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NRBE3 promotes metastasis of breast cancer by down-regulating E-cadherin expression

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ABSTRACT

NRBE3 acts as an E3 ligase of RB to promote RB's polyubiquitination and degradation. In addition, NRBE3 is up-regulated in human breast cancer (BC) tissues. However, how NRBE3 functions in BC is unknown. Here, we show that up-regulation of NRBE3 is correlated with lymphatic metastasis in human BC tissues. Ectopic expression of NRBE3 promotes migration and invasion in BC cells. Accordingly, knockdown of NRBE3 inhibits migration and invasion in BC cells. Depletion of NRBE3 inhibits lung metastasis of BC cells *in vivo*. Knock-down of NRBE3 causes increase of E-cadherin protein levels. Interestingly, Flag-NRBE3 decreases E-cadherin level in RB-expressing and RB-null BC cells, demonstrating that there exist RB-independent mechanisms for NRBE3-mediated E-cadherin expression regulation. However, the E3 ligase deficient deletion mutant Flag-NRBE3 (ΔU-box) modestly decreases E-cadherin level in RB-expressing cells, indicating that NRBE3 controls E-cadherin expression mainly through RB-dependent pathways in RB-expressing cells. We further demonstrate that NRBE3 inhibits the transcription of E-cadherin in BC cells. Significantly, NRBE3 expression is negatively correlated with E-cadherin expression in human BC tissues and cell lines. Collectively, we demonstrate that NRBE3 promotes metastasis of BC and possesses the potential as a therapeutic target in BC.

1. Introduction

Breast cancer (BC) is the most frequently diagnosed cancer in women. It accounts for 30% of cancer cases newly diagnosed in 2017 [1]. BC is also one of the most common causes of cancer deaths, contributing to 14% of cancer deaths in female in 2017 [2,3]. It is noteworthy that metastasis is responsible for 90% of BC deaths. In spite of early diagnosis and therapy, metastasis still occurs in 30% of BC patients [4]. Therefore, further identification of key factors promoting BC metastasis will provide more therapeutic targets for BC.

NRBE3 (a novel RB E3 ubiquitin ligase, also known as KIAA0649) was identified as a potential oncoprotein as it transforms NIH3T3 fibroblast cells [5]. Recently, we demonstrated that NRBE3 binds to RB (retinoblastoma protein) and promotes RB's degradation through its E3 ubiquitin ligase activity [6]. Furthermore, we found that NRBE3 is up-regulated in human BC tissues [6], suggesting NRBE3 is involved in the development of BC. However, if NRBE3 functions in the metastasis of BC remains unknown.

RB is the first identified tumor suppressor protein, which inhibits cell cycle progression by controlling G1/S transition [7,8]. RB regulates various cellular processes and is inactivated in some types of cancer [9–11]. In BC, loss of RB occurs in approximately 20%–35% of patients and is closely related to poor survival of patients [12,13]. Loss of RB promotes invasion of ErbB2-positive or MYC-positive BC cells [14,15], and stable depletion of RB enhances the invasive potential of ErbB2-positive BC cells *in vivo* in mouse models [15]. Loss of RB has been reported to be associated with metastasis in human BC [16]. Besides, RB deficiency has been found in the metastasis of a variety of cancers including hepatocellular cancer [17], esophagus cancer [18], bladder cancer [19], colorectal cancer [20] and gastric cancer [21]. Moreover, RB deficiency alters the expression of key molecules such as protocadherin, TGF, FGF and ZEB1 involved in cell adhesion and invasion in BC [14,15,22]. In particular, RB was identified as an upstream regulator of E-cadherin expression [23,24]. RB binds to the promoter of E-cadherin and activates its transcription.

E-cadherin is a transmembrane protein mediating calcium-dependent cell-cell adhesion. Thus, E-cadherin plays an indispensable role in maintaining the epithelial integrity [25–27]. Importantly, loss of E-

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Abbreviations

NRBE3	novel RB E3 ubiquitin ligase
RB	retinoblastoma protein
BC	breast cancer
EMT	epithelial-mesenchymal transition
TGF	transforming growth factor
EGF	epidermal growth factor

cadherin is the hallmark of epithelial-mesenchymal transition (EMT) and associated with metastasis in multiple types of cancer including BC and prostate cancer [28–33]. Especially, it has been well documented that loss of E-cadherin leads to metastasis in BC through EMT [34,35].

Thus, we speculate that NRBE3 might regulate BC metastasis through promoting RB degradation. In present study, we show that NRBE3 expression positively correlates with lymphatic metastasis of BC patients. We further uncovered the function of NRBE3 in BC metastasis.

2. Materials and methods

2.1. Antibodies

Polyclonal antibody against NRBE3 was produced in our laboratory [36]. Antibody against RB was purchased from BD Pharmingen. Antibody against E-cadherin was purchased from CST (Cell Signaling Technology) and Abclonal. Antibody against β-actin was purchased from Abclonal. Antibody against Flag was purchased from Sigma Aldrich. Antibody against smad2 was purchased from Abcam. Antibody against phospho-smad2 was purchased from Bioss. Antibodies against smad3 and phospho-smad3 were purchased from Abclonal.

2.2. Cell culture and regents

BC cell lines T47D, MDA-MB-231 and BT549 were obtained from cell bank of Chinese Academy of Medical Sciences. MDA-MB-231-Luc-D3H2LN stably expressing firefly luciferase was a gift from Dr. YF Shang. MDA-MB-231-Luc-control shRNA and MDA-MB-231-Luc-NRBE3 shRNA cells were generated by infection of MDA-MB-231-Luc-D3H2LN cells with lentiviruses carrying control shRNA or NRBE3 shRNA. These cells were grown in DMEM medium containing 10% fetal bovine serum (FBS) and incubated at 37 °C in a humidified atmosphere with 5% CO2.

2.3. Transfection and RNA interference

Transfection was performed using jetPRIME (Polyplus) and Lipofectamine 2000 (Invitrogen) according to the instructions provided by the manufacturers. The sequences of siRNAs were as follows: control siRNA, 5′ -CGUACGCGGAAUACUUCGA-3′; NRBE3 siRNA-1, 5′ -CGCU UCUCCAGUGGUUGCU-3′; NRBE3 siRNA-2, 5′ -AACUUGUACCUGGAU CAGGUG-3′.

2.4. Western blot and immunofluorescence staining

Western blot was performed as described previously [28]. Target proteins were detected with antibodies against NRBE3 (1:5000), E-cadherin (1:500), Flag (1:8000) or β-actin (1:40000). Immunofluorescence staining was performed as described previously [28,31]. E-cadherin was detected with a dilution of 1:200 of the antibody (Cell Signaling Technology). Smad2, phospho-smad2, smad3 and phospho-smad3 were detected with a dilution of 1:200 of the antibodies.

2.5. Real-time PCR

Total RNA was extracted from cells by Trizol reagent (Invitrogen) and reversely transcribed into cDNA. Real-time PCR was performed with FastStart Universal SYBR Green Master (Roche). Normalization was performed with GAPDH. Each real-time PCR experiment was independently repeated for at least three times in duplicates. The primers were as follows: E-cadherin, forward 5′-ATTTTTCCCTCGACACCC GAT-3′ and reverse 5′-TCCCAGGCGTAGACCAAGA-3′; GAPDH forward 5′-ACAACTTTGGTATCGTGGAAGG-3′ and reverse 5′-GCCATCACGCCA CAGTTTC-3′.

2.6. Wound-healing and transwell experiments

Wound-healing experiment was performed as previously described [37]. Distance of migration was measured by imageJ software. Transwell migration and invasion experiments were performed using Millicell chambers (Millicell® Cell Culture Inserts, Millipore) with or without Matrigel coating. Briefly, the Millicell chambers were placed in 24-well plates containing DMEM with 10% FBS. Cells were serum-starved overnight and transferred to the Millicell chamber containing serum-free DMEM. After 24-hour incubation at 37 °C, the migratory or invasive cells were stained by crystal violet and counted in five random fields.

2.7. In vivo metastasis experiment

MDA-MB-231-Luc-control shRNA cells, MDA-MB-231-Luc-NRBE3 shRNA cells, MDA-MB-231-Luc-Flag-vector cells or MDA-MB-231-Luc-Flag-NRBE3 cells were injected into the lateral tail vein (1×10^6 cells) of Nod/SCID mice (5-6 weeks, female, purchased from Peking University Laboratory Animal Center). After 4 or 5 weeks, luciferase substrate D-luciferin was injected intraperitoneally into mice and the metastasis of BC cells in mice were observed under bioluminescence imaging system (IVIS; Xenogen). The bioluminescence imaging and analysis were performed as previously described [38].

BT549-control shRNA or BT549-NRBE3 shRNA cells were injected into the lateral tail vein (5×10^5 cells) of Nod/SCID mice. The mice were sacrificed at 4 weeks after injection and the lungs of mice were sectioned. At least five sections with a 200 μm interval were stained with hematoxylin and eosin (H&E) and imaged under a microscope. The total lung area and metastatic area on the lung section were measured and the relative metastatic area were calculated. All animal experiments were approved by the Peking University Health Science Center Institutional Animal Care and Use Committee.

2.8. Patient tissues and immunohistochemistry (IHC) staining

The patient tissues were obtained from the Third Affiliated Hospital of Peking University (Beijing, China). The clinical factors of patients are shown in Table 1. IHC was performed to evaluate NRBE3 and E-cadherin expression in BC tissues. Expression levels of NRBE3 and E-cadherin were determined according to the extent score and intensity score as described previously [39,40]. Briefly, the intensity score was valued as 0 (negative), 1 (low), 2 (medium) or 3 (high). The extent score was evaluated according to the percentage of staining positive cancer cells as 0 (0%), 1 (1–25%), 2 (26–50%) or 3 (51–100%). The final scores (intensity score × extent score) of 0 or 1 indicate negative (−), of 2 or 3 indicate low level (+), of 4 to 6 indicate medium level (++), scores > 6 indicate high level (+++). Concerning patient's samples, informed consents were obtained and approved by the Peking University Health Science Center Ethics Committee.

2.9. Statistical analysis

The data were presented as mean ± SEM. Student *t*-test and ANOVA were used to analyze statistical significance. P value < 0.05 was regarded as statistically significance.