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Research Article

Zhang Yang, Wang Lu, Li Ning, Dai Hao, Sun Jian, Cai Hai-Feng*

Downregulation of long non-coding RNA MALAT1 induces tumor progression of human breast cancer through regulating CCND1 expression

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Abstract: *Objective*: Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is already known to be involved in the development and progression of many types of tumors. In the present study, we set to seek the role of MALAT1and the molecular mechanisms in breast cancer. *Methods*: MALAT1 mRNA expression level was measured by real-time PCR in selected tissues and breast cancer cell lines. SiRNAs targeting MALAT1 were employed to knockdown the endogenous MALAT1. Then cell counting method and colony formation method were applied to reveal the proliferation changes after MALAT1 was suppressed. Afterwards, the mRNA and protein expression of growth related gene cyclinD1 (CCND1) were detected by RT-PCR and western blotting, respectively. *Results*: We found a downregulation of MALAT1 expression in breast cancer cell lines and tissues. Inhibition of its expression led to enhanced cell proliferation and colony formation. Importantly, the mRNA and protein expression of CCND1 was significantly increased in MALAT1-depleted cells. *Conclusion*: MALAT1 is a potential tumor suppressive long non-coding RNA that negatively regulates cell proliferation in breast cancer progression, via suppressing CCND1 expression.

Keywords: Breast cancer, MALAT1, proliferation, CCND1, tumor progression

***Corresponding author: Cai Hai-Feng,** Department of Breast Surgery, Tangshan City Workers Hospital of Hebei Province, 063000, China, E-mail: caihf_ts@163.com

Zhang Yang, Wang Lu, Li Ning, Dai Hao, Sun Jian, Department of Breast Surgery, Tangshan City Workers Hospital of Hebei Province, 063000, China

1 Introduction

Breast cancer is the most common malignancy among females, and is also the second leading cause of cancer-related mortality in the world [1,2]. During the past few years advances have been made in its treatment. However, the incidences of breast cancer are on the rise recently [3]. The prognosis of patients with breast cancer is still poor, particularly due to the frequent vital organ metastasis [4-6]. Once metastasis occurs, the 5-year survival rate is less than 25% [7]. Therefore, it is urgent to identify novel biomarkers to serve as possible therapeutic targets for the treatment of breast cancer. In addition, a deeper understanding of the molecular mechanisms that govern tumor progression of breast cancer is required.

Long non-coding RNAs (lncRNAs) were recently identified to play as important oncogenes or tumor suppressors in human malignancies [8-10]. They are genomic transcripts longer than 200 nucleotides that do not code for proteins [11-12]. According to the recent advances of cellular and molecular biology, it is clear that lncRNAs participate in gene regulation at least through transcriptional, post-transcriptional and epigenetic levels [13-14]. Among them, most lncRNAs are expressed at a low level, and share poor primary sequence similarity over evolution15. Long non-coding RNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), is outstanding due to its evolutionarily high conservation and abnormal expression within many different malignancies [16-19]. In breast cancer, it has been shown that MALAT1 is downregulated in breast cancerous tissues and breast cancer cell lines, and could modulate the epithelial-to-mesenchymal transition (EMT) program via phosphatidylinositide-3 kinase-AKT pathways [20]. Nevertheless, it was still unclear whether MALAT1 influenced cell growth of breast cancer cells.

2 Materials and methods

2.1 Humanbreast tissue samples

Human breast cancer tissues were provided by Tangshan City Workers Hospital after approval was obtained. The tissues were obtained from 20 individuals undergoing surgery due to normal adjacent tissues (NATs) and breast cancer tissues (BCTs). The sampled tissues were immediately snap-frozen in liquid nitrogen and stored at –80°C.

2.2 Cell culture and regents

The human normal breast cell line MCF-10A was cultured in DMEM/F-12 medium with 5% HS, 2 μg/ml EGF, 10μg/ml insulin at 37°C with 5% CO_2. The human breast cancer BT549 and SK-BR-3 cell lines were cultured in RPMI-1640 medium with 10% FBS. The human breast cancer MDA-MB-231 and MDA-MB-453 cell lines were cultured in Leibovitz's L-15 medium with 10% FBS at 37°C in 100% air. Human MCF7 adenocarcinoma cells were cultured in EMEM with 10% FBS and 0.01 mg/ml human recombinant insulin. Cells were cultured at 37°C with 5% CO_2. All cell lines were provided by the Chinese Academy of Sciences (Shanghai, China).

2.3 Transfections and siRNAs

Transfections were performed using Lipofectamine 2000 (Invitrogene) as per the manufacturer's instructions. MALAT1 siRNAs and control siRNAs were purchased from GenePharma (Shanghai, China).

2.4 Real-time PCR

To quantitatively determine the mRNA level of MALAT1 in breast cancer tissue and cell lines, real-time PCR was used. Total RNA was extracted with TRIzol according to the manufacturer's protocol. The genes were amplified using specific oligonucleotide primer, and human GAPDH gene was used as an endogenous control. Data were analyzed using the comparative Ct method (2^{-DDCt}).

2.5 Immunoblotting and antibodies

MCF7 Cells were lysed in protein RIPA buffer and cellular debris was cleared by centrifugation at 12,000 rpm for 30 min at 4°C. Protein concentration was measured by the BCA Protein Assay Kit (Beyotime, Haimen, China). Protein was denatured at 95°C for 5 min prior to loading onto 10% SDS-PAGE. After gel separation, proteins were transferred to PVDF membranes and incubated with primary antibodies over night at 4°C. The membranes were washed five times, 5 min per washing and incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at RT, followed by another five washing steps. Proteins were visualized by chemiluminescence detection imaging. CCND1 and β-actin antibodies were purchased from Santa Cruz.

2.6 Cell proliferation assay

Cell proliferation was analyzed with the cell count assay using WST-8 kits. Briefly, MCF7 cells transfected with MALAT1 siRNA were incubated in 96-well plates at a density of 1×10^5 per well. And then the cells were treated with 10 ml WST at 12, 24, 48, and 72h. Color reaction was measured at 570 nm with enzyme immunoassay analyzer. The proliferative activities were calculated for each clone.

2.7 Colony formation assay

Non-transfected and transfected MCF7 cells (500 cells/well) were seeded in 6-well plates. The cells were cultured for 12 days and fixed with 4% paraformaldehyde. After washing by PBS, the plates were air dried, and the colonies were photographed using a microscope. The total number of colonies was counted and the experiments were performed in triplicate.

2.8 Statistical analysis

All data were presented as mean ± standard deviation. Statistical significance was analysed using a Student's t-test. $P < 0.05$ indicated significant difference.

3 Results

3.1 MALAT1 expression was reduced in breast cancer cell lines and patients

First we conducted the real-time PCR analysis to determine the MALAT1 expression in breast cancer. As shown in **Figure 1A**, we found a significant downregulation of MALAT1 in breast cancer tissues (BCTs), compared to that in normal adjacent tissues (NATs). In addition, all five tested breast cancer cell lines showed consistent reduced MALAT1 expression, when compared with the non-malignant breast epithelial cell MCF-10A. Our results presented here were accordant with the previous findings in breast cancer cell lines and tissues. Hence, our data indicated that abnormal lower levels of MALAT1 might serve as a tumor suppressor in breast cancer.

3.2 Knockdown of MALAT1 promoted cell proliferation and colony formation

To further investigate the role of MALAT1 in breast cancer cell growth, MCF7 cell line was selected for functional analysis, which including WST assay and colony formation assay. We inhibited MALAT1 expression in MCF7 cell line by transfecting siRNAs targeting MALAT1 or its negative control (NC) oligoes following the manufacture's instructions. WST assay showed that after MALAT1 was inhibited, cell proliferation rate was elevated significantly (**Figure 2A**). Results from the colony formation assay revealed that MALAT1-delepted MCF7 cells displayed more colonies than the NC group (**Figure 2B**). The efficiency of MALAT1 knockdown was quantified in **Figure 3A**, by using real-time PCR method. Therefore, we concluded here that MALAT1 indeed impacted cell growth, with a negative effect.

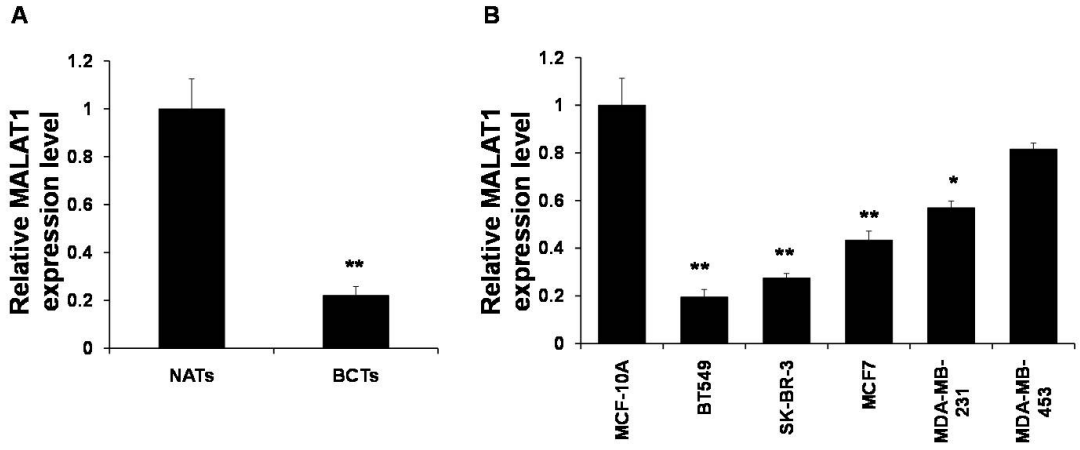

Figure 1 LncRNA MALAT1 was down-regulated in human breast cancer tissues and cell lines. (A) The level of MALAT1 was decreased in breast cancer tissues compared with NATs; **P < 0.01 vs. NATs. **(B)** MALAT1 was absent in breast cancer cells compared with MCF-10A; *P < 0.05, **P < 0.01 *vs*. MCF-10A.

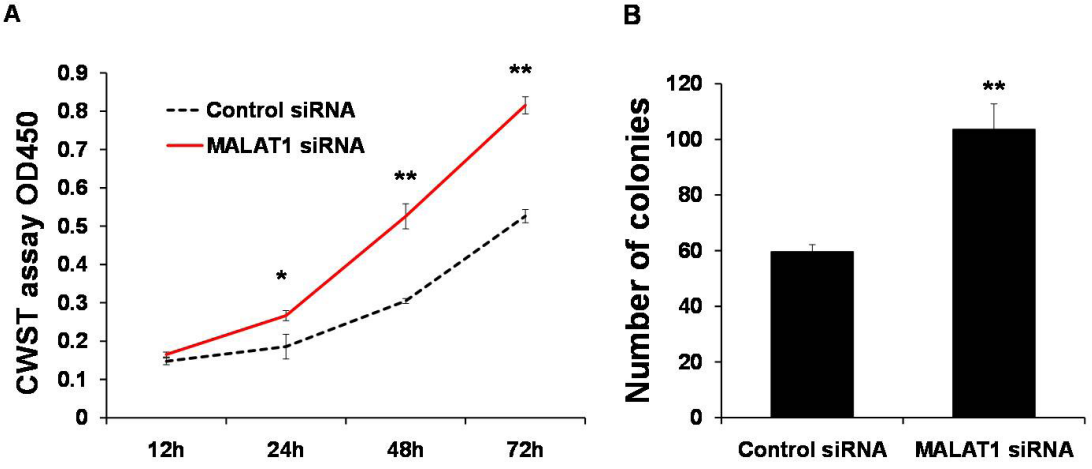

Figure 2 MALAT1 knockdown accelerated proliferation in MCF7 cell lines. (A) Cellular proliferation of untransfected or transfected MCF7 cells was measured using WST assay for 12-72 h. **(B)** MCF7 cells were seeded at 500cells/well, and the cells were allowed to form colonies. The colony numbers were counted and recorded. Results are expressed as mean ± SD from three independent experiments. **P < 0.01 *vs*. control.

3.3 MALAT1 induced CCND1 expression at both mRNA and protein levels

To uncover the related mechanisms by which MALAT1 inhibited cell proliferation, we examined the growth-related cyclinD1 (CCND1) expression by real-time PCR and western blotting, to reveal its mRNA and protein expression, respectively. As shown in **Figure 3A** and **Figure 3B**, knockdown of MALAT1 significantly increased both mRNA and protein expression of CCND1, compared to the NC group. These results demonstrated that CCND1 might be the linkage of MALAT1 to cell proliferation changes, which further contributes to breast cancer progression.

4 Discussion

Since lncRNAs are novel and important gene regulators in human cancers, including breast cancer [21], it is popular and of significance to characterize the role and the underlined mechanisms of specific lncRNAs in breast cancer. Accumulating evidence has reported that lncRNAs are involved in the malignant biological behavior of cancer cells, including enhanced proliferation, survival, apoptosis resistance and metastasis induction [22]. MALAT1 has high conservation over evolution. In breast cancer research, it was proved that downregulated MALAT1 could induce EMT transition via the PI3K-Akt pathway [20]. This observation supported a tumor suppressive role of MALAT1 in breast cancer, however, the proliferation effect of its downregulation in breast cancer is still lacking.

In the present study, we first confirmed the reduced expression of MALAT1 in breast cancer tissues and. cell lines. Then we performed functional assays. We found its inhibition could promote cell proliferation as well as colony formation abilities in vitro. We further analyzed the growth-related molecule CCND1 expression in our system, and found that after MALAT1 was knocked down, the expression of CCND1 was indeed elevated significantly. Hence, we speculated that, the growth-inhibitory functions of MALAT1 might be mediated by CCND1, at least partially.

Nevertheless, Dong et al had reported that MALAT1 was highly expressed in human osteosarcoma tissues, and its overexpression promoted proliferation and metastasis of osteosarcoma cells by activating PI3K/Akt pathway [17]. In esophageal squamous cell carcinoma, it was also shown that upregulation of MALAT1 contributed to enhanced cell proliferation and metastasis processes [16]. These reports seem to conflict with our findings in breast cancer, but can be explained by the cancer type or microenvironment differences. Also, in recent research, it had been reported that MALAT1 could regulate other targets in many different types of cancers, eg. EGFL7 in gastric cancer [23] and MCL-1 in bladder cancers [24]. We had done related experiments to check these known targets in our study (data not shown). However, comparing with the CCND1 targeting, we observed that other targets seemed insignificantly changed after MALAT1 was deleted in breast cancer cells. We thought it could be explained by the cancer type-dependent targeting.

In conclusion, we confirmed that MALAT1 was an anti-tumoral lncRNA that inhibited cell proliferation through

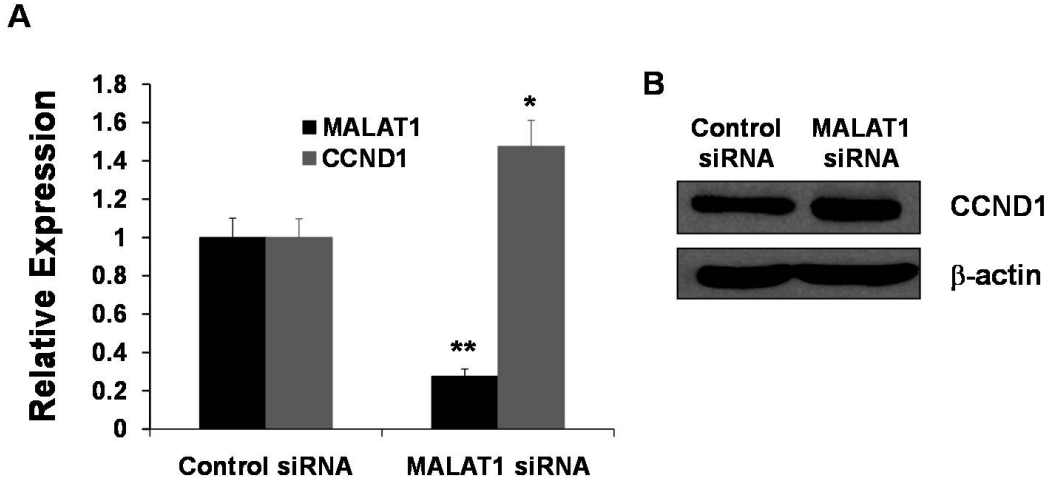

Figure 3 Loss of MALAT1 induced CCND1 expression in MCF7 cells. (A) qPCR assay was used to analysis of the efficiency of MALAT1 knock-down and the expression of ccnd1 gene in MCF7 cell lines. **(B)** Immunoblot assay of CCND1 in MCF7 cells following MALAT1 knockdown. b-actin was used as an internal control. Experiments were performed in triplicate (**P < 0.01 *vs*. control).

regulating CCND1 expression in breast cancer cells. Although the data is limited we still provided important clues to enrich its roles in breast cancer cells, which might further be utilized for therapeutic target development.

Conflict of interest: Authors declare nothing to disclose.

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