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Expression of Nischarin negatively correlates with estrogen receptor and alters apoptosis, migration and invasion in human breast cancer

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ABSTRACT

Nischarin, a novel integrin binding protein, has been demonstrated its negative effects on cell migration and invasion. However, the biological role of Nischarin in breast cancer has not been fully elucidated yet. Our study aimed to analyze the association between Nischarin expression and clinical features of breast cancer patients, and further investigate the role of Nischarin in breast cancer cells apoptosis, migration and invasion. Results showed that Nischarin expression was significantly lower in breast cancer tissues (37.8%, 23/67) than in normal tissues (61.8%, 21/34; $P < 0.05$), and the expression of Nischarin significantly negatively correlated with estrogen receptor status. Similarly, Nischarin expression was highest in normal breast cell line HBL-100 while triple-negative breast cancer cell line MDA-MB-231 had the lowest expression of Nischarin. Further experiments demonstrated that overexpression of Nischarin may induce apoptosis, and inhibit cell migration and invasion. The present data confirmed that Nishcharin might be a novel tumor suppressor and plays an important role in breast cancer cell apoptosis and metastasis, which can be used as a potential therapeutic target for breast cancer treatment.

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1. Introduction

Nischarin gene is located at chromosome 3p21.1 and encodes a ~190 kDa protein, which is an intracellular protein and was identified as an integrin $\alpha5\beta1$ interacting protein, selectively binding to the integrin $\alpha5$ subunit cytoplasmic tail [1,2]. It is well known that integrins play a critical role in diverse cellular processes including cell migration, growth, apoptosis, and signal transduction [3,4]. Nischarin interacts with integrin $\alpha5$ subunit and affects the integrin $\alpha5$ mediated events: (1) Nischarin inhibits Rac-mediated activation of the c-fos promoter, and can reverse the Rac-induced cell migration and invasion in breast epithelial cells [5–7]. (2) Nischarin interacts with LIM kinase to inhibit LIM kinase activation, cofilin phosphorylation, and LIM kinase-mediated cell invasion [8,9]. (3) Nischarin reduces integrin $\alpha5$ expression resulting in reduction of FAK phosphorylation and Rac GTP loading, which in turn reduces tumor growth [10]. Nischarin has been reported that it may be a novel tumor suppressor and its overexpression may be associated with poor survival in human breast cancer [11,12]. However, the biological role of Nischarin in breast cancer has not been fully elucidated yet.

To further clarify the effects of Nischarin in breast cancer, we examined Nischarin expression in 67 breast cancer specimens and 34 noncancerous breast tissues by immunohistochemistry, and analyzed the association between Nischarin expression and clinical features of breast cancer patients. Furthermore, we examined the negative effects of Nischarin in breast cancer progression using various *in vitro* biological experiments.

2. Materials and methods

2.1. Tissue used

Formalin-fixed paraffin-embedded tissue sections including 67 breast cancer specimens and 34 noncancerous breast tissues were collected retrospectively from the files of the Department of Pathology, Union Hospital (Wuhan, China). Clinical and pathological data including age, tumor stage, lymph node involvement, hormone receptor and human epidermal growth factor receptor 2

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(HER2) status were acquired from the medical record. None of the patients received radiotherapy or chemotherapy before surgery. For this type of study no informed consent from the participants is required.

2.2. Cell culture

Human breast cancer cell lines MCF-7, T47D and MDA-MB-231 and normal breast epithelial cell line HBL-100 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MCF-7, HBL-100 and MDA-MB-231 cells were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 1% antibiotic/antimycotic solution, while T47D cells were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal calf serum, 2 mM L-glutamine and10 μg/ml insulin. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO2.

2.3. Immunohistochemistry

Nischarin immunostaining was done as elsewhere [11]. Briefly, after deparaffinization, rehydration and antigen retrieval, tissue sections were incubated with rabbit anti-human Nischarin polyclonal antibody (Santa Crutz, 1:200 dilution) at room temperature overnight followed by incubation with goat anti-rabbit IgG-HRP (horseradish peroxidase; Santa Cruz) for 30 min at 37 °C. Colorimetric detection was performed with the EnVision™ Kit (DAKO), using diaminobenzidine as the chromogen. A normal mouse IgM antibody (1:200 dilution, Proteintech, USA) was used as negative control. All slides were evaluated independently by two pathologists blinded to patient information. The expression of Nischarin was considered positive if staining intensity was moderate or strong and the percentage of positively stained cells was more than 20%.

2.4. Construction of pcDNA3.1-Nischarin and transient transfection

The full length of Nischarin was amplified by RT-PCR using following primers: forward 5′-AATTCGGCACGAGGGTGGCGGCG-GAGACCCGAACA-3′ and reverse 5′-CTCGAGTTA ATGGTGGT GATGGTGATGGCCGGTGAG-3'. The PCR fragment was designed to contain 5′ *Eco*R I and *Xho*I restriction sites. The fragment was cut with *Eco*RI and *Xho*I, gel purified, and ligated in frame into pcDNA3.1. The product pcDNA3.1- Nischarin construct was sequence-verified. Transient transfection of breast cancer cell lines MDA-MB-231 and MCF-7 were usually done with Lipofectamine (Gibco) for 48 h according to the manufacturer's manual.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from breast cancer cell lines with the use of Trizol reagent (Invitrogen). cDNA was synthesized using MMLV reverse transcriptase (Invitrogen). The following gene-specific primer pairs were used for PCR: NISCH (forward 5′-TGGTCCTCGGTGGTGTTCTA-3′ and reverse 5′-TCTTC CCTGTGGTCTTGTTCC-3′) and β-actin (forward 5′-AGTCCT CTCCCAAGTCCACA-3'; reverse 5′-AGGGAGACCAAAAGCCTTCA-3′). PCR was conducted in a total reaction volume of 25 μl that contained 2 μl of cDNA solution, 0.4 μM sense and antisense primers, and 12.5 μl *EasyTaq* PCR SuperMix (TansGen Biotech). PCR conditions were as follows: initial denaturation for 10 min at 94 °C, followed by 32–35 cycles of 30 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C. The amplification products (5 μl) were resolved on a 2% agarose gel, stained with ethidium bromide, and visualized and photographed using UVP gel imaging system.

2.6. Cell lysis and western blot

Cells were lysed with RIPA cell lysis buffer (Boster, China) supplemented with 1 mM PMSF. Protein content was assessed using BioRad assay. Equal amounts of protein (50 μg) were separated by 8% SDS PAGE and then transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia). After blocking with 5%BSA in TBST, the membranes were incubated overnight at 4 °C with rabbit anti-human Nischarin (1:500 dilution, Proteintech, USA), Bax(1:500 dilution, Proteintech, USA), MMP-1(1:200 dilution, Santa Cruz, USA) and MMP-3(1:200 dilution, Santa Cruz, USA) before subsequent incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution, Boster, China) for 1 h at 37 °C. Protein was visualized using enhanced chemiluminescence reagent (Pierce, USA) and the expression level was normalized to that of β-actin.

2.7. Immunofluorescence staining

Cells were cultivated in 24-well plates, transfected with pcDNA3.1-Nischarin and pcDNA3.1-null plasmids. Then the cells were washed with PBS twice and fixed with 4% paraformaldehyde for 30 min. After permeabilization with 0.1% Triton X-100, cells were blocked with 5% BSA for 1 h, subsequently incubated with Nischarin(1:100 dilution, Proteintech, USA) overnight. After washed with PBS, the cells were incubated with Cy3-conjugated goat anti-rabbit or anti-mouse IgG antibody (1:150 dilution, Boster, China) in the dark for 1 h at 37 °C, counterstained with DAPI. Images were collected using an Olympus Fluoview FV1000 laser-scanning confocal microscope.

2.8. Apoptosis assay

Cell apoptosis was analyzed by Annexin V-FITC/propidium iodide (PI) assay. Briefly, cells were washed twice with cold PBS and then stained with Annexin V-FITC/PI using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manual and subjected to flow cytometric analysis.

2.9. Wound-healing assay

Cells were seeded in a 6-well plate to form a confluent monolayer. The monolayer was transfected with pcDNA3.1-Nischarin and pcDNA3.1-null plasmids for 48 h before being scratched by a plastic tip. Wounded monolayers were washed by PBS to remove cell debris, and taken photos under microscopy after 24 h.

2.10. Transwell assay

Transwell (Corning Costar) upper chamber were coated with matrigel for invasion assay or without matrigel for migration assay. Cells at a final concentration of 3×10^5 cells/ml were seeded in serum-free medium in the upper chamber, while medium containing 5% fetal calf serum in the lower chamber. After incubating for 24 h at 37 °C, cells in the upper chamber were carefully removed with a cotton swab, and the invaded cells that had traversed to reserve face of the membrane were fixed with 100% methanol and stained with Giemsa.

2.11. Statistical analysis

Statistical analyses were conducted using SPSS 20.0 software. Pearson's x^2 test was used to evaluate statistical significance of Nischarin expression differences between tumor and non-tumor tissue. *Chi*-square test was also used to determine the association

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