Upregulation of Small Nucleolar RNA 78 Promotes Cell Proliferation and Invasion of Breast Cancer

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Research

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Abstract

OBJECTIVE: Recent studies suggest that abnormal snoRNA expression may play crucial role in tumor development and progression. In the study, we aim to explore the clinical significance and functional role of Small nucleolar RNA 78 (SNORD78) in breast cancer.

PATIENTS AND METHODS: The expression of SNORD78 in 92 breast cancer and adjacent non tumor tissues was analyzed by quantitative real time polymerase chain reaction (qRT-PCR). The association between SNORD78 expression and clinicopathological factors was evaluated. The biological functions in vitro were examined by MTT cell proliferation, colony formation and transwell invasion assays. QRT-PCR and Western blot assays were used to analyze the mRNA and protein expression of WNT1, GSK3 β and β -catenin.

RESULTS: SNORD78 expression showed a significant increase in breast cancer tissues and cell lines. Higher SNORD78 expression positively was related to lymph node metastasis and TNM stage in patients. Furthermore, knockdown of SNORD78 inhibited the abilities of cell proliferation, colony formation, and cell invasion in MDA-MB-231 and SKBR-3 cells. Moreover, we demonstrated that knockdown of SNORD78 inhibited WNT/ β -catenin signaling pathway in MDA-MB-231 and SKBR-3 cells via downregulating the relative protein expression of WNT1 and β -catenin and upregulating the GSK3 β expression.

CONCLUSION: Our results indicated that SNORD78 may be potential therapeutic target for breast cancer.

Introduction

Breast cancer is the most common malignancy in women worldwide. In 2015, 231,840 women were diagnosed with breast cancer, and approximately 40,290 died of the disease [1, 2]. Tumor recurrence and metastasis cause larger cancer related morbidity and mortality [3]. In recent decades, large advances in the diagnosis and therapy for breast cancer have been improved, however, patients who are diagnosed at advanced stage present poor outcome [4]. Therefore, to investigate tumor makers of early detection or therapeutic target of breast cancer is important.

Small nucleolar RNAs (snoRNAs) are a class of RNA without protein coding capacity and about 60–300 nucleotides in length [5]. Recent studies have indicated that snoRNAs were involved in some disease process including tumor development [6]. For instance, small nucleolar RNA U91 is a new internal control for accurate microRNAs quantification in pancreatic cancer [7]. SNORD76, not its host gene, is selectively downregulated in glioblastoma (WHO grade IV) and acts as a tumor suppressor in glioblastoma [8]. SNORD113-1 suppresses tumorigenesis in hepatocellular carcinoma by inactivating the phosphorylation of ERK1/2 and SMAD2/3 in MAPK/ERK and TGF- β pathways [9]. However, the clinical role and potential functions of small nucleolar RNA 78 (SNORD78) in breast cancer remain unknown.

In the study, we demonstrated that SNORD78 expression was significantly upregulated in breast cancer. SNORD78 silencing obviously inhibited breast cancer cells proliferation, cell invasion and Wnt/ β -catenin

signaling pathway. Thus, our results indicated that SNORD78 may be potential therapeutic target for breast cancer.

Patients And Methods

Human patient tissue specimens

Freshly breast tissue specimens obtained from breast cancer patients who underwent curative surgical treatment between April 2010 and March 2012 were immediately frozen in liquid nitrogen for further RNA extraction. All of patients were selected at Department of Oncology, Peking University Shenzhen Hospital. The study was approved by Peking University Shenzhen Hospital and written informed consent was obtained from all of the patients.

Cell lines culture

Three human breast cancer cell lines (MDA-MB-231, MCF-7, and SKBR-3) and one normal mammary epithelial cell line (MCF-10A) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All of breast cancer cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) added with 10% fetal bovine serum at 37 °C with 5% CO₂.

Quantitative real-time polymerase chain reaction (QRT-PCR)

The TRIzol® (Invitrogen, Carlsbad, CA, USA) was used for RNA extraction from tissues and cells according to the manufacturer's instructions. RNA was subjected to cDNA synthesis using a Prime Script RT Regent kit (Takara, Dalian, China). The SYBR Green PCR Master Mix (Takara, Dalian, China) was used for qRT-PCR assay on ABI PRISM 7900 Real-time PCR system (Applied Biosystems, Foster City, CA). GAPDH were used for normalization. The primer sequences were as follows: SNORD78(Forward:5'-GTGTAATGATGTTGATCAAATGTCTGAC-3';Reverse: 5'-CACATTACTACAACCTAGTTTACAGACTGG-3'),GAPDH(Forward:5'-CTCAAGGGCATCCTGGGCTAC-3';Reverse:5'-CAGCCCCAGCGTCAAAGGT-3').QRT-PCR results were analyzed using $2^{-\Delta\Delta CT}$ Methods.

RNA interference and cell transfection

Two siRNAs against SNORD78 was designed to knockdown SNORD78 expression and one negative control (si-NC) was purchased from Ribobio (Guangzhou, China). Cell transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The siRNAs targeted SNORD78 sequences were as follow: si-SNORD78-1(5'-GTTGATCAAATGTCTGACCTG-3'), si-SNORD78-2 (5'-GACCTGAAATGAGCATGTAGA-3').

MTT assay

Cell growth rate was investigated using a MTT assay. 3×10^3 transfected cells/well were seeded into 96-well plates. At indicated time 0, 24, 48, and 72 h, cells were incubated with 20 µl MTT (5 mg/ml; Sigma-

Aldrich, St. Louis, MO, USA) and cultured for 4 h at 37 °C with 5% CO₂. The cell proliferation was analyzed using an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the absorbance at 490 nm.

Cell clonogenic assay

300 transfected cells were placed in 12-well plates cultured in medium added with 10% fetal bovine serum at 37 °C with 5% CO₂ for 14 days. Colonies were fixed with 100% methanol, stained with 0.1% crystal violet, and then were counted under a microscope.

Cell invasion assays

Transwell chambers with an 8 µm pore polycarbonate membrane (Costar; Corning Incorporated, Corning, NY, USA) were used to assess cell invasion ability. 1×10^5 cells were added on the upper chamber supplemented with 300 µl culture medium without FBS and 500 µl culture medium supplemented with 20% FBS was added on the lower chamber. After cell cultured for 24 h, cells were fixed with 100% methanol, and then stained with 0.1% crystal violet. Cells on the lower chamber were counted in five random fields under a light microscope.

Western blot analysis

Cells were lysed using radioimmunoassay precipitation assay (RAPA) lysis buffer with a proteinase inhibitor. The quantity of approximately 40 µg proteins in the lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with anti-WNT1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), GSK3β (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), β-catenin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 12 h at 4 °C and then washed three times for 5 min. Following horseradish peroxidase-conjugated secondary antibodies were incubated. The blot was visualized by an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

Statistical analysis

All of results are presented as means ± SD from at least three or more independent experiments. The association between SNORD78 expression and clinical characteristics was evaluated using the chi-square test. Differences between groups were compared using Student's *t*-test or an analysis of variance (ANOVA). A *p* < 0.05 were considered statistically significant.

Results

SNORD78 expression is upregulated in breast cancer tissues and associates with clinicopathologic features

In the study, SNORD78 expression was determined by qRT-PCR in 92 breast cancer tissues compared with adjacent normal tissues. As shown in Fig. 1A, results analysis demonstrated that SNORD78 expression in

breast cancer tissues was significantly higher compared with adjacent normal tissues ($p < 0.05$). According to the median expression rate of SNORD78 expression in breast cancer tissues, we divided patients into higher expression group and lower expression group. The association between the SNORD78 expression and the clinical characteristics of breast cancer patients were analyzed. We demonstrated that higher SNORD78 expression was significantly associated with the lymph node status ($p = 0.001$, Table I) and advanced TNM stage ($p = 0.023$, Table I), but was not associated with the other characteristics in breast cancer patients ($p > 0.05$, Table I).

Table I
Association of SNORD78 expression with clinicopathological features

Clinicopathological features	Total (n = 92)	Lower SNORD78 (n = 44)	Higher SNORD78 (n = 48)	<i>p</i> -value
Age (year)				0.548
≤ 50	43	22	21	
> 50	49	22	27	
Tumor size (cm)				0.714
< 2 cm	40	20	20	
> 2 cm	52	24	28	
Tumor grade				0.676
G1-G2	65	32	33	
G3	27	12	15	
HER-2 status				0.064
Negative	49	19	30	
Positive	43	25	18	
Lymph node status				0.001*
Negative	43	29	14	
Positive	49	15	34	
Distant metastasis				0.944
No	75	36	39	
Yes	17	8	9	
PR				0.485
Negative	53	27	26	
Positive	39	17	22	
TNM stage				0.023*
I-II	58	33	25	
III	34	11	23	
* <i>p</i> < 0.05.				

Downregulation of SNORD78 expression suppresses cell growth and invasion in MDA-MB-231 and SKBR-3 cells in vitro

Additionally, we evaluated the expression of SNORD78 in three human breast cancer cell lines (MDA-MB-231, MCF-7, and SKBR-3) and one normal mammary epithelial cell line (MCF-10A). As shown in Fig. 1B, the expression of SNORD78 was higher in three human breast cancer cell lines than MCF-10A cells ($p < 0.05$). To further analyze the biological effects of on cell growth and invasion, two siRNAs targeting SNORD78 oligos were transfected into MDA-MB-231 and SKBR-3 cells (Fig. 2A-2B). The si-SNORD78-1 was chosen to knock down SNORD78 expression in following experiments due to its higher silencing efficiency. MTT analysis results showed that knockdown of SNORD78 in MDA-MB-231 and SKBR-3 cells notably suppressed cell proliferation at 48 h and 72 h, compared with control group (Fig. 2C-2D, $p < 0.05$). Cell colony formation results demonstrated that knockdown of SNORD78 in MDA-MB-231 and SKBR-3 cells notably reduced cell colony formation ability at 14 days, compared with control group (Fig. 3A-3B, $p < 0.05$). Moreover, cell invasion assay results also showed that knockdown of SNORD78 in MDA-MB-231 and SKBR-3 cells notably reduced cell invasion ability after cell transfection at 48 h, compared with control group (Fig. 3C-3D, $p < 0.05$). These results indicated that downregulation of SNORD78 expression suppressed cell growth and invasion in MDA-MB-231 and SKBR-3 cells.

Downregulation of SNORD78 expression inhibits Wnt/ β -catenin signaling pathway in MDA-MB-231 and SKBR-3 cells

It has been reported that abnormal Wnt/ β -catenin signaling pathway functions as a crucial signaling cascade in breast cancer [10]. After knockdown of SNORD78 in MDA-MB-231 and SKBR-3 cells at 48 h, we performed qRT-PCR and western blot analysis to examine the mRNA and protein expression of WNT1, GSK3 β and β -catenin. The results demonstrated mRNA and protein expression levels of WNT1 and β -catenin was significantly downregulated, while GSK3 β expression was upregulated after knockdown of SNORD78 in MDA-MB-231 and SKBR-3 cells at 48 h (Fig. 4A-4D, $p < 0.05$). Thus, these results indicated that downregulation of SNORD78 expression inhibited Wnt/ β -catenin signaling pathway in breast cancer.

Discussion

Small nucleolar noncoding RNAs (snoRNAs) regulate function of ribosomes and increasing evidence indicates that dysregulation of small nucleolar RNAs (snoRNAs) were associated with tumorigenesis [11]. Such as, integrated analysis of the prostate cancer small-nucleolar transcriptome reveals SNORA55 as a driver of prostate cancer progression [12]. SNORD50A and SNORD50B deletion and oncogenic KRAS mutation co-occurred significantly in multiple human tumor types [13]. Small nucleolar RNA 47 promotes tumorigenesis by regulating EMT markers in hepatocellular carcinoma [14]. Small nucleolar RNA ACA11 promotes cell proliferation, migration and invasion in hepatocellular carcinoma by targeting the PI3K/AKT signaling pathway [15]. In the study, we found that SNORD78 expression in breast cancer tissues was significantly higher compared with adjacent normal tissues. Higher SNORD78 expression was significantly associated with the lymph node status and advanced TNM stage. Moreover, knockdown

of SNORD78 inhibited cell proliferation, colony formation and cell invasion capacities in breast cancer. Consistent with our findings, SNORD78 was observed to be upregulated in NSCLC tissues compared to their adjacent normal tissues. SNORD78 promoted cell proliferation and invasion of NSCLC cells, which indicated SNORD78 function as an oncogene [16].

Furthermore, we demonstrated that WNT/ β -catenin signaling pathway related protein expression levels of WNT1 and β -catenin was significantly downregulated, but GSK3 β expression were upregulated when SNORD78 was knocked down in MDA-MB-231 and SKBR-3 cells. The WNT pathway has emerged as a key signaling cascade participating in mammary organogenesis and breast oncogenesis [10]. Aberrantly activated Wnt/ β -catenin signaling could affect diverse biological processes including cell motility, migration, differentiation, proliferation, and invasion [17]. Overexpression of Wnt/ β -catenin protein Lrp6 or stabilized β -catenin, or loss of APC, resulted in mouse mammary hyperplasia or tumors [18]. To elucidate the underlying mechanisms of SNORD78 affecting cell proliferation and invasion, we demonstrated that knockdown of SNORD78 inhibited cell proliferation and invasion by regulating WNT/ β -catenin signaling pathway.

Conclusions

In conclusion, our results found that SNORD78 expression was significantly upregulated in breast cancer. Knockdown of SNORD78 inhibited cell proliferation, invasion and WNT/ β -catenin signaling pathway. Thus, our results indicated that SNORD78 may be potential therapeutic target for breast cancer.

Declarations

Ethics approval and consent to participate

The study was approved by Peking University Shenzhen Hospital and written informed consent was obtained from all of the patients.

Consent for publication

All the authors agreed that the article should be published.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare no conflict of interest.

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N/A

Authors' contributions

Qiaohong Nong and Shubin Wang carried out the study concepts, study design, manuscript editing and manuscript review; Zhu Li and Gangling Tong was dedicated to the literature research, data acquisition and data analysis; Shaokang Yu and Hui Hu was involved in the manuscript preparation and manuscript editing. All authors read and approved the final manuscript.

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Figures

Figure 1 to 4 are missing in this version of the manuscript.