and possible real-world applications in breast cancer. Breast Cancer-Tokyo 2016; 23: 33-38.
2) Woolston C. Breast cancer. Nature 2015; 527: S101.
3) Colavito SA. AXL as a target in breast cancer therapy. J Oncol 2020; 2020: 5291952.
4) Li T, Mello-Thoms C, Brennan PC. Descriptive epidemiology of breast cancer in China: incidence, mortality, survival and prevalence. Breast Cancer Res Treat 2016; 159: 395-406.
5) Fan L, Strasser-Weippl K, Li JJ, St LJ, Finkelstein DM, Yu KD, Chen WQ, Shao ZM, Goss PE. Breast cancer in China. Lancet Oncol 2014; 15: e279-e289.
6) Wang SK, Xiao HM, Xia H, Sun GJ. Tea consumption and risk of breast cancer: a meta-analysis. Int J Clin Pharmacol Ther 2018; 56: 617-619.
7) Sotiriou C, Pusztai L. Gene-expression signatures in breast cancer. N Engl J Med 2009; 360: 790-800.
8) Narod SA. Cancer risk estimates for study of multiple-gene testing after diagnosis of breast cancer. JAMA Oncol 2018; 4: 1787-1788.
9) Dumitrescu RG. Interplay between genetic and epigenetic changes in breast cancer subtypes. Methods Mol Biol 2018; 1856: 19-34.
10) Byler S, Goldgar S, Heerboth S, Leary M, Housman G, Moulton K, Sarkar S. Genetic and epigenetic aspects of breast cancer progression and therapy. Anticancer Res 2014; 34: 1071-1077.
11) DeMichele A, Yee D, Esserman L. Mechanisms of resistance to neoadjuvant chemotherapy in breast cancer. N Engl J Med 2017; 377: 2287-2289.
12) Li G, Hu J, Hu G. Biomarker studies in early detection and prognosis of breast cancer. Adv Exp Med Biol 2017; 1026: 27-39.
13) Patop IL, Wust S, Kadener S. Past, present, and future of circRNAs. EMBO J 2019; 38: e100836.
14) Patop IL, Kadener S. CircRNAs in cancer. Curr Opin Genet Dev 2018; 48: 121-127.
15) Das A, Gorospe M, Panda AC. The coding potential of circRNAs. Aging (Albany NY) 2018; 10: 2228-2229.
16) Lei M, Zheng G, Ning Q, Zheng J, Dong D. Translation and functional roles of circular RNAs in human cancer. Mol Cancer 2020; 19: 30.
17) Jeck WR, Sorrentino JA, Wang K, Slevin MK, Burd CE, Liu J, Marzluff WF, Sharpless NE. Circular RNAs are abundant, conserved, and associated with ALU repeats. RNA 2013; 19: 141-157.
18) Li X, Lin S, Mo Z, Jiang J, Tang H, Wu C, Song J. CircRNA_100395 inhibits cell proliferation and metastasis in ovarian cancer via regulating miR-1228/p53/epithelial-mesenchymal transition (EMT) axis. J Cancer 2020; 11: 599-609.
19) Chen Q, Chen Z, Cao S, Guo B, Chen Y, Feng Z, Wang J, Guo G, Chen X, Huang X. Role of circRNAs_100395 in proliferation and metastases of liver cancer. Med Sci Monit 2019; 25: 6181-6192.