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MiR-491 suppresses migration and invasion *via* directly targeting TPX2 in breast cancer

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Abstract. – OBJECTIVE: Breast cancer (BC) is one of the primary causes of tumor-related female mortalities. Although in recent years, we have made great progress in the systemic therapy and earlier diagnosis for BC patients, recurrence or distant metastasis remains leading obstacles for the successful therapy of BC. Therefore, a comprehensive understanding of the molecular mechanism underlying the progression may be crucial in developing an effective strategy against BC. The current research aimed to explore the expressions, fu and molecular mechanism of microR (miR-491) in BC.

PATIENTS AND METHODS: Quantitative al Time-Polymerase Chain Reaction (qRT-P was performed to examine the l of miR-4 expression in 52 pairs of BC and pa ra-cancerous specimens, a he ion between miR-491 level and clinic eatures of BC patient prognos s ana swell invasion and mi rat s we ducted to determin ethe 491 had effects on the regula of BC m sis. Potential target genes R-491 were out using TargetSca e the mole ar functions of miR in in g breast cancer cell invasion a migration. cidate the mechanism of 2 in suppress l invasion and migrati nedicated by miR- in breast cancer, urther nsfected TPX2 siRNAs into MC ells t lete endogenous TPX2, along with ctions miR-491 inhibitor into MCF nes.

RESULTS: The findings demonstrated that mi 1 exp s were significantly decrea d in BC sues and cells. The miR-491 res n suppressed the invasion and migrati . In addition, we identified the targeting protein for Xklp2 (TPX2) as a direct target miR-491 in BC. The knockdown of TPX2 markeversed miR-491-medicated inhibition of ce asion and migration in BC cell lines.

CONCLUSIONS: In short, all the results suggested that miR-491 functioned as a tumor suppressor b g TPX2 in B nd the miR-491 rest on m an effective therapy for the BC treatment in ure.

K *ds:*
R-491, Invasion, Migration, TPX2, Breast cancer.

Introduction

cancer (BC), a common tumor in fe- ale, one of the leading causes of tumor-related female mortality[1]. Early BC has a 5-year survival rate of 99%, but for patients with distant metastasis, the 5-year survival rate is only 24%[2]. Hence, early detection of patients with breast cancer is very important. In addition, even the same therapies, including chemotherapy or radiotherapy[3], are provided for breast cancer patients with the same TNM stage, some patients may benefit from the therapy but others may have tumor metastasis or recurrence[4]. Therefore, it is still hard to predict the consequence of the treatment for every single patient accurately. As a result, we know that it is urgent to find out new biomarker for the earlier diagnosis and prognosis for BC.

Recently, increasing evidence has demonstrated a significant relation between tumors and microRNAs (miRNAs). As we all know, miRNAs are small non-coding RNAs, consisting of approximately17-24 nucleotides[5]. It has been reported that miRNAs play critical regulatory roles via directly base pairing with the 3'UTR of their target mRNAs, contributing to the suppression of the mRNA expressions[6]. Notably, several studies have shown that miRNAs are related to extensive biological processes of cancer cells, such as cell differentiation and proliferation, leading to

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increasing studies focusing on targeting miRNAs to improve the efficacy of breast cancer treatments[7, 8]. For example, Zhang et al[9] found that miR-129-5p could inhibit cell invasion and proliferation via binding to E-cadherin, vimentin, and microspherule protein 1 in lung cancer. MiR-495 was reported to suppress cell proliferation and migration by targeting FAM83D in colorectal cancer[10]. MiR-320a was found to have function in inhibiting tumor proliferation and invasion in human hepatocellular carcinoma by targeting c-Myc[11]. Additionally, miR-491 has been identified to express aberrantly in different human tumors[12,13]. However, the functions of miR-491 in modulating BC cell invasion and migration needs to be fully elucidated.

New evidence emerging in gene expression profiling suggested that differentially expressed genes in tumors can provide new targeted therapeutic strategies for patients with tumors and used as biomarkers for detecting early-stage tumor or predicting prognosis[14]. Based on this, current research has focused on the targeting protein for Xklp2 (TPX2). TPX2 has essential functions in mitotic spindles formation[15]. In recent years, several researches have shown that TPX2 is related to the development of various cancers such as cervical and colon cancer[16,17]. However, there are few studies on the association between TPX2 and miR-491 in BC. The insightful information about TPX2 presented in this research will provide a therapeutic target and prognostic marker for BC

Patients and Methods

Human Tissue Samples and Cell Culture

Specimens of patients with BC (n=52) and adjacent normal tissues were collected from our hospital from 2015 to 2017. All patients involved in this study had never been subjected to any previous treatment. All tissue samples were snap-frozen in liquid nitrogen immediately after surgery and further analysis. This study was approved by the Ethics Committee of Jinan City People's Hospital. Additionally, signed written informed consents were obtained from all participants before the study. Human BC cell line MCF-7 and mammary epithelial cell line MCF-10A were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells involved in this study were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) which contained 10% of fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an atmosphere with 5% CO_2 at 37°C.

Cell Transfection

MiR-491 mimics, miR-491 inhibitor, TPX2 siRNAs and corresponding controls were all obtained from RiBoBio (Guangzhou, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was applied to transiently transfect into MCF-7 cells in line with the manufacturer's instruction. 48 h after transfections, cells were collected for further study.

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

Total RNA in BC and normal tissue samples and cell lines were prepared by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. Then, the Prime Script RT reagent kit (TaKaRa, Dalian, China) was used to synthesize the cDNA. QRT-PCR analysis for miR-491 and TPX2 were conducted with TaqMan MicroRNA Assays Kit (Applied Biosystems, Foster City, CA, USA) and SYBR® Premix Ex Tag™ II (TaKaRa, Dalian, China) on the ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The conditions for PCR were as follows: 95°C (30 s), 40 cycles at 95°C (5 s) and 60°C (34 s). The miR-491 expression was normalized to U6 while the TPX2 was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The relative expression ratios of Genes were evaluated by the $2^{-\Delta\Delta CT}$ method. The primers involved in this assay were shown in Table I.

Western Blots

MCF-7 cells were collected 72 h after the transfections and the total protein was prepared by a radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, Shanghai, China). Then, the protein concentrations were measured by bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Subsequently, the protein sample was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) which was blocked in Tris-Buffered Saline and Tween (TBST) with 5% non-fat dry milk for 2 h. Next, we incubated the membrane with primary antibodies at 4°C overnight. After that, wash it

Table I. Primer sequences for qRT-PCR.

Primer	Sequence
miR-491 forward	5'-ACACTCCAGCTGGGAGTGGGGAACCC
miR-491 reverse	5'-TGGTGTCGTGGAGTCG-3'
U6 forward	5'-CTCGCTTCGGCAGCACA-3'
U6 reverse	5'-AACGCTTCACGAATTTGCGT-3'
TPX2 forward	5'-ACCTTGCCCTACTAAGATT-3'
TPX2 reverse	5'-AATGTGGCACAGGTTGAGC
GAPDH forward	5'-ACCTGACCTGCCGTCTAG
GAPDH reverse	5'-TCCACCACCCTGTTGCT

U6: small nuclear RNA, snRNA; TPX2: targeting protein for Xklp2; GAPDH: glyceraldehy osph ehydrog se.

with TBST, followed by secondary antibody incubation for 2 h at room temperature. Primary antibodies involved in this assay were as follows: anti-TPX2 (1:1000; ab71816; Abcam, Cambridge, MA, USA); anti-GAPDH (1:1000; ab9485; Abcam, Cambridge, MA, USA). The secondary antibody was Anti-Rabbit IgG (1:4000; ab150077; Abcam, Cambridge, MA, USA). Protein levels were measured by a chemiluminescent detection system (Beyotime, Shanghai, China). GAPDH was an internal reference.

Transwell Assays

After treated with miR-491 mimics, inh or TPX2 siRNA, MCF-7 cell lines were see in the top chamber. For invasio migratio assays, transwell chambers w ted with or without matrigel (BD ience ranklin Lakes, NJ, USA), respec . The t mber contained a serum-fre tom chamber conta DM edium with FBS. After incub for 48h a the cells remained on the face were e ed by a cotton swab. time, those adhered to the bottom surface were using 4% formaldehyde a ained with 0.1 rystal violet for detecti e images using a roscope (Olympus oratio kyo, Japan).

Luci eporte say

The a ed T wild- or mutant-type cont miR-491 target sites was inse into th MIR-GLO luciferase reporter vec (Ambion, Foster City, CA, USA). Then, a he manufacturer's protocols, Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA,) was used to transfect miR-491 mimics a iferase reporter vectors of the wide type or mutant type 3'UTR of TPX2 gene into MCF-7 cells. Dual-Luciferase Reporter assay system (Promega, M , WI, USA) ed to measure the l ra tivities of th MCF-7 48 h after the t nsfectio

stical Analysis

ll the above periments were performed 3 s. The statis l analysis was evaluated by t aphPad P n 6 (GraphPad Software, La Jolla together with Statistical Product and ce Solutions (SPSS) 18.0 version (SPSS Inc. Chicago, IL, USA). Student's *t*-test lied to evaluate the differences between e t groups. The data was indicated as means ± SD (standard deviation). The differences were identified as statistically significant when $p<0.05$.

Results

MiR-491 Expressions Was Reduced and TPX2 Expressions Was Up-Regulated in BC

To confirm the effects of miR-491 in BC, we first measured the miR-491 expressions in BC tissues and cell lines respectively. The results of qRT-PCR indicated that the miR-491 expressions in BC tissues declined significantly in contrast to that in normal breast tissue samples (Figure 1A). Additionally, to confirm miR-491 expressions were downregulated in BC, we further detected the miR-491 expression in breast cancer MCF-7 cells using qRT-PCR, the results indicated that miR-491 expression was also decreased significantly in contrast with the normal MCF10A cells (Figure 1B). Furthermore, the TPX2 expression level was measured in BC cell lines. The qRT-PCR analysis showed significantly higher mRNA levels of TPX2 in MCF-7 cells in contrast with that in normal MCF10A cells (Figure 1C). Spear-

man's correlation analysis disclosed a negative correlation between miR-491 and TPX2 expressions (Figure 1D).

MiR-491 Inhibited BC Cell Invasion and Migration

Transwell invasion and migration assays were conducted to determine whether miR-491 had effects on the regulation of BC metastasis. First, miR-491 mimics or inhibitor was transfected into MCF-7 cell lines and the efficiency was confirmed by RT-qPCR. Results revealed that the miR-491 expressions in MCF-7 cell lines treated with miR-491 mimics were up-regulated while that in MCF-7 cell lines with miR-491 inhibitor transfections was down-regulated (Figure 2A). We found that, compared to control group, treatment with the miR-491 mimics could significantly reduce the invasion ability of MCF-7 cells (Figure 2B and 2D). Moreover, by comparison with the control group, miR-491 overexpression also could repress the migration capacity of MCF-7 cells significantly (Figure 2C and 2E). [illegible] the above findings suggested that miR[illegible] a suppressive role in BC cell lines.

MiR-491 Targeted TPX2 and [illegible]ced TPX2 Expression in BC [illegible]l Line

Potential target genes [illegible]iR-491 we[illegible] out using TargetScan. [illegible]xplore [illegible]he mole[illegible] functions of miR-491 [illegible]nhibit[illegible] breast cancer cell invasion and mig[illegible] [illegible]own i[illegible]gure 3A, the TPX2 3[illegible]R [illegible] a put[illegible] miR-491 binding si[illegible] ubsequen[illegible]cif[illegible]e report-er assays w[illegible]ried out to d[illegible]ne whether TPX2 wa[illegible]ar[illegible]ne of miR-49[illegible]. MCF-7 cell lines were [illegible]otransf[illegible] with luciferase reporter vector[illegible]ntaining T[illegible]'UTR-Wt or TPX2-3'U[illegible]t, along with m[illegible]491 mimics. Results i[illegible]ated that, compared with the control group,

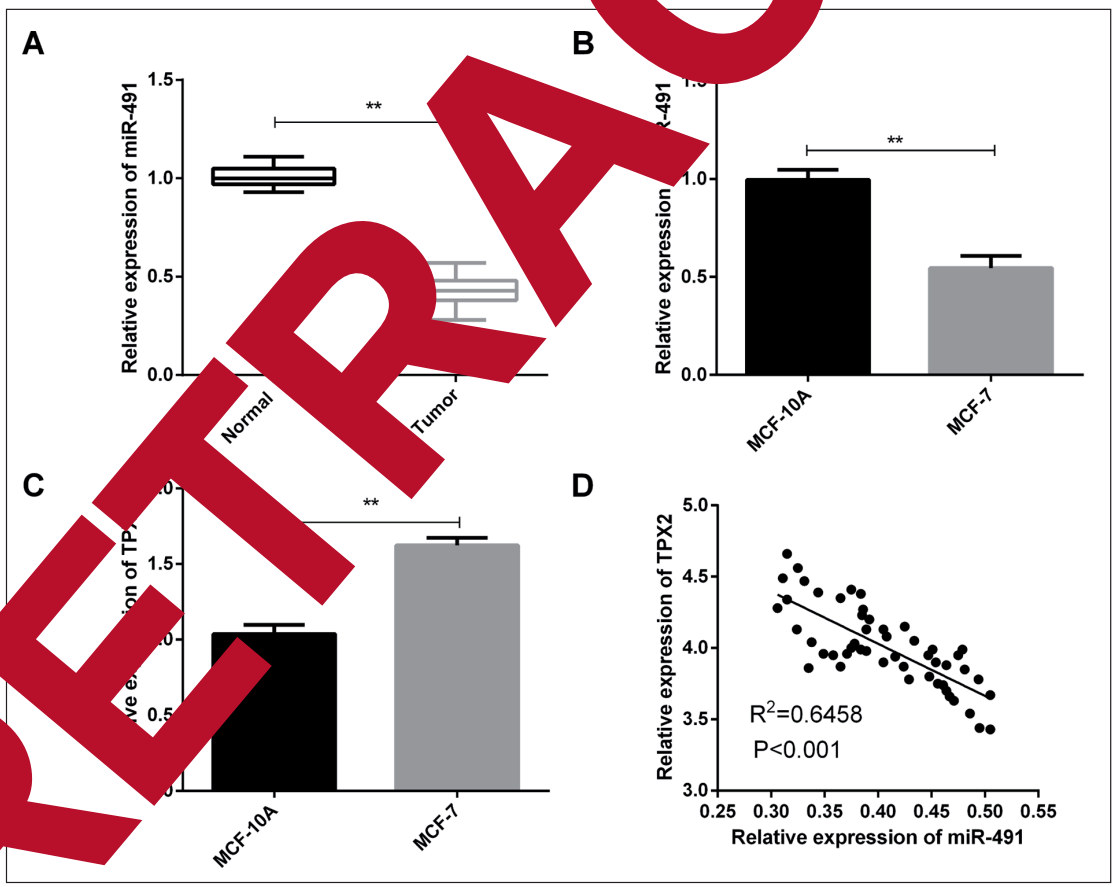

Fig. 1. MiR-491 expression reduced and TPX2 expression elevated in BC. **A,** QRT-PCR was applied to measure the miR-491 [illegible]essions in BC tissues (n=52) (**p<0.01). **B,** MiR-491 expressions in BC cells were decreased by qRT-PCR (**p<0.01). **C,** TPX2 expression was measured using qRT-PCR in breast cancer cells (**p<0.01). **D,** Regression analysis of correlation between miR-491 and TPX2 expressions.

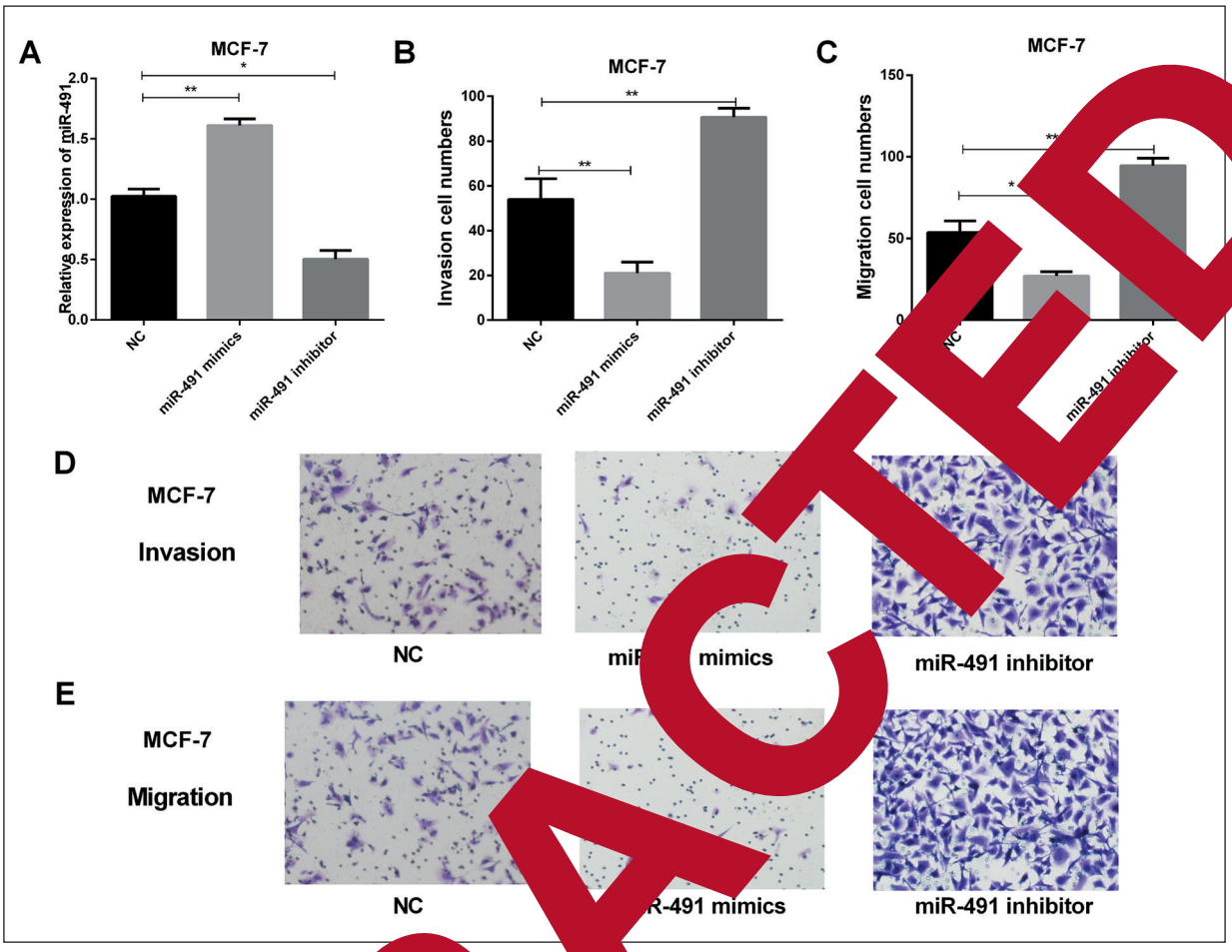

Figure 2. MiR-491 suppressed BC ... and mig... **A,** MiR-491 expressions in transfected breast cancer cells were measured using qRT-PCR ...05, * ...01). **B,** In... on cell numbers of BC cells were counted (**$p<0.01$). **C,** Migration cell numbers of BC c... ere coun... $^*p<0.05$, **$p<0.01$). **D,** Cell invasion was observed by the transwell assay in transfected BC cells. **E,** Trans... ay w... fect cell migration in transfected BC cells (magnification: 40×).

miR-491 mimic... significantly ...ase the luciferase act... irGLO-TPX...-3' UTR Wt (Figure 3B), nevert... the luciferase activities of ...irGLO-TPX2 ...R Mut were not affecte... miR-491 mimics, suggesting that miR... boun... the TPX2 3'UTR directly. M... r, qR... CR an... Western blotting were next c... to mea... the TPX2 expressions ...MCF-... lines ...ch were transfected with ...91 mi... ...ibitor. Results of qRT-PCR and ...estern b... g both indicated that, compar... with that in control group, miR-491 restor... / cell lines suppressed the TPX2 ...pression; on the contrary, the down expression ...iR-491 in MCF-7 cell lines resulted in a sig... increase in TPX2 expressions (Figure 3C and ...D). Taken together, TPX2 was identified to be a new target of miR-491 in breast cancer.

Knockdown of TPX2 Markedly Reversed MiR-491-Medicated Inhibition of Cell Invasion and Migration in BC Cell Lines

To elucidate the mechanism of TPX2 in suppressing cell invasion and migration medicated by miR-491in breast cancer, we further transfected TPX2 siRNAs into MCF-7 cells to delete endogenous TPX2, along with the transfections with miR-491 inhibitor into MCF-7 cell lines. We next examined the mRNA and protein expressions in miR-491 down-regulated MCF-7 cells or miR-491 down-regulated and TPX2-silenced MCF-7 cells. Results showed that, compared to the control group, TPX2 expressions in MCF-7 cells cotransfected with TPX2 siRNAs and miR-491inhibitor were significantly decreased (Figure 4A and 4B). Then, we next performed transwell assays to detect the effects of TPX2

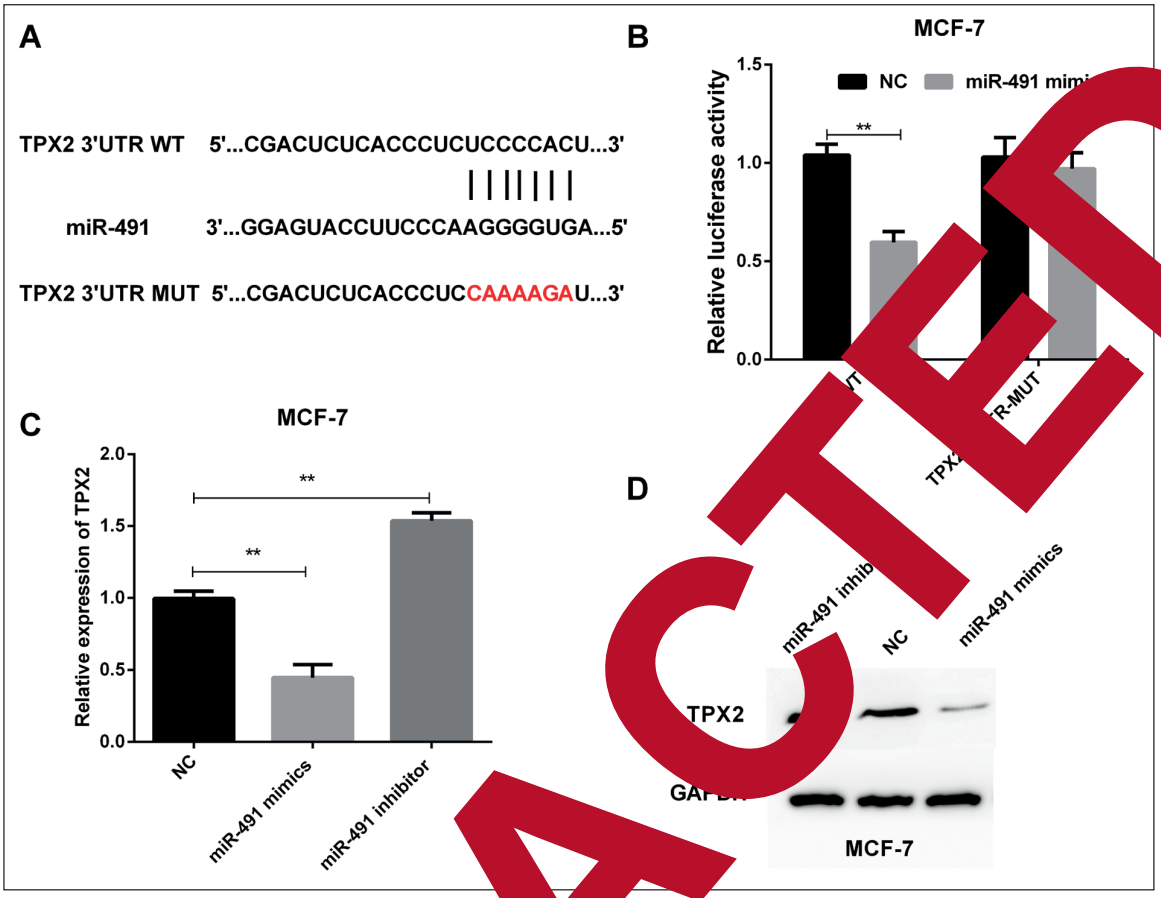

Figure 3. MiR-491 de-regulated TPX2 expression throu ing its 3'UTR directly. **A,** Target site of miR-491 in the TPX2 3'UTR. **B,** Luciferase reporter gene as arried out ct the fluorescence activities of the TPX2 3'UTR in BC cells which were co-transfected with wil 3'UTR or ional type TPX2 3'UTR and miR-491 mimics, respectively (**p<0.01). **C,** TPX2 mRNA exp ns in t fected BC was determined by qRT-PCR (**p<0.01). **D,** TPX2 protein expressions in transfected BC were eval by Western blotting.

on BC invasion a migration. retely, the invasion and mi capacities F-7 cell lines co-tran ed TPX2 siRNAs and miR-491inhibitor were red and the results demonstr that the delet TPX2 markedly reverse R-491 inhibitor-m cated promotion of ce vasion migration in MCF-7 cell lines (Fi 4D a E). Col ctively, these data sug-gested 2 may rse the partial function iR-4 MCF ll lines.

Discussion

As we all know, BC is one of the primary es of tumor deaths in females worldwide[18]. P ing tumor growth and metastasis is the centr problem in breast cancer treatments. However, sometimes the examination is too late on account of metastasis may have already occurred at that moment[19]. Hence, exploring a new biomarker for earlier diagnosis of BC with metastasis or recurrence is an emergency. miRNAs are new approaches to develop the novel tumor therapies, suppressing tumor relapse, treatment resistance, and metastasis[20]. MiRNAs have been considered to have crucial functions in cancer development because expressions of miRNAs in tumors are different from normal tissues, and miRNAs may have a unique role in tumor regulation[21].

Li et al[22] indicated that the function of miRNA varies with tumor type, even in the same tumor type. MiR-491 has relevance to tumorigenesis in a wide range of tumors, such as colorectal cancer[23], gastric cancer[24], and hepatocellular cancer[25]. The current study aimed to explore the functions of miR-491 in BC and the findings

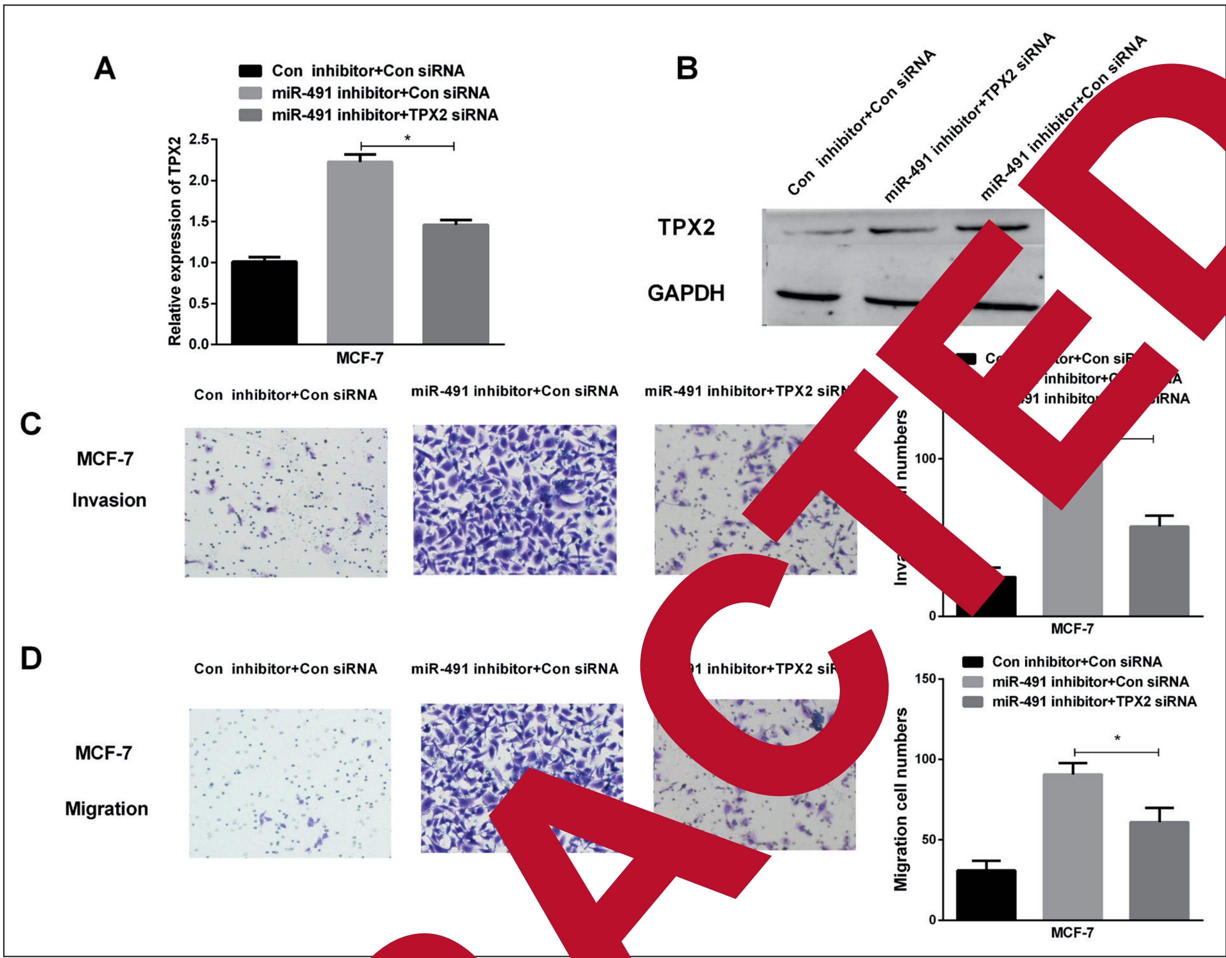

Figure 4. Depletion of TPX2 cou[illegible]erse p[illegible] function [illegible]R-491 in BC cells. **A, B,** TPX2 mRNA or protein expression level was detected by western bl[illegible]qRT-PC[illegible]BC cells co-[illegible]nsfected with TPX2 siRNA and miR-491 inhibitor (*p<0.05). **C, D,** Transwell assays were c[illegible]out to de[illegible]migration abilities of BC cells co-transfected with TPX2 siRNA and miR-491 inhibitor (ma[illegible]40×[illegible]0.01).

revealed that [illegible] was down[illegible]ated in BC cells; mo[illegible]er, [illegible]rther deter[illegible]ned that over-expres[illegible] of miR-[illegible]uld inhibit the activities of [illegible] cells by targ[illegible] TPX2. In short, the fin[illegible] of this research [illegible]monstrated that miR-[illegible] plays i[illegible]rtant roles in tumor developme[illegible]ludi[illegible]reast c[illegible]ncer. Qi et al[26] support our fi[illegible]r exam[illegible] miR-491 was reported [illegible]have [illegible]ctio[illegible] regulating glioma cells [illegible]ratio[illegible]ing TRIM28; Wang et al[27] fou[illegible]at miR-[illegible] inhibited lung metastasis and che[illegible]istance in osteosarcoma via regulating a[illegible]in directly.

[illegible]PX2 is a microtubule-associated protein, [illegible]recently, increasing studies have shown that T[illegible]s associated with the tumorigenesis of many tumors including breast cancer[28]. Being reported to be over-expressed in many cancers, TPX2 is considered to be a new candidate for malignant tumors diagnosis as well as prognosis[29]. Therefore, seeking a TPX2 gene-targeted therapy for breast cancer may be a curative method which would alleviate associated side effects. The current study demonstrated that TPX2 was a direct target of miR-491, in addition, the findings of this study also found that knockdown of TPX2 significantly inhibited human BC cell invasion and migration.

Conclusions

We found that the down-regulation of miR-491 in our 52 BC tissue samples and in one human BC cell line. In addition, we identified that TPX2 was a functional and direct target

of miR-491. Moreover, the findings also revealed that TPX2 inhibition may reverse the partial function of down-regulated miR-491-induced cell migration and invasion in BC. All the above findings demonstrated a regulatory function of miR-491 on TPX2 by base-pairing with its 3'UTR, suggesting that miR-491 might be an effective biomarker and therapeutic strategy for breast cancer treatment in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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