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Upregulation of NUAK2: A novel prognostic marker in breast cancer

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Summary. Background. Breast cancer is the most commonly diagnosed neoplasm in women worldwide. New molecular biomarkers and effective prognostic models are being developed. This study aimed to investigate the clinical and prognostic significance of NUAK2 expression in patients with breast cancer.

Methods. The expression of NUAK 2 was examined in breast cancer cells and tissues by real-time PCR, western blotting, and immunohistochemical staining. CCK-8 and colony formation assays were performed to verify the effect of NUAK2 on the proliferation and tumor progression of breast cancer cells. A tumor formation assay in nude mice was performed to analyze the effect of NUAK2 on the tumorigenicity of breast cancer cells.

Results. The expression of NUAK2 in breast cancer tissues was higher than that in paracarcinoma and normal breast tissues. The overall survival of patients with high NUAK2 expression was significantly lower than that of patients with low NUAK2 expression. Multivariate analyses indicated that NUAK2 was an independent prognostic indicator of survival in breast cancer. *In vitro* experiments demonstrated that knocking down NUAK2 in breast cancer cells inhibited cell proliferation and tumor-forming ability, and over-expression of NUAK2 showed the opposite effects. NUAK2 overexpression promoted the tumorigenicity of breast cancer cells *in vivo*.

Conclusion. These findings suggest that NUAK2 is involved in breast cancer development and progression. NUAK2 may be a valuable prognostic indicator in patients with breast cancer.

Key words: NUAK2, Breast cancer, Prognosis, Biomarker

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Introduction

Among the various life-threatening diseases in women, the incidence of breast carcinoma has been gradually increasing. Breast cancer easily metastasizes to the bones and the lungs (DeSantis et al., 2019). Breast cancer is the most commonly diagnosed neoplasm among women worldwide and is the principal cause of cancer-related mortality in the female population (Bray et al., 2018). The high incidence and mortality of breast cancer remains a global health challenge, and the worldwide burden is still increasing (Bray et al., 2018). Despite significant progress in chemotherapy, endocrine therapy, molecular-targeted therapies, and other newly developing therapeutic strategies (Prat et al., 2015), multidrug resistance-related recurrence and distant metastasis remain the main obstacles to the successful control of breast cancer (Tang et al., 2016; Marinello et al., 2019). To enhance the clinical management of breast cancer, it is important to establish new biomarkers that can promote molecular subtyping, risk stratification, recurrence prediction, and prognostic assessment. The application of prognostic models is essential for clinical

Abbreviations. HMEC, Human mammary epithelial cells; qRT-PCR, Real Time quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; IHC, Immunohistochemistry; EDTA, ethylenediaminetetraacetic acid; SNARK, SNF1-like kinase2; AMPK, adenosine monophosphate-activated protein kinase; EMT, epithelial-to-mesenchymal transition.

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decision-making and precision treatment and is also crucial for prolonging the survival time. Therefore, there is an urgent need to explore new molecular biomarkers and develop effective prognostic models for predicting clinical outcomes and guiding clinical practice in breast cancer patients.

The human NUAK2 gene is located on chromosome 1q32.1 and encodes a protein of 628 amino acids with a molecular weight of 69 kDa (Lefebvre et al., 2001). NUAK2 is usually activated in a cell type-specific pattern by multiple stimuli, including DNA damage, hyperosmotic stress, oxidative stress, and nutrient deprivation, including glucose and glutamine deficiency (Lefebvre et al., 2001; Lefebvre and Rosen, 2005). NUAK2, which forms part of the 1q32 amplicon, is frequently upregulated in various cancers in humans such as glioblastoma, melanoma, and other types of cancers (Namiki et al., 2011). However, the role of NUAK2 in breast cancer has not been well demonstrated. The present study aimed to investigate the role of NUAK2 in breast cancer.

In the present study, we found that NUAK2 expression was upregulated in breast cancer samples and we illuminated its prognostic significance. To further explore the role of NUAK2 in breast cancer, CCK-8, colony formation assays, and animal experiments were performed to determine whether NUAK2 influences the proliferation and tumor formation of breast cancer cells *in vitro* and *in vivo*.

Materials and methods

Patients and tissue specimens

All clinical samples were collected between March 2001 and December 2012 at the Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China. A total of 126 paraffin-embedded tissue samples were obtained from patients who were histopathologically diagnosed with primary breast cancer and had undergone curative surgery. The median age at the time of diagnosis was 44 years. Clinicopathological classification and TNM stage were defined based on the American Joint Committee on Cancer (AJCC), 8th edition staging system. There were 10 (7.94%), 76 (60.32%), and 40 (31.75%) patients belonging to stages I, II, and III, respectively. The tumor (T) stage was classified on the basis of tumor size. T1 indicates that the tumor size is ≤2 centimetres (cm) across. T2 indicates that the tumor size is more than 2 cm, but no more than 5 cm across. T3 indicates that the tumor size is larger than 5 cm. Node (N) staging describes whether the cancer has spread to the lymph nodes and is as follows: N0: Cancer has not spread to nearby lymph nodes; N1: Cancer has spread to 1-3 axillary lymph node(s), and/or found in the internal mammary lymph nodes on sentinel lymph node biopsy; N2: Cancer has spread to 4-9 axillary lymph nodes or internal mammary lymph nodes; N3: greater than the extent of N1 and N2. The number of patients with T1, T2, and T3 stage was 26, 87, and 13, respectively, and the number of patients with N0, N1, N2, and N3 was 49, 39, 30, and eight, respectively. The follow-up duration ranged from 2 to 131 months (median follow-up duration, 111 months). The clinicopathological characteristics of the patients are summarized in Table 1.

Of the 126 patients with breast cancer, ten paired adjacent non cancerous tissues were obtained immediately after surgery (adjacent non cancerous tissue was defined as at least 2 cm away from the edge of the tumor) to detect the differential expression of NUAK2 in breast tumor tissues and adjacent non cancerous tissue by western blotting. Also, 30 normal breast tissues and 30 breast tumor tissues were obtained from patients with benign breast diseases and breast cancer to detect the differential expression of NUAK2 in normal breast tissues and breast tumor tissues using RT-PCR. Consent was obtained from each patient for the use of these clinical specimens for research purposes. The study protocol was approved by the Institutional Research Ethics Committee of Sun Yat-sen University (approval number: [2015]2-51).

Table 1. Correlation of NUAK2 expression with clinicopathologic features.

Characteristics	Total	NUAK2 expression		P value
	(n=126)	Low(n=73)	High(n=53)	
Age(years)				0.863
≥60	37(29.37%)	21(56.8%)	16(43.2%)	
<60	89(70.63%)	52(58.4%)	37(41.6%)	
Clinical stage				0.000
I	10(7.94%)	9(90.0%)	1(10.0%)	
II	76(60.32%)	55(72.4%)	21(27.6%)	
III	40(31.75%)	9(22.5%)	31(77.5%)	
T classification				0.103
T1	26(20.63%)	15(57.7%)	11(42.3%)	
T2	87(69.05%)	54(62.1%)	33(37.9%)	
T3	13(10.32%)	4(30.8%)	9(69.2%)	
N classification				0.000
N0	49(38.89%)	43(87.8%)	6(12.2%)	
N1	39(30.95%)	20(51.3%)	19(48.7%)	
N2	30(23.81%)	9(30.0%)	21(70.0%)	
N3	8(6.35%)	1(12.5%)	7(87.5%)	
Differentiation				0.829
Well	13(10.32%)	8(61.5%)	5(38.5%)	
Moderate	94(74.6%)	53(56.4%)	41(43.6%)	
Poor	19(15.08%)	12(63.2%)	7(36.8%)	
Expression of ER				0.687
Negative	45(35.71%)	25(55.6%)	20(44.4%)	
Positive	81(64.29%)	48(59.3%)	33(40.7%)	
Expression of PR				0.639
Negative	54(42.86%)	30(55.6%)	24(44.4%)	
Positive	72(57.14%)	43(59.7%)	29(40.3%)	
Expression of HER2				0.732
Negative	90(71.43%)	53(58.9%)	37(41.1%)	
Positive	36(28.57%)	20(55.6%)	16(44.4%)	

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

Cells

Human mammary epithelial cells (HMEC) were obtained from primary cultured fresh normal breast tissues as described in our previously published articles (Wang et al., 2012) and cultured in Keratinocyte-SFM medium (Invitrogen, Grand Island, NY) supplemented with bovine pituitary extract at 37°C in humidified 5% CO_2 incubator. Human breast cancer cell lines, MDA-MB-435 (ER-PR-HER2+), MCF-7 (ER+PR+HER2-), SK-BR-3 (ER-PR-HER2+), MDA-MB-453 (ER-PR-HER2+), and T47D （ER+PR+HER2-) (purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM/F12 (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT), penicillin (100 units/ml), and streptomycin (100 units/ml) at 37°C in humidified 5% CO_2 incubator.

Real Time quantitative polymerase chain reaction (qRT PCR) analysis.

Total RNA was extracted from fresh human tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Two µg of total RNA treated with DNAase was used for complementary DNA (cDNA) synthesis using the SuperScriptH III First-Strand Synthesis System (Invitrogen). The cDNAs were subjected to qRT-PCR analysis using the CFX384 Real-Time System (Bio-Rad Laboratories, Inc.) with iQTM SYBR® Green Supermix (Bio-Rad Laboratories, Inc.). All reactions were amplified in a 10-µl volume reaction according to the manufacturer's instructions, and performed in triplicate repeats in three independent experiments. The geometric mean of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The thermocycling conditions included an initial hold step (95°C for 20 s) and 40 cycles of a two-step PCR (95°C for 30 s and then 55°C for 30 s), followed by a dissociation step (95°C for 15 s, 60°C for 30 s, and then a sequential increase to 95°C). The primer sequences were as follows: NUAK2 sense 5'-ATGGAGTCGCTGGTTTTCGC-3', antisense 5'-TCCCTCCGTATGTGCATCAG-3, 'GAPDH sense 5'-TGTTGCC ATCAATGACCCCTT-3', antisense 5'-CTCCACGACGTACTCAGCG-3'. The relative mRNA expression was quantified using the comparative $2^{-\Delta\Delta Cq}$ method.

Western Blotting Analysis

Cells and fresh tissue samples were solubilized in sodium dodecyl sulfate (SDS) lysis buffer, and protein concentrations were determined using the BCA protein assay kit (PIERCE, Rockford, IL, USA) according to the manufacturer's protocol. Denaturing solution (β-mercaptoethanol and bromophenol blue, 5 µl) was added to 100 µl protein at 95°C for 10 min and then cooled on ice for over 10 min. Equal amounts of protein samples (20 µg/lane) were separated by electrophoresis on 10.0% resolving SDS-polyacrylamide gel for 2 h, and then transferred to PVDF membranes (EMD Millipore) for 3 h at 2000 mA. After blocking the non-specific binding with 5% non-fat dry milk in TBS-T solution (TBS+0.5% Tween-20) for 2h at room temperature, the membranes were incubated with a primary polyclonal antibody against NUAK2 (Affinity Biosciences Ltd.. DF6224), overnight at 4°C. After washing in TBS-T thrice, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibody (diluted 1:1000 in TBS-T) for 1h at room temperature and then washed with TBST thrice. The localization of antibodies was detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech), according to the manufacturer's protocol. GAPDH (1:1000; Cell Signaling, Billerica, MA, USA) was used to confirm equal loading of the lysates. Image J software was used to analyze the gray values.

Immunohistochemistry (IHC) staining and scoring

Formalin-fixed paraffin-embedded blocks of postoperative tissues were collected for IHC examination. After being baked at 60°C for 3h, the slides were deparaffinized with xylene and rehydrated through a series of graded alcohols before being immersed in 0.3% hydrogen peroxide for 20 min at room temperature to block endogenous peroxidase activity. To retrieve antigens, the slides were boiled in ethylenediamine-tetraacetic acid (EDTA) buffer (pH 8.0) in a microwave for 30 min. After the solution returned to room temperature, slides were incubated with rabbit polyclonal anti-NUAK2 antibody (DF6224, 1:150 dilution, Affinity Biosciences Ltd.) in a moist chamber overnight at 4°C. Subsequently, the slides were incubated with horseradish peroxidase (DAKO ChemMate™ EnVision™ Detection Kit, Copenhagen, Danmark) for 30 min at 37°C and after washing in PBST thrice, the slides were stained in 3,3′-diaminobenzidine (DAB) solution at room temperature for 2 min for visualization. Finally, nuclear counterstaining with Mayer's hematoxylin was performed for better visualization of the tissue structure. Negative control was obtained by using normal rabbit IgG (preimmune serum) instead of the primary antibody. To ensure the objectivity of the score assessment, three independent pathologists blinded to the patients' clinicopathological data conducted the score evaluation for NUAK2 expression in the nuclei and cytoplasm of breast tumor cells. NUAK2 was expressed in the nucleus and cytoplasm. The staining intensity of NUAK2 in cancer cells was graded as follows: 0 (negative staining , no staining), 1 (weakly positive staining, weak yellow), 2 (positive staining, yellow), and 3 (strongly positive staining, brown). The number of NUAK 2 (+) cells was

graded according to the percentage of counted cells in 3-5 microscopic fields: 0 (0-5%), 1 (6-25%), 2 (26-50%), 3 (51-75%), 4 (76-100%). The sum of both the grades was defined as the final NUAK2 staining score. The optimal cutoff value was determined as follows: a score ≤4 was defined as NUAK2 low expression and >4 was defined as NUAK2 high expression.

Plasmid Construction and Retroviral Infection

The Plncx2 plasmid vector was used to generate the plncx2-NUAK2. After oligoduplex synthesis, the pSUPER.retro.puro plasmid vector was digested with AgeI and EcoRI restriction endonucleases. NUAK2-specific shRNAs were cloned into the pSUPER.retro.puro. Plasmid construction was verified by DNA sequencing. Retrovirus were generated by transient transfection 293FT cells as described (Lu et al., 2021). Briefly, NUAK2 expression plasmid or knockdown plasmids and the packing plasmid pCL were transfected into 293FT cells. At 18h post-transfection, the culture media were changed, and at 48h post-transfection, the media was collected and clarified using a 0.45-μm filter. The retrovirus was used to infection or cryopreservation. MCF-7 and MDA-MB-435 cells were infected with a retrovirus expressing NUAK2 or vector. SKBR-3 and T47D cells were infected with retroviruses expressing NUAK2-shRNA1, NUAK2-shRNA2, or an empty vector. MCF-7 and MDA-MB-435 cells expressing NUAK2 or the vector were selected for 14 days with G418 after infection. SKBR3 and T47D cells infected by NUAK2-shRNA1, NUAK2-shRNA2, and NUAK2-CTR were cultured with puromycin for three days to produce a stable NUAK2 knockdown cell line. The targeted NUAK2 sequences were as follows: NUAK2-SH1, CTCTCCAACCTCTACCATCAA; NUAK2-SH2: CCATGGCC CATCTGATAAA.

Proliferation Assay

CCK-8 assay was performed to evaluate the effect of NUAK2 on cellular proliferation. Cells were seeded onto 96-well plates at 1.5×10^3 cells/well with 200 μl DMEM supplemented with 10% FBS. Each sample was analyzed in triplicate. After culturing for 0, 24, 48, 72, 96, and 120h, the cells were incubated with 20 μl CCK 8 solution (Beyotime Institute of Biotechnology, Shanghai, China) for 30 min at 37°C. The cells were counted using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at an absorbance of 450 nm.

Colony formation assay.

Cells were seeded evenly in 6-well plates (5×10^3 cells/well) and cultured for 12 days. The medium was replaced every three days. Then the colonies were fixed with methanol for 10 min at room temperature and stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 15 min at room temperature. Each independent experiment was performed at least three times. The number of colonies was counted under an optical microscope.

Overexpression of NUAK2 promotes the tumor growth in nude mice

To evaluate the effect of NUAK2 overexpression, Balb/C nude mice were subjected to *in vivo* experiments. MCF-7-NUAK2 and MCF-7-vector cells (5×10^6/mouse) mixed with 25% Matrigel (BD) were injected into the mammary fat pad of 4- to 6-week-old female Balb/C nude mice. One estrogen pellet (17β-estradiol, 0.72 mg/pellet, Innovative Research of America, Sarasota, FL) was implanted subcutaneously into each mouse one day before injection. Tumor sizes were measured twice a week using the formula: $V=(\text{length}\times\text{width}^2)/2$. All mice were sacrificed on day 26 after implantation.

All experiments on mice were performed in accordance with the guidelines of the Animal Care and Use regulations of Sun-Yat sen University, and the protocol was approved by the Biomedical Ethics Committee of Sun-Yat sen University.

Statistical analysis

All statistical analyses were performed using a statistical software package (SPSS22.0, Chicago, IL) and GraphPad Prism 5. The measurement data of real-time PCR relative values are expressed as the mean ±SD. Student's t-test was used to evaluate the statistical significance of the differences in NUAK2 expression between the two tissue categories. To assess the correlation between NUAK2 expression and other clinicopathological features, P-values were calculated using the χ^2 test or Fisher's exact test. Survival outcomes were evaluated using Kaplan-Meier survival analysis, and differences between the two groups were calculated using the log-rank test. Relative risks (RRs) associated with NUAK2 expression and other clinicopathological features were estimated using univariate and multivariate Cox proportional hazard models. Variations in the proliferation assay, colony formation assay, tumor volume, and tumor weight were assessed using two-tailed Student's t-test. All tests were two-sided, and a p value less than 0.05 was considered statistically significant.

Results

The expression of NUAK2 is elevated in breast cancer tissues and cell lines

To determine the function of NUAK2 in breast cancer, we first investigated the expression of NUAK2 in 30 breast cancer tissues and 30 normal breast tissues by qRT-PCR. The results revealed that the expression level of NUAK2 mRNA was higher in breast cancer tissues than in normal breast tissues (Fig. 1A). Western

blotting was performed on ten breast cancer tissues with paired adjacent non-tumoral tissues. Upregulation of NUAK2 expression was detected in nine breast tumor tissues compared to adjacent non-tumoral tissues (Fig. 1B). To further detect endogenic NUAK2 expression in breast cancer cell lines, we cultured MDA-MB-435, MCF-7, SKBR3, MDA-MB-453, and T47D cell lines, and primary cultured human mammary epithelial cells (HEMC). The expression of NUAK2 was detected by western blotting, revealing that the expression of NUAK2 were higher in breast cancer cell lines than in normal breast epithelial cells (Fig. 1C).

NUAK2 overexpression is associated with patient survival

The pattern of NUAK2 immunostaining was cytoplasmic and nuclear. The different intensities of staining are shown in Fig. 2B-D. Seventy-three (73 of 126, 57.9%) paraffin-embedded breast cancer tissues showed low expression of NUAK2, whereas 53 (42.1%) breast cancer tissues showed high expression of NUAK2. Clinicopathological characteristics of the patients are shown in Table 1. There were no statistical differences in mean age (p=0.863), T classification (p=0.103), differentiation (p=0.829), expression of ER (p=0.687), expression of PR (p=0.639), and expression of Her-2 (p=0.732) between the NUAK2 high and low expression groups. NUAK2 expression was significantly associated with the clinical stage (p<0.001) and N classification (p<0.001) (Table 1). The follow-up duration ranged from 2 to 131 months (median follow-up duration, 111 months). Kaplan-Meier curves and log-rank tests revealed that patients with high NUAK2 expression had a shorter OS (p=0.022) than those with low NUAK2 expression (Fig. 3A). Univariate analysis indicated that ER expression (hazard ratio [HR]=2.584, p=0.005), PR expression (HR=2.996, p=0.002), and NUAK2 expression (HR=0.465, p =0.025) were prognostic factors for OS. In the multivariate Cox regression model, PR (HR=3.066, p=0.002) and NUAK2 expression (HR=0.451, p=0.020) were independent prognostic factors for OS (Table 2).

Furthermore, we analyzed the prognostic value of NUAK2 in selected patient subgroups stratified by T classification, N classification, and clinical stage. For patients in the N0 subgroups, the expression of NUAK2 was strongly associated with OS duration (Fig. 3B; log-rank test, p=0.002), but not in the N1-3 subgroups (Fig. 3C; log-rank test, p=0.187). The expression of NUAK2 was not associated with OS duration in patients in the T1 2 (Fig. 3D; log-rank test, p=0.127) or T3 subgroups (Fig. 3E; log-rank test, p=0.085). When evaluated according to clinical stage, the impact on the OS associated with the expression of NUAK2 was not significant in early stage tumors (Fig. 3F; log-rank test, p=0.496) and late-stage tumors (Fig. 3G; log-rank test, p=0.232).

NUAK2 might affect the proliferation and colony formation ability of breast cancer cells

To further investigate whether NUAK2 could affect

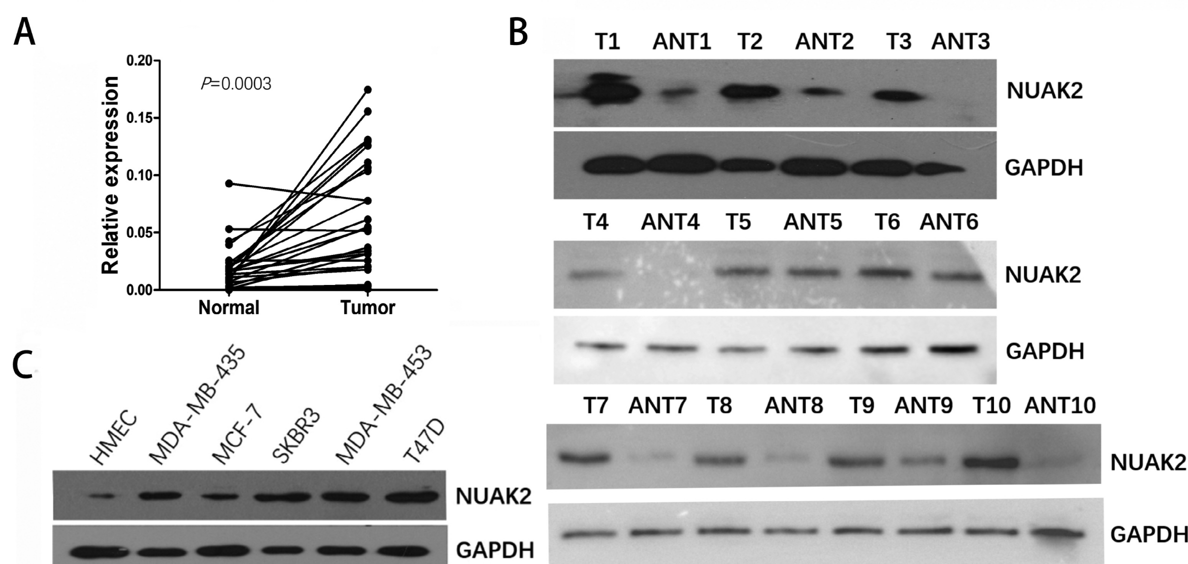

Fig. 1. Expression of NUAK2 is elevated in breast cancer tissues and cell lines. **A.** RT-qPCR was performed to determine the expression of NUAK2 in the breast cancer and normal tissues. **B.** Western blotting assays were performed to assess the protein levels of NUAK2 in 10 breast cancer tissues with paired adjacent non-tumoral tissues. **C.** Western blotting analysis of NUAK2 protein expression in breast cancer cell lines and primary cultured human mammary epithelial cell (HEMC).

the proliferation and colony formation ability of breast cancer cells, we established stable MCF-7 and MDA-MB-435 cell lines that expressed NUAK2 (MCF-7-NUAK2 or MDA-MB-435-NUAK2) or empty vector (MCF-7- vector or MDA-MB-435-vector). As shown in Fig. 4A, the expression level of NUAK2 was significantly increased in MCF-7-NUAK2 and MDA-MB-435-NUAK2 cells compared with that in empty vector control cells.

CCK-8 assay was performed to determine the effect of NUAK2 on cellular proliferation and it showed that the overexpression of NUAK2 increased cell proliferation compared with that in the control (Fig. 4C). Next, we examined the effect of NUAK2 on the tumorigenic activity of breast cancer cells using a colony formation assay. As shown in Fig. 4D, NUAK2 overexpression in MCF-7 and MDA-MB-435 cells significantly promoted colony formation, as indicated by the increase in colony size and colony number. To further explore the impact of NUAK2 on proliferation and colony formation ability, a short hairpin RNA for NUAK2 was generated to stably reduce NUAK2 expression in the SK-BR-3 and T47d cells. As shown in Fig. 4B, western blot data showed decreased expression in SK-BR-3-NUAK2 shRNA#1 and SK-BR-3- NUAK2 shRNA#2 cells compared to that in control cells (SK-BR-3-Scramble), as well as T47D cells, suggesting that the knockdown cells were successfully established. Depletion of endogenous NUAK2 in SK-BR-3 or T47D cells significantly inhibited their proliferation (Fig. 4E) and colony formation (Fig. 4F).

Over-expression of NUAK2 promotes the tumor growth in Balb/C nude mice

An *in vivo* experiment was performed to investigate the effects of NUAK2 overexpression in Balb/C nude mice. As shown in Fig. 5, the growth rate and tumor weight of the MCF7-NUAK2 group were much higher than those of the MCF7-vector group. These results indicate that NUAK2 overexpression strongly promotes the tumorigenicity of breast cancer cells *in vivo*.

Table 2. Cox-regression analysis of various prognostic parameters in patients for all patients.

Factor	Univariate		Multivariate	
	HR(95%CI)	P value	HR(95%CI)	P value
Age				
<60	Reference			
≥60	0.794(0.395-1.597)	0.518	—	—
Clinical stage				
I	Reference	0.045	—	—
II	2.612(0.349-19.573)	0.35	—	—
III	5.436(0.721-41.011)	0.101		
T classification				
T1	Reference	0.296		
T2	1.601(0.613-4.182)	0.337	—	—
T3	2.682(0.776-9.269)	0.119	—	—
N classification				
N0	Reference	0.123		
N1	0.694(0.277-1.739)	0.435	—	—
N2	1.695(0.759-3.784)	0.198	—	—
N3	2.407(0.784-7.389)	0.125	—	—
Differentiation				
Well	Reference	0.187		
Moderate	4.036(0.548-29.743)	0.171	—	—
Poor	6.255(0.782-50.025)	0.084	—	—
Expression of ER				
Negative	Reference			
Positive	2.584(1.328-5.028)	0.005	—	—
Expression of PR				
Negative	Reference		Reference	
Positive	2.996(1.490-6.025)	0.002	3.066(1.524-6.170)	0.002
Expression of HER2				
Negative	Reference		—	—
Positive	0.994(0.477-2.069)	0.987	—	—
NUAK2 expression				
Low	Reference		Reference	
High	0.465(0.238-0.909)	0.025	0.451(0.230-0.881)	0.020

Discussion

Breast cancer is a malignant tumor that develops from mammary tissue and easily metastasizes to the bones and lungs (Urooj et al., 2020). It is the most commonly diagnosed malignancy among women, with more than 2,100,000 new cases worldwide and more than 626,000 deaths (Ferlay et al., 2019) and is the principal cause of cancer-related deaths among women worldwide (Bray et al., 2018; Siegel et al., 2018; DeSantis et al., 2019). Among the various life-threatening diseases affecting the female population, the incidence of breast cancer has been gradually increasing and is now considered a worldwide health challenge.

SNF1-like kinase2 (NUAK2, also known as SNARK), encoding SNF1/59-adenosine mono phosphate-activated protein kinase (AMPK)-related kinase, was originally identified in keratinocytes as a UVB-induced gene (Rosen et al., 1995). NUAK2 is a member of the SNF1/AMPK (serine/threonine kinases or AMP-activated protein kinase) family, which is regulated by the putative tumor suppressor liver kinase B1(LKB1) as well as by death receptor signaling through nuclear factor-κB (Lefebvre et al., 2001;

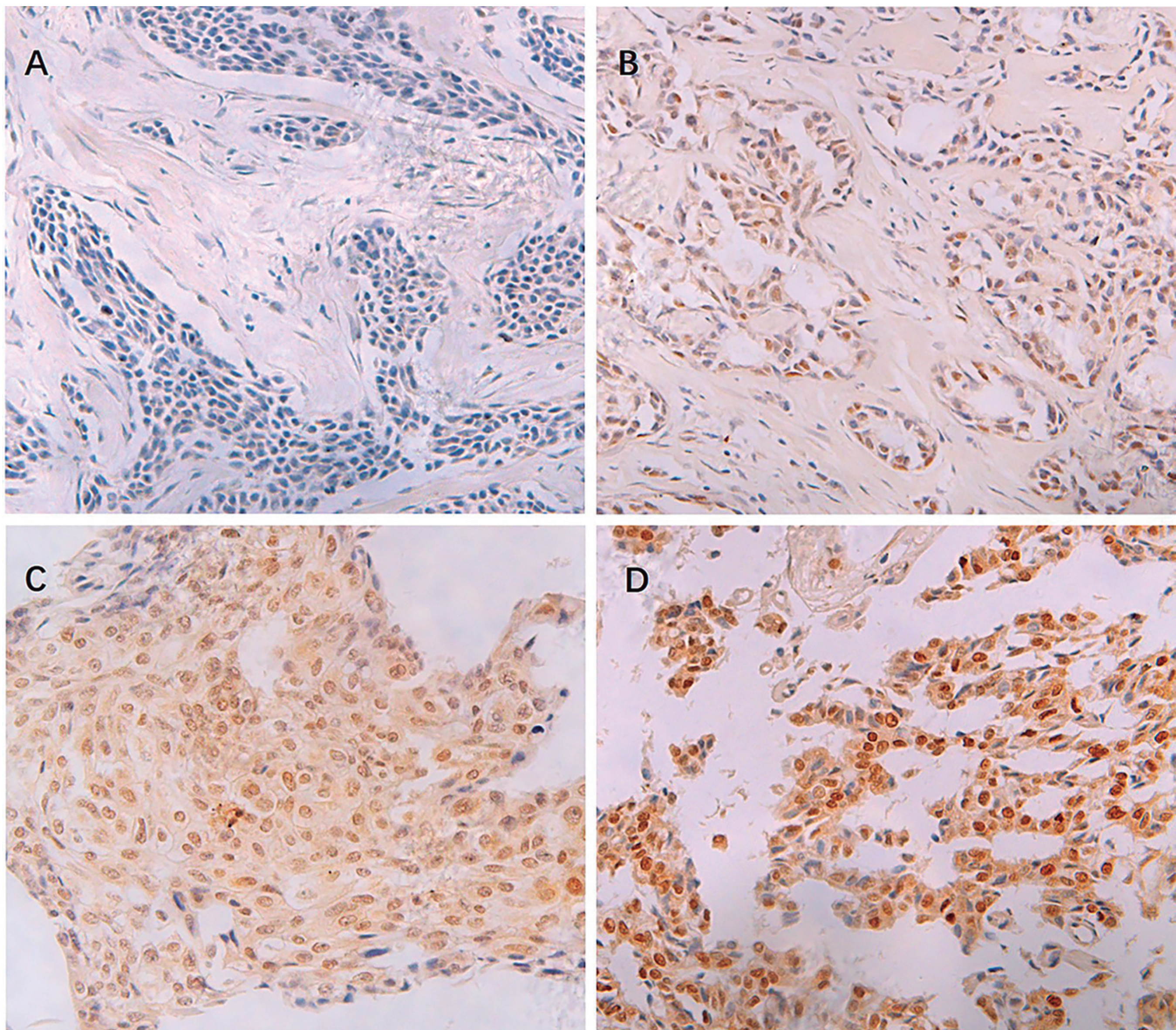

Fig. 2. Immunochemistry analyses of NUAK2 expression in breast cancer tissue samples. Representative images of negative staining of NUAK2 **(A)**, weakly positive staining (+) of NUAK2 **(B)**, positive staining (++) of NUAK2 **(C)** and strongly positive staining (+++) of NUAK2 **(D)**. x 400.

Legembre et al., 2004; Lizcano et al., 2004; Zagorska et al., 2010).

NUAK2 interacts with USP9X (ubiquitin-specific protease 9, X chromosome), a deubiquitinating enzyme that catalyzes deubiquitination of the kinase. Non-USP9X binding mutants of NUAK2 mutants are inactive (Al-Hakim et al., 2008). It is also capable of autophosphorylation, and immunoprecipitated NUAK2 exhibits phosphotransferase activity with SAMS, a synthetic peptide as a kinase substrate (Lefebvre et al., 2001). The functions of NUAK2 were identified in heterozygous mice, which show metabolic disorders and mature-onset obesity as well as a higher risk of colorectal cancer (Yang et al., 2021). A recent study reported that NUAK2 expression is up-regulated in the skeletal muscle of humans subjected to obesity and metabolic stressors (Rune et al., 2009). NUAK2 can mediate glucose transport and its expression is regulated by diabetes and metabolic stress (Koh et al., 2010; Egan and Zierath, 2009). NUAK2 has also been reported to be

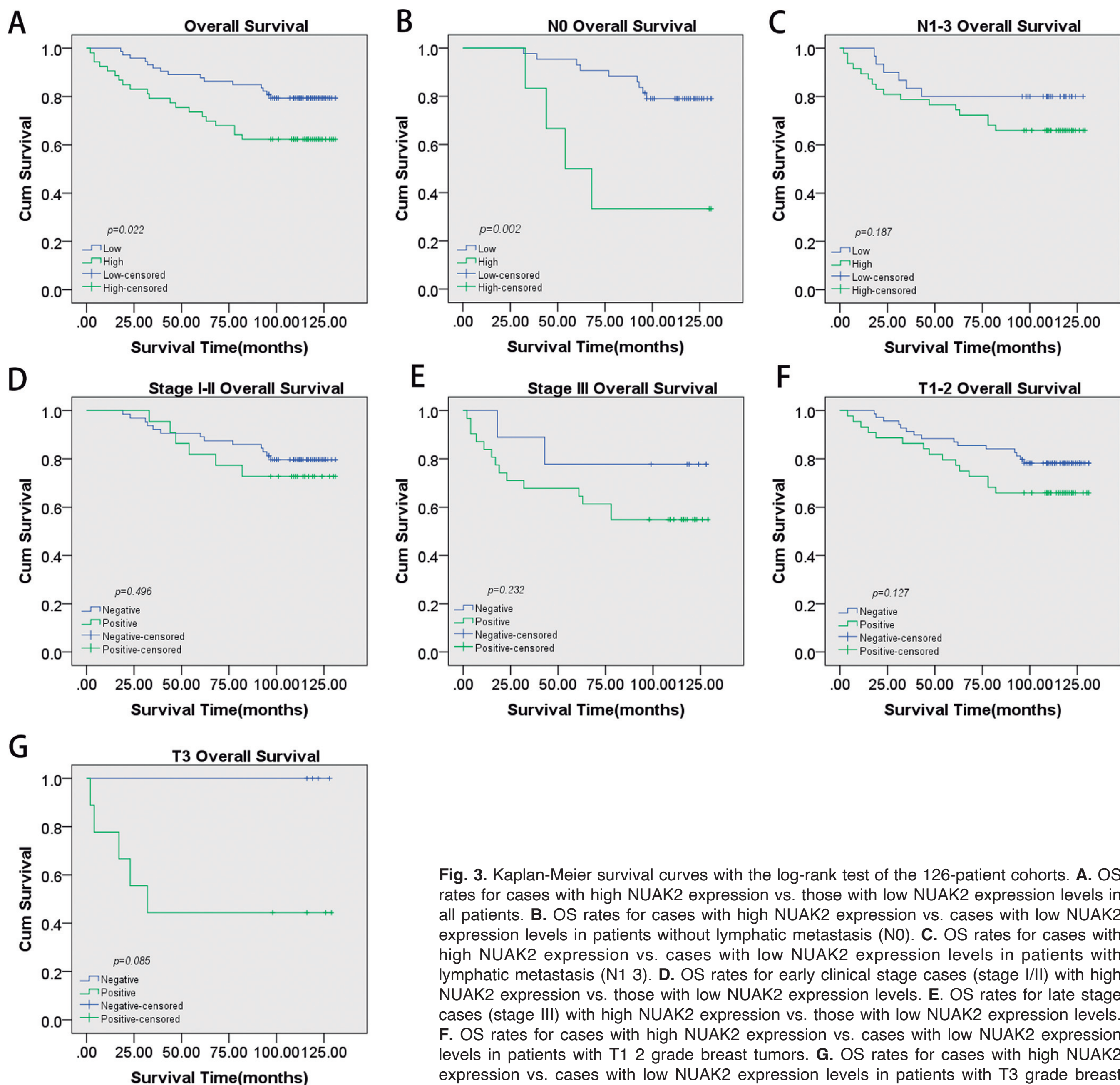

Fig. 3. Kaplan-Meier survival curves with the log-rank test of the 126-patient cohorts. **A.** OS rates for cases with high NUAK2 expression vs. those with low NUAK2 expression levels in all patients. **B.** OS rates for cases with high NUAK2 expression vs. cases with low NUAK2 expression levels in patients without lymphatic metastasis (N0). **C.** OS rates for cases with high NUAK2 expression vs. cases with low NUAK2 expression levels in patients with lymphatic metastasis (N1 3). **D.** OS rates for early clinical stage cases (stage I/II) with high NUAK2 expression vs. those with low NUAK2 expression levels. **E.** OS rates for late stage cases (stage III) with high NUAK2 expression vs. those with low NUAK2 expression levels. **F.** OS rates for cases with high NUAK2 expression vs. cases with low NUAK2 expression levels in patients with T1 2 grade breast tumors. **G.** OS rates for cases with high NUAK2 expression vs. cases with low NUAK2 expression levels in patients with T3 grade breast tumors.

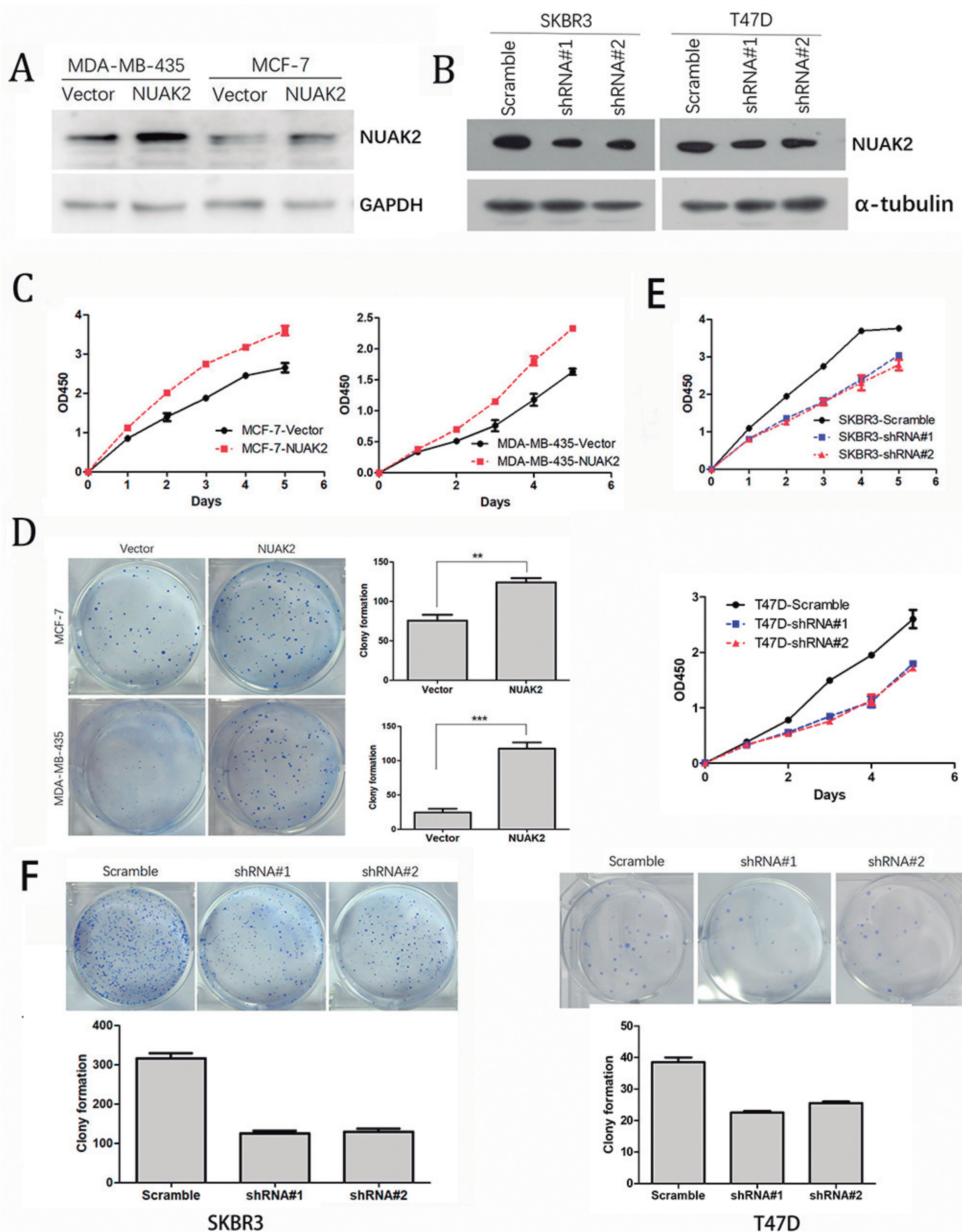

Fig. 4. NUAK2 promotes cell proliferation and colony formation in breast cancer cells. **A.** Western blotting analysis of NUAK2 protein expression in NUAK2-overexpressed MCF-7 cell and MDA-MB-435 cell or empty vector cell lines. **B.** Down-regulation of NUAK2 in SKBR-3 and T47D cells by NUAK2 shRNA. **C.** CCK8 assay was performed between control and NUAK2 overexpression cells (MCF-7 cell and MDA-MB-435). **D.** Colony formation assay was performed between control and NUAK2 overexpression cells (MCF-7 cell and MDA-MB-435). **E.** CCK8 assay was performed between control and NUAK2 knockdown cells (SKBR-3, T47D). **F.** Colony formation assay was performed between control and NUAK2 knockdown cells (SKBR-3,T47D).

implicated in the regulation of other vital cytological processes, such as cell motility (Sun et al., 2013), and its expression levels are regulated by disease states, such as obesity (Rune et al., 2009), and malignant cancer (Namiki et al., 2011).

NUAK2 could also regulate cell-cell and cell-matrix adhesion (Suzuki et al., 2003a). Besides, NUAK1 and NUAK2 were reported to suppress cell apoptosis (Suzuki et al., 2003a,b; Legembre et al., 2004). NUAK2 has been reported to exhibit anti-apoptotic properties; it protects cells from TNF-related apoptosis and ligand-induced apoptosis. It also intervenes in CD95-induced motility and invasiveness of breast cancer cells (Legembre et al., 2004). Yamamoto et al. has reported NUAK2 regulated cell migration and cell cycle progression in melanoma cells (Yamamoto et al., 2008). Namiki et al. reported that NUAK2 participates in the regulation of cell proliferation in melanoma. It also regulates the cyclin-dependent kinase2 (CDK2) expression, increases the S-phase population, and increases cell proliferation in melanoma cells (Namiki et al., 2015). Public databases, such as GeneCards (www.genecards.org), reveal that NUAK2 is upregulated in various types of malignant cancers, including lung, breast, and lymphoid tissue cancers, implying that it may play an important role in cancer development and progression (Lefebvre et al., 2001; Legembre et al., 2004; Lizcano et al., 2004). NUAK2 was reported to be upregulated in cervical cancer tissues and cells, and knockdown of NUAK2 inhibited proliferation, invasion, migration, and epithelial-to-mesenchymal transition (EMT) in cervical cancer cells (Li et al., 2021). NUAK2 has also been reported to promote cell proliferation in gastric cancer (Tang et al., 2017). However, to the best of our knowledge, the expression and function of NUAK2 in breast cancer is yet to be elucidated.

In the present study, NUAK2 was found to be upregulated in breast cancer tissues and cells compared with adjacent noncancerous tissues, normal breast tissues, and normal breast epithelial cells, indicating that NUAK2 might play an important role in the development and progression of breast cancer. As shown by Immunohistochemical analysis, 57.9% paraffin-embedded breast cancer tissues showed low expression of NUAK2, while 42.1% breast cancer tissues showed high expression of NUAK2. High NUAK2 expression was seen in 77.5% of the late-stage (stage III) patients, whereas only 25.6% of early stage (stages I/II) patients showed high NUAK2 expression. The results revealed that higher NUAK2 expression was related to more aggressive tumor behavior, which was further supported by its expression in lymphatic metastases. Among the patients without lymphatic metastasis, 12.2% showed high NUAK2 expression in contrast to 61.0% of patients

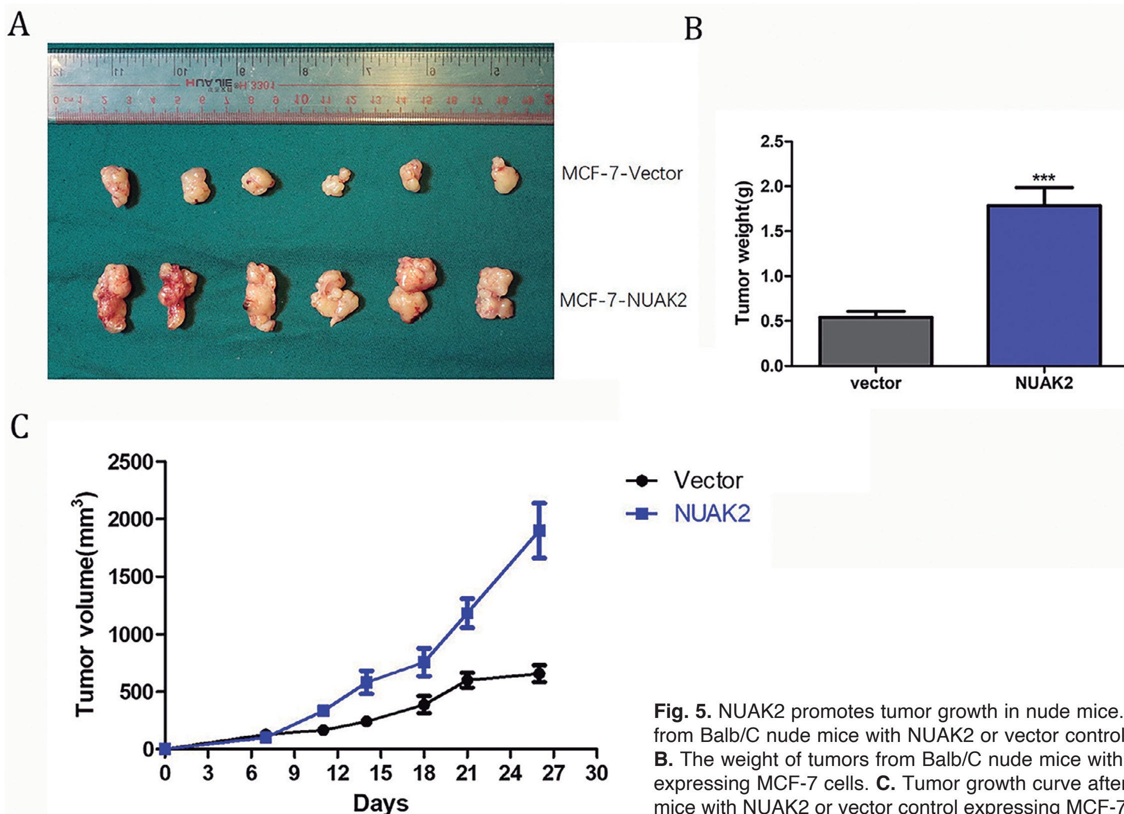

Fig. 5. NUAK2 promotes tumor growth in nude mice. **A.** The picture of tumors from Balb/C nude mice with NUAK2 or vector control expressing MCF-7 cells. **B.** The weight of tumors from Balb/C nude mice with NUAK2 or vector control expressing MCF-7 cells. **C.** Tumor growth curve after injection of Balb/C nude mice with NUAK2 or vector control expressing MCF-7 cells.

with lymphatic metastasis. This indicates that a high level of NUAK2 protein might contribute to the invasion of breast cancer.

Furthermore, multivariate analyses showed that patients with high NUAK2 expression had a worse prognosis than those with low NUAK2 expression and that the status of NUAK2 was an independent prognostic index influencing overall survival. We also analyzed the prognostic value of NUAK2 in selected patient subgroups stratified by T and N classification and clinical stage. The expression of NUAK2 was strongly associated with OS duration in patients in N0 subgroups, but not in patients in N1-3 subgroups, indicating that NUAK2 might play a more important prognostic role in patients without lymphatic metastases.

Many factors are involved in tumor progression, including cell proliferation, migration, invasion, and colony forming-ability. In this study, NUAK2 was shown to contribute to the proliferation and colony formation abilities of cell lines. To address the role of NUAK2 in tumor progression, NUAK2 was overexpressed in MCF-7 and MDA-MB-435 cell lines and knocked down in SK-BR-3 and T47d cell line.

CCK-8 assay showed that the overexpression of NUAK2 increased cell proliferation. Colony formation assay showed that NUAK2 overexpression significantly promoted colony formation. The depletion of endogenous NUAK2 significantly inhibited proliferation and colony formation. In addition, we examined the potential oncogenic role of NUAK2 by injection NUAK2- expressing MCF-7 cells into nude mice. NUAK2 overexpression promoted tumorigenicity of MCF-7 cells in nude mice. These results suggest that NUAK2 may be involved in the development of breast cancer by promoting cell proliferation, colony formation, and tumorigenicity. NUAK2 expression status, combined with other clinicopathological indices and other biomarkers of breast cancer, may be useful for stratifying patients with more aggressive behavior for individual treatment.

Conclusion

This study provides the first evidence of the clinical significance of NUAK2 in breast cancer, suggesting that NUAK2 might be involved in the occurrence and progression of breast cancer and thereby may serve as a valuable prognostic index for patients with breast cancer. Further investigations are required to clarify the potential use of NUAK2 as a therapeutic target and the mechanisms by which it is involved in the progression of breast cancer.

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Conflict of interest. The authors declare that they have no competing interests.

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