

# NRSN2 promotes breast cancer metastasis by activating PI3K/AKT/mTOR and NF- $\kappa$ B signaling pathways

FEI REN<sup>1</sup>, WEI ZHANG<sup>1</sup>, SHUAI LU<sup>1</sup>, HONG REN<sup>2</sup> and YANTONG GUO<sup>1</sup>

<sup>1</sup>Department of Medicine, Peking University, Beijing 100191; <sup>2</sup>Department of Breast Surgery, Affiliated Hospital, Shanxi Traditional Chinese Medical University, Taiyuan, Shanxi 030024, P.R. China

Received October 24, 2018; Accepted June 11, 2019

DOI: 10.3892/ol.2019.11152

**Abstract.** Breast cancer is a leading cause of cancer-associated mortality globally amongst gynecologic tumors due to aggressive metastasis. A previous study reported that neurensin-2 (NRSN2) was implicated in human cancer cells, and that NRSN2 gene and protein expression levels were significantly upregulated in human breast cancer tissues compared with adjacent non-tumor tissues. The purpose of the present study was to analyze the role of NRSN2 in the metastasis of breast cancer cells and explore its potential mechanism. Reverse transcription-quantitative PCR, MTT, western blotting and immunohistochemistry was used to analyze the role of NRSN2 both *in vitro* and *in vivo*. The present study demonstrated that NRSN2 knockdown inhibited the proliferation, migration and invasion of breast cancer cells *in vitro*. NRSN2 upregulation promoted breast cancer cell proliferation and tissue growth *in vitro* and *in vivo*. In addition, the results demonstrated that the regulatory effects of NRSN2 on breast cancer cells were associated with PI3K/AKT/mTOR and NF- $\kappa$ B signaling pathway dysregulation. Furthermore, NRSN2 overexpression in mice significantly promoted breast cancer cell proliferation. In conclusion, the results from the present study indicated that NRSN2 may be considered as a novel oncogenic protein and may represent a potential therapeutic target for breast cancer.

## Introduction

Breast cancer is one of the most common gynecologic tumors, mainly occurring in the elderly (1). A previous review reported the most common genes involved in epigenetic modifications in patients with breast cancer (2). Most breast cancer-associated mortalities are caused by local breast cancer cell migration and distant metastasis (3). A review reported

genetic analyses and inherited gene mutations in patients with breast cancer (4,5). Although advances in molecular diagnosis and medical treatments, including surgical techniques, radiation, chemotherapy and gene target therapy, have improved the 5-year survival rate of patients with breast cancer, the overall clinical outcomes remain poor (6-9). It is therefore essential to determine potential target proteins to inhibit breast cancer growth and metastasis.

Neurensin-2 (NRSN2) is a small neuronal membrane protein that is localized in small vesicles of neural cells (10). A previous study revealed that NRSN2 can promote non-small cell lung cancer cell proliferation via the PI3K/AKT/mTOR pathway (11). Tang *et al* (12) reported that NRSN2 overexpression was associated with malignant phenotypes in ovarian cancer, suggesting that it could be considered as a target for ovarian cancer treatment. However, Wang *et al* (13) demonstrated that NRSN2 upregulation inhibited cell proliferation and survival via the PI3K/AKT/mTOR pathway in hepatocellular carcinoma. These findings encouraged the present study to further investigate the role of NRSN2 in breast cancer cells.

The PI3K/AKT/mTOR, p65 and NF- $\kappa$ B signaling pathways contribute to breast cancer progression. Therefore, the present study hypothesized that NRSN2 may regulate breast cancer cell proliferation via the PI3K/AKT/mTOR and NF- $\kappa$ B pathways (14). The results from the present study demonstrated that NRSN2 overexpression significantly increased the proliferation, invasion and metastasis of breast cancer cells, suggesting that NRSN2 may be considered as a potential therapeutic target for breast cancer treatment via downregulation of the PI3K/AKT/mTOR and NF- $\kappa$ B signaling pathways.

## Materials and methods

**Ethical statements.** This study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Tianjin Medical University. The protocol was approved by the Chinese Association for Laboratory Animal Operations. All surgery and euthanasia were performed under sodium pentobarbital anesthesia (intravenous, 35 mg/kg). Mice were sacrificed via cervical decapitation.

**Patients and tissues.** A total of 24 patients with breast cancer were recruited in Peking University between May 2015

---

**Correspondence to:** Professor Yantong Guo, Department of Medicine, Peking University, 38 Xueyuan Road, Haidian, Beijing 100191, P.R. China  
E-mail: yantong\_guo@yeah.net

**Key words:** neurensin-2, breast cancer, proliferation, PI3K/AKT/mTOR, NF- $\kappa$ B

and October 2016. Their average age was  $54.5 \pm 24.5$  years (range, 30–79 years). Breast cancer and adjacent noncancerous tissues were obtained from patients who underwent tumor resection and stored at  $-80^{\circ}\text{C}$  prior to immunohistochemistry (IHC) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses. Patients who had previously undergone radiotherapy, chemotherapy or administration of any other drug were excluded from this study. All patients provided written informed consent prior to any procedures of this study. The patient study was approved by the Ethics Committee of Peking University (approval no. PEK20150524).

**Cell line, chemicals and reagents.** The breast tumor cell lines MDA-MB-231 and BT549, and the normal breast cell line MCF-10A were purchased from the American Type Culture Collection. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) and placed at  $37^{\circ}\text{C}$  in a humidified incubator containing 5%  $\text{CO}_2$ . Cells were treated with the NF- $\kappa$ B inhibitor caffeic acid phenethyl ester (CAPE; 70 mM; Apex Biotechnology Corp.), PI3K inhibitor (70 mM; Sigma-Aldrich; Merck KGaA) or PBS as control for 12 h at  $37^{\circ}\text{C}$  for further experiments (15).

**Small interfering RNA (siR)-NRSN2 transfection.** All siRs (siR-NRSN2, 5'-CAATCTTCTGTGCAGACTATC-3'; siR-NC, 5'-CGAGGACAGGCTGATCTTCC-3') were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. MDA-MB-231 cells ( $1 \times 10^6$  cells/well) were cultured in six-well plates and transfected with 150 pM siR-NRSN2 or si-control using a Cell Line Nucleofector kit (cat. no. VCA-1003; Lonza Group, Ltd.) according to manufacturer's protocol. The efficiency of siR-NRSN2 transfection was verified via western blotting at 72 h following transfection, prior to subsequent experiments.

**NRSN2 overexpression.** An expression plasmid (pRK5-hNRSN2) with a Flag tag at the C-terminus was constructed by Invitrogen (Thermo Fisher Scientific, Inc.). MDA-MB-231 cells ( $1 \times 10^4$ ) were seeded in 6-well plates (Corning Inc.) and transiently transfected with pRK5-hNRSN2 (2  $\mu\text{g}$ ) or pRK5-control (pControl) (2  $\mu\text{g}$ ) using Lipofectamine<sup>®</sup> 2000 (cat. no. 11668-027; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The efficiency of NRSN2 overexpression was verified by western blotting at 72 h following transfection, prior to subsequent experiments.

**RT-qPCR.** Total RNA was isolated from tissues or cells by using an RNAeasy Mini kit (Qiagen, Inc.). The expression of NRSN2 in tissues and cells was measured using a Hairpin-it<sup>™</sup> RT-qPCR kit (Invitrogen; Thermo Fisher Scientific, Inc.). NRSN2 expression levels were measured in an iCycler thermal cycler (Bio-Rad Laboratories, Inc.) using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.). The thermocycling conditions were:  $95^{\circ}\text{C}$  for 120 sec; followed by 45 cycles at  $95^{\circ}\text{C}$  for 30 sec,  $56^{\circ}\text{C}$  for 20 sec and  $65^{\circ}\text{C}$  for 30 sec. The primers were designed as follows: NRSN2, forward 5'-CGGAGACGCAGGTCCAGAGGGAT-3', reverse 5'-TATGCATCAACTGTTTATTGAAAGG-3'; and  $\beta$ -actin, forward 5'-GTG

GGCGCCCAGGCACCA-3' and reverse 5'-CTCCTTAATGTCACGCACGATTT-3'. Relative mRNA expression changes were calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method (16). The results are expressed compared to  $\beta$ -actin expression.

**Cell proliferation assay.** Cell proliferation was determined using a Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. Briefly, pRK5-hNRSN2 or siR-NRSN2-transfected MDA-MB-231 cells and their controls were seeded in 96-well plates at a density of  $1 \times 10^3$ /well and cultured at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere for 48 h. CCK-8 solution (10  $\mu\text{l}$ ) was added to each well for 2 h. The cell proliferation was monitored by measuring the absorbance at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

**Colony formation assay.** For the colony formation assay, pRK5-hNRSN2 or siR-NRSN2-transfected MDA-MB-231 cells were seeded in 6-well plates at a density of  $1 \times 10^3$ /well and cultured at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere for 7 days until visible colonies were formed. Cells were stained with 5% crystal violet for 10 min at room temperature. The numbers of colonies were then counted using a light microscope at  $\times 20$  magnification.

**Cell migration and invasion assay.** MDA-MB-231 cells were transfected with pRK5-hNRSN2 or siR-NRSN2. Matrigel-uncoated and -coated migration inserts (8- $\mu\text{m}$  pore size; Corning Inc.) were used for migration and invasion assays, respectively.

For the migration assay, MDA-MB-231 cells ( $1 \times 10^4$ ) in DMEM were plated into the upper chamber with the non-coated membrane. For the invasion assay, pRK5-hNRSN2 or siR-NRSN2-transfected cells were prepared at a density of  $1 \times 10^4$  cells in 500  $\mu\text{l}$  serum-free DMEM in the upper chamber and the lower chamber contained DMEM with 5% FBS. Cells were seeded in the upper chamber of a BD BioCoat Matrigel Invasion Chamber (BD Biosciences) according to the manufacturer's instructions. Following incubation for 48 h, cells were fixed with 4% paraformaldehyde for 1 h at room temperature and stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA) for 10 min at  $37^{\circ}\text{C}$ . The membranes were mounted onto a glass slide with antifade mounting medium (cat. no. P0126; Beyotime Institute of Biotechnology), and the number of migrating and invading tumor cells was counted in at least three randomly selected fields under a light microscope (Olympus Corporation) at  $\times 200$  magnification.

**Western blotting.** MDA-MB-231 cells were transfected with pRK5-hNRSN2 or siR-NRSN2. Following transfection, cells were lysed using radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA) containing protease-inhibitor (Sigma-Aldrich; Merck KGaA) and were centrifuged at  $12,000 \times g$  at  $4^{\circ}\text{C}$  for 10 min. Breast cancer tissues (10 mg) were lysed using radioimmunoprecipitation assay buffer containing protease inhibitor and were centrifuged at  $1,000 \times g$  at  $4^{\circ}\text{C}$  for 10 min. Supernatant was collected for protein analysis. The concentrations of protein were measured using a BCA Protein Concentration Assay kit (Beyotime Institute of Biotechnology). Protein samples (50  $\mu\text{g}$  per lane) were separated by sodium

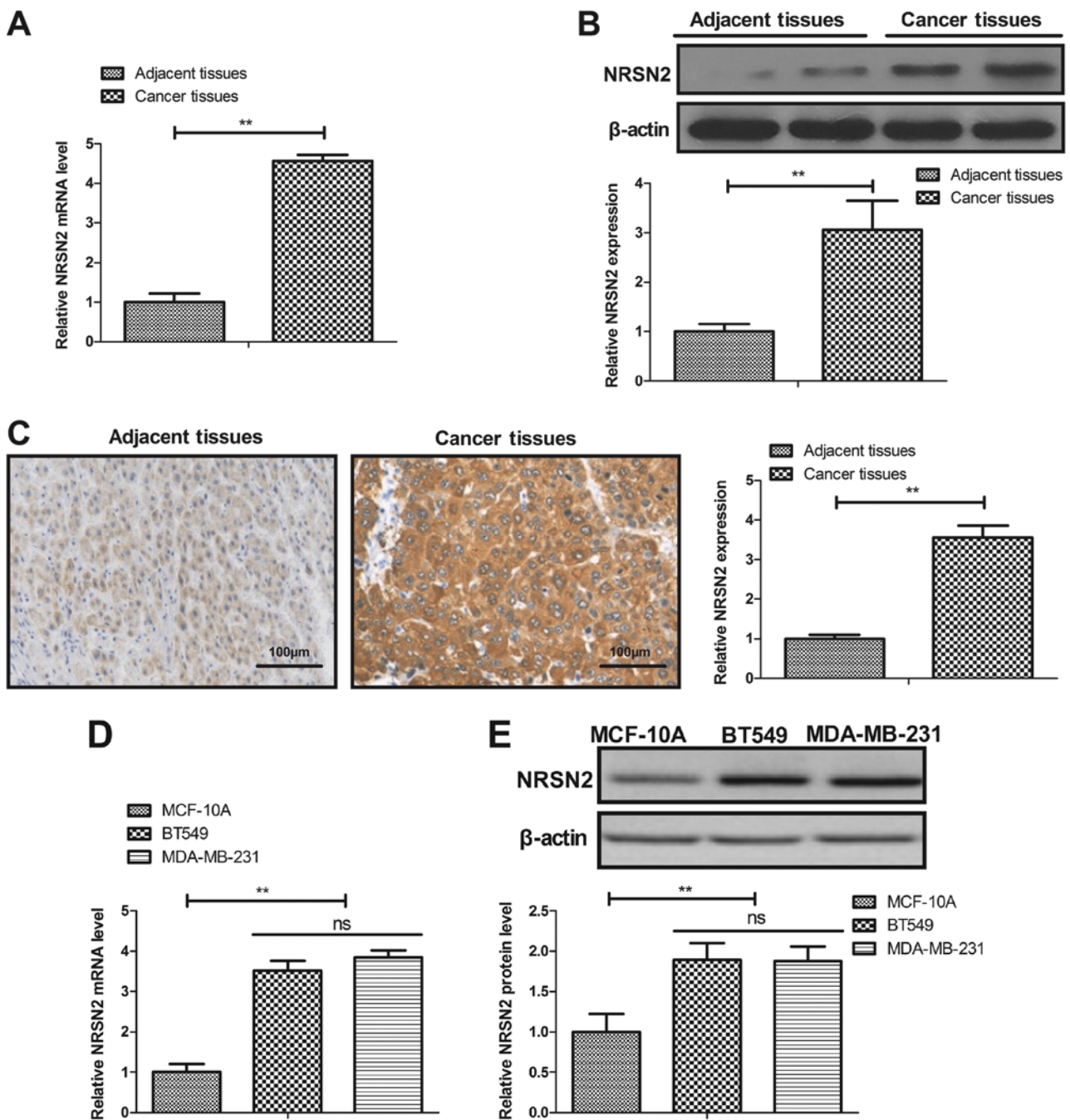


Figure 1. NRSN2 expression is upregulated in breast cancer tissues and cell lines. (A) mRNA and (B) protein levels of NRSN2 were upregulated in cancer tissues compared with match adjacent tissues. (C) NRSN2 was highly expressed in breast cancer tissues determined by immunohistochemistry. (D) mRNA and (E) protein levels of NRSN2 in breast cancer cells were upregulated compared with in MCF-10A cells. NRSN2, neuensin-2; ns, not significant. \*\* $P < 0.01$ .

dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) on a 10% gel and transferred to polyvinylidene difluoride (PVDF; Bio-Rad Laboratories, Inc.) membrane. Membranes were incubated with rabbit anti-human primary antibodies against NRSN2 (1:1,000; cat. no. ab237739; Abcam), PI3K (1:1,000; cat. no. ab32089; Abcam), phosphorylated (p)-PI3K (1:1,000; cat. no. ab154598; Abcam), p65 (1:1,000; cat. no. ab16502; Abcam), p-p65 (1:1,000; cat. no. ab86299; Abcam), I $\kappa$ B $\alpha$  (1:1,000; cat. no. ab7217; Abcam) and p-I $\kappa$ B $\alpha$  (1:1,000; cat. no. ab133462; Abcam),  $\beta$ -actin (1:5,000; cat. no. 20536-1-AP; ProteinTech Group, Inc.), AKT (1:1,000; cat. no. 51077-1-AP; ProteinTech Group, Inc.), p-mTOR

(1:1,000; cat. no. ab2731; Abcam), and mTOR (1:1,000; cat. no. ab109268; Abcam), overnight at 4°C. The membranes were subsequently incubated with HRP-conjugated anti-rabbit IgG secondary antibodies (diluted 1:5,000; cat. no. A9169; Sigma-Aldrich; Merck KGaA) for 24 h at 4°C. Enhanced chemiluminescence reagent (Millipore; Merck KGaA) was used to visualize the bands. Quantitation of the signal intensities were analyzed using the Quantity One software package (version 2.0; Bio-Rad Laboratories, Inc.).

**Animal study.** Pathogen-free female Balb/c (8-week-old; 20-25 g body weight) nude mice were purchased from Slack

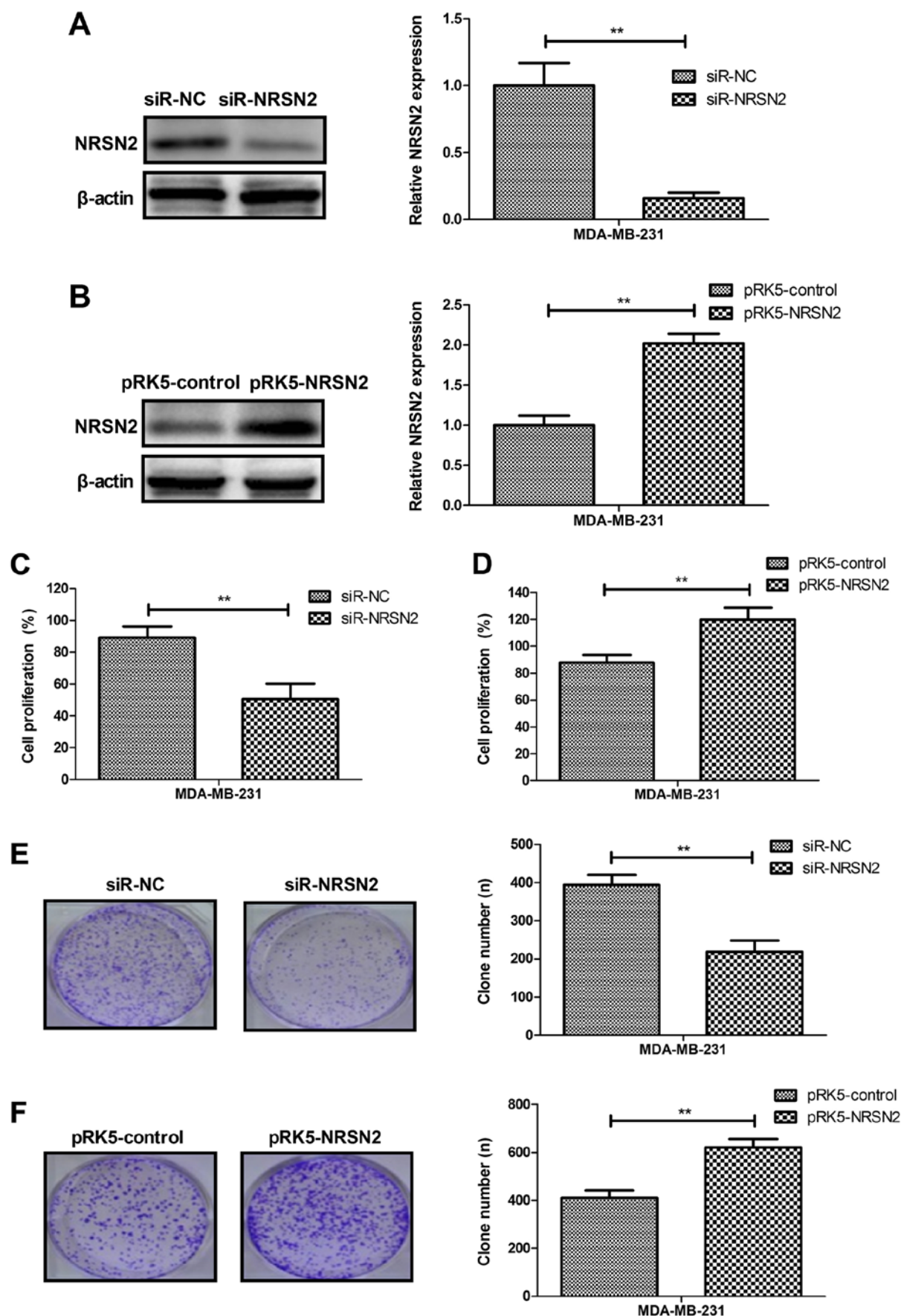


Figure 2. NRSN2 knockdown suppresses and NRSN2 overexpression increases the proliferation of breast cancer cells *in vitro*. (A) NRSN2 knockdown decreased NRSN2 expression in MDA-MB-231 cells. (B) NRSN2 overexpression increased NRSN2 expression in MDA-MB-231 cells. (C) NRSN2 knockdown inhibited MDA-MB-231 cell proliferation. (D) NRSN2 overexpression promoted MDA-MB-231 cell proliferation. (E) NRSN2 knockdown inhibited the colony formation of MDA-MB-231 cells. (F) NRSN2 overexpression promoted the colony formation of MDA-MB-231 cells. NC, negative control; NRSN2, neurensin-2; ns, not significant; si, small interfering (RNA); siR-NRSN2, siRNA against NRSN2; pRK5-NRSN2, NRSN2 overexpression vector. \*\* $P < 0.01$ .



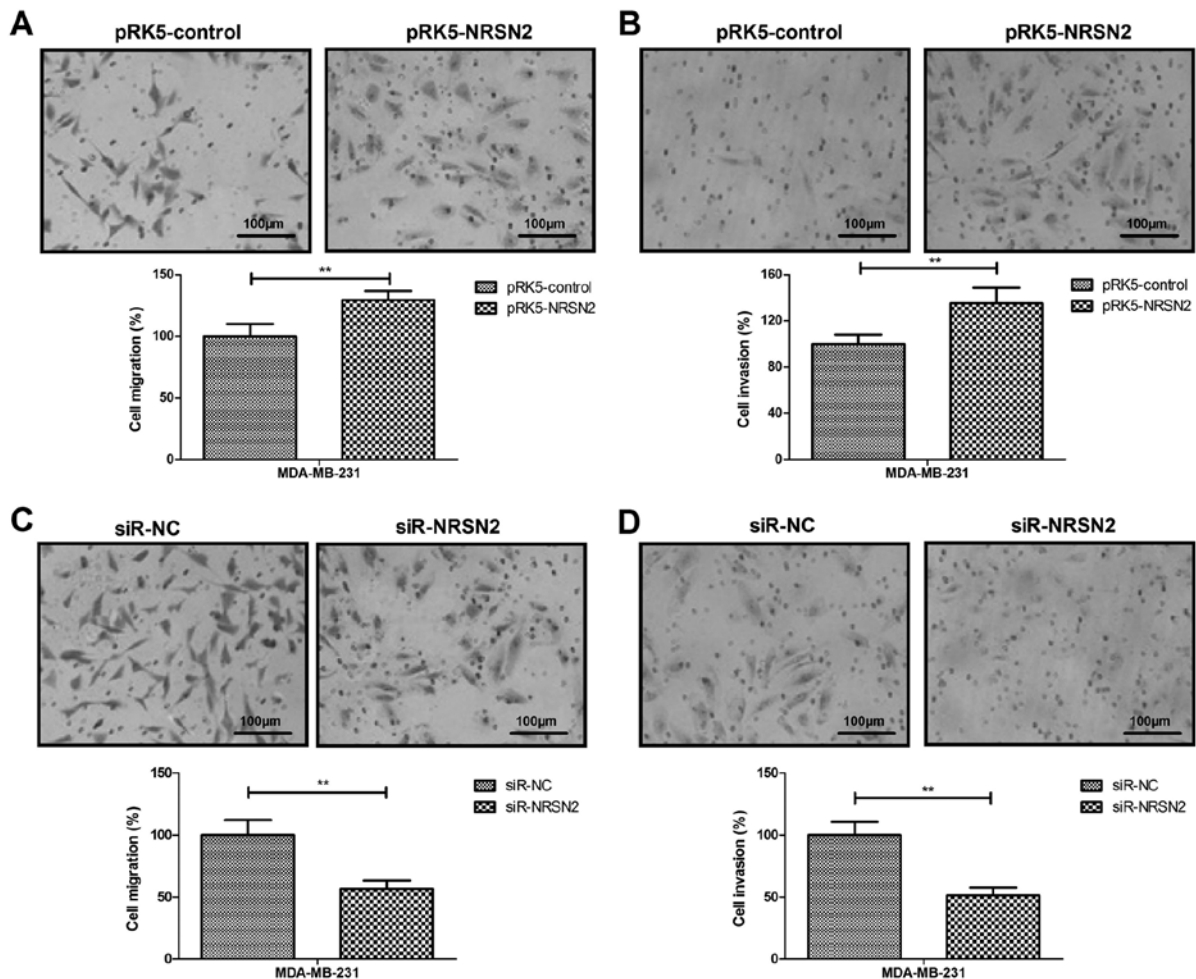


Figure 3. NRSN2 promotes tumor cell migration and invasion *in vitro*. NRSN2 overexpression promoted the (A) migration and (B) invasion of MDA-MB-231 cells. NRSN2 knockdown inhibited the (C) migration and (D) invasion of MDA-MB-231 cells. NRSN2, neuensin-2; si, small interfering (RNA); siR-NRSN2, siRNA against NRSN2; pRK5-NRSN2, NRSN2 overexpression vector. \*\*P<0.01.

Co., Ltd. All mice were treated in accordance with the China Legislation on the Protection of Animals and the Guide for the Care and Use of Laboratory Animals. The study was approved by the ethics committees of Shanxi Traditional Chinese Medical University. Animals were housed in a temperature-controlled facility at  $23 \pm 1^\circ\text{C}$  with  $50 \pm 5\%$  humidity under a 12-h light/dark cycle. All mice had free access to food and water. The siR-NRSN2-transfected MDA-MB-231, pRK5-control vector-transfected MDA-MB-231 cells or MDA-MB-231 cells ( $1 \times 10^7$ ) were subcutaneously injected into the flanks of female Balb/c mice. The tumor volume was calculated every 3 days and calculated as  $\text{Volume} = (D \times d^2)/2$  (D represents the maximal diameter, d represents the minimal one). The mice were sacrificed on day 30 following anesthesia.

**IHC.** Human breast tissues or mouse cancer tissues were analyzed for NRSN2 expression using IHC, as previously described (17). Briefly, tumor tissue samples were fixed in 10% formalin for 12 h at room temperature and embedded in paraffin blocks for 8 h at room temperature. Thin tissue sections ( $4\text{-}\mu\text{m}$  thick) were de-waxed and rehydrated. Tissue sections were immersed for 15 min at room temperature in  $0.3\% \text{H}_2\text{O}_2$  (diluted with 100% methanol) to block endogenous peroxidase activity. The tumor sections were incubated with

specific primary antibodies: Rabbit anti-human or mouse antibodies against rabbit anti-human/mouse NRSN2 (1:1,000; cat. no. ab237739; Abcam) overnight at  $4^\circ\text{C}$ . Tumor tissues were then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:5,000; cat. no. PV-6001; OriGene Technologies, Inc.) for 2 h at room temperature. Finally, tissue sections were stained with 3-3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature and lightly counter-stained by hematoxylin for 30 min at room temperature. A Ventana Benchmark automated staining system was used to detect NRSN2 protein expression in tumor tissues (Olympus BX51; Olympus Corporation) at x200 magnification. The quantification of NRSN2 density was analyzed using Image J software (version 4.6; National Institutes of Health).

**Statistical analysis.** All data were expressed as the mean  $\pm$  standard deviation. Experiments were repeated at least three times. Statistical analyses were performed using Student's t-test or one-way ANOVA followed by Tukey's honestly significant difference post hoc test. Data were analyzed using SPSS Statistics 19.0 software (IBM Corp.) and GraphPad Prism 5.0 (GraphPad Software, Inc.).  $P < 0.05$  was considered to indicate a statistically significant difference.

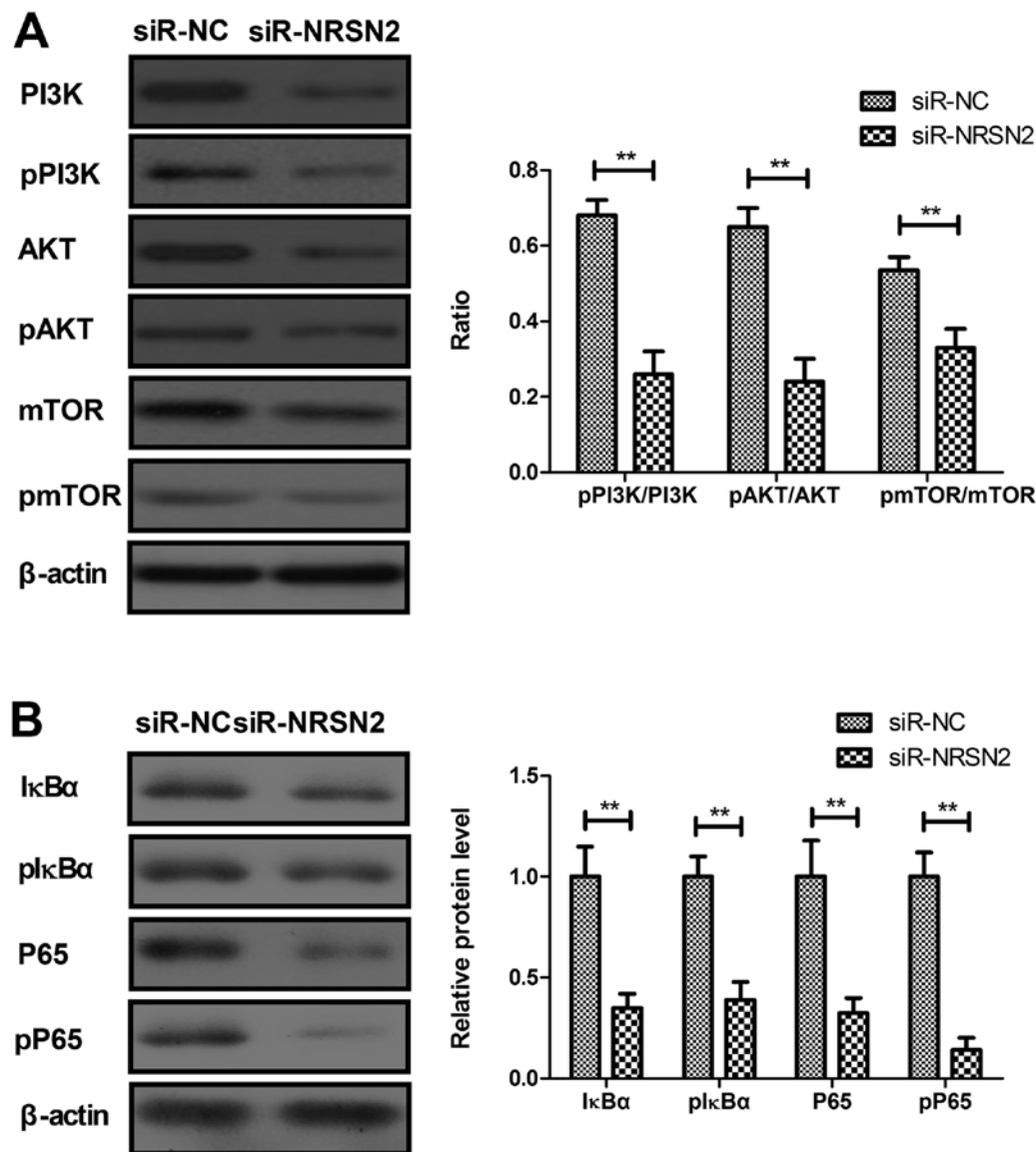


Figure 4. NRSN2 promotes proliferation, migration and invasion of breast cancer cells by activating PI3K/AKT/mTOR and NF- $\kappa$ B signaling pathways. (A) NRSN2 knockdown significantly inhibited the phosphorylation of PI3K, AKT and mTOR in MDA-MB-231 cells. (B) NRSN2 knockdown significantly decreased the levels of I $\kappa$ B $\alpha$  and P65 phosphorylation in MDA-MB-231 cells. Control, PBS-treated cells. NC, negative control; NRSN2, neurensin-2; ns, not significant; p-, phosphorylated; si, small interfering (RNA); si-RNRSN2, siRNA against NRSN2; pRK5-NRSN2, NRSN2 overexpression vector; NF- $\kappa$ BIR, NF- $\kappa$ B inhibitor; PI3KIR, PI3K inhibitor. \*\* $P < 0.01$ .

## Results

*NRSN2 expression is elevated in breast cancer tissues and cell lines.* The mRNA and protein levels of NRSN2 in breast cancer tissues and cell lines were determined. As presented in Fig. 1A and B, mRNA and protein levels of NRSN2 were significantly increased in breast cancer tissues compared with adjacent tissues. Furthermore, the results from IHC demonstrated that NRSN2 was highly expressed in breast cancer tissues compared with adjacent tissues (Fig. 1C). In addition, as presented in Fig. 1D and E, the mRNA and protein levels of NRSN2 were significantly increased in the breast cancer cell lines BT549 and MDA-MB-231 compared with the MCF-10A cell line. These results demonstrated that NRSN2 was highly expressed in breast cancer tissues and cell lines, which suggested that NRSN2 may have a role in breast cancer progression.

*NRSN2 knockdown inhibits the proliferation of breast cancer cells in vitro.* The role of NRSN2 in the breast cancer cell line MDA-MB-231 was further investigated. As presented in Fig. 2A and B, NRSN2 knockdown (siR-NRSN2) and overexpression (pRK5-NRSN2) significantly decreased and increased, respectively, NRSN2 expression in MDA-MB-231 cells. Furthermore, NRSN2 knockdown inhibited MDA-MB-231 cell proliferation (Fig. 2C), whereas NRSN2 overexpression promoted MDA-MB-231 cell proliferation (Fig. 2D). Colony formation assays demonstrated that NRSN2 overexpression increased the numbers of MDA-MB-231 cell colonies, whereas NRSN2 knockdown decreased the numbers of MDA-MB-231 cells colonies formed (Fig. 2E and F). These results demonstrated that NRSN2 knockdown could inhibit breast cancer cell proliferation *in vitro*.

*NRSN2 promotes tumor cell migration and invasion in vitro.* The effects of NRSN2 on cell motility were analyzed using

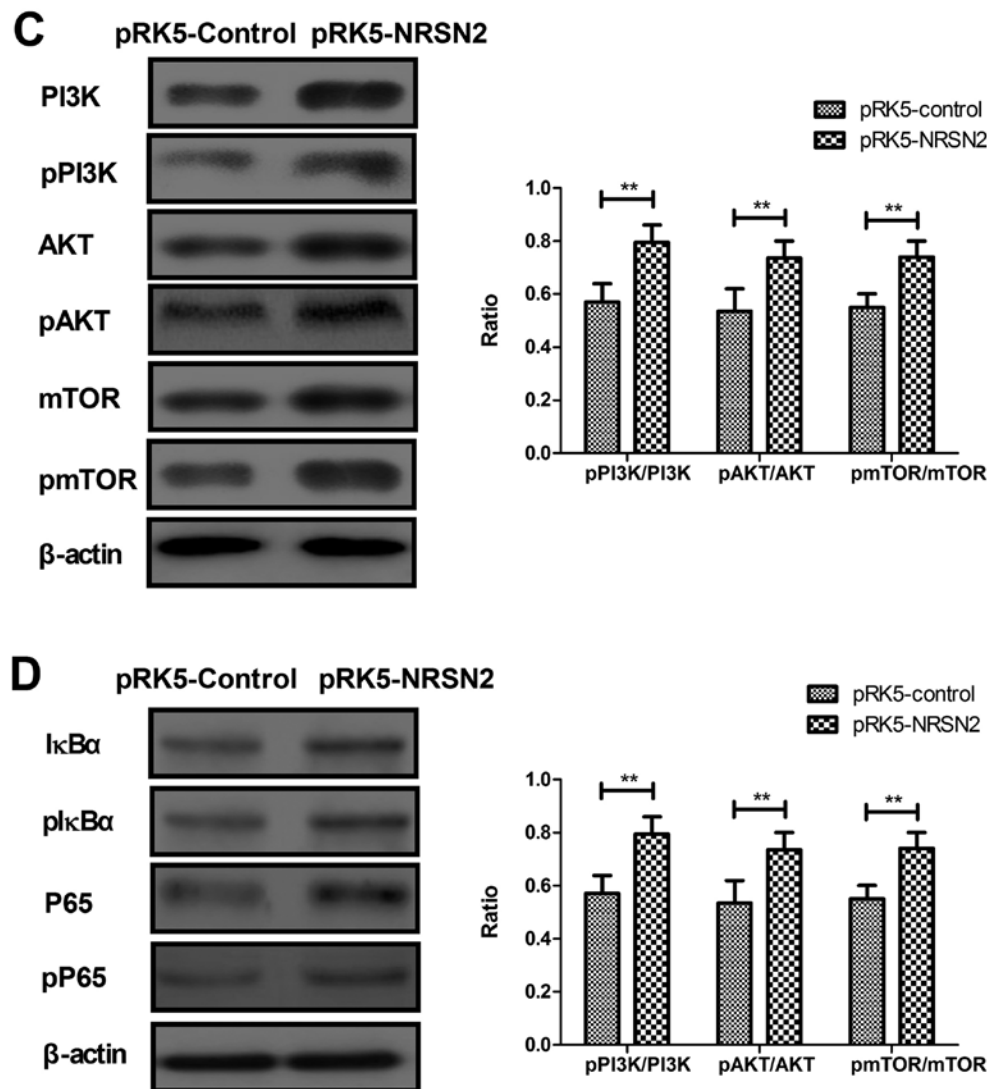


Figure 4. Continued. NRSN2 promotes proliferation, migration and invasion of breast cancer cells by activating PI3K/AKT/mTOR and NF- $\kappa$ B signaling pathways. (C) NRSN2 overexpression promoted the phosphorylation of PI3K, AKT and mTOR in MDA-MB-231 cells. (D) NRSN2 overexpression promoted the levels of p-I $\kappa$ B $\alpha$  and p-P65 in MDA-MB-231 cells. Control, PBS-treated cells. NC, negative control; NRSN2, neuensin-2; ns, not significant; p-, phosphorylated; si-, small interfering (RNA); si-NRSN2, siRNA against NRSN2; pRK5-NRSN2, NRSN2 overexpression vector; NF- $\kappa$ BIR, NF- $\kappa$ B inhibitor; PI3KIR, PI3K inhibitor. \*\*P<0.01.

migration and invasion assays. The results demonstrated that NRSN2 overexpression promoted the migration and invasion of MDA-MB-231 cells (Fig. 3A and B), whereas NRSN2 knockdown inhibited the migration and invasion of MDA-MB-231 cells (Fig. 3C and D).

*NRSN2 promotes proliferation, migration and invasion of breast cancer cells by activating PI3K/AKT/mTOR and NF- $\kappa$ B signaling pathways.* The potential molecular mechanisms mediated by NRSN2 were investigated in MDA-MB-231 cells. The results demonstrated that NRSN2 knockdown significantly inhibited the phosphorylation of PI3K, AKT and mTOR in MDA-MB-231 cells (Fig. 4A). Furthermore, NRSN2 knockdown significantly decreased I $\kappa$ B $\alpha$  and P65 phosphorylation in MDA-MB-231 cells (Fig. 4B). NRSN2 overexpression induced opposing effects (Fig. 4C and D). In addition, the NF- $\kappa$ B inhibitor CAPE (NF- $\kappa$ BIR) inhibited the pro-proliferation effects of NRSN2 (NF- $\kappa$ BIR) in MDA-MB-231 cells (Fig. 4E). Furthermore,

NF- $\kappa$ BIR suppressed the NRSN2 overexpression-induced (NF- $\kappa$ BIR-NRSN2) migration and invasion of MDA-MB-231 cells (Fig. 4F and G). Treatment of NRSN2 overexpressing cells with PI3K inhibitor (PI3KIR-NRSN2) inhibited NRSN2 overexpression-induced proliferation, migration and invasion of MDA-MB-231 cells (Fig. 4H-J). However, PI3K inhibitor or NF- $\kappa$ B inhibitor induced no effects on the proliferation, migration or invasion of MDA-MB-231 cells. These results suggested that NRSN2 may regulate the proliferation and aggressiveness of MDA-MB-231 cells through PI3K/AKT/mTOR and NF- $\kappa$ B signaling pathways.

*NRSN2 overexpression promotes tumor growth in vivo.* The role of NRSN2 in tumor growth was further investigated *in vivo* in subcutaneous breast cancer xenograft nude mice. The results demonstrated that tumor growth was faster in nude mice injected with pRK5-NRSN2 plasmid-transfected MDA-MB-231 cells than in nude mice injected with pRK5-control vector (control; Fig. 5A and B). However,

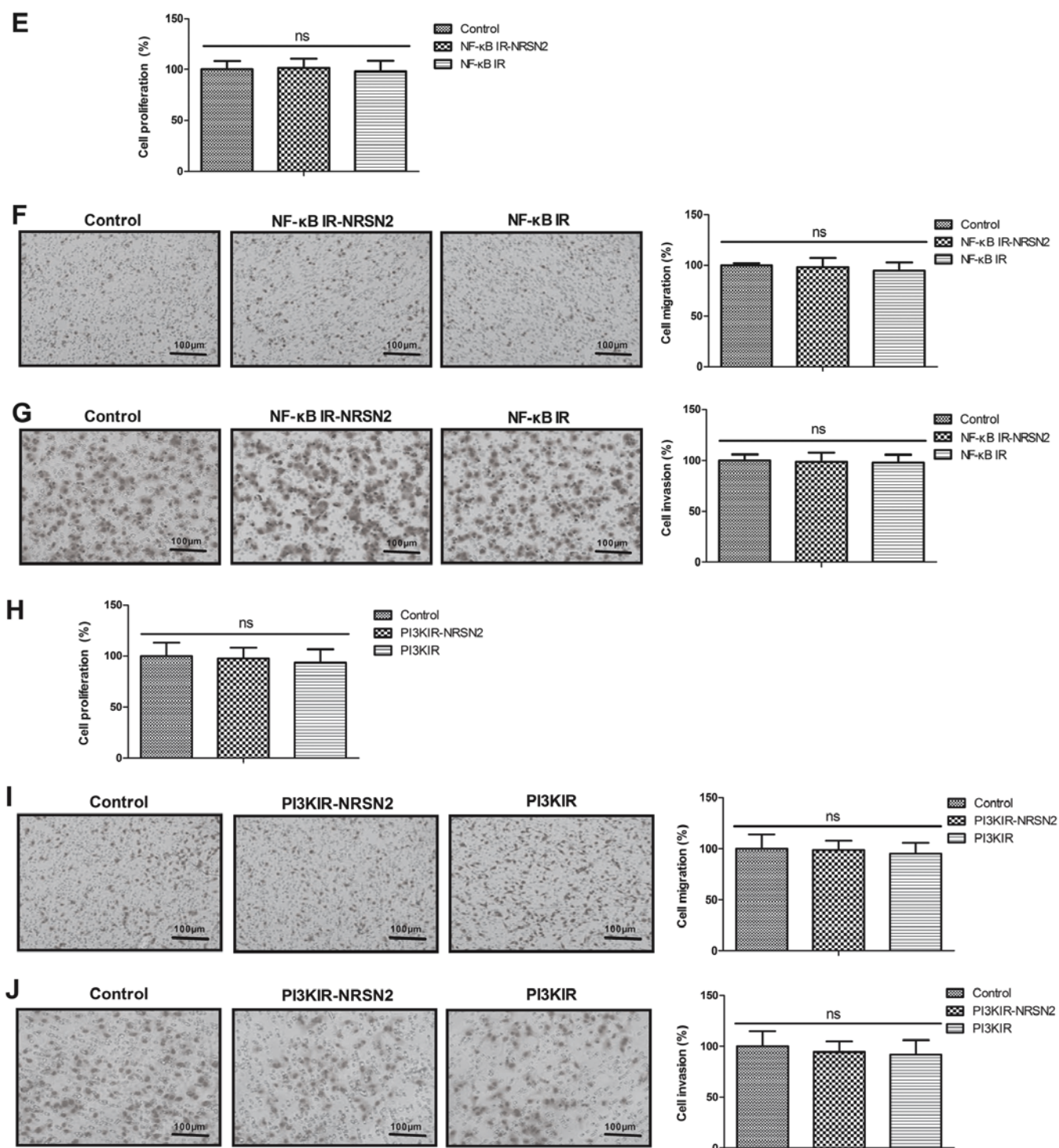


Figure 4. Continued. NRSN2 promotes proliferation, migration and invasion of breast cancer cells by activating PI3K/AKT/mTOR and NF- $\kappa$ B signaling pathways. (E) NF- $\kappa$ BIR inhibited the pro-proliferation effects of NRSN2 in MDA-MB-231 cells. NF- $\kappa$ BIR suppressed the migration (F) and invasion (G) of MDA-MB-231 cells. PI3KIR suppressed NRSN2-promoted (H) proliferation, (I) migration and (J) invasion of MDA-MB-231 cells. Control, PBS-treated cells. NC, negative control; NRSN2, neuensin-2; ns, not significant; p-, phosphorylated; si, small interfering (RNA); si-RNRSN2, siRNA against NRSN2; pRK5-NRSN2, NRSN2 overexpression vector; NF- $\kappa$ BIR, NF- $\kappa$ B inhibitor; PI3KIR, PI3K inhibitor. \*\*P<0.01.

injection of siR-NRSN2 plasmid-transfected MDA-MB-231 cells induced smaller tumor volume and weight compared with in nude mice injected with cells transfected pRK5-control vector (Fig. 5A and B). The results from western blotting and IHC demonstrated that NRSN2 protein expression was increased in pRK5-NRSN2 plasmid-transfected tumor tissues compared with in siR-NRSN2 plasmid-transfected tumor

tissues (Fig. 5C and D). These results suggested that NRSN2 may promote tumor growth *in vivo*.

## Discussion

Breast cancer is the most common female cancer worldwide. It is commonly diagnosed at advanced stages and exhibits



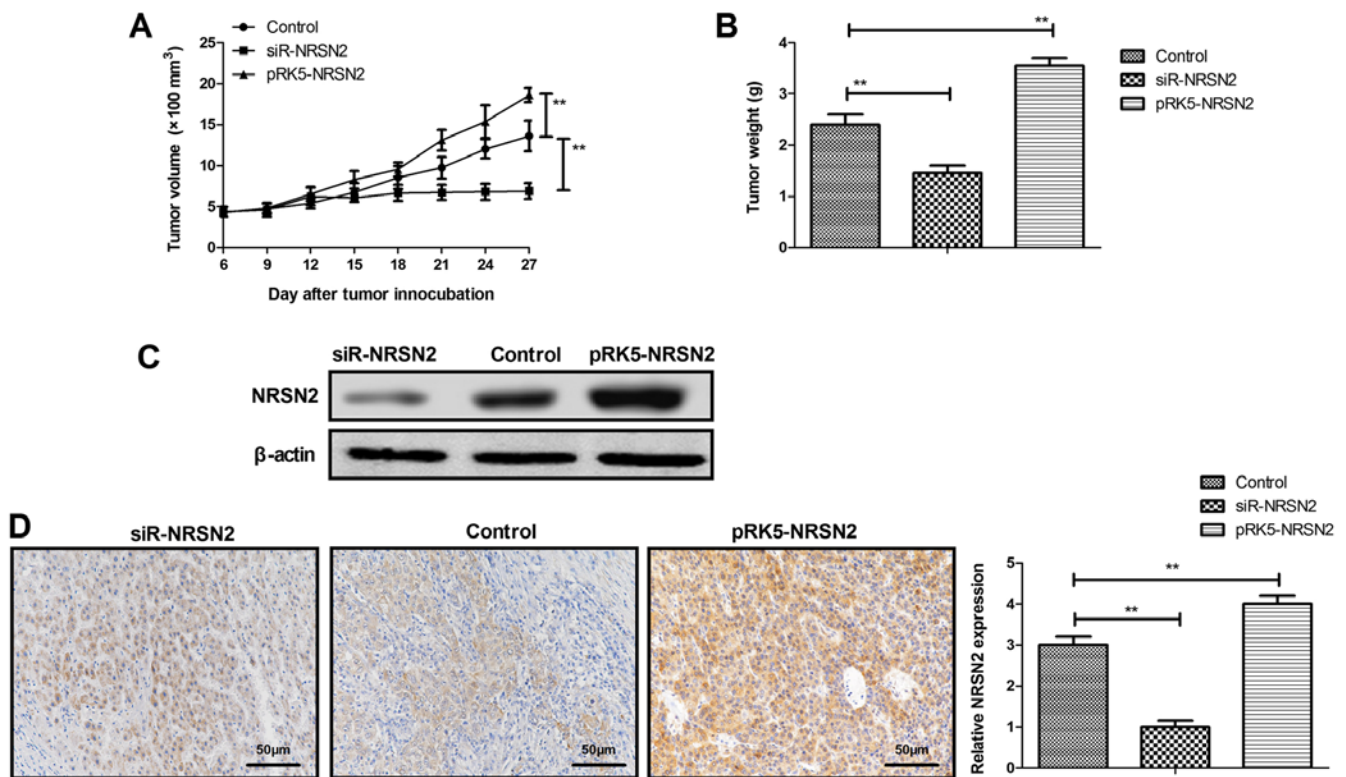


Figure 5. NRSN2 overexpression promotes tumor growth *in vivo*. NRSN2 overexpression (A) promoted tumor growth and (B) increased tumor weight in a xenograft mouse model. (C) NRSN2 protein expression was upregulated in pRK5-NRSN2 plasmid-transfected tumor tissues. (D) Immunohistochemistry analyzed NRSN2 protein expression in tumor tissues. NRSN2, neuensin-2; si, small interfering. \*\* $P < 0.01$ .

rising incidence and mortality rates (18). Previous studies have indicated that NRSN2 is highly expressed in numerous human cancer cells, including lung cancer and hepatocellular carcinoma, and may therefore be considered as a potential target for human cancer treatment (10,11,13). The results from the present study suggested that NRSN2 may serve an important role in the carcinogenesis and progression of breast cancer *in vitro* and *in vivo*. The results demonstrated that NRSN2 was highly expressed in breast cancer tissues and cells, and may therefore stimulate the progression of breast cancer and promote the proliferation of breast cancer cells. These findings also indicated that NRSN2 knockdown may inhibit the migration and invasion of breast cancer cells *in vitro*. Notably, NRSN2 knockdown potentially regulated human breast cancer proliferation via PI3K/AKT/mTOR and NF- $\kappa$ B signaling pathway inactivation.

Tumor markers have been widely used for the diagnosis of early stage breast cancer in patients (19-21). The present study demonstrated that NRSN2 was significantly upregulated in breast cancer tissues compared with adjacent noncancerous tissues. Although a previous study reported that NRSN2 downregulation promoted cell proliferation and survival via the PI3K/AKT/mTOR pathway in hepatocellular carcinoma (13), the results from the present study demonstrated that NRSN2 downregulation significantly inhibited breast cancer cell proliferation and aggressiveness. Notably, NRSN2 controlled human breast cancer proliferation via the regulation of PI3K/AKT/mTOR and NF- $\kappa$ B signaling pathways; however, NF- $\kappa$ B inhibitor or PI3K inhibitor had no effects on the proliferation, migration and invasion of MDA-MB-231

cells. These results suggested that NRSN2 may regulate the proliferation and aggressiveness of breast cancer cells via PI3K/AKT/mTOR and NF- $\kappa$ B signaling pathways.

Currently, the PI3K/AKT/mTOR signaling pathway is considered as a potential target for breast cancer therapy (22). A recent study reported that intermittent hypoxia induces the overexpression of prometastatic genes in breast cancer cells via NF- $\kappa$ B, such as tenascin-C (an essential factor of the metastatic niche) and matrix metalloproteinase 9, and induces pro-inflammatory processes, via cyclooxygenase-2 (COX-2) for example (23). According to previous studies (24-26), alterations of the PI3K/AKT/mTOR and NF- $\kappa$ B signaling pathways were investigated in breast cancer cell lines following NRSN2 overexpression or knockdown.

Shen *et al* (27) reported that the tumor volume in 6-week-old female NOD/SCID mice injected with wild-type MDA-MB-231 cells ( $1 \times 10^6$  cells in  $30 \mu\text{l}$  PBS) was  $\sim 200 \text{ mm}^3$  at day 22. In the present study, specific pathogen-free female Balb/c nude mice were injected with MDA-MB-231 cells transfected with pRK5-hNRSN2- or pRK5-vector (control;  $1 \times 10^7$  cells) or MDA-MB-231 cells to analyze the role of NRSN2 in breast cancer growth. The tumor volume of wild-type MDA-MB-231-injected mice at day 27 was  $\sim 2,100 \text{ mm}^3$ . The difference in the tumor volumes between the present study and the study by Shen *et al* (27) may be due to the types of mice used and the number of cells injected. The results from the present study demonstrated that NRSN2 stimulated PI3K/AKT/mTOR and NF- $\kappa$ B phosphorylation in MDA-MB-231 cells, which further promoted breast cancer cells growth both *in vitro* and *in vivo*. However, only 8 mice were used in this study to explore the inhibitory effects of NRSN2.

Further investigation using a larger sample size would therefore be needed to determine other signaling pathways associated with NRSN2. In addition, the present study did not use ideal controls when investigating the effects of NF- $\kappa$ B inhibitor or PI3K inhibitor on the proliferation, migration and invasion of breast cancer cells, as an overexpression-only group was not included. The effects of NF- $\kappa$ B inhibitor or PI3K inhibitor on NRSN2 expression will also be further analyzed in the future.

In conclusion, the results from the present study demonstrated that NRSN2 was overexpressed in breast cancer tissues and cells, and that it significantly promoted the proliferation and aggressiveness of breast cancer by activating PI3K/AKT/mTOR and NF- $\kappa$ B signaling pathways. These findings suggested that NRSN2 may be considered as a potential therapeutic target for breast cancer.

### Acknowledgement

Not applicable.

### Funding

No funding was received.

### Availability of data and materials

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

### Authors' contributions

FR and WZ performed all experiments. SL and HR prepared for the experiments, analyzed and collected data. YG designed the study and wrote the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The animal study was approved by the Chinese Association for Laboratory Animal Operations. The patient study was approved by the Ethic Committee of Peking University (approval no. PEK20150524). All patients provided written informed consent prior to the study.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

### References

- DeSantis CE, Fedewa SA, Goding Sauer A, Kramer JL, Smith RA and Jemal A: Breast cancer statistics, 2015: Convergence of incidence rates between black and white women. *CA Cancer J Clin* 66: 31-42, 2016.
- Iranshahi N, Zafari P, Yari KH and Alizadeh E: The most common genes involved in epigenetics modifications among Iranian patients with breast cancer: A systematic review. *Cell Mol Biol (Noisy-le-grand)* 62: 116-122, 2016.
- Araki K and Ito Y: A review multigene assays for clinical utility in breast cancer. *Gan To Kagaku Ryoho* 43: 1332-1340, 2016 (In Japanese).
- Lucius K and Trukova K: Integrative therapies and cardiovascular disease in the breast cancer population: A review, part 2. *Integr Med (Encinitas)* 14: 33-40, 2015.
- Lucius K and Trukova K: Integrative therapies and cardiovascular disease in the breast cancer population: A review, part 1. *Integr Med (Encinitas)* 14: 22-29, 2015.
- Leuteritz K, Weißflog G, Barthel Y, Brähler E, Zwerenz R, Wiltink J and Beutel ME: Therapeutic alliance and treatment outcome in psychodynamic psychotherapy of depressed breast cancer patients: The same old story or different from other populations? *Breast Cancer* 24: 765-773, 2017.
- Moreno Ayala MA, Gottardo MF, Asad AS, Zuccato C, Nicola A, Seilicovich A and Candolfi M: Immunotherapy for the treatment of breast cancer. *Expert Opin Biol Ther* 17: 797-812, 2017.
- Allaire BT, Ekwueme DU, Poehler D, Thomas CC, Guy GP Jr, Subramanian S and Trogon JG: Breast cancer treatment costs in younger, privately insured women. *Breast Cancer Res Treat* 164: 429-436, 2017.
- Holzel D, Eckel R, Bauerfeind I, Baier B, Beck T, Braun M, Ettl J, Hamann U, Kiechle M, Mahner S, *et al*: Improved systemic treatment for early breast cancer improves cure rates, modifies metastatic pattern and shortens post-metastatic survival: 35-year results from the munich cancer registry. *J Cancer Res Clin Oncol* 143: 1701-1712, 2017.
- An Y, Amr SS, Torres A, Weissman L, Raffalli P, Cox G, Sheng X, Lip V, Bi W, Patel A, *et al*: SOX12 and NRSN2 are candidate genes for 20p13 subtelomeric deletions associated with developmental delay. *Am J Med Genet B Neuropsychiatr Genet* 162B: 832-840, 2013.
- Zhang XY, Kuang JL, Yan CS, Tu XY, Zhao JH, Cheng XS and Ye XQ: NRSN2 promotes non-small cell lung cancer cell growth through PI3K/Akt/mTOR pathway. *Int J Clin Exp Pathol* 8: 2574-2581, 2015.
- Tang W, Ren A, Xiao H, Sun H and Li B: Highly expressed NRSN2 is related to malignant phenotype in ovarian cancer. *Biomed Pharmacother* 85: 248-255, 2017.
- Wang X, Han L, Zhang J and Xia Q: Down-regulated NRSN2 promotes cell proliferation and survival through PI3K/Akt/mTOR pathway in hepatocellular carcinoma. *Dig Dis Sci* 60: 3011-3018, 2015.
- Jeong YJ, Choi Y, Shin JM, Cho HJ, Kang JH, Park KK, Choe JY, Bae YS, Han SM, Kim CH, *et al*: Melittin suppresses EGF-induced cell motility and invasion by inhibiting PI3K/Akt/mTOR signaling pathway in breast cancer cells. *Food Chem Toxicol* 68: 218-225, 2014.
- Kuo YY, Jim WT, Su LC, Chung CJ, Lin CY, Huo C, Tseng JC, Huang SH, Lai CJ, Chen BC, *et al*: Caffeic acid phenethyl ester is a potential therapeutic agent for oral cancer. *Int J Mol Sci* 16: 10748-10766, 2015.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-</sup>(Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Fernandez-Pol S, Ma L, Ohgami RS and Arber DA: Immunohistochemistry for p53 is a useful tool to identify cases of acute myeloid leukemia with myelodysplasia-related changes that are TP53 mutated, have complex karyotype, and have poor prognosis. *Mod Pathol* 30: 382-392, 2017.
- Gupta A, Shridhar K and Dhillon PK: A review of breast cancer awareness among women in India: Cancer literate or awareness deficit? *Eur J Cancer* 51: 2058-2066, 2015.
- Brodsky AS, Xiong J, Yang D, Schorl C, Fenton MA, Graves TA, Sikov WM, Resnick MB and Wang Y: Identification of stromal ColXalpha1 and tumor-infiltrating lymphocytes as putative predictive markers of neoadjuvant therapy in estrogen receptor-positive/HER2-positive breast cancer. *BMC Cancer* 16: 274, 2016.
- Tan H, Zhang H, Yang W, Fu Y, Gu Y, Du M, Cheng D and Shi H: Breast-specific gamma imaging with Tc-99m-sestamibi in the diagnosis of breast cancer and its semiquantitative index correlation with tumor biologic markers, subtypes, and clinico-pathologic characteristics. *Nucl Med Commun* 37: 792-799, 2016.
- Adamczyk A, Niemiec J, Ambicka A, Mucha-Matecka A, Ryś J, Mituś J, Wysocki WM, Cichocka A and Jakubowicz J: Survival of breast cancer patients according to changes in expression of selected markers between primary tumor and lymph node metastases. *Biomark Med* 10: 219-228, 2016.

22. Araki K and Miyoshi Y: Mechanism of resistance to endocrine therapy in breast cancer: The important role of PI3K/Akt/mTOR in estrogen receptor-positive, HER2-negative breast cancer. *Breast Cancer* 25: 392-401, 2018.
23. Gutsche K, Randi EB, Blank V, Fink D, Wenger RH, Leo C and Scholz CC: Intermittent hypoxia confers pro-metastatic gene expression selectively through NF- $\kappa$ B in inflammatory breast cancer cells. *Free Radic Biol Med* 101: 129-142, 2016.
24. Guerrero-Zotano A, Mayer IA and Arteaga CL: PI3K/AKT/mTOR: Role in breast cancer progression, drug resistance, and treatment. *Cancer Metastasis Rev* 35: 515-524, 2016.
25. Li Z, Qian J, Li J and Zhu C: Knockdown of lncRNA-HOTAIR downregulates the drug-resistance of breast cancer cells to doxorubicin via the PI3K/AKT/mTOR signaling pathway. *Exp Ther Med* 18: 435-442, 2019.
26. Liu B, Sun L, Liu Q, Gong C, Yao Y, Lv X, Lin L, Yao H, Su F, Li D, *et al*: A cytoplasmic NF- $\kappa$ B interacting long noncoding RNA blocks I $\kappa$ B phosphorylation and suppresses breast cancer metastasis. *Cancer Cell* 27: 370-381, 2015.
27. Shen Q, Cohen B, Zheng W, Rahbar R, Martin B, Murakami K, Lamorte S, Thompson P, Berman H, Zúñiga-Pflücker JC, *et al*: Notch shapes the innate immunophenotype in breast cancer. *Cancer Discov* 7: 1320-1335, 2017.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.