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SNHG16 contributes to breast cancer cell migration by competitively binding miR-98 with E2F5

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ABSTRACT

Long noncoding RNAs (lncRNAs) have been proved to play important roles in cellular processes of cancer, including the development, proliferation, and migration of cancer cells. In the present study, we demonstrated small nucleolar RNA host gene 16 (SNHG16) as an oncogene on cell migration in breast cancer. Expression levels of SNHG16 were found to be frequently higher in breast cancer tissues than in the paired noncancerous tissues. Gain- and loss-of-function studies proved that SNHG16 significantly promoted breast cancer cell migration. We predicted SNHG16 as a competitive endogenous RNA (ceRNA) of E2F transcription factor 5 protein (E2F5) via competition for the shared miR-98 through bioinformatics analysis, and proved this regulation using relative quantitative real-time PCR (qRT-PCR), western blot, RNA immunoprecipitation (RIP) assay and luciferase reporter assay. In addition, we identified a positive correlation between SNHG16 and E2F5 in breast cancer tissues. Furthermore, we demonstrated that forced expression of miR-98 could partially abrogate SNHG16-mediated increase of breast cancer cells migration, suggesting that SNHG16 promoted cell migration in a miR-98 dependent manner. Taken together, our findings indicated that SNHG16 induces breast cancer cell migration by competitively binding miR-98 with E2F5, and SNHG16 can serve as a potential therapeutic target for breast cancer treatment.

1. Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death among women in China, accounting for 15% of all new cancers among females in 2015. In addition, there is a significant upward trend in incidence and mortality rates of breast cancer [1]. Morbidity and mortality in patients with solid tumors always result from the disruption of normal biological function by disseminating tumor cells, and tumor cell migration has been intensely investigated as the underlying cause of cancer metastasis [2]. However, the molecular mechanisms which mediate tumor cell migration remain largely unclear.

The long noncoding RNAs (lncRNAs) are a category of noncoding RNAs with over 200 nucleotides that do not encode for proteins, and the emerging impact of lncRNAs in cancer research has been discovered in prevalent cancer types [3]. Through diverse mechanisms, lncRNAs play critical roles as drivers of tumor suppressive and oncogenic functions [4]. Modes of direct post-transcriptional interaction among lncRNAs and miRNAs include miRNA-triggered lncRNA decay, lncRNA as miRNA sponge/decoy, and lncRNA generating miRNAs [5].

Small nucleolar RNA host gene 16 (SNHG16), also known as non-coding RNA expressed in aggressive neuroblastoma (ncRAN), was newly identified as a potential oncogene in colorectal cancer [6], neuroblastoma [7] and bladder cancer [8]. In the current study, we investigated how SNHG16 contributes to the progression of breast cancer and explored the underlying mechanisms.

2. Materials and methods

2.1. Cell lines and clinical specimens

Human breast cancer cell lines MDA-MB-231, MCF-7, MDA-MB-468 and HEK293T were purchased from American Type Culture

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Collection (ATCC, Manassas, VA, USA), and were routinely cultured in DMEM/high glucose medium (Gibco, Rockville, IN, USA) supplemented with 10% FBS (Clark Bioscience, Seabrook, MD, USA), 100 U/ml penicillin and 100 mg/ml streptomycin. SK-BR-3 cells were cultured in RPMI 1640 (GIBCO) supplemented with 10% FBS and antibiotics. All the cells were cultured at 37 °C and 5% CO2.

Breast cancer specimens and the corresponding adjacent noncancerous tissues were obtained from Ethics Committee on Scientific Research of Shandong University Qilu Hospital with informed consent. The protocols used in the study were approved by the Ethical Committee of Shandong University.

2.2. Overexpression and RNA interference

The full length of SNHG16 was amplified from human cDNA by PCR (forward primer 5′- CCCAAGCTTGCGTTCTTTTCGAGGTCGGC-3′ and reverse primer 5′- CCGGAATTCTGACGGTAGTTTCCCAAGTTTATTGTAAGT-3′) and inserted into pcDNA3.1 vector (Invitrogen, USA). Small interfering RNAs of SNHG16 and the negative control siRNA were obtained from Sigma-Aldrich (USA). MiR-98 mimics and small interfering RNAs of E2F5 as well as the negative control were purchased from RiboBio (Guangzhou, China). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

2.3. Relative quantitative real-time PCR analysis

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol and cDNA was synthesized from total RNA by a PrimerScript RT Reagent kit (Takara, Japan). MiRNA from total RNA was reverse transcribed using the Prime-Script miRNA cDNA Synthesis Kit (TaKaRa). Real-time PCR (RT-PCR) was performed with the SYBR green Premix Ex Taq II (Takara) on Applied Biosystems Step One Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). GAPDH was used as the endogenous control for detection of mRNA expression level, while U6 was used as endogenous control for miRNA expression analysis. Relative quantification analysis was performed using the comparative C (T) ($2^{-\Delta\Delta Ct}$) method. Primer information used in the study is provided in Supplementary Table S1.

2.4. Western blot analysis

Cells were collected and lysed with lysis buffer (1 × PBS, 1% NP40, 0.1% SDS, 5 mM EDTA, 0.5% sodium deoxycholate and 1 mM sodium orthovanadate) containing protease inhibitors, and protein concentration was determined using the BCA Assay Kit (Thermo Scientific). Equal amounts of protein samples were separated by SDS-PAGE and then electrotransferred onto PVDF membrane (Millipore) using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA, USA). After blocking with 5% non-fat milk at room temperature for 1 h, the membrane was incubated overnight at 4 °C with the primary antibody and then with horseradish peroxydase-coupled secondary antibody. Signal was detected with enhanced chemiluminescence (ECL) (PerkinElmer) by ImageQuant LAS 4000 (GE Healthcare Life Sciences). Antibodies used include *anti*-E2F5 (Santa Cruz), and *anti*-β-actin (Sigma-Aldrich).

2.5. Transwell migration assay

For the Transwell (24-well insert, 8 mm pore size with polycarbonate membrane; Corning Costar, Lowell, MA, USA) migration assays, 700 μL media supplemented with 20% FBS was added to the lower chamber and 1 × 10^5 of cells resuspended in serum-free media were added to the upper insert after transfection. Transwell membranes were fixed and stained using crystal violet after specified time. The cells adhering to the lower surface of the membrane were counted under a light microscope (Olympus).

2.6. RNA immunoprecipitation assay

The MS2bs-MS2bp-based RNA immunoprecipitation (RIP) assay was carried out according to previous reports [9]. The plasmid pSL-MS2-12X (Addgene plasmid # 27119) [10] and pMS2-GFP (Addgene plasmid # 27121) [11] were gifts from Robert Singer. The sequences of SNHG16 and MS2-12X were inserted into pcDNA3.1 plasmid, and the miR-98 complementary site with the sequence **CUACCUC** in SNHG16 was mutated by deletions to remove complementarity to miR-98 as described previously [12]. Cells were cotransfected with pcDNA3.1-SNHG16-MS2, pcDNA3.1-SNHG16-mut-MS2, or pcDNA3.1-MS2 together with pMS2-GFP. After 48 h, cells were used to perform RIP using a GFP antibody (Roach) and the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). Normal mouse IgG and SNRNP70 were used as negative control and positive control, respectively. Then the miR-98 level was analyzed by qRT-PCR. Total RNAs (input controls) and IgG were assayed simultaneously to demonstrate that the detected signals were the result of miR-98 specifically binding to SNHG16.

2.7. Dual luciferase reporter assay

The SNHG16 sequence containing putative miR-98 binding site was cloned and inserted into pmirGLO vector (Promega, Madison, WI, USA). A mutant reporter plasmid was constructed the same way as pcDNA3.1-SNHG16-mut-MS2. The 3′-UTR of E2F5 containing putative miR-98 binding site was obtained from GENEWIZ (Suzhou, China) and cloned into pmirGLO vector. Cells were seeded in 96-well plates and co-transfected with recombinant plasmids or empty pmirGLO vector, and miR-98 mimics or negative control. Luciferase activity was measured with the dual luciferase reporter assay system (Promega) 24 h after transfection. Firefly luciferase activity was normalized against Renilla luciferase activity.

2.8. Statistical analysis

Data were presented as mean ± SD from at least three separate experiments. The software GraphPad Prism 6 V6.01 was used for statistical analysis. Student's t-test and one-way ANOVA were used to determine the significance of differences between two groups or among multiple groups, respectively. Bivariate correlations between study variables in tissues were calculated by Pearson's rank correlation coefficients. The level of significance was set at $p < 0.05$, $p < 0.01$ and $p < 0.001$. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. SNHG16 is upregulated in breast cancer tissues and can promote breast cancer cell migration

We assayed SNHG16 expression in a panel of cancerous tissues and adjacent noncancerous tissues of each donor with breast cancer, revealing that SNHG16 was expressed at higher levels in tumor tissues compared with adjacent tissues (Fig. 1A). SNHG16 expression was measured in MDA-MB-231, MCF-7, MDA-MB-468 and SK-BR-3 cell lines, which was obviously higher in MDA-MB-231 and MCF-7 cells (Fig. 1B). Then MDA-MB-231 and MCF-7 cells were selected for SNHG16 knockdown by three siRNAs (SNHG16si1, SNHG16si2, SNHG16si3). Moreover, we constructed a plasmid containing SNHG16 sequence, and MDA-MB-468 and SK-BR-3 cells

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