NUSAP1 promotes the metastasis of breast cancer cells via the AMPK/PPARγ signaling pathway

Juanjuan Qiu[1,2#^], Li Xu[1#], Xiaohong Zeng[1#], Zhenru Wu[2], Yu Wang[1], Yao Wang[1], Jiqiao Yang[1], Hao Wu[1], Yanyan Xie[1], Faqing Liang[1], Qing Lv[1], Zhenggui Du[1^]

[1]Department of Breast Surgery, West China Hospital, Sichuan University, Chengdu, China; [2]Key Laboratory of Transplant Engineering and Immunology NHC, West China Hospital, Sichuan University, Chengdu, China

Contributions: (I) Conception and design: Z Du; (II) Administrative support: None; (III) Provision of study materials or patients: Q Lv, Z Du, F Qing, Y Xie; (IV) Collection and assembly of data: Yu Wang, Z Wu, X Zeng; (V) Data analysis and interpretation: J Qiu, L Xu, Yao Wang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Zhenggui Du. Department of Breast Surgery, West China Hospital, Sichuan University, Chengdu, China. Email: docduzg@163.com.

Background: The biological function and molecular mechanism of nucleolar spindle-associated protein 1 (NUSAP1) in breast cancer remain controversial, this study aimed to reveal the mechanism of NUSAP1 in breast cancer cell metastasis and survival.

Methods: The expression of NUSAP1 expression was evaluated by immunohistochemistry and quantitative real-time polymerase chain reaction (RT-qPCR) in breast tissue samples. The correlation of NUSAP1 expression with the clinicopathological parameters of the patients and overall survival (OS) was evaluated. The protein expression was detected by Western blotting, the cell proliferation was evaluated by Edu staining and MTT assay, migration and invasion were tested by transwell and migration assay. Female BALB/c nude mice models for tumor growth and metastasis of breast cancer were evaluated *in vivo*.

Results: NUSAP1 is up-regulated in multiple cancers and is associated with a poor prognosis in breast cancer patients. Further analysis of the Gene Expression Omnibus (GEO) database and our included patients revealed that NUSAP1 expression gradually increased with pathological changes in breast tissue. Cell function assays confirmed that NUSAP1 was related to the proliferation, migration, and invasion of breast cancer cells. *In vivo*, NUSAP1 promoted lung metastasis in nude mice. We found that the NUSAP1 protein can promote tumor proliferation and metastasis by activating the AMPK/PPARγ signaling pathway.

Conclusions: Our findings show that NUSAP1 promotes breast cancer proliferation and metastasis by activating the AMPK/PPARγ signaling pathway.

Keywords: Breast cancer; nucleolar spindle-associated protein 1 (NUSAP1); AMPK; PPARγ; proliferation; metastasis

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^ ORCID: Juanjuan Qiu, 0000-0003-3852-5243; Zhenggui Du, 0000-0002-7412-0270.

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Introduction

The GLOBOCAN 2020 report of the International Agency for Cancer Research showed that breast cancer overtook lung cancer for the first time as cancer with the highest incidence rate worldwide (1). Local invasion and distant metastasis are the main risk factors associated with survival. There is no effective method to completely cure breast cancer metastasis, and prevention and control are the focus of treatment for metastatic breast cancer.

Nucleolar spindle-associated protein 1 (NUSAP1) has recently been recognized as a cell cycle regulating protein that binds microtubules and dominates mitotic progression, spindle formation, and stability (2,3). The structural integrity of spindle ensures an equal division of the chromosomes, which can make a prerequisite for cell division. Abnormal spindle structure can result in wrong chromosome separation (also known as chromosomal instability), which will lead to tumorigenesis (4).

In recent years, with the in-depth study of NUSAP1, its biological function has been gradually identified. Several studies have shown that NUSAP1 plays an important regulatory role in tumors, including colorectal cancer (5,6), prostate cancer (7), breast cancer (8), lung cancer (9,10) and cervical cancer (11), and that a poor prognosis of breast cancer and melanoma may be significantly associated with high expression of NUSAP1 (7,11,12). Further studies revealed that NUSAP1 plays an important role in cancer development, progression, and metastasis. For example, NUSAP1 is expressed in cervical cancer, and cancer stem cell traits and epithelial-mesenchymal transition progression can be enhanced by high expression of NUSAP1, resulting in cancer metastasis (13). A previous study confirmed that compared with normal tissue samples, NUSAP1 presented high expression in breast cancer tissue samples. In additional, NUSAP1 overexpression promoted the growth, migration, and invasion of MCF-7 cells (14). This study complemented the subcutaneous tumorigenesis and tail vein injection metastasis model in nude mice to make the study more reliable and complete. Furthermore, microarray analysis has revealed that NUSAP1 increased during from normal tissue to invasive ductal carcinoma, suggesting that NUSAP1 played an important role in tumor occurrence and progression perspective. These results suggest that high NUSAP1 expression is associated with the occurrence and progression of tumors.

The above results show that NUSAP1 plays an important role in the development, survival, and metastasis of various cancers. However, the biological function and molecular mechanism of NUSAP1 in breast cancer remain controversial. This study aimed to reveal the mechanism of NUSAP1 in breast cancer cell metastasis and survival.

We present the following article in accordance with the ARRIVE reporting checklist (available at https://dx.doi.org/10.21037/atm-21-5517).

Methods

Breast cancer patient microarray gene expression data from Gene Expression Omnibus (GEO) and survival analysis

The expression profile GSE5364 was obtained from GEO (https://www.ncbi.nlm.nih.gov/geo/), a free and publicly available database. The set comprises 341 samples (270 tumors and 71 matched normal samples) from patients with breast, colon, liver, lung, esophageal, and thyroid cancer. We used the "limma" R package to detect NUSAP1 between breast cancer samples and normal breast tissue samples. Survival analysis was performed on the Kaplan-Meier Plotter website (15), whose sources for the databases included the GEO, the European Genome-Phenome Archive (EGA), and The Cancer Genome Atlas (TCGA).

Tissue specimens and follow-up of patients

The 80 breast cancer samples analyzed in our study were from patients who underwent surgery between August and November 2015 at the Department of Breast Surgery at the West China Hospital of Sichuan University (Chengdu, China). The other 30 pairs of breast cancer and adjacent tissues for quantitative real-time polymerase chain reaction (RT-qPCR) analyses were collected from patients who underwent surgery between June and July 2019 in the same hospital. Patients who had received neoadjuvant chemotherapy and those without a complete pathology diagnostic report were excluded. We also included 40 patients [10 usual ductal hyperplasia (UDH), 10 atypical ductal hyperplasia (ADH), 10 ductal carcinoma *in situ* (DCIS) and 10 invasive ductal carcinoma (IDC)] for tissue microarray analysis (the normal tissues were also from the 10 UDH patients). Each patient was followed up every year to ensure reliable prognostic information. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All patients provided their written informed consent for inclusion before they participated in the study. The study was approved by the Ethics Committee

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of the Sichuan University [No. (2015)108].

Cell culture

Human breast cancer cell lines BT-474 and MDA-MB-231 were purchased from the American Type Culture Collection. Dulbecco's modified Eagle's medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA) was used to culture MDA-MB-231 cells, and RPMI 1640 (Invitrogen, USA) supplemented with 10% fetal bovine serum was used for BT-474 cells. All cell lines were cultured in a humidified incubator at 37 ℃ with 5% CO_2.

RT-qPCR, immunohistochemistry, and Western blot

RT-qPCR, western blotting, and immunohistochemistry were performed as previously reported (16). The following primers were used to amplify cDNA: NUSAP1: 5'-ATGAATGAACTGAAGCAGCCCA-3'; NC shRNA: 5'-ACTTAGGTGCGATACACGCTT-3' (Sangon Biotech, China), all sequences of used NUSAP1 shRNA and NC shRNA were showed in Table S1.

Immunohistochemistry was performed as previoussly reported (17). Primary antibodies (NUSAP1) coupled with an HRP-polymer kit (Maixin Biotechnology, China) were used, followed by incubation with 3,3'-diaminobenzidine for coloration. NUSAP1 was observed in breast cancer patients and pulmonary metastatic nodules of BALB/c nude mice. Immunohistochemical intensity scores were classified as follows: 0, without staining; 1, weak staining (light yellow); 2, moderate staining (yellow-brown); or 3, strong staining (brown); the percentage of stained cells was scored as follows: 0, <5%; 1, 6–25%; 2, 26–50%; 3, 51–75%; or 4, 76–100% positive cells (16). The total score was calculated by adding the density and percentage scores; high expression was defined as a total score of ≥4, while low expression was defined as a total score of <4. Two pathological experts conducted the evaluation.

Proteins in breast cancer cell lines and tissues were extracted with the Membrane and Cytosol Protein Extraction Kit (Beyotime, China). Protein concentrations were determined with the Enhanced BCA Protein Assay Kit (Beyotime). In this study, NUSAP1 antibodies (Affinity, USA), AMPK (Thermo Fisher Scientific, USA), PPAR (Thermo Fisher Scientific, USA), β-actin antibodies (Affinity), and horseradish peroxidase conjugated anti-mouse secondary antibodies (Santa Cruz Biotechnology, USA) were applied.

Transfection

Lentiviral vectors containing NUSAP1 shRNA, NC shRNA, and NUSAP1 OE plasmid were obtained from HanBio (China) and transfected into cells by following the manufacturer's instructions. Stable cells transfected with lentivirus were tested with puromycin (2 μg/mL) for use in subsequent experiments.

Cell proliferation, migration, and invasion assay

The MTT assay measured Cell proliferation (Beyotime Biotechnology, China) and the EdU assay (Ribo Life Science, China). In the Cell Counting Kit-8 (CCK-8) assay, MDA-MB-231 and BT-474 cells (1.0×10^3/well) were seeded in a 96-well plate. After 0, 24, 48, 72, and 96 h of incubation, cells were washed with PBS and dimethylsulfoxide was used to dissolve the purple formazan product. Then, 10 μL MTT was added to each well for 2 h. The absorbance of the resulting solution was assessed at a wavelength of 450 nm with a microplate reader (Thermo Scientific, USA). For the EdU assay, cells were subcultured in a 24-well plate for 1 day. The cell-Light EdU Apollo567 In Vitro Imaging Kit (RiboBio, China) was used for the EdU incorporation assay following the manufacturer's instructions 48 h after transfection. Images were captured under the fluorescence microscope.

Wound healing assays evaluated MDA-MB-231 cells. The cell line was seeded in six-well plates and scratched with a sterile pipette tip, and then the cells were washed with PBS. DMEM containing 5% FBS was added to each well. The images were sequentially captured at 0, 24, and 48 h of cultivation. The same areas of each wound were sequentially captured and the relative migration rate of the fused cells was calculated. The Transwell assay determined the migration ability of BT-474 cells due to their special growth characteristics. For BT-474 cells, migration assays, 6×10^4 cells suspended in 200 μL serum-free medium were added to the upper chamber of Transwell, each of which included a Tewksbury multi-porous polycarbonate membrane (8-mm pore size) insert, and medium containing 10% FBS was placed as a chemical attractant at the bottom of the chamber.

According to the manufacturer's instructions, cell invasion was detected using a Transwell chamber coated with Matrigel (1:100 in DMEM; BD Biosciences, USA). The 2×10^5 cells in serum-free medium were added to the upper chamber, and the lower chamber contained 500 μL of 20%

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FBS-supplemented medium. After culturing for 24 h, the cells were fixed with 4% paraformaldehyde for 30 min. The migrated cells were stained with crystal violet for 20 min and washed with PBS three times.

Animal studies

Twenty female BALB/c nude mice (4–5 weeks old) were purchased from Gem Pharmatech (Nanjing, China). All animal studies and procedures followed ethical guidelines and were approved by the Sichuan University Animal Ethics Committee (No. [2020]203), in compliance with the current national guidelines for Animal Welfare of China (GB/T 35892-2018) for the care and use of animals. A protocol was prepared before the study without registration. All mice were maintained on a regular diurnal lighting cycle (12:12 light:dark) with ad libitum access to food and water at a temperature of 22 to 26 ℃. Wood shavings was used as bedding. The weight loss caused by tumor overload was logged as "adverse events" in the experiment, which would terminate the experiment and the mice was immediately killed. The sample size was determined from a similar experiment reported in the literature (15,17). Animals were excluded if the animal died prematurely, preventing tumor and lung collection. For the subcutaneous inoculation model, ten mice were randomly divided into two groups (5 mice/group): the NC group and the KD group, 1×10^7 cells in 200 µL PBS were injected subcutaneously into the right dorsal flank, random numbers were generated using the standard = RAND() function in Microsoft Excel (18). The mice were sacrificed and tumor volume was measured after 6 weeks; the formula calculated tumor volume:

$$\text{Tumor volume} = \frac{\pi}{6} \times L \times W^2 \quad [1]$$

where L is the longest diameter and W is the shortest diameter of the excised tumor. For the metastasis model, the grouping method is the same as before; 2×10^6 cells in 200 µL PBS were injected into the tail veins of 6-week-old female nude mice. The mice were sacrificed after 6 weeks, and the tumor nodules formed in the lungs were embedded in paraffin for H&E staining and counted. No animals were excluded from this study. Three different investigators were involved in this study as follows: the first investigator injected cells, who was aware of the allocation of the treatment group. The second investigator performed the surgical procedure, while the third analyzed the data.

Statistical analysis

SPSS 20.0 software (IBM Corporation, Armonk, NY, USA) was used for statistical analyzes. Data are presented as mean ± standard deviation and were compared using the Student's *t* test or analysis of variance. Survival curves were estimated using the Kaplan-Meier method and the logarithmic rank test was used to assess the difference in survival curves. $P<0.05$ (two-sided) was considered statistically significant.

Results

The expression of NUSAP1 in multiple tumors, including breast cancer

GEO database analysis showed that NUSAP1 was one of the hub genes in multiple cancers (breast, colon, liver, lung, esophageal, and thyroid cancer) (*Figure 1A*). In various tumors, NUSAP1 was highly expressed in tumor tissues compared to adjacent tissues (*Figure 1B*), which was also verified in breast cancer (*Figure 1C*). Furthermore, we assessed the expression of NUSAP1 in 30 breast cancer tissues and adjacent tissues using RT-PCR. As expected, the expression of NUSAP1 mRNA was higher in breast cancer tissues than in adjacent tissues (*Figure 1D*).

In the analysis of the other 80 breast cancer tissues, we found that the expression of NUSAP1 was associated with diameter (P=0.039), estrogen receptor status (ER) (P=0.024), progesterone receptor status (PR) (P=0.038), Ki67 (P=0.011), molecular phenotype (P<0.001) and lymph node metastasis (P=0.008) (*Table 1*); 59% (29/49) of patients with high expression of NUSAP1 were diagnosed with lymph node metastasis, and 83% (20/24) of patients with lymph node metastasis had high expression of NUSAP1, indicating that NUSAP1 could promote metastasis and prognosis of breast cancer.

The gradual increase in NUSAP1 expression with breast tissue pathological changes and its influence on the overall survival (OS) and recurrence-free survival (RFS) of breast cancer patients

To investigate the potential role of NUSAP1 in breast cancer progression, we examined the expression of NUSAP1 in different pathological types of breast tissue. Compared with that in normal breast tissue, the expression of NUSAP1 increased stepwise in UDH, ADH, and DCIS, according to GEO database analysis. The expression of

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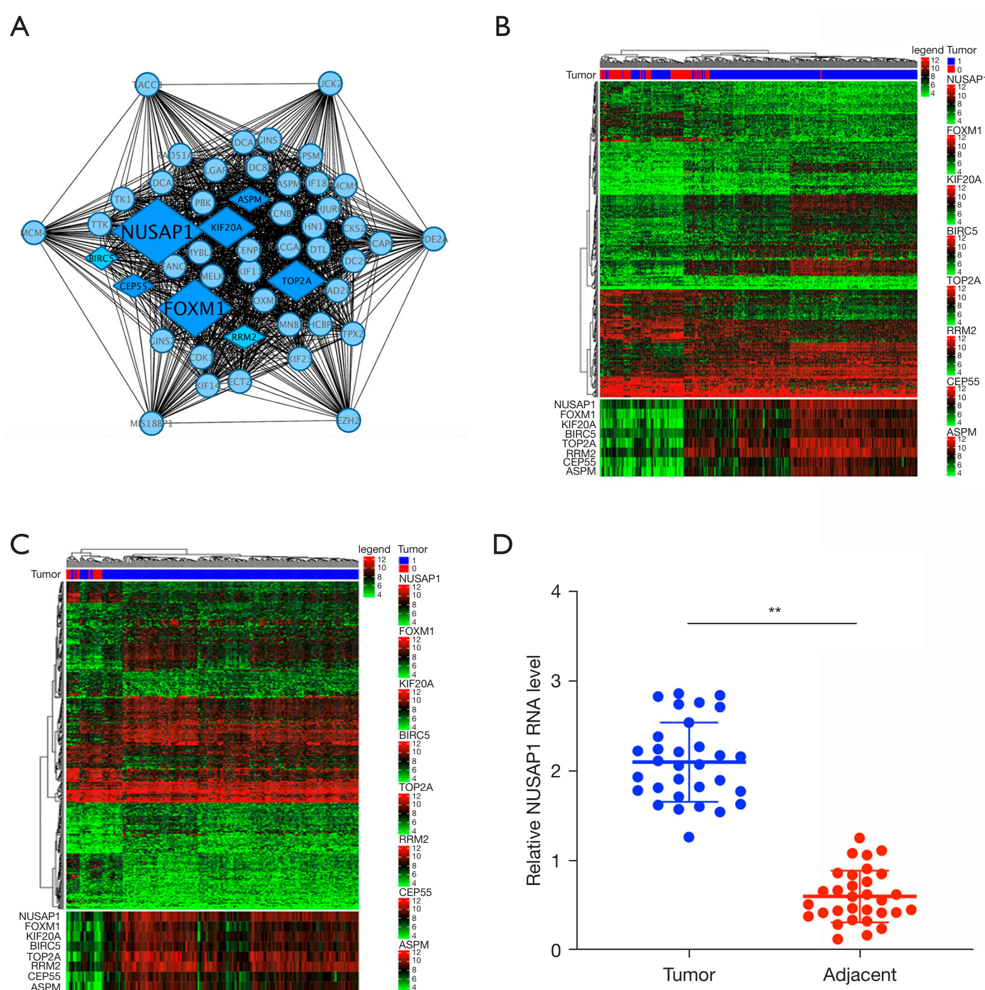

Figure 1 NUSAP1 expressed highly in a variety of tumors. (A) NUSAP1 was one of the hub genes in multi-cancer (breast, colon, liver, lung, oesophagal and thyroid cancer) progression in GEO database; NUSAP1 in tumor tissues was higher than that in adjacent tissues in multi-cancer (B) and in breast cancer (C) in GEO database. (D) NUSAP1 mRNA expression in breast cancer tissues and adjacent tissues. **, P<0.01. NUSAP1, nucleolar spindle-associated protein 1; GEO, Gene Expression Omnibus.

NUSAP1 in IDC was slightly lower than that in DCIS, but was still significantly higher than that in other tissue types (*Figure 2A,2B*). To verify this conclusion, we stained a tissue microarray of normal breast, UDH, ADH, DCIS, and IDC tissues and obtained a similar conclusion (*Figure 2C,2D*).

We analyzed the effect of NUSAP1 expression on the prognosis of breast cancer patients by using online tools. The results showed that patients with high expression of NUSAP1 had worse OS and RFS than patients with low expression (P value was 1.9×10^{-7} and $<1\times10^{-16}$, respectively) (*Figure 3A,3B*). Kaplan-Meier analysis of the 80 breast cancer patients was performed and yielded a similar conclusion (P values were 0.037 and 0.047, respectively) (*Figure 3C,3D*).

NUSAP1 significantly affected the proliferation of breast cancer cell lines

To detect the roles of NUSAP1 in breast cancer, we first detected NUSAP1 expression in breast cancer cell lines with differences in ER, PR, and HER2 status. The expression of NUSAP1 was higher in the MDA-MB-231 breast cancer cell line and lower in the BT-474 cell lines than in other cell lines (*Figure 4A*). Then, gain-of-function

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Table 1 Demographic characteristics and association between NUSAP1 expression and clinicopathological features

Parameters	Total (n=80)	NUSAP1		χ^2	P
		Low expression (n=31)	High expression (n=49)		
Age				1.311	0.252
≤40	25 (31.25)	12	13		
>40	55 (68.75)	19	36		
Diameters				6.479	0.039
≤2	22 (27.50)	13	9		
>2 and <5	47 (58.75)	13	34		
≥5	11 (13.75)	5	6		
ER				5.074	0.024
Negative	20 (25.00)	12	8		
Positive	60 (75.00)	19	41		
PR				4.296	0.038
Negative	23 (28.75)	13	10		
Positive	57 (71.25)	18	39		
Her2				0.216	0.642
Negative	48 (60.00)	18	30		
Positive	32 (40.00)	13	19		
Ki-67				6.492	0.011
<30%	30 (37.50)	17	13		
≥30%	50 (62.50)	14	36		
Molecular phenotypes				27.781	<0.001
Luminal A	19 (23.75)	13	6		
Luminal B	41 (51.25)	6	35		
HER2 positive	12 (15.00)	10	2		
Triple negative	8 (10.00)	2	6		
Histological classification				1.965	0.374
Low grade	9 (11.25)	5	4		
Moderate grade	43 (53.75)	14	29		
High grade	28 (35.00)	12	16		
Lymph node metastasis				7.045	0.008
Negative	56 (70.00)	27	29		
Positive	24 (30.00)	4	20		

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

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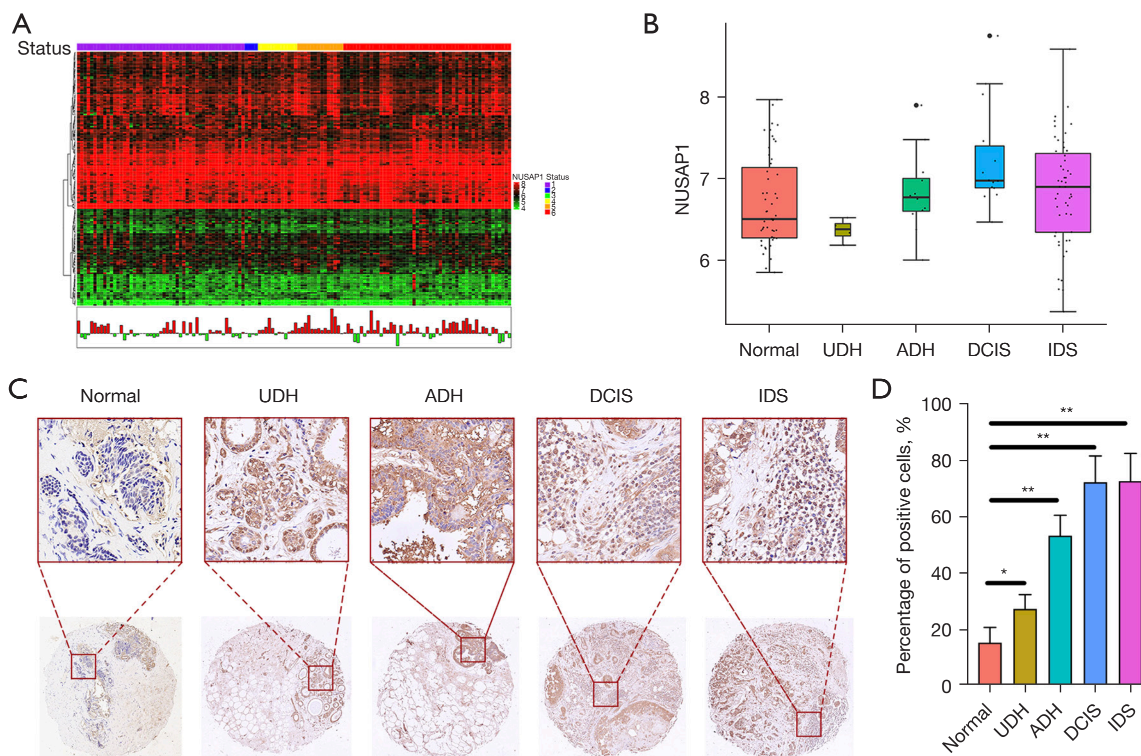

Figure 2 The expression of NUSAP1 increased gradually in normal breast tissue, UDH, ADH, DCIS and IDC. Compared with normal breast tissue, the expression of NUSAP1 in UDH, ADH and DCIS increased gradually and in IDC was also higher than other types in GEO database (A,B); immunohistochemical validation of tissue microarray results showed that NUSAP1 expression increased gradually from normal breast tissue to DCIS and IDC (C,D) (×40). *, P<0.05; **, P<0.01. NUSAP1, nucleolar spindle-associated protein 1; UDH, usual ductal hyperplasia; ADH, atypical ductal hyperplasia; DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; GEO, Gene Expression Omnibus.

and loss-of-function methods were used. We generated stable MDA-MB-231 and BT-474 cells with NUSAP1 knockdown (KD) or overexpression (OE). Western blotting was conducted to verify the protein expressions of NUSAP1 after transfection (*Figure 4B*). Subsequently, we further assessed cell proliferation. MTT and 5-ethynyl-2'-deoxyuridine (EdU) assays showed that KD of NUSAP1 remarkably inhibited the growth of breast cancer cells compared with cells without transfection. However, OE of NUSAP1 significantly enhanced the proliferation of breast cancer cells (*Figure 4C,4D*).

NUSAP1 significantly affected the migration and invasion of breast cancer cell lines

We evaluated the impact of NUSAP1 on the migration and invasion of MDA-MB-231 and BT-474 cells. Wound healing assays evaluated the migration of MDA-MB-231 cells, and Transwell assays evaluated the migration of BT-474 cells. Transwell assays evaluated the invasion of MDA-MB-231 and BT-474 cells. The results showed that the migration and invasion ability of MDA-MB-231 cell lines decreased after NUSAP1 KD (P<0.05; *Figure 5A,5B*), while NUSAP1 OE significantly enhanced the migration and invasion of BT-474 cells (P<0.05; *Figure 5C,5D*).

Knockdown of NUSAP1 inhibited breast cancer growth in vivo

To further verify the effect of NUSAP1 on breast cancer tumorigenesis *in vivo*, we established subcutaneous tumor and tail vein metastasis models in BALB/c-nu nude mice. MDA-MB-231-NUSAP1-KD and MDA-MB-231-NC cells were injected subcutaneously or via the tail vein. After

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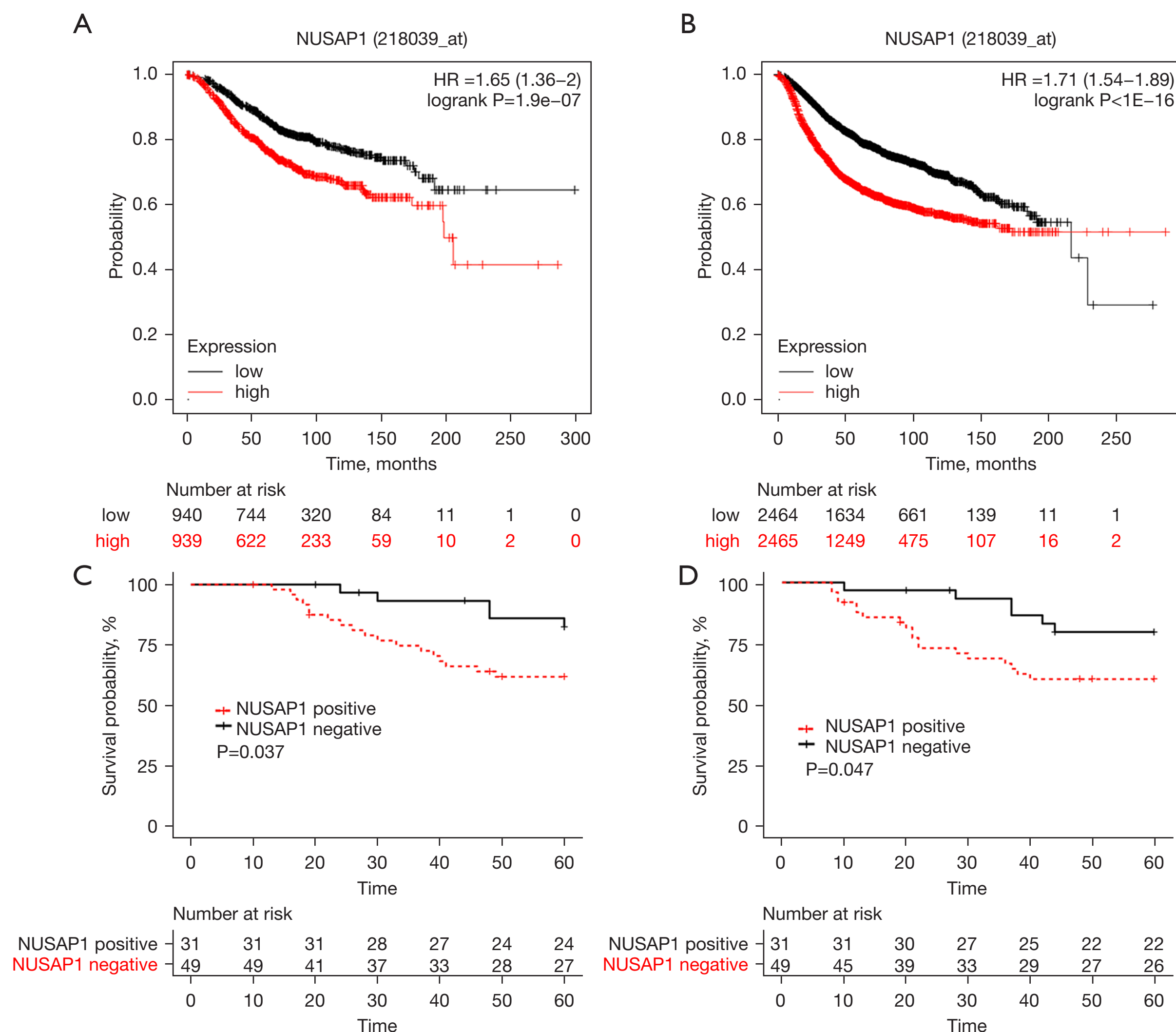

Figure 3 Positive expression of NUSAP1 was associated with poor OS and RFS in patients with breast cancer in GEO, EGA, and TCGA database (A,B), the similar results were found in our included breast cancer patients (C,D). NUSAP1, nucleolar spindle-associated protein 1; OS, overall survival; RFS, relapse free survival; GEO, Gene Expression Omnibus; EGA, European Genome-Phenome Archive; TCGA, The Cancer Genome Atlas.

42 days, mice were sacrificed, and the subcutaneous tumors and lungs were enucleated and analyzed.

The results revealed that NUSAP1 KD could significantly inhibit tumor growth (NC and KD group were 953.0±100.6, 513.6±156.3, respectively) (*Figure 6A*, P=0.0011), and western blotting confirmed nUSAP1 expression after isolating the tumor at the end of the seventh week (*Figure 6B*). NUSAP1 KD also strongly suppressed the metastasis of MDA-MB-231 cells (*Figure 6C*), which was further confirmed by counting the visible metastatic lesions by hematoxylin and eosin (H&E) staining (NC and KD group were 24.4±8.9, 13.0±4.9, respectively) (*Figure 6D*, P=0.0354). Immunohistochemistry confirmed the expression of NUSAP1 expression (*Figure 6E*).

NUSAP1 activates the AMPK/PPAR pathway in breast cancer cells

The Kyoto Encyclopedia of Genes and Genomes (KEGG)/ Gene Ontology (GO) analysis of differentially expressed genes was used to explore the mechanism by which NUSAP1 promotes breast cancer progression. Specifically,

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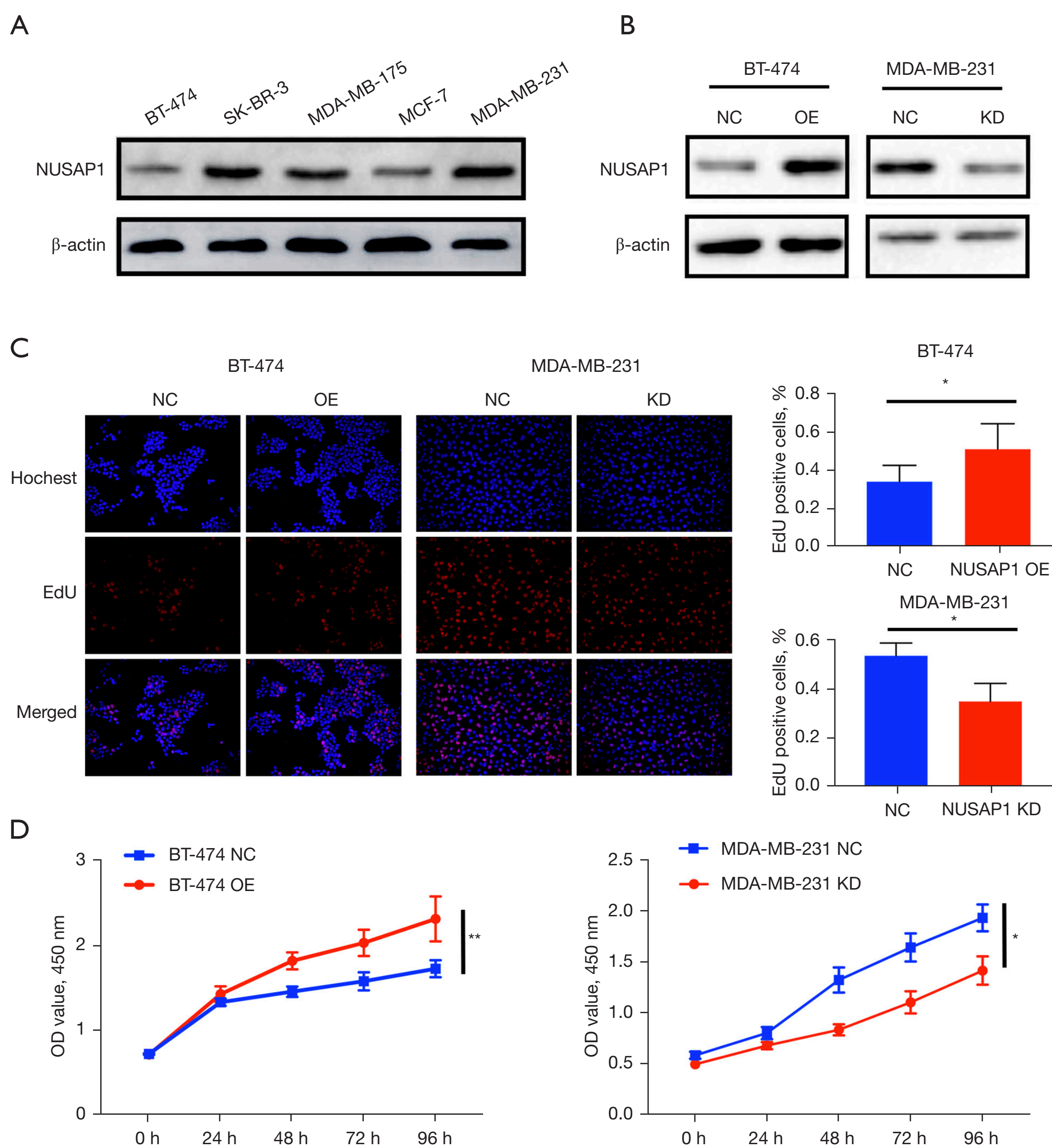

Figure 4 NUSAP1 affected proliferation of breast cancer cells. (A) NUSAP1 expression in different breast cancer cell lines; (B) Western blotting was performed to verify the protein levels of NUSAP1 after transfection; EDU (C) (×50) and MTT (D) assay were performed in stable KD or OE NUSAP1 group of MDA-MB-231 and BT-474 cell lines. *, P<0.05; **, P<0.01. NUSAP1, nucleolar spindle-associated protein 1; NC, negative control; OE, overexpression; KD, knockdown.

465 downregulated genes and 188 upregulated genes were selected for further analysis. The results indicated that NUSAP1 affected PPAR signaling, metabolic pathways, fatty acid pathways, etc. (*Figure 7A*). The AMPK/PPAR pathway is related to cell metabolism (18-21), which could affect the occurrence and development of tumors. Thus, to elucidate whether the AMPK/PAR pathway is involved in NUSAP1-induced breast cancer progression, Western blotting examined the expression of AMPK and PPARγ in MDA-MB-231-NUSAP1-KD cells, MDA-MB-231-NC cells, BT-474-NUSAP1-OE cells and BT-474-NC cells. *Figure 7B* shows that AMPK and PPARγ were downregulated in NUSAP1-KD cell lines and upregulated in NUSAP1 OE cell lines. In summary, the pathway by

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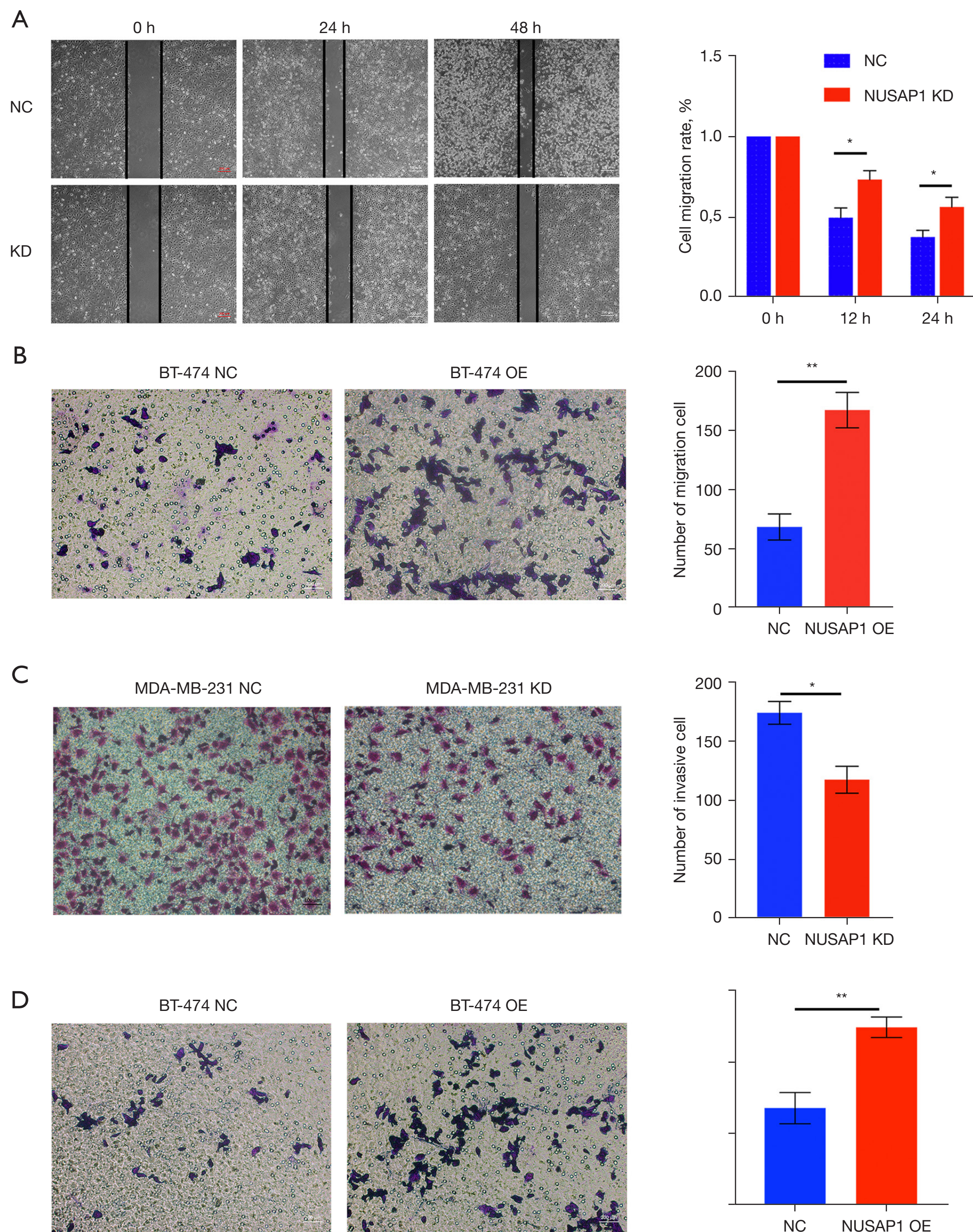

Figure 5 NUSAP1 expression regulates the breast cancer cells migration and invasion (×50). (A) Cell migration of the MDA-MB-231 cell line via wound healing assay in NC and KD NUSAP1 group; (B) cell invasion of the MDA-MB-231 cell line via transwell assay in NC and KD NUSAP1 group; cell migration (C) and invasion (D) of the BT-474 cell line via transwell assay in NC and OE NUSAP1 group; (B-D) was determined by crystal violet staining. *, P<0.05; **, P<0.01. NUSAP1, nucleolar spindle-associated protein 1; NC, negative control; OE, overexpression; KD, knockdown.

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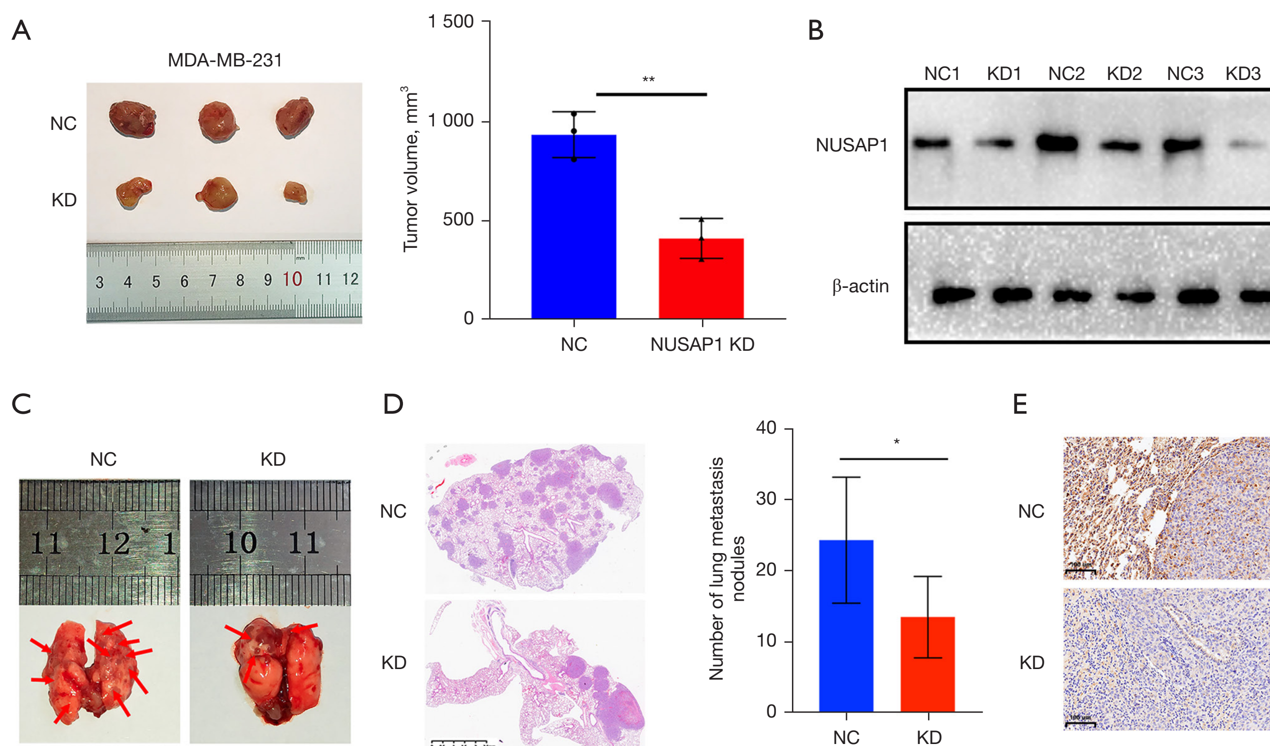

Figure 6 NUSAP1 promoted breast cancer growth and lung metastasis *in vivo*. (A) Representative images of xenografts and a summary of tumor weight in nude mice; (B) NUSAP1 expression in xenografts via western blot; (C) representative images of metastasis nodules (arrows) on the lung surface 8 weeks after tail vein injection of KD-NUSAP1 and NC-MDA-MB-231 cells; (D) representative images of hematoxylin and eosin staining sections derived from lung metastatic, the number of nodules was quantified in the right panel; (E) representative immunohistochemistry images of NUSAP1 expression in lung metastasis nodules, *, P<0.05; **, P<0.01. NUSAP1, nucleolar spindle-associated protein 1; NC, negative control; KD, knockdown.

which NUSAP1 affects breast cancer progression may be as follows. NUSAP1 affects the metabolism of breast cancer cells through the AMPK/PPARγ pathway, further affecting their proliferation and invasion abilities to regulate the progression of breast cancer (*Figure 7C*).

Discussion

The present study systematically assessed the expression of NUSAP1 in breast cancer tissues and investigated its role in the development of breast cancer. We found that NUSAP1 expression was higher in breast cancer tissues compared to adjacent tissues and that NUSAP1 was negatively correlated with OS and RFS. Interestingly, NUSAP1 expression increased with the transition from normal breast tissue to invasive breast cancer. Functionally, NUSAP1 OE promoted, while NUSAP1 KD inhibited, the proliferation of breast cancer cells *in vitro* and *in vivo*. This study demonstrated that NUSAP1 plays a critical role in breast cancer cell invasion *in vitro* and lung metastasis *in vivo*. We also found that NUSAP1 may affect the proliferation and metastasis of breast cancer through the AMPK/PPARγ pathway.

NUSAP1 is a 55 kD vertebrate protein that plays a key role in spindle assembly and normal cell cycle progression (22), and its expression is strictly regulated. When NUSAP1 expression increases abnormally, cells proliferate indefinitely (23), explains the high expression of NUSAP1 in various tumors, including breast cancer. NUSAP1 expression is associated with tumor diameter, ER status, PR status, Ki67 expression, molecular phenotypes, and lymph node metastasis. Western blot assays also showed that NUSAP1 was correlated with a cell line type. Furthermore, our study on breast cancer cell lines confirmed that NUSAP1 could significantly affect breast cancer cell proliferation. Many other studies have confirmed

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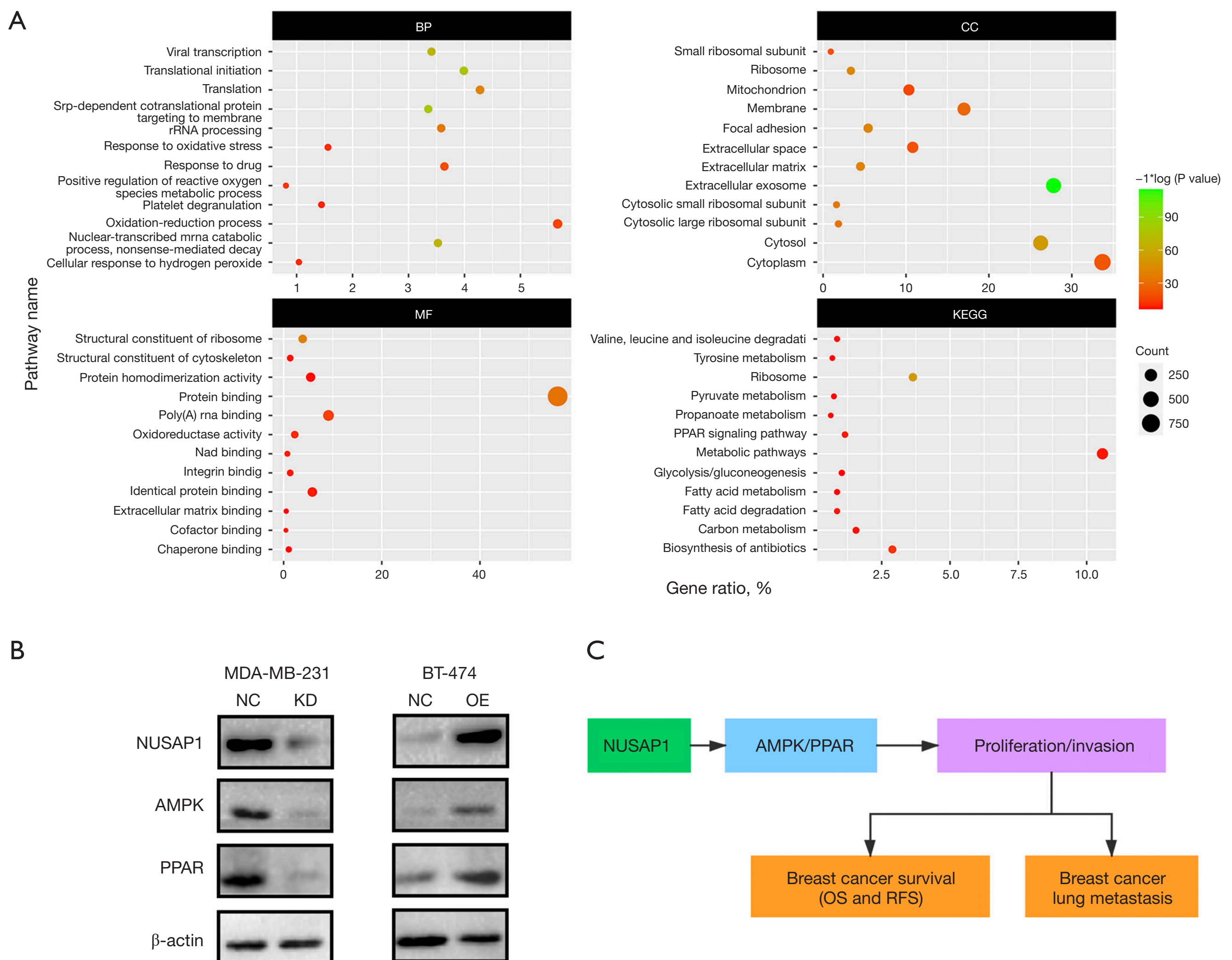

Figure 7 The promotion of NUSAP1 on breast cancer is related to AMPK signaling pathway. (A) KEGG/GO enrichment analysis showed that NUSAP1 may be related to PPAR signaling pathway in breast cancer; (B) western blot analysis of NUSAP1, AMPK and PPAR protein expression in NC and stable KD or OE NUSAP1 cell lines; (C) schematic diagram of the effect of NUSAP1 on breast cancer. BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; NUSAP1, nucleolar spindle-associated protein 1; NC, negative control; KD, knockdown; OE, overexpression.

that NUSAP1 could affect the growth of other cancers. For example, NUSAP1 can induce the proliferation and migration of colorectal cancer cells (5) and induce non-small-cell lung cancer cell growth and metastasis *in vitro* and *in vivo* (10).

In addition to cell proliferation, NUSAP1 was also involved in invasion and lymph node metastasis in breast cancer cell lines and in patients, which was confirmed in an animal study. The association between NUSAP1 and metastasis was confirmed in previous studies. For example, NUSAP1 silencing inhibits DNMT1 to inhibit colorectal cancer cell proliferation, migration, and invasion (5). In non-small-cell lung cancer, NUSAP1 KD inhibits cell metastasis by regulating BTG2/PI3K/Akt signaling (10). Additionally, we observed that NUSAP1 was highly expressed in patients with lymph node metastasis, indicating that NUSAP1 promotes lymph node metastasis in breast cancer. Further survival analysis showed worse OS and RFS in the NUSAP1-positive group, which was confirmed in the database. All the above results support that NUSAP1 may

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be a potential therapeutic target for breast cancer.

PPARγ belongs to the PPAR family of nuclear hormone receptors, which regulate various biological processes, including cell differentiation, cell proliferation, and glucose and fatty acid metabolism (23,24). In addition to acting as a transcription factor to regulate glycolysis and lipid metabolism to meet the energy needs of cancer cells (25,26), PPARγ can also act as a tumor protooncogene to promote tumorigenesis (27). PPARγ can increase ROS levels and inhibit tumor growth by regulating metabolism (28,29), inducing apoptosis (29), and inhibiting the proliferation, invasion, and migration of tumor cells (30,31). As one of the targets of aspirin, AMPK plays an important role in homeostasis, autophagy, and redox balance (32). However, increasing evidence also shows that AMPK has various substrates involved in different biological processes (33) and plays an inhibitory or promoting role in various tumors (34). For example, AMPK can lead to cell cycle arrest by promoting the phosphorylation of CDKN1 and p53 (35). In addition, AMPK promotes the growth of KRAS-mutant lung cancer by activating TFE3 (36). Our study first found that PPARγ may play a role in the occurrence and development of breast cancer through enrichment analysis. We also found that NUSAP1 affects the proliferation and metastasis of breast cancer through AMPK/PPARγ. This is the first study to show that NUSAP1 can promote breast cancer through the AMPK/PPARγ signaling pathway, which provides a new idea for targeting the NUSAP1/AMPK/PPARγ axis in breast cancer research and treatment. However, *in vitro* experiment the animal model does not adequately represent the molecular basis for, or the clinical features of cancer metastasis.

Conclusions

In summary, we demonstrate that NUSAP1 overexpression in breast cancer promotes breast cancer proliferation and metastasis via activating the AMPK/PPARγ signaling pathway, which provides evidence for NUSAP1 as a diagnostic and therapeutic target for breast cancer.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All patients provided their written informed consent for inclusion before they participated in the study. The study was approved by the Ethics Committee of the Sichuan University (No. [2015]108). All animal studies and procedures followed ethical guidelines and were approved by the Sichuan University Animal Ethics Committee (No. [2020]203), in compliance with the current national guidelines for Animal Welfare of China (GB/T 35892-2018) for the care and use of animals.

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Supplementary

Table S1 The sequences of NUSAP1 shRNAs used in this study

Gene	Sequences for shRNAs
NUSAP1 shRNA-1	5'-GCACCAAGAAGCTGAGAATGC-3'
NUSAP1 shRNA-2	5'-GGAAATGGAGTCCATTGATCA-3'
NUSAP1 shRNA-3	5'- ATGAATGAACTGAAGCAGCCCA -3'
NC shRNA	5'-ACTAAGTTGCGTTACACCCTT-3'

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