

Synaptopodin-2 plays an important role in the metastasis of breast cancer via PI3K/Akt/mTOR pathway

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Introduction: Synaptopodin 2 (SYNPO2) is a functioning protein. It has been detected in many malignancies. But the relation between SYNPO2 and breast cancer (BC) is unclear.

Materials and methods: In this study, we explored the expression and function of SYNPO2 in BC. We found that *SYNPO2* gene in BC was downregulated at the transcriptional level in both validated and TCGA cohorts.

Results: The results revealed that age, lymph node metastasis, and clinical stage in the validated cohort were related to the expression of SYNPO2 negatively. Kaplan–Meier analysis showed that patients with lower SYNPO2 expression had a worse overall survival.

Discussion: We found that migration and invasion were promoted after knocking down SYNPO2 in MCF-7, MDA-MB-231, BT-549, and MDA-MB-468. Meanwhile, knockdown of SYNPO2 could enhance PI3K/AKT/mTOR signaling pathway, which may induce migration and invasion. Our findings reveal that SYNPO2 was associated with BC.

Keywords: breast cancer, SYNPO2, metastasis, PI3K/AKT/mTOR signaling pathway

Introduction

Breast cancer (BC) is the most common malignancy in the world in women.¹ Previous studies proved that metastasis of cancer cells was the major reason for cancer-related death.^{2,3} In recent years, advanced progress has been made, and the treatment options proposed included surgery, and endocrine, cytotoxic, or targeted therapies. However, the treatment is not always satisfactory.^{4–7} Therefore, it is important to probe the underlying mechanism of BC and find new biomarkers at an early stage.

In order to find new biomarkers in BC, we performed RNA sequencing of 8 pairs of BC and adjacent noncancerous tissues in our unpublished study. Thus, we found that synaptopodin 2 (SYNPO2) is lower in BC compared with adjacent noncancerous tissues. We downloaded and analyzed the RNA-seq data of BC from TCGA. In the differential genes analysis, we found the SYNPO2 was lower in BC compared with the normal tissues as well. So, we selected the SYNPO2 in BC for further experiments. SYNPO2, also known as myopodin, is the second identified member of the podin family, and the coding gene is located on chromosome 4q26. Previous studies have proved that SYNPO2 is expressed in numerous cancers including ovarian cancer, kidney cancer, and melanoma.^{9–11} SYNPO2 could enhance the assembly of peripheral actin bundles and immature focal adhesions to encourage prostate cancer cell migration.¹² De Ganck et al¹³ found that downregulation of SYNPO2 expression could reduce invasion and motility of PC-3 prostate cancer cells. SYNPO2 could induce prostate cancer cell migration,

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and it is associated with the formation of morphologically and biochemically distinct actin networks.¹⁴ In triple negative breast cancer, SYNPO2 could suppress metastasis via inhibition of YAP/TAZ activity.¹⁵ However, the biological functions of SYNPO2 in BC are not fully investigated.

In this study, we found that SYNPO2 was lower in BC tissues compared to adjacent nontumor tissues. The results revealed that age, lymph node metastasis, and clinical stage in the validated cohort were related to the expression of SYNPO2. Kaplan–Meier analysis showed that patients with lower SYNPO2 expression had a worse overall survival. Cell line experiments were performed to explore the role of SYNPO2 in BC. Thus, our results indicated that SYNPO2 may be a prognostic biomarker and therapeutic target for BC.

Materials and methods

Patients and breast tissue samples

We selected 38 BC patients who underwent primary surgical resection at the Department of Thyroid and Breast Surgery, The First Affiliated Hospital of Wenzhou Medical University. Patients signed informed consents, and research protocols for the use of tissues were approved by and conducted in accordance with the ethical standards of the Institutional Review Board of The First Affiliated Hospital of Wenzhou Medical University (approval no. 2012-57). All fresh tissues were snap-frozen in liquid nitrogen immediately and stored at -80°C until further RNA detection. We downloaded BC mRNA expression data from the TCGA data portal (<https://tcga-data.nci.nih.gov/docs/publications/tcga/>).

Cell cultures and growth conditions

MDA-MB-231, BT-549, SK-BR-3, MDA-MB-468, BT-474, T-47D, MCF-7, MDA-MB-453, MDA-MB-436, and MCF-10A cells were used in this study. These cells were obtained from Shanghai Cell Biology, Institute of the Chinese Academy of Sciences (Shanghai, People's Republic of China). MDA-MB-231, SK-BR-3, T-47D, and MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco). MDA-MB-468, MDA-MB-453, and MDA-MB-436 cells were cultured in L-15 medium (Gibco) supplemented with 10% FBS (Gibco). BT-549 and BT-474 cells were cultured in Roswell Park Memorial Institute 1640 medium (Gibco) supplemented with 10% FBS (Gibco). MCF-10A cells were cultured in Dulbecco's Modified Eagle Medium-F12 (Gibco) supplemented with 100 U/mL of penicillin, 100 $\mu\text{g}/\text{mL}$ of streptomycin, 2 mM of L-glutamine,

20 ng/mL of epidermal growth factor, and 10% FBS (Gibco). MDA-MB-468, MDA-MB-453, and MDA-MB-436 cells were incubated in a standard cell culture incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C without CO_2 . The others were incubated in a humidified incubator at 37°C with 5% CO_2 .

Cell transfection

MCF-7, MDA-MB-231, and BT-549 cells were transfected with the Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific). Around 100,000 BC cells were plated 24 h before transfection. SYNPO2 was silenced by siRNA. The siRNA sequences used in the study are SYNPO2 siRNAs that target the following sequences: forward 5'-GCCUCCAGAG-GAUUGGAAUTT-3' and reverse 5'-AUUCCAAUCCUCUG-GAGGCTT-3'. Both siRNAs were provided by GenePharma (Shanghai, People's Republic of China).

RNA extraction and quantitative real-time PCR

According to the manufacturer's instructions (Thermo Fisher Scientific), total RNA was extracted using TRIzol[®] reagent. The isolated RNA was measured at 260/280 nm using spectrophotometry (Thermo Fisher Scientific). RNA samples were stored at -80°C . Real-time reactions were run and analyzed by using the ABI 7500 quantitative PCR System (Applied Biosystems, Foster City, CA, USA). The relative expression of mRNA was calculated using the comparative cycle threshold (CT) ($2^{-\Delta\text{ACT}}$) method with GAPDH as the endogenous control to normalize the data. The sequences of the primers used were as follows:

SYNPO2 forward: 5'-ATGAAGCACCAACCGTATC-3' and reverse: 5'-CTGAATTGACCTTGACTGATG-3'; GAPDH forward: 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse: 5'-ACCACCCTGTTGCTGTAGCCAA-3'.

Invasion and migration assay

For cell invasion assays, cells were collected in a medium containing 10% FBS. Invasion of cells was measured in Matrigel (BD Bioscience, Franklin Lakes, NJ, USA). A total of 40,000 cells ($\sim 250 \mu\text{L}$) transfected with si-NC or si-SYNPO2 were transferred into the upper chamber, and 600 μL medium containing 20% FBS was added to the lower chamber. Then, the plate was placed in the incubator with 5% CO_2 atmosphere. After 24 h, the membrane was carefully removed and fixed with 4% paraformaldehyde and stained with 0.4% crystal violet solution for 15 min. Motility assays

were similar to the invasion assay. Cell migration and invasion were counted in 5 random fields, and images were captured under a microscope at a magnification of 20 \times .

Western blot analysis

Whole-cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (BioRad, Berkeley, CA, USA) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The blots were then blocked with 5% nonfat milk for 2 h at room temperature. According to the manufacturer's protocol, the blots were probed with polyclonal antibody at 4°C overnight. The blots were then incubated with the secondary antibody (Abcam, Cambridge, MA, USA) for 1 h at room temperature. Primary antibodies used were as follows: PI3K, p-PI3K, AKT, p-AKT (Ser473), mTOR, and p-mTOR (Cell Signaling Technology, Danvers, MA, USA).

Statistical analysis

All statistical analyses were performed using SPSS 23.0 software (IBM Corporation, Armonk, NY, USA); data are presented as mean \pm standard error; differences were considered to be statistically significant at $P < 0.05$. Student's *t*-test (2-tailed) was performed to analyze differences between groups.

Results

SYNPO2 is downregulated in BC

The expression of SYNPO2 was determined in 38 pairs of BC samples and matched adjacent noncancerous tissues.

The results showed that the expression of SYNPO2 was significantly downregulated in the BC samples compared with the matched adjacent noncancerous tissues (T: $N = 2.38 \pm 2.58$; 15.24 ± 16.58 , $P < 0.001$) (Figure 1A). To further validate the downregulated nature of SYNPO2, we download RNA sequencing data of BC from TCGA database. We found the same trend in TCGA cohort (T: $N = 97,779.22 \pm 19,8062.31$; $34,4798.48 \pm 226713.44$, $P < 0.001$) (Figure 1B). These results indicated that SYNPO2 was related to BC.

The relationship between SYNPO2 expression and clinical features

To better understand the relation between SYNPO2 and BC, we investigated the relationship of SYNPO2 with clinicopathologic features. In the validated cohort, we divided all patients into the low-expression group ($n = 19$) and the high-expression group ($n = 19$) according to the median value. The results showed age ($P = 0.036$), lymph node metastasis ($P = 0.023$), and clinical stage ($P = 0.027$) were related with the expression of SYNPO2 negatively (Table 1). In the TCGA cohort, the patients were divided into low-expression group and high-expression group according to the same median value. The results revealed that clinical stage ($P = 0.026$) and age at diagnosis ($P = 0.0000$) were significantly related to the SYNPO2 expression (Table 2). We downloaded the overall survival of 1,227 BC patients from TCGA and found that the difference between the survival curves of the 2 groups was significant ($P = 0.0381$) (Figure 2). These results indicated that low SYNPO2 expression may influence the ability of

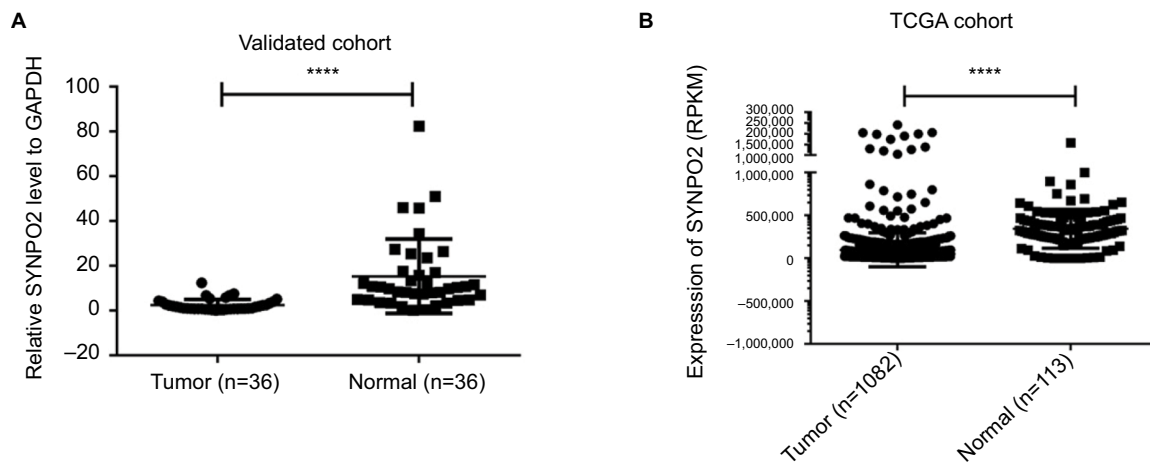


Figure 1 SYNPO2 expression in BC in validated cohort and TCGA cohort.

Notes: (A) SYNPO2 expression was examined by qRT-PCR in 38 paired human BC tissues and adjacent noncancerous tissues (paired *t*-test, $P < 0.001$). A logarithmic scale of $2^{-\Delta\Delta C_t}$ is used to represent the fold change in quantitative real-time PCR detection. (B) The TCGA cohort contained 1,100 breast tumor tissues and 113 normal tissues. RPKM was represented for expression of SYNPO2. The analysis was done using the Mann–Whitney *U*-test. **** $P < 0.0001$.

Abbreviations: BC, breast cancer; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RPKM, reads per kilobase per million; SYNPO2, synaptopodin 2.

Table 1 The relationship between SYNPO2 expression and clinicopathological characteristics in the validation cohort (n=38)

Clinicopathologic characteristics	Low expression (%)	High expression (%)	X ²	P-value
Age (years)			4.378	0.036*
≤60	18 (94.7)	13 (68.4)		
>60	1 (5.3)	6 (31.6)		
Tumor size (cm)			1.029	0.598
<2	12 (63.2)	9 (47.4)		
2–5	6 (31.6)	9 (47.4)		
>5	1 (5.3)	1 (5.3)		
Lymph node metastasis			5.158	0.023*
No	6 (31.6)	13 (68.4)		
Yes	13 (68.4)	6 (31.6)		
Distant metastasis			1.027	0.311
No	18 (94.7)	19 (100)		
Yes	1 (5.3)	0		
Estrogen receptor			0.106	0.744
Negative	8 (42.1)	9 (47.4)		
Positive	11 (57.9)	10 (52.6)		
Progesterone receptor			0.106	0.744
Negative	9 (47.4)	8 (42.1)		
Positive	10 (52.6)	11 (57.9)		
Her-2 receptor			0.432	0.511
Negative	7 (36.8)	9 (47.4)		
Positive	12 (63.2)	10 (52.6)		
Clinical stage			4.886	0.027*
I–II	11 (57.9)	17 (89.5)		
III–IV	8 (42.1)	2 (10.5)		

Note: *P<0.05.

Abbreviation: SYNPO2, synaptopodin 2.

Table 2 The relationship between SYNPO2 expression and clinicopathological characteristics in the TCGA cohort (n=1,071)

Clinicopathologic characteristics	Low expression (%)	High expression (%)	X ²	P-value
Age (years)			12.927	0.000*
≤60	265 (49.4)	323 (60.4)		
>60	271 (50.6)	212 (39.6)		
Tumor size (cm)			5.368	0.068
<2	122 (22.8)	151 (28.2)		
2–5	329 (61.4)	293 (54.8)		
>5	85 (15.8)	91 (17.0)		
Lymph node metastasis			6.339	0.096
N0	280 (52.5)	241 (48.6)		
N1	170 (31.7)	185 (33.1)		
N2	52 (9.7)	68 (11.2)		
N3	34 (6.3)	41 (7)		
Distant metastasis			1.659	0.198
No	522 (97.4)	527 (98.5)		
Yes	14 (2.6)	8 (1.5)		
Clinical stage			4.945	0.026*
I–II	408 (76.1)	375 (70.1)		
III–IV	128 (23.9)	160 (29.9)		

Note: *P<0.05.

Abbreviation: SYNPO2, synaptopodin 2.

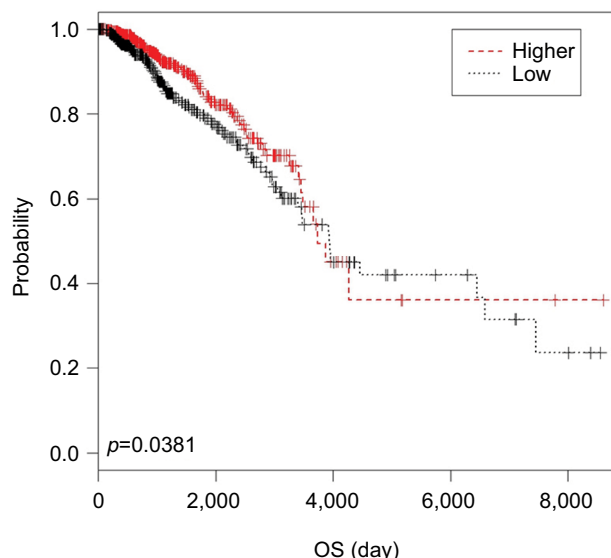


Figure 2 Kaplan–Meier analysis for the overall survival (OS) of BC patients with different expressions of SYNPO2 in TCGA cohort.

Note: Patients with low SYNPO2 expression had a shorter OS than those with high expression.

Abbreviations: BC, breast cancer; SYNPO2, synaptopodin 2.

migration in BC cell, and it was associated with unfavorable prognosis in BC.

Downregulation of SYNPO2 could inhibit MCF-7, MDA-MB-231, BT-549, and MDA-MB-468 migration and invasion

To further confirm the function of SYNPO2 in BC, we assessed SYNPO2 expression level in BC cell lines and normal breast cell line through quantitative reverse transcription (qRT)-PCR. We found SYNPO2 expressed at a higher level in MCF-7, MDA-MB-231, BT-549, and MDA-MB-468 than in other breast cell lines (Figure 3A). So, we selected MCF-7, MDA-MB-231, BT-549, and MDA-MB-468 as the research cell lines. Then we knocked down SYNPO2 expression in MCF-7, MDA-MB-231, BT-549, and MDA-MB-468 using siRNA. As shown in Figure 3B and C, both mRNA and protein levels of SYNPO2 were significantly reduced.

In the clinical feature analysis, SYNPO2 expressed at low levels was associated with lymph node metastasis, so we continued to demonstrate the role of SYNPO2 in BC through migration and invasion assay. Our study indicated that knocking down SYNPO2 could significantly enhance the migration capacity of MCF-7, MDA-MB-231, BT-549, and MDA-MB-468 compared with the control groups (Figure 4). The invasion assays also showed that downregulated SYNPO2

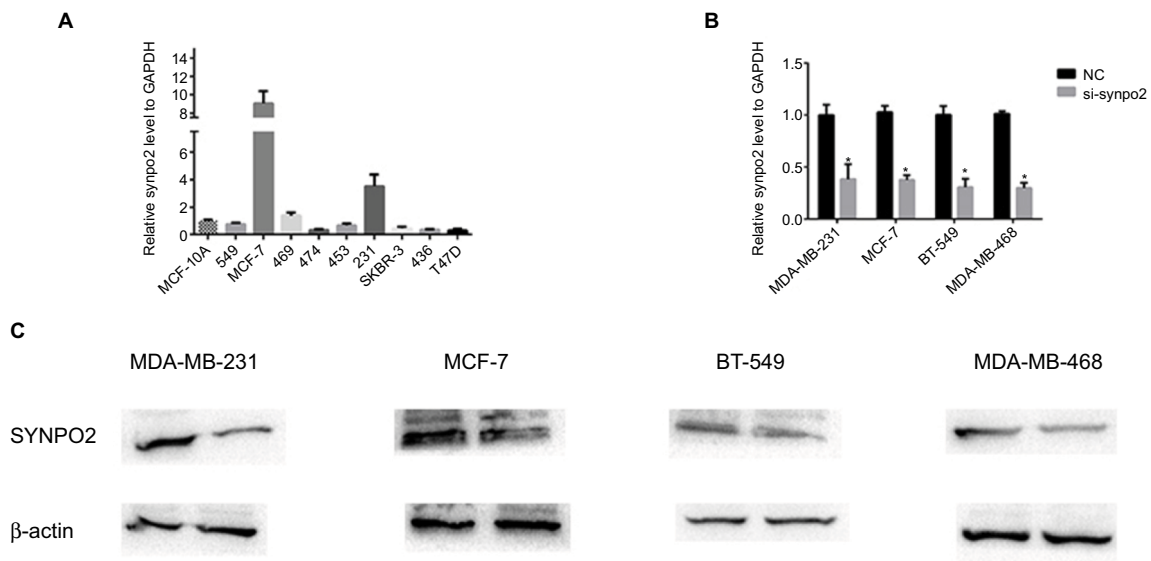


Figure 3 SYNPO2 has low expression in BC cell lines.

Notes: (A) The relative expression of SYNPO2 gene (compared with the GAPDH gene) using qRT-PCR. Compared to the other cell lines, SYNPO2 was expressed at a higher level in MCF-7, MDA-MB-231, BT-549, and MDA-MB-468. (B and C) The relative expression of SYNPO2 gene (compared with the GAPDH gene) in MCF-7, MDA-MB-231, BT-549, and MDA-MB-468 and both mRNA and protein levels of SYNPO2 were significantly reduced. Compared with the corresponding control group, the expression of SYNPO2 gene in the siRNA group was lower. * $P < 0.05$.

Abbreviations: BC, breast cancer; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SYNPO2, synaptodin 2.

effectively enhanced invasion capacity in MCF-7, MDA-MB-231, BT-549, and MDA-MB-468 (Figure 5).

SYNPO2 regulates migration and invasion via PI3K/AKT/mTOR signaling pathway

The PI3K/AKT/mTOR pathway plays an important role in cancer cell metastasis. In order to know whether SYNPO2 regulates migration and invasion that are related to the PI3K/AKT/mTOR signaling pathway, we used Western blot. The results showed that cells with knocked down SYNPO2 could significantly enhance the phosphorylation levels of PI3K, AKT, and mTOR (Figure 6A and B), which may induce cell migration and invasion. To further test whether PI3K/AKT/mTOR mediated SYNPO2-dependent invasion and migration, we cotransfected with Akt inhibitor MK-2206 and si-SYNPO2. Consistent with our hypothesis, treatment with MK-2206 could reduce the SYNPO2-mediated metastasis significantly (Figures 7 and 8). Collectively, these results suggested that downregulated SYNPO2 activates the PI3K/AKT/mTOR signaling pathway to enhance the metastasis of BC.

Discussion

As one of the commonest cancers in women, BC has a huge impact on the health burden in the world. The latest published clinical data indicates that BC forms a large proportion of cancer-related deaths in women.¹ Therefore, it is imperative to

develop some novel and effective therapies for BC. However, before that, we need to explore the underlying mechanisms of BC progression better.

In this study, we performed RNA sequencing of 8 pairs of BC and adjacent noncancerous tissues in our unpublished study and selected the gene *SYNPO2* for further research. We found that SYNPO2 was downregulated in BC tissues compared to paired adjacent nontumor tissues. This was further confirmed by the TCGA cohort. Then we found that the expression of SYNPO2 was related to age, lymph node metastasis, and clinical stage negatively in the validated cohort, while clinical stage and age at diagnosis were significantly related to the SYNPO2 expression. Kaplan–Meier analysis indicated that the difference between the survival curves of the low- and high-expression groups was obviously significant. These findings suggest that SYNPO2 may have pivotal roles in BC, and thus, we explored the SYNPO2 gene in cell lines. Knocking down SYNPO2 could enhance migration and invasion in MCF-7, MDA-MB-231, BT-549, and the MDA-MB-468 cell lines, consistent with the clinicopathologic features. This indicated that low expression of SYNPO2 may enhance the metastasis of BC cells and induce the development of BC through more lymph node metastasis and worse clinical stage.

The PI3K/AKT/mTOR pathway plays an important role in cell physiology.⁸ PI3K/AKT/mTOR signaling pathway

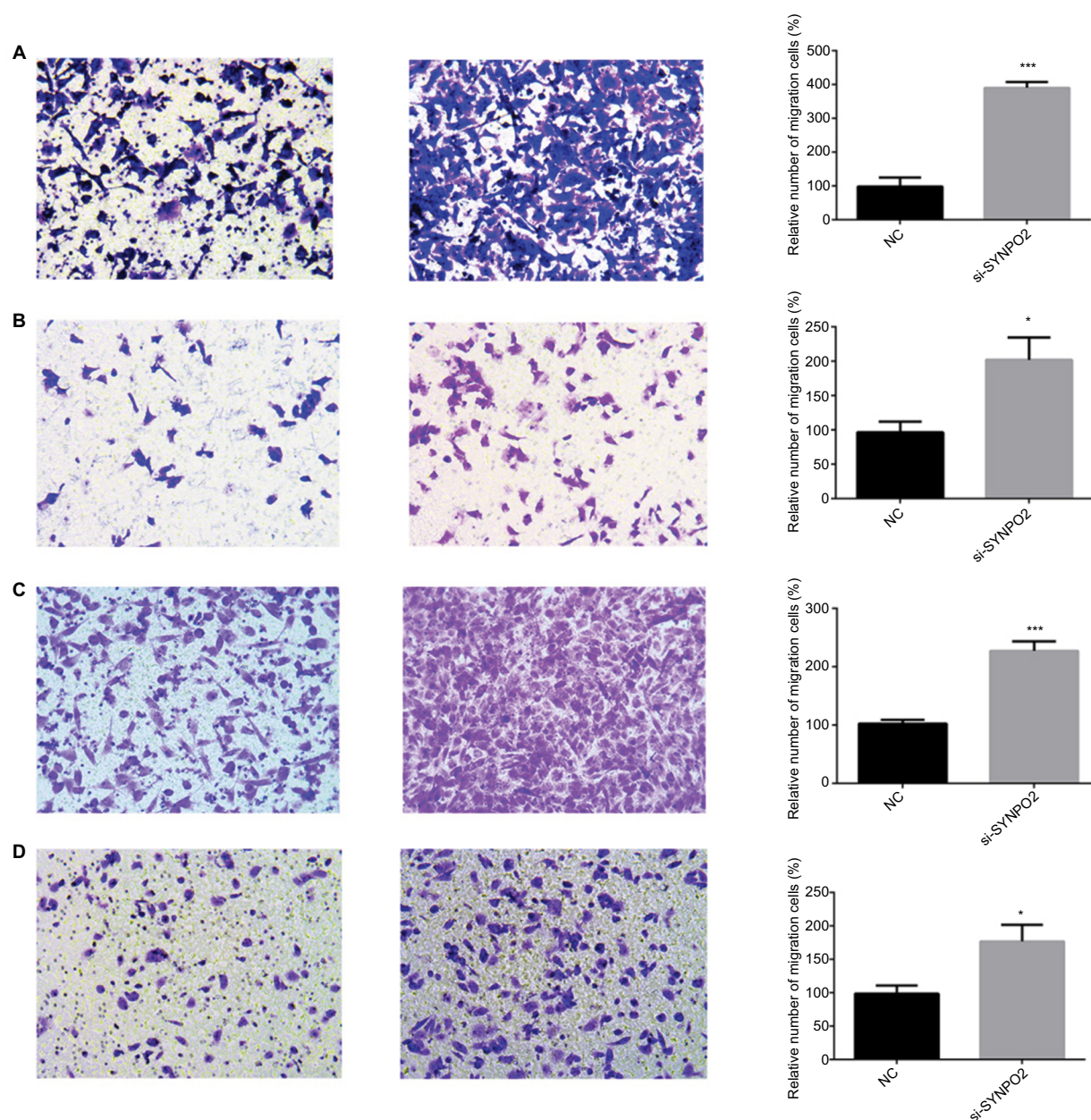


Figure 4 Downregulation of SYNPO2 gene expression in MCF-7, MDA-MB-231, BT-549, and MDA-MB-468 cells inhibited migration.

Notes: (A) In MCF-7, transwell migration assays in downregulation of SYNPO2 cells compared with their corresponding control cells. (B) In MDA-MB-231, transwell migration assays in downregulation of SYNPO2 cells compared to their corresponding control cells. (C) In BT-549, transwell migration assays in downregulation of SYNPO2 cells compared with the corresponding control cells. (D) In MDA-MB-468, transwell migration assays in downregulation of SYNPO2 cells compared with the corresponding control cells. All results are quantitative results of migration assays. The stained cells were manually counted from 5 randomly selected fields and normalized with cell proliferation. * $P < 0.05$; and *** $P < 0.001$ in comparison with the NC group using Student's *t*-test. Magnification 20 \times .

Abbreviations: NC, negative control; SYNPO2, synaptopodin 2.

has emerged as a powerful platform that plays a role in many human diseases. For example, abnormal activation of PI3K/AKT/mTOR signaling pathway often influences the process of BC.^{16,17} Previous studies showed that addition of PI3K/AKT/mTOR signaling inhibitors could inhibit the metastasis of BC, and some inhibitors have shown potential effects in

BC.^{18,19} In this study, we showed that cells with knocked down SYNPO2 could significantly enhance the phosphorylation levels of PI3K, AKT, and mTOR expression, which may induce cell migration and invasion. We observed that after cotransfection with Akt inhibitor, MK-2206 and si-SYNPO2, SYNPO2-mediated metastasis was partly rescued.

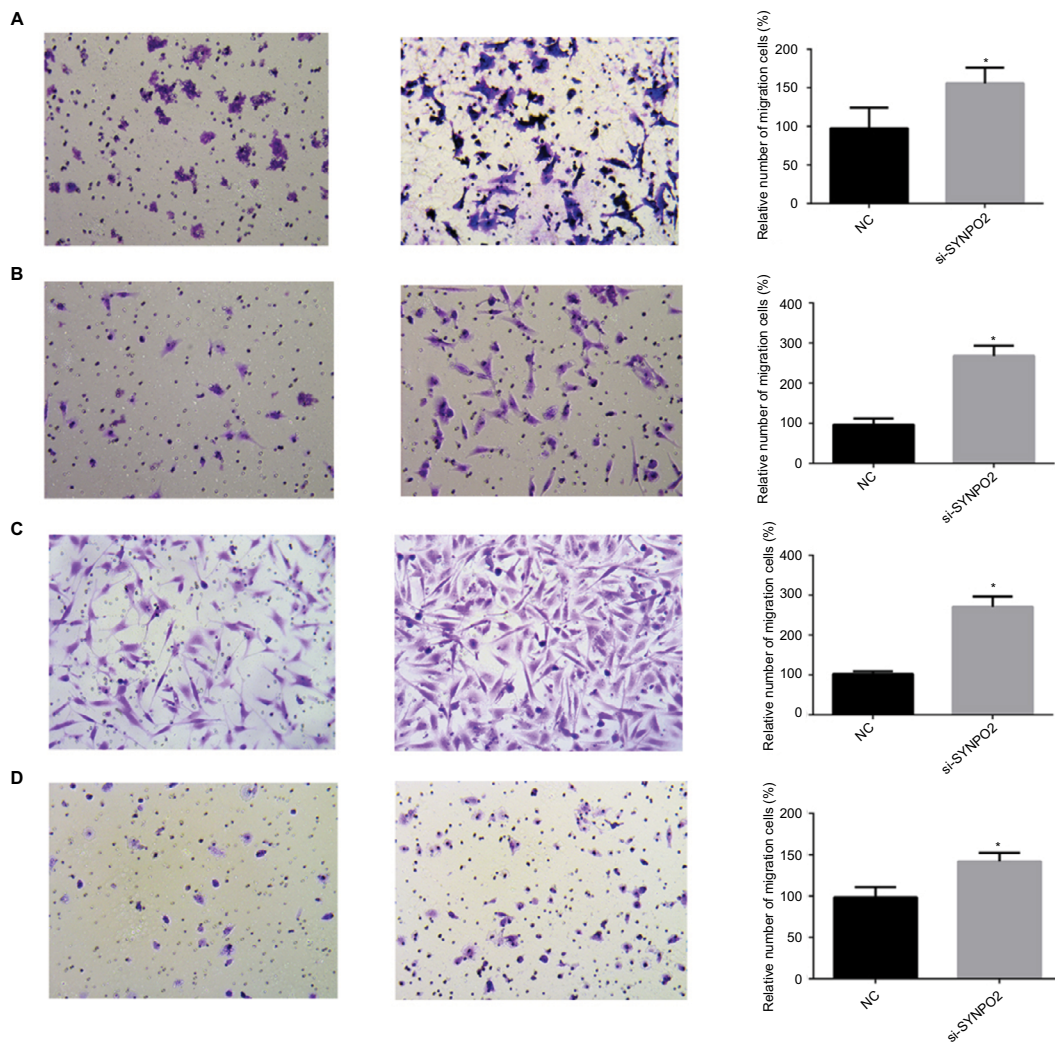


Figure 5 Downregulation of SYNPO2 gene expression in MCF-7, MDA-MB-231, BT-549, and MDA-MB-468 cells inhibited invasion. **Notes:** (A) In MCF-7, transwell invasion assays in downregulation of SYNPO2 cells compared to their corresponding control cells. (B) In MDA-MB-231, transwell invasion assays in downregulation of SYNPO2 cells when tested with their corresponding control cells. (C) In BT-549, transwell invasion assays in downregulation of SYNPO2 cells against their corresponding control cells. (D) In MDA-MB-468, transwell invasion assays in downregulation of SYNPO2 cells compared with their corresponding control cells. All results are quantitative results of migration assays. The stained cells were manually counted from 5 randomly selected fields and normalized with cell proliferation. *P<0.05; in comparison with the NC group using Student's t-test. Magnification 20x. **Abbreviations:** NC, negative control; SYNPO2, synaptopodin 2.

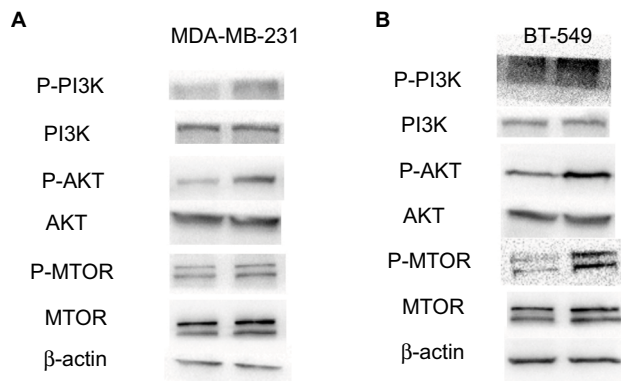


Figure 6 Downregulation of SYNPO2 regulates migration and invasion by inducing the phosphorylation of PI3K, AKT, and mTOR. **Notes:** (A, B) The influence of SYNPO2 expression on the phosphorylation levels of PI3K, AKT, and mTOR expression in MDA-MB-231 and BT-549 cells by Western blot. **Abbreviations:** SYNPO2, synaptopodin 2.

Despite our remarkable findings, this study still has some limitations. First, in vivo experiments need to be executed to better validate the biological effects of SYNPO2. Second, although we confirmed that PI3K/AKT/mTOR signaling pathway is related to the effect of SYNPO2, we still need to explore the mechanism of SYNPO2 in breast tumorigenesis clearly. Third, the samples of BC tissues should be expanded to certify the clinicopathological features and findings.

All in all, our study showed that SYNPO2 might negatively affect the tumorigenesis of BC. Low expression of SYNPO2 was associated with clinicopathological features. The results of our study provide a potential marker and a target for gene therapy in BC.

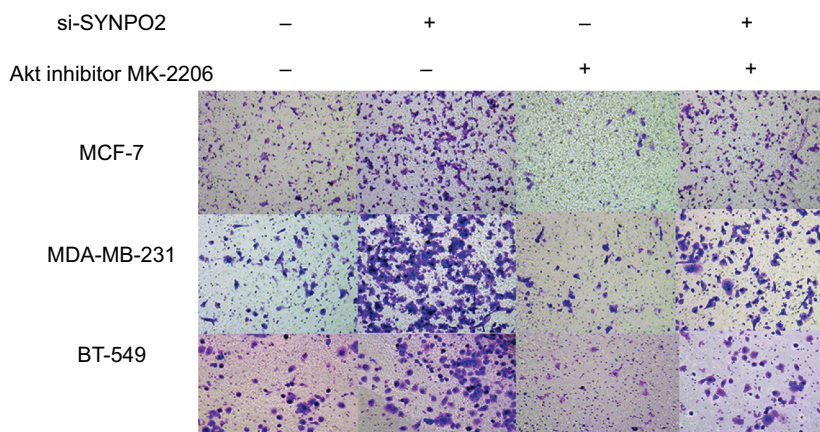


Figure 7 Transfection with Akt inhibitor MK-2206 could partly rescue SYNPO2-mediated migration.

Notes: Transfection with Akt inhibitor MK-2206 prevented SYNPO2-induced migration. Magnification 20 \times .

Abbreviation: SYNPO2, synaptopodin 2.

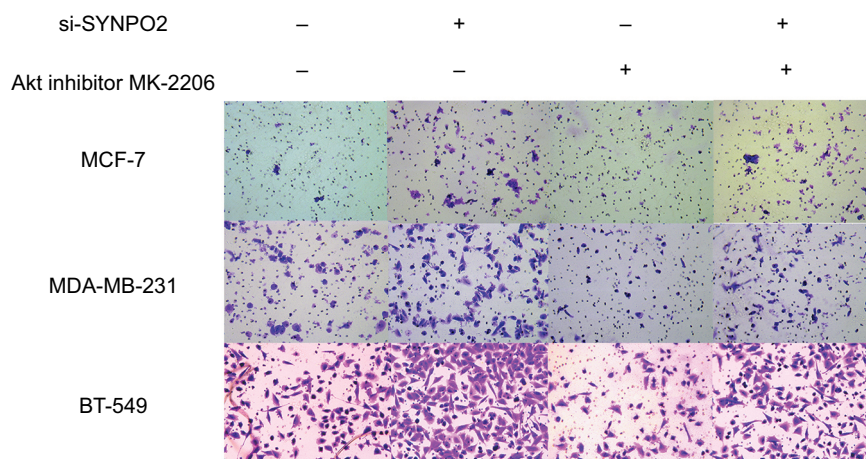


Figure 8 Transfection with Akt inhibitor MK-2206 could partly rescue SYNPO2-mediated invasion.

Note: Magnification 20 \times .

Abbreviation: SYNPO2, synaptopodin 2.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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