

# Long non-coding RNA BANCR indicates poor prognosis for breast cancer and promotes cell proliferation and invasion

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**Abstract.** – **OBJECTIVE:** We investigated the expression of human long non-coding ribonucleic acid (lncRNA), BRAF-activated non-coding RNA (BANCR) in breast cancer tissues and its effects on the in vitro proliferation, apoptosis, invasion and metastasis of breast cancer cells; therefore, we investigated its possible mechanism.

**PATIENTS AND METHODS:** The expression of BANCR in 65 pairs of breast cancer tissues and para-carcinoma normal breast tissues were detected by Real-time fluorescence quantitative polymerase chain reaction (qRT-PCR). Expressions of BANCR in normal breast tissues (MCF10A) and breast cancer cell lines (MCF-7, MDA-MB-231, SKBR3 and BT-20) were also detected. The knockdown efficiency of BANCR in all interfering RNA (siRNA) MCF-7 cells was detected by qRT-PCR. The effects of BANCR knockdown on proliferation, apoptosis, invasion and metastasis capacities of MCF-7 cells were explored via methyl thiazol tetrazolium (MTT) proliferation assay, cell colony formation assay, fluorescence-activated cell sorting (FACS) and transwell migration assay. Western blotting was used to detect the changes in expression of apoptosis-related proteins, epithelial-mesenchymal transition (EMT)-related proteins and matrix metalloproteinases (MMPs) after knockdown of BANCR.

**RESULTS:** The expression level of lncRNA BANCR in breast cancer tissues was significantly higher than that in para-carcinoma normal tissues. The prognosis of patients in low-expression BANCR group was significantly superior to that of patients in high-expression BANCR group. After BANCR knockdown in breast cancer MCF-7 cells, the cell proliferation and colony formation capacities were significantly inhibited. Further mechanism analysis showed that inhibiting BANCR could promote the apoptosis of MCF-7 cells. Results of Western blotting revealed that the expressions of B-cell lymphoma 2 associated X protein (BAX), cleaved-Caspase-3 and cleaved-poly adenosine diphosphate-ribose polymerase (PARP) in knockdown group were significantly up-regulated compared with those in control group. Both wound-healing assay and transwell migration assay showed that the down-regulation of lncRNA BANCR could inhibit the invasion and metastasis capacities of MCF-7 cells, whose mechanism was related to the inhibition of EMT process and down-regulation of MMP expressions in cells.

**CONCLUSIONS:** LncRNA BANCR is highly expressed in breast cancer, which is significantly correlated with the prognosis of patients; moreover, it can promote the growth, invasion and metastasis of ovarian cancer cells. The down-regulation of BANCR can inhibit the proliferation, invasion and metastasis capacities of MCF-7 cells.

**Key Words**

Breast cancer, lncRNA, BANCR, Proliferation, Invasion.

## Introduction

Breast cancer is one of the common malignant tumors in women, whose incidence rate is increasing year by year<sup>1</sup>. According to the global tumor data released by the American Cancer Society,

there were about 1,676,600 new cases of breast cancer in 2012, accounting for 25% of all cancers; about 521,900 patients died of breast cancer, accounting for 15% of all cancers<sup>2</sup>. The prognosis of patients with breast cancer is poor, and metastases to the lymph nodes, liver, lung and other tissues occur easily. Therefore, screening and studying the new molecular targets for the treatment of breast cancer are still hot spots currently. Long non-coding ribonucleic acid (lncRNA) is a new type of RNA molecule that does not encode proteins, with about 200-200,000 nucleotides in length, which regulates the gene expression in the epigenetic inheritance, gene transcription and post-transcriptional level, and can interact with proteins and nucleic acids to be involved in the regulation of a variety of physiological and pathological processes<sup>3</sup>. lncRNA has become one of the hot spots in the life sciences field in recent years. Scholars<sup>4,5</sup> have shown that lncRNA is abnormally expressed in a variety of cancer cells and plays an important role in the occurrence, development, invasion, metastasis, etc., of cancer.

The abnormally-expressed BRAF-activated non-coding RNA (BANCR) is found in the lung cancer, lung cancer, gastric cancer, colorectal cancer, bladder cancer and esophageal cancer<sup>6-8</sup>. BANCR can regulate the cell proliferation, migration and invasion in these tumors, which may potentially exert carcinogenic or anti-cancer effect<sup>9</sup>. However, there have been no reports about the correlation between BANCR and breast cancer up to now. This study aimed to analyze the expression of BANCR in breast cancer and the correlation between its expression level and prognosis of patients with breast cancer. Besides, the effects of BANCR on biological functions of breast cancer cells and its mechanism were further explored via *in vitro* experiments.

## Patients and Methods

### Patients

105 pairs of breast cancer and para-carcinoma tissue samples were collected from 65 breast cancer patients receiving operation in our hospital from January 2008 to December 2014. All patients were pathologically diagnosed, and underwent no neoadjuvant chemotherapy, radiotherapy or other forms of specific tumor treatment before operation. All cases in this study were staged according to the breast cancer staging (8<sup>th</sup> edition) of American Joint Committee on Cancer (AJCC).

After surgical resection, samples were immediately frozen in liquid nitrogen, and then cryopreserved in a refrigerator at -80°C. This study was approved by the Ethics Committee of our hospital; all patients signed the informed consent.

### Materials

Anti-B-cell lymphoma 2 (Bcl-2), anti-Bcl-2 associated X protein (BAX), anti-Caspase-3, anti-poly adenosine diphosphate-ribose polymerase-1 (PARP), anti-E-cadherin, anti-N-cadherin, anti-epithelial cell adhesion molecule-1 (E-cadherin), anti-matrix metalloproteinase-2 (MMP-2), anti-MMP-9 and anti-actin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA); Hoechst 33342 and DAPI/propidium iodide staining kits were purchased from BD (Franklin Lakes, NJ, USA); methyl thiazolyl tetrazolium (MTT) kits were bought from Beyotime (Shanghai, China); Dexamethasone 2000 was purchased from Invitrogen (Carlsbad, CA, USA); real-time fluorescence quantitative polymerase chain reaction (qRT-PCR) SYBR Green kits were purchased from Roche (Basel, Switzerland); non-coding small interfering RNA (si-NC) and BANCR siRNA (si-BANCR) were synthesized by Nanjing GenScript Biotechnology Company (Nanjing, China); other experimental reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Cell Culture

MCF-7, MDA-MB-231, SKBR3, BT-20 and normal mammary epithelial cells MCF-10A were purchased from American Type Cell Culture (ATCC, Manassas, VA, USA). Cells were incubated using Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin double-antibody solution and 1% non-essential aminoacids in an incubator with 5% CO<sub>2</sub> at 37°C. The cell growth was observed every day, and the solution was replaced once every 2 d. When about 80-90% cells were fused, cells were sub-cultured at a ratio of 1:1, and cells in the logarithmic growth phase were taken for experiments.

### qRT-PCR

The total mRNA was extracted from cells using TRIzol and reversely transcribed into complementary DNA (cDNA). The conditions of reverse transcription reaction are as follows: 25°C for 10 min, 50°C for 30 min and 85°C for 5 min. The detection was done by using the fluorescence quantitative PCR kit. Primer sequences of BANCR: forward primer:

5'-ACAGGACTCCATGGCAAACG-3', reverse primer: 5'-ATGAAGAAAGCCTGGTGCAGT-3'. Primer sequences of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (internal reference): forward primer: 5'-GGTCTCCTCTGACTTCAA-CA-3', reverse primer: 5'-AGCCAAATTCGTTGT-CATAC-3'. Conditions of fluorescence quantitative PCR are as follows: 95°C for 5 min, 95°C for 15 s and 60°C for 1 min; a total of 40 cycles. The solubility curve temperature was set to 60-95°C, and three repeated wells were set for each sample.

#### **Detection of Cell Proliferation**

MCF-7 cells were transfected with si-NC and si-BANCR. After 48 h of transfection, cells were collected, and the concentration of cell suspension was adjusted. Then, cells were added into a 96-well plate, making the cell densities in si-NC group and si-BANCR group 6000 cells/well; cells were cultured in an incubator with 5% CO<sub>2</sub> at 37°C. The cell proliferation in each group was detected using MTT kit at 0 h, 24 h, 48 h and 72 h. The optical density value was read at the wavelength of 490 nm, and the cell growth curve was drawn. The cell proliferation in both groups was detected by cell colony formation assay. At 48 h of transfection of MCF-7 cells, cells were collected, inoculated into a 6-well plate (300 cells/well), and cultured in an incubator with 5% CO<sub>2</sub> at 37°C for another 12 d. After 12 d of culture, cells were washed with phosphate buffered saline (PBS), fixed with 10% formaldehyde, stained with Giemsa and photographed. The cell colony containing more than 50 cells was counted.

#### **Hoechst 33342 Staining**

MCF-7 cells were transfected with si-NC and si-BANCR. After 48 h of transfection, cells were collected and inoculated into a 12-well plate. After culture for 24 h, the supernatant was discarded and cells were washed twice with PBS. Hoechst 33258 fluorescent dye was added, followed by incubation at 37°C for 15 min. After that, the fluorescent supernatant was discarded, and cells were washed twice with PBS, and photographed under an inverted fluorescence microscope.

#### **Detection of Apoptosis by Flow Cytometry**

MCF-7 cells were transfected with si-NC and si-BANCR. After 48 h of transfection, cells were collected, washed twice with PBS, and resuspended using 400  $\mu$ L binding buffer. Annexin V-fluorescein isothiocyanate (FITC) reagent was

added into the cell suspension and mixed evenly, followed by incubation in a dark place at room temperature for 15 min. Next, PI reagent was added and mixed evenly, followed by incubation in a dark place at room temperature for 15 min and submission for inspection within 1 h.

#### **Wound-healing Assay**

Cells were inoculated into a 6-well plate, and the plate was replaced with the serum-free DMEM after cell adherence. When 90-100% cells were fused, wounds were made uniformly using a 10  $\mu$ L spearhead perpendicular to the bottom of the 6-well plate. Then, cells were washed with PBS for three times to clear the floating cells, followed by incubation. At 24 and 72 h after wounding and culture, the migration distance of cells in the wound was observed under a microscope, and several different fields of view were randomly selected and photographed.

#### **Transwell Migration and Invasion Assay**

In migration assay, cells in two groups were collected, counted and resuspended in the serum-free DMEM solution. 100  $\mu$ L cell suspension was added into the upper chamber, while 100  $\mu$ L complete medium containing 10% fetal bovine serum (FBS) was added into the lower chamber, followed by incubation for 24 h. After fixation and staining, five fields of view were randomly selected and photographed under an inverted microscope. The number of cells passing through the membrane was counted, and the average was taken. In invasion assay, what was different from migration assay was that the transwell chamber was coated with a layer of Matrigel gel. After air-drying in a sterile environment, cells were inoculated. The remaining operations were the same as those in migration assay.

#### **Western Blotting**

Cells were washed with pre-cooled PBS, fully cleaved using radioimmunoprecipitation assay (RIPA) cell lysis buffer, and then centrifuged to take the supernatant. After protein quantification, 20  $\mu$ g total protein sample was taken and mixed with 5  $\times$  sodium dodecyl sulfonate (SDS) protein loading buffer, followed by denaturation at 100°C for 5 min. Then, the protein was loaded for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under 350 mA, after which the gel and activated polyvinylidene difluoride (PVDF) membrane were placed onto the membrane trans-

fer frame for membrane transfer under constant current for 2 h. PVDF membrane was removed and blocked with 5% skim milk powder for 1 h. The primary antibody was added for incubation at 4°C overnight, the membrane was washed with Tris-buffered saline and Tween 20 (TBST). Horseradish peroxidase (HRP)-labeled secondary antibody was added for incubation at room temperature for 1 h, and the membrane was washed again with TBST, followed by color development and gray value analysis using ImageJ software. The target protein/ $\alpha$ -actin represented the relative expression level of target protein.

### Cell Transfection

MCF-7 cells in the logarithmic growth phase were taken, digested and cultured in a 6-well plate. When about 70% cells were fused, 500  $\mu$ L transfection medium already prepared, opti-MEM, containing Lipofectamine 2000 and siRNA were added into each well. Cells in experiment group and control group were transfected with si-BANCR and si-NC, respectively, shaken evenly and incubated for 6 h. The original medium was discarded, and 2 mL complete medium were added into each well, followed by incubation for another 48 h for protein application. Sequence of si-BANCR: 5'-GCA-

CUCCAUGGCAAACGUUTT-3'; sequence of si-NC: 5'-AACGUUUGCCAUGGAUUCCTT-3'.

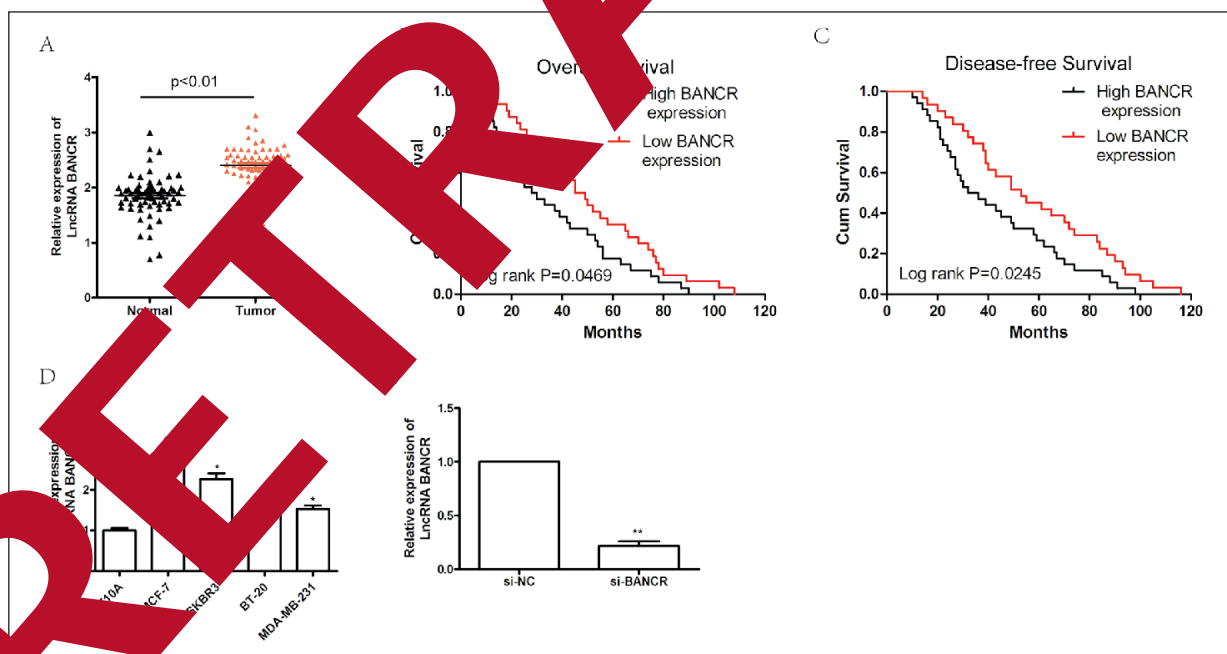
### Statistical Analysis

Statistical product and service solutions (SPSS) 10.0 software (Chicago, IL, USA) was used for statistical analysis. *t*-test was used for enumeration data. The survival rate was calculated using Kaplan-Meier method, and the survival curve was drawn. The survival rate was compared using Log rank test, and factors affecting prognosis were analyzed via Cox regression analysis. Significance level  $\alpha=0.05$ .

## Results

### BANCR was Highly Expressed in Breast Cancer Tissues and Cells

To explore the expression of BANCR in breast cancer, qRT-PCR was used to detect the expression level of lncRNA BANCR in 65 pieces of cancer tissues and para-carcinoma tissues. Results showed that, compared with that in normal tissues, the expression of BANCR was significantly increased in breast cancer tissues (Figure 1A,  $p<0.01$ ). After further analysis



**Figure 1.** Relative BANCR expression in breast cancer tissues and its clinical significance. **A**, Relative expression of BANCR in breast cancer tissues (n=65) and adjacent non-cancerous tissues (n=65) was examined by qPCR and normalized to GAPDH expression. **B-C**, Kaplan-Meier overall survival and disease-free survival curves according to BANCR expression levels. **D**, qRT-PCR analysis of relative BANCR expression levels in the MCF10A cell line and four breast cancer cell lines. **E**, Effective knockdown of BANCR in MCF-7 cells 48 h after siRNA treatment. (\* $p<0.05$ , \*\* $p<0.01$ ).

**Table IV.** Correlation between BANCR expression and clinicopathological characteristics of BC patients.

Characteristics	BANCR		$\rho$ X <sup>2</sup> -test p-value
	High No. cases (34)	Low No. cases (31)	
Age (years)			0.559
≤ 50	20	16	
> 50	14	15	
Tumor size			0.019 *
≤ 2 cm	12	20	
> 2 cm	22	11	
TNM stage			0.005 *
I + II	16	25	
III	18	6	
Lymph node metastasis			0.020 *
Negative	10	18	
Positive	24	13	
Her-2 status			0.772
Negative	23	22	
Positive	11	9	
ER status			0.615
Negative	13	10	
Positive	21	21	
PR status			0.123
Negative	15	8	
Positive	19	23	

of the relationship between BANCR expression level and clinicopathological features of breast cancer, it was found that the expression level of BANCR was significantly correlated with the size, tumor lymph node metastasis (TNM) staging and lymph node metastasis of breast cancer, but had no significant correlation with other clinical features (Table IV). Further survival analysis showed that the prognosis of patients in low-expression BANCR group was significantly superior to that of patients in high-expression BANCR group, and both disease-free survival (DFS) and overall survival were longer in low-expression BANCR group (Figure 1B-C). *In vitro* experiments also proved that the expression level of BANCR in normal breast epithelial MCF-10 cells was significantly lower than that in breast cancer cells, while the expression level in MCF-7 cells was relatively the highest (Figure 1D). In this study, MCF-7 cells with the highest expression level of BANCR were selected for subsequent transfections and studies. The inhibition efficiency at 48 h after si-BANCR transfection was verified by qRT-PCR. As shown in Figure 1E, si-BANCR was effective in inhibiting the expression of BANCR in ovarian cancer MCF-7 cells, and there was a statistically significant

difference compared with that in negative control group ( $p < 0.01$ ), so si-BANCR was used for the transfection in subsequent experiments.

#### **Down-regulation of BANCR Expression Inhibited the *In Vitro* Proliferation of MCF-7 Cells**

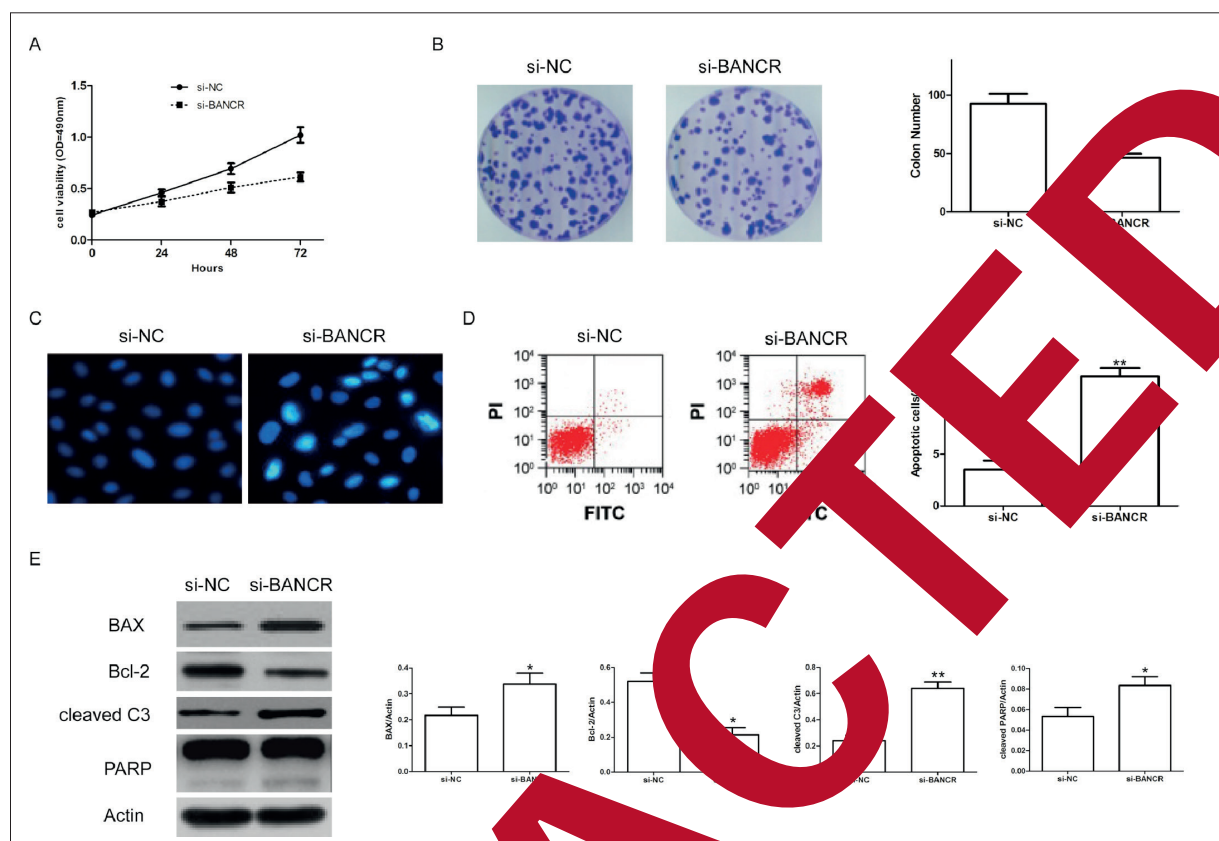
The effect of BANCR on *in vitro* proliferation of MCF-7 cells was verified via MTT assay and colony formation assay. After intervention with si-BANCR and si-NC, MCF-7 cells were cultured for 24 h, 48 h, and 72 h respectively, followed by MTT assay. Results showed that the growth rate of MCF-7 cells was inhibited after BANCR was knocked down (Figure 2A). Results of colony formation assay revealed that the number of colony formation in BANCR knockdown group was significantly decreased compared with that in control group (Figure 2B,  $p < 0.05$ ), suggesting that the down-regulation of BANCR expression can significantly inhibit the cell colony formation.

#### **Down-regulation of BANCR Expression Promoted the Apoptosis of MCF-7 Cells**

To further investigate the mechanism of BANCR1 knockdown in inhibiting the proliferation of MCF-7 cells, the effect of BANCR knockdown on cell apoptosis was verified. Hoechst 33342 staining results showed that the proportion of apoptotic cells in knockdown group was significantly increased compared with that in control group (Figure 2C). Results of flow cytometry revealed that the total apoptosis rate of cells in knockdown group was significantly increased compared with that in control group (Figure 2D,  $p < 0.05$ ). Moreover, Western blotting results (Figure 2E) showed that the expressions of Bax, cleaved-Caspase-3 and cleaved-PARP were up-regulated after knockdown of BANCR, while the expression of Bcl-2 was down-regulated, which were consistent with the above results. They suggest that inhibiting the BANCR expression can significantly up-regulate the ratio of Bax/Bcl-2, thereby increasing the expressions of cleaved-Caspase-3 and cleaved-PARP, activating the mitochondrial apoptosis pathway and promoting the cell apoptosis.

#### **Down-regulation of BANCR Expression Inhibited the Migration and Invasion Capacities of MCF-7 Cells**

The effects of BANCR on the migration and invasion of breast cancer cells were further analyzed. First, the wound-healing assay was used to detect the migration capacities of cells in control



**Figure 2.** Effects of BANCR on MCF-7 cells proliferation and apoptosis in vitro. **A**, MTT assays were performed to determine the proliferation of si-BANCR and si-NC transfected MCF-7 cells. **B**, Colony-forming assays were conducted to determine the proliferation of si-BANCR and si-NC transfected MCF-7 cells. **C-D**, Hoechst 33342 staining and flow cytometry were conducted to determine the apoptosis of si-BANCR transfected MCF-7 cells. **E**, The proteins levels of BAX, Bcl-2, cleaved-caspase3 and PARP were detected by Western blotting in si-BANCR transfected MCF-7 cells (\* $p < 0.05$ , \*\* $p < 0.01$ ).

group and BANCR knockdown groups. The results showed that after 72 h, the migration distance of cells in BANCR knockdown group was significantly shorter than in control group ( $p < 0.05$ , Figure 3A-B). Next, Transwell migration assay was performed, obtaining consistent results with the in wound-healing assay. After knockdown of BANCR, the number of cells passing through the filter membrane in the lower chamber was significantly reduced, and the difference was statistically significant ( $p < 0.05$ , Figure 3C-D).

**Knockdown of BANCR Expression Inhibited the Epithelial-mesenchymal Transition (EMT) Process and Expressions of MMP-2 and MMP-9 in MCF7 Cells**

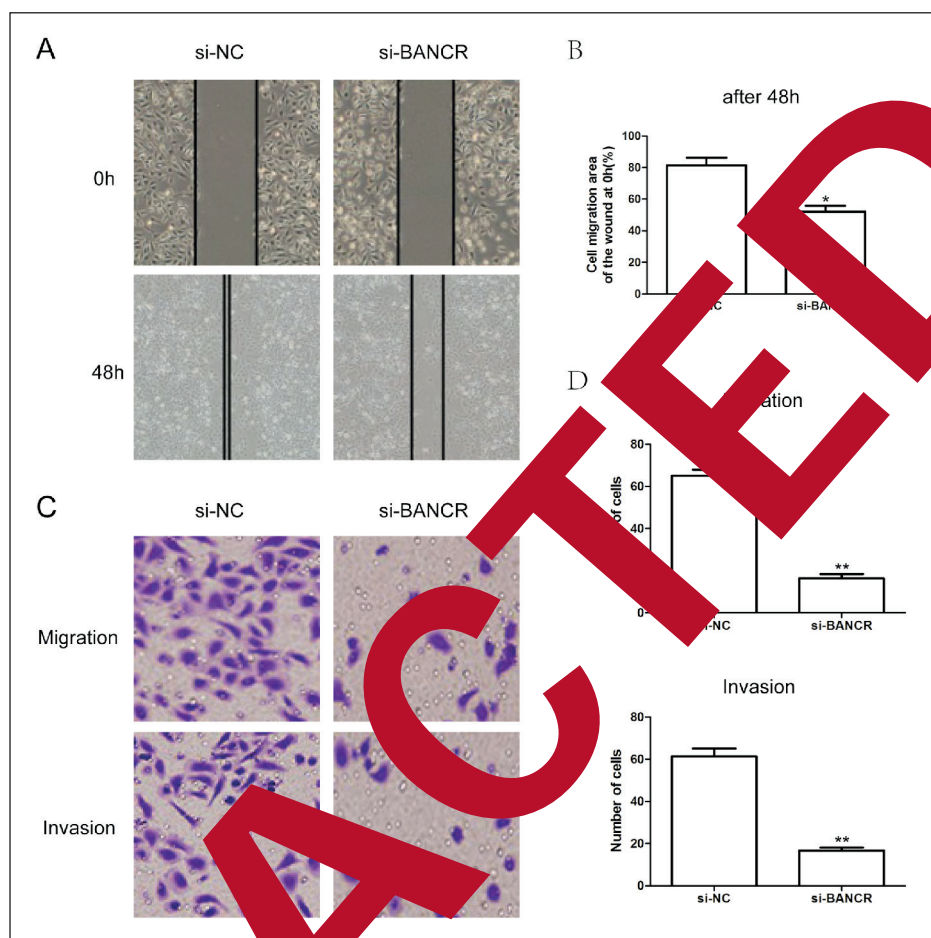
To further investigate the mechanism of BANCR knockdown in inhibiting the migration and invasive capacities of MCF-7 cells, the expressions of relevant molecules were further detected by Western blotting. As shown in Figure 4 after knock-

down of BANCR, the expression of E-cadherin was significantly up-regulated, but the expressions of N-cadherin and vimentin were down-regulated. It was further found that, after knockdown of BANCR, MMP-2 and MMP-9, expressions were also down-regulated in MCF-7 cells. The above results indicated that knockdown of BANCR can inhibit the cell migration through inhibiting the EMT process and expressions of MMPs in MCF-7 cell.

**Discussion**

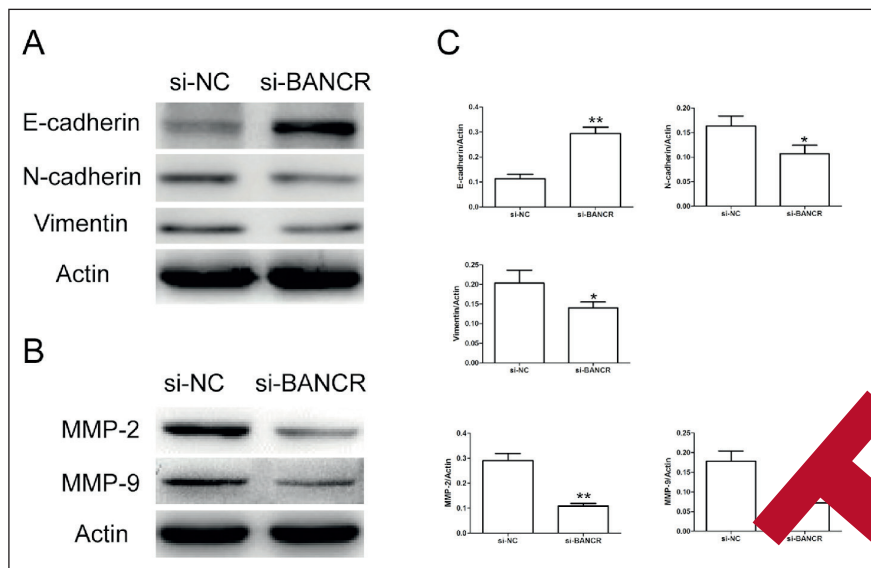
In recent years, lncRNAs, as a hot spot, has attracted widespread concern and research. BANCR was originally found in melanoma cells by Flockhart et al<sup>10</sup> in 2012, which is a lncRNA with 693 bp in length on chromosome 9. BANCR is abnormally expressed in a variety of cancers, such as lung cancer, hepatocellular carcinoma and colorectal cancer, which can regulate the cell proliferation,

**Figure 3.** Effects of BANCR on MCF-7 cells migration and invasion in vitro. **A-B**, MCF-7 cells were treated with si-BANCR and si-NC, and the effects on cell migration were determined with cell scratch test. **C-D**, The effects on cell migration and invasion were determined with cell transwell test. (\* $p < 0.05$ , \*\* $p < 0.01$ ).



migration and invasion. Besides, it is closely related to the occurrence and development of tumor and prognosis of patients, which is a novel type of biomarker for early diagnosis of cancer, and a new target for clinical diagnosis, treatment and prognosis prediction [11-13]. Li et al. [14] found that BANCR can reduce the proliferation of malignant melanoma cells via regulating the mitogen-activated protein kinase (MAPK) signaling pathway. Some studies have found that the expression of BANCR in liver cancer tissues is positively correlated with the expression of Vimentin, but negatively correlated with the expression of E-cadherin, suggesting that BANCR is highly expressed in liver cancer tissues, which is correlated with the expressions of EMT-related proteins, which may be involved in the migration and metastasis processes of liver cancer cells. Li et al. [12] found that BANCR can also induce EMT through MEK/ERK signaling pathway and promote the tumor cell invasion. Despite of the amazing results obtained in the research on BANCR, there have been no reports on the role of BANCR in breast cancer yet. In the present study,

it was confirmed by the detection of clinical samples of breast cancer that the expression of BANCR was significantly high in breast cancer, and the further analysis of clinicopathologic features of tumor showed that BANCR had significant correlations with the TNM staging, size and lymph node metastasis of breast cancer, suggesting that BANCR plays a potential role in promoting the occurrence and development of breast cancer. In cell experiments, it was found that knockdown of BANCR in breast cancer MCF-7 cells could inhibit the cell proliferation, whose mechanism was related to the mitochondrial apoptotic pathway induced after knockdown of BANCR. To further verify the relationship between BANCR and cell invasion and metastasis, wound-healing assay and transwell assay were performed to detect changes in cell invasion and migration capacities after knockdown of BANCR. The results revealed that knockdown of BANCR could inhibit the EMT process and the expressions of MMP-2 and MMP-9 in cells, thus inhibiting the invasion and migration capacities of MCF-7 cells.



**Figure 4.** Effects of BANCR on the proteins expression related to migration and invasion of MCF-7 cells. **A-B,** The levels of target proteins (E-cadherin, N-cadherin, vimentin, MMP-2 and MMP-9) measured by Western blotting in BANCR cells transfected with si-BANCR or si-NC. **C,** Downward are representative of 3 independent experiments. Data are presented as means  $\pm$  SE (n = 3). (\* $p < 0.05$ , \*\* $p < 0.01$ )w

### Conclusions

We showed that BANCR was highly expressed in breast cancer, and it is significantly associated with the prognosis of breast cancer. Moreover, knockdown of BANCR inhibited the proliferation, invasion and migration capacities of breast cancer, suggesting that BANCR plays a role as an oncogene in the occurrence and development of breast cancer, which is expected to become a molecular marker for the diagnosis and prognostic evaluation of breast cancer and a potential target for treatment.

### Acknowledgement

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### Conflict of Interest

The authors declared no conflict of interest.

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