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Original Article

Clinical significance of NOB1 expression in breast infiltrating ductal carcinoma

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Abstract: Background: NIN/RPN Binding protein 1 homologue (NOBp1), encoded by NOB1 gene, was reported to play an essential role in the oncogenesis and prognosis of carcinomas. We conducted a study to reveal its expression and clinical significance in breast infiltrating ductal carcinoma. Methods: To explore the relationship between NOB1 expression and the clinical TNM (cTNM), 162 patients who undergone surgery were involved in the study. Compared to healthy tissues, abnormal localization and higher level of NOB1 in tumor cells was observed by Immunohistochemistry staining. Real-time PCR and western-blotting verified the up-regulation of NOB1 in carcinoma individuals. Results: A significant correlation between high level of NOB1 and the T stage, lymph node metastasis and cTNM was shown. Furthermore, patients with higher level of NOB1 predicted a declined overall survival (OS). Notably, multivariate analyses by Cox's proportional hazard model revealed that expression of NOB1 was an independent prognostic factor in breast infiltrating ductal carcinoma. Conclusions: In summary, our present study clarify that the aberrant expression of NOB1 in breast infiltrating ductal carcinoma is possibly involved with tumorigenesis and development, and the NOB1 protein could act as a potential biomarker for prognosis assessment of breast infiltrating ductal carcinoma. Related mechanism is worthy of further investigation.

Keywords: Breast cancer, NOB1 protein, immunohistochemistry, tissue microarray

Introduction

Breast cancer is one of the leading causes of death among women and threatens the lives of 1.2 million women worldwide [1]. It accounts for 10% of all newly diagnosed cancers, 32% of female malignancies and result in 15% of death of victims [2]. Although there is a lower incidence in China, an obvious increase has been shown recently [3]. The most common pathological type of the breast cancer is Breast Infiltrating Ductal Carcinoma (BIDC), which has a different 5-year survival rates depending on the classification of Clinical TNMs. It is reported that I, II and III BIDC has a 91.9%, 90% and 39.8% 5-year survival rates respectively [4]. Therefore it's important to develop a strategy for its early detection, prevention and treatment. Reliable markers of BIDC are highly needed for both prognosis evaluation and treatment.

NIN/RPN Binding protein 1 homologue (NOBp1), encoded by NOB1 gene, is located on chromosome 16q22.1 [5]. NOBp1 is a subunit of the 26S proteasome and plays an essential role in protein degradation pathway. It serves as a chaperone to join the 20S proteasome with 19S regulatory particle in the nucleus and facilitates the maturation of the 20S proteasome [6-8]. NOB1 protein composed of a PIN (PilT amino terminus) domain and a C terminal zinc ribbon domain [9], which has been reported to function as a transcription regulator. It is indicated that NOB1 may play a role in cell cycle progression, drug resistance and tumor genesis [10, 11]. NOB1 is an important regulator of the tumorigenic properties of human breast and hepatocellular carcinoma and could be used as a potential therapeutic target [12-14]. Those findings indicate that NOB1 may be involved in various tumors and may serve as an oncogenic factor. To explore the correlation

Table 1. Clinical-pathological characteristics of BIDC

Number	N=162	%
Age		
<35	36	22.2
≥35	126	77.8
Tumor size (cm)		
<4	108	66.7
≥4	54	33.3
Histological grading		
I-II	97	59.9
III	65	40.1
Hormone-receptor		
ER (+)/PR (+)	72	44.4
ER (-)/PR (+)	31	19.1
ER (+)/PR (-)	14	8.6
ER (-)/PR (-)	45	27.9
Lymph node metastasis		
+	117	72.2
-	45	27.8
Distance metastasis		
+	153	94.4
-	9	5.6
Prognosis		
Death	21	13.0
Alive/Lost connection	141	87.0

between NOB1 and breast cancer, we studied the potential role of NOB1 in BIDC.

Materials and methods

Patients

A total of 162 patients who underwent surgery at hospitals for histologically proven BIDC that cooperated with National Engineering Center for Biochip at Shanghai during 2007-2010 are selected in this research (**Table 1**). Freshly removed samples of tumor and the healthy breast tissues (≥5 cm away from the tumor) were embedded with paraffin for further analysis. All the patients have been follow-up visited till Sep. 2012 (median, 35 months). This study approved by Ethics Committee of Tenth People's Hospital-Affiliated to Tongji University. Written-informed consent signed by study participants and/or their legal guardians.

Construction and section of tissue microarray

Briefly, a tissue arraying instrument (Beecher Instruments, Inc) was used to create cylinder shaped holes in a square recipient paraffin block, then to remove tissue cores from the donor block such as clinical biopsies or tumor samples by a hollow needle with an inner diameter of 1.5 mm, held in an X-Y axis precision guide. The cylindrical sample from a region of the donor block, which is selected by an experienced pathologist, was then inserted into a recipient paraffin block in a precisely spaced, array pattern. After the construction of arrayed block, a 5-μm section was cut with a microtome continuously with a high speed and picked a perfect piece to place on polylysine-coated slides. There is 1 tissue array block in this research containing totally 162 samples for monoclonal antibody against NOB1 protein.

Immunohistochemistry

IHC staining for NOB1 on sections of the formalin-fixed samples on the tissue microarray was carried out by using the Envision ready-to-use methods. Slides were deparaffinized in xylene and rehydrated through graded concentrations of ethanol to distilled water and endogenous peroxidase activity was blocked by incubation with 30 mL/L H_2O_2 in methanol for 10 min at room temperature. Then sections were submitted to antigen retrieval in a pressure cooker containing 0.01 mmol/L sodium citrate buffer for 10 min. Slides were subsequently incubated in 100 mL/L normal goat serum for 20 min at room temperature. Sections were permeabilized in PBS-Triton and incubated overnight with primary antibody at 4°C. The antibodies were used in PBS-Triton with variable dilution. Rabbit anti-human polyclonal antibody to NOB1 was used. Each section was then incubated with Envision rabbit peroxidase (GeneTech) for 30 min. Finally, the sections were treated with 0.02% 3,3'-diaminobenzene and 0.005% H_2O_2 in 0.05 mmol/L Tris-HCl buffer and counterstained by hematoxylin.

The evaluation of the immunohistochemical staining was performed independently by two authors without knowledge of the clinic pathological information. The immunoreactive scores besides NOB1 was determined by the sum of extension and intensity as literature reported previously [15]. The intensity of IHC staining was scored using the following scale: 0, no staining of the tumor cells; +, mild staining; ++, moderate staining and +++, marked staining. The area of staining was evaluated and record-

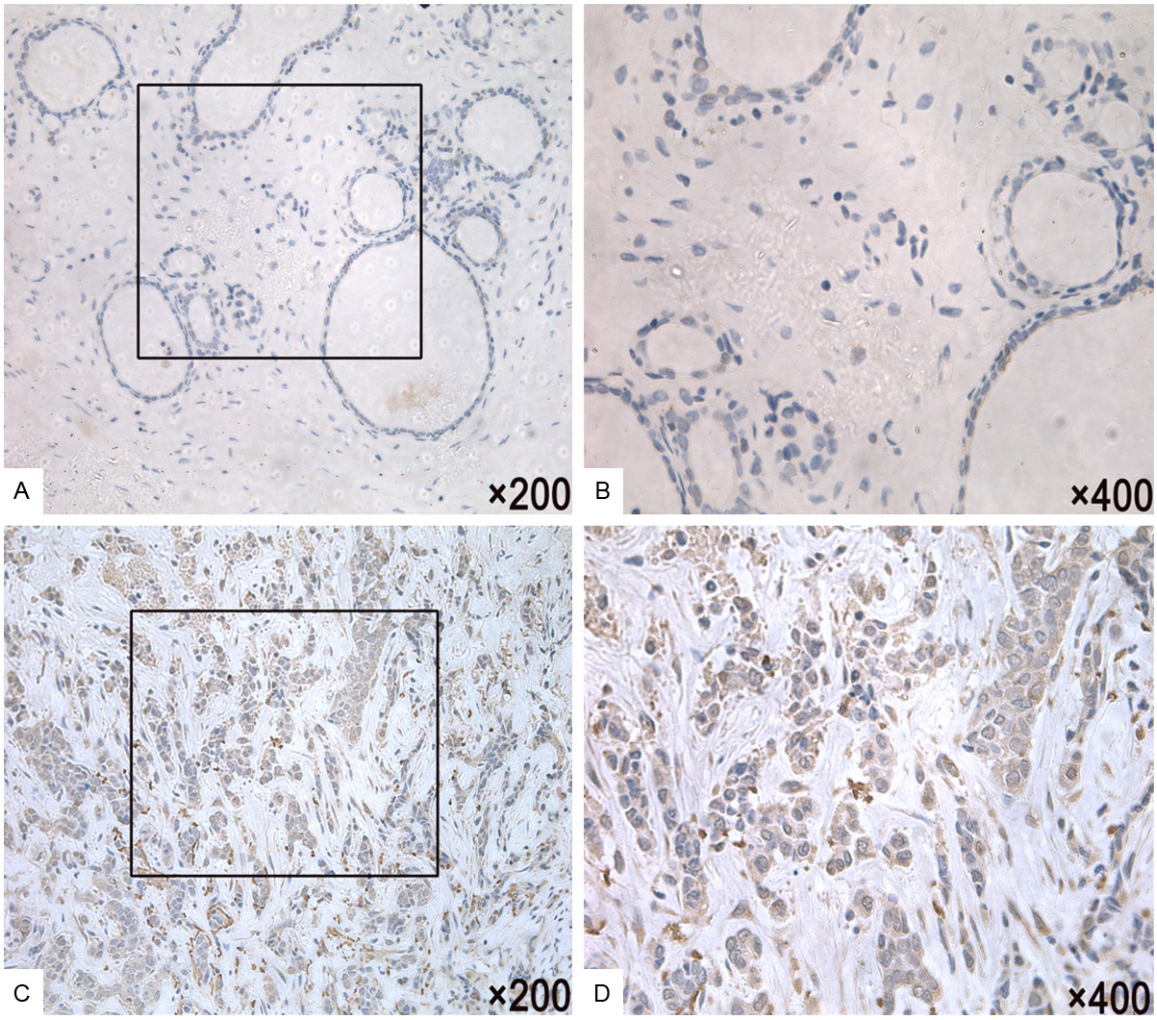

Figure 1. A: Immunohistochemistry expression of NOB1 in tumor tissue (×200). B: Immunohistochemistry expression of NOB1 in tumor tissue (×400). C: Immunohistochemistry expression of NOB1 in adjacent non-tumor tissue (×200). D: Immunohistochemistry expression of NOB1 in adjacent non-tumor tissue (×400).

Table 2. NOB1 expression in tumor tissue and adjacent non-tumor tissue

Marker	tumor tissue	adjacent non-tumor tissue	p*	Z
NOB1	4.47±2.105	3.14±1.217	0.001	-5.732

*p<0.05.

ed as a percentage: 0, less than 5%; +, 5%-25%; ++, 26%-50%; 3+, 51%-75% and 4+, more than 75%. The combined scores were recorded and graded as follows: -, 0; +, 1-2; ++, 3-5; +++, 6-7.

Real-time PCR

Real-time PCR performed using a commercial kit (TAKARA). Total RNA was isolated using Trizol reagent (Invitrogen) according to manufacturer's instructions. Single-stranded cDNAs were synthesized using the RNAs as the template following the manufacturer's instructions. Actin was used as the reference housekeeping gene for RT-PCR analysis. The primers were used as follows: NOB1-F 5'-ATCTGCCCTACAAGCCTAAAC-3' and NOB1-R 5'-TCCTCCTCCTCCTCCTCAC-3', Actin-F 5'-CGGCATTGTCACCAACTG-3' and Actin-R 5'-CGCTCGGTCAGGATCTTC-3'. Data were analyzed using ABI Prism 7900 Sequence Detection System software (Applied Biosystems). Relative gene expression levels compared to Actin was calculated using $2^{-\Delta\Delta CT}$ analyze method. All samples were examined in triplicate.

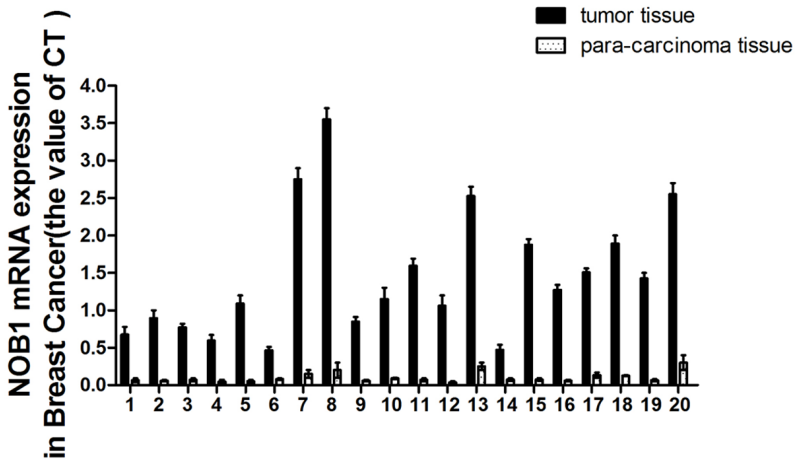

Figure 2. Level of *NOB1* mRNA in breast cancer tissue determined by real-time PCR.

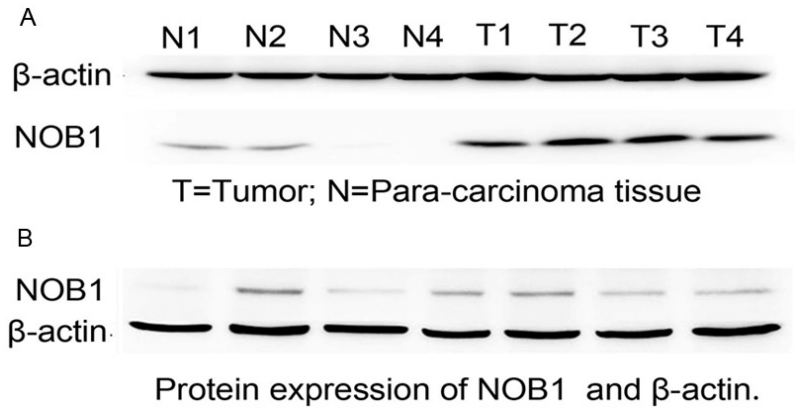

Figure 3. Expression of NOB1 in breast tissues detected by Western-blotting. (T=tumor; N=normal).

Western blotting analysis

Freshly removed tissues were homogenized with liquid nitrogen followed by centrifuge at 12,000 r/min 5 min at 4°C. Total protein was isolated from homogenized tissues using ice-cold protein lysis buffer. Protein samples were run by SDS-page then transferred to PVDF membrane. After blocking with 5% nonfat milk for 1 h, the membrane was incubated with Rabbit-anti Human monoclonal NOB1 antibody (CST) or Mouse-anti Human β-actin antibody (GenScript) at 4°C for overnight or 1 h. Following thoroughly washing the membrane was incubated with Goat-anti Rabbit polyclonal antibody (GenScript) at room temperature for 1 h. The antibodies used as follows: Rabbit-anti Human monoclonal NOB1 antibody 1:1000, Mouse-anti Human β-actin antibody 1:5000. And the membrane was developed with Chemiluminescence method.

Statistical analysis

Computerized statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 13.0. Clinical and histopathology information and the results from the IHC studies of the tissue microarray were entered into a database. The variances of NOB1 expression among different tissues was analyzed using Mann-Whitney U-test and the clinic pathological data was analyzed with Spearman's correlation test. In all statistical analyses, a two-tailed p value ≤0.05 was considered statistically significant.

Results

The expression of NOB1 was significantly higher in tumorous tissue than in adjacent tissue

By using of a large tissue microarray (162 cores) we investigated the protein expression of NOB1 in breast cancer specimens and adjacent non-tumor tissue. The tumorous or healthy staining was semi-quantitatively scored by the intensity and the percentage of positive staining. NOB1 proteins expressions were detected mainly in cytoplasm of malignant cells (**Figure 1**). The positive expression of NOB1 (p<0.01) in tumor tissue was significantly higher than in adjacent non-tumor tissue. Images of representative immuno-staining are presented in **Figure 1**. The results are shown in **Table 2**.

Quantitative real-time PCR and western blotting analysis

In order to confirm the expression level of NOB1 in tumor and healthy tissues, Real-time PCR

Table 3. Relationship between the expression of NOB1 protein and clinicopathological parameters

Marker	Correlation coefficient (r)					
	Age	Tumor size	cTNM	LN metastasis	Distance metastasis	Histological grading
NOB1	0.052	0.081	0.412*	0.314*	0.078	-0.052

*p<0.05; LN: lymph node.

Table 4. The 5-year survival rate of the NOB1 expression and other clinicopathologic features

		5-year survival rate		
		Survival rate	SD	*p* value
NOB1	-	0.639	0.071	0.037
	+	0.397	0.048	
Age	<65	0.622	0.057	0.006
	≥65	0.351	0.059	
Tumor size	<4 cm	0.674	0.070	0.000
	≥4 cm	0.362	0.049	
Histological grading	I-II	0.675	0.074	0.021
	III	0.358	0.042	
cTNM	TNM1	0.664	0.118	0.017
	TNM2	0.361	0.073	

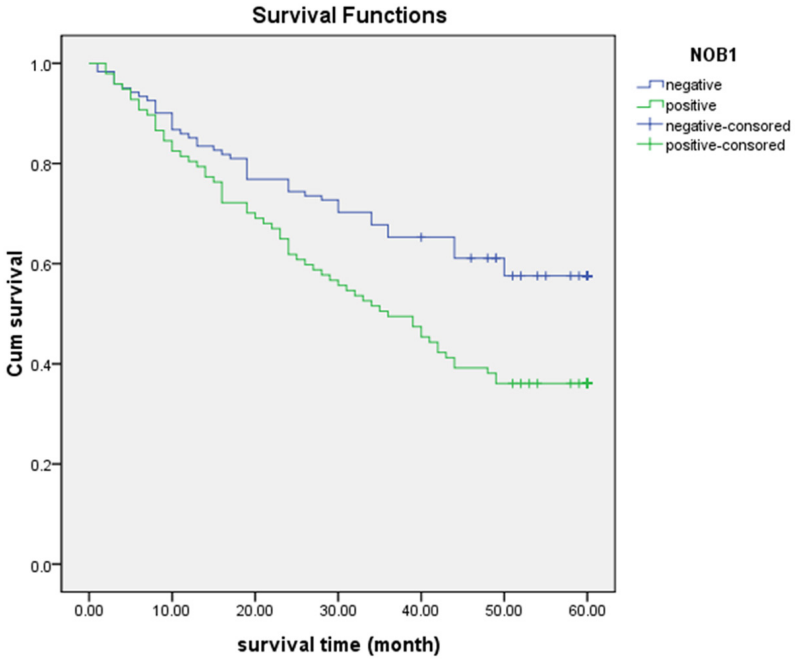

Figure 4. The survival analysis of NOB1. Patients with higher NOB1 expression in tumor tissue were closely correlated with poorer overall survival than patients with tumor with lower expression (*p*=0.038).

and Western-blotting were performed to quantify mRNA and protein levels of NOB1 respectively. Real-time PCR indicated a significant increase of NOB1 mRNA in 20 patients ($p<0.05$, **Figure 2**). Accordingly, Western-blotting analysis showed high expression in 80% carcinoma tissues compared to healthy tissues ($p<0.05$, **Figure 3**).

Relationship between the expression of NOB1 proteins and clinic pathological parameters

Based on the up regulation of NOB1 in tumors, we tried to further investigate the potential clinical significance of NOB1 in breast cancer. The correlations between NOB1 and T stage, lymph node metastasis or cTNM were analyzed. The expression of NOB1 has significant correlations with T stage (r=0.052, $p=0.014$), lymph node metastasis (r=0.314, $p=0.015$) and cTNM (r=0.412, $p=0.032$). But the correlations between NOB1 expression and age, gender, size of tumor or distance metastasis was not found (**Figure 2**, **Table 3**).

NOB1 expression and survival analysis: univariate survival analysis

The follow-up visit with 162 BIDC patients was conducted until Sep. 2012. Of all the patients, 21 died of cancer (median, 20 months), and the left 141 patients were alive or could not be tracked (median, 55 months). Survival analysis

Table 5. Multivariate Cox's proportional hazards regression analysis of prognostic factors for breast cancer

Variate								
							95.0% CI for Exp (B)	
	B	SE	Wald	df	Sig.	Exp (B)	Lower	Upper
NOB1	0.960	0.449	4.572	1	0.031	2.761	1.102	6.447
Tumor size	0.069	0.026	5.742	1	0.019	1.071	1.016	1.134
Histological grading	0.571	0.231	5.957	1	0.017	1.648	1.104	2.457
Age	0.039	0.023	4.497	1	0.042	1.027	1.021	1.045
cTNM	0.537	0.286	8.819	1	0.004	1.752	1.219	2.521

by Kaplan-Meier survival curve and log-rank test demonstrated a declined overall survival (OS) for individuals with higher level of NOB1 (39.7% VS 63.9%, *P*=0.037) (**Table 4**, **Figure 4**).

Multivariate cox regression analysis

To avoid the influence caused by univariate analysis, the expression of NOB1 as well as other parameters was examined in multivariate Cox analysis, interpretation of result revealed that the expression of NOB1 was an independent prognostic factor in BIDC (B=0.96, *p*=0.04, Exp (B)=2.76) (**Table 4**). All the findings above suggest NOB1 as a biomarker for the prognostic of breast cancer. In addition, the size of tumor, age, pathological grading and cTNM also contribute to the survival rate (**Table 5**).

Discussion

The majority of intracellular proteins are degraded by ubiquitin-proteasome pathway (UPP), which is one of the most important destinations for proteins [16]. Much has been learned about the 16S proteasome, a very large multicatalytic protease complex, by which the ubiquitinated proteins are degraded to smaller peptides [17]. Interestingly, UPP regulates the somatic mutations and tumor incidence by selectively degrading oncogene or tumor suppressors, and apoptosis regulator [18]. The 26S complex is composed of a 20S proteasome with a 19S regulatory particle at either or both of its ends [19-21]. Being an important component of 26S proteasome, NOB1 is critical for the normal function of 26S proteasome. Altered UPP functions may result in accumulation of oncogene product, abnormal degradation of tumor suppressors, apoptosis decline or over proliferation of somatic mutations [22]. The critical role of NOB1 in the process of various carcinomas has been revealed [12, 13]. Down-regulation of NOB1 in ovarian cancer cell line SKOV3 and HEY can suppress the cell viability, proliferation and cause G1 arrest [12]. NOB1 plays a key role in the colorectal carcinoma through the dysfunction of UPP, from which the abnormal apoptosis would be resulted [13, 14]. Recently, RNA interference technology enables researchers to explore the potential role of NOB1 in the ontogenesis of breast cancer. Down-regulation of NOB1 in breast cancer cell line MCF-7 results in the inhibition of the cell proliferation and tumor genesis. In addition, by arresting the tumor cells in G0/G1 phase, NOB1 may function as a novel oncogene [5, 25]. To our knowledge, the molecular mechanism of NOB1 in the process of breast carcinoma is still obscure. Therefore NOB1 and target proteins may enable us on the further investigation of its influence in the oncogenesis.

To test our hypothesis, we performed immunohistochemistry staining to localize the NOB1 in breast tissues. 162 samples removed from breast cancer surgery and according counterparts were examined. The results suggested up-regulation of NOB1 in the tumor tissues compared to the healthy tissues ($p<0.05$). Furthermore, we also checked mRNA and protein levels of NOB1 by Real-time PCR and western-blot respectively. Because the patients involved in our study were pathogenic grad I-II, the over-expression of NOB1 took place in the early stage of oncogenesis. In addition, the up-regulation of NOB1 has a significant correlation with cTNM (r=0.379, *p*=0.032) and lymph node metastasis (r=0.257, *p*=0.015). We assumed that NOB1 may participated in the formation

and migration of tumor by affecting the cellular signal pathways, angiogenesis or cell cycle, and tumor genesis will be altered eventually. The findings of follow-up visit demonstrated that higher level of NOB1 corresponds to high malignance and poorer prognosis. Compared to patients with higher NOB1 expression, individuals who have a lower level of NOB1 had a higher overall survival rate (p=0.038). In this study we demonstrated for the first time that NOB1 is an independent prognostic factor of breast cancer.

Conclusions

In summary, our study demonstrated that the over-expression of NOB1 may play a role in the oncogenesis and may serve as a biomarker for prognosis. Based on our findings, NOB1 gene could be a potential target for the treatment of breast cancer. The findings of this study present a novel knowledge of NOB1 and a potential future prospect for breast cancer treatment.

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Disclosure of conflict of interest

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

Abbreviations

BIDC, Breast Infiltrating Ductal Carcinoma; TMA, tissue microarray; OS, overall survival; IHC, Immunohistochemistry; PBS, Phosphate buffer solution; HRMs, hypoxia-responsive microRNAs; SCID, severe combined immune deficiency; MDA-MB-231, a type of frequently-used breast cancer cell line.

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