Long non-coding RNA BLACAT1 promotes cell proliferation, migration and invasion in cervical cancer through activation of Wnt/β-catenin signaling pathway


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Abstract. – OBJECTIVE: Long noncoding RNAs (lncRNAs) are aberrantly expressed in various cancers. The purpose of this study was to determine the association of lncRNA (BLACAT1) with the prognosis of cervical cancer (CC) patients, and to further investigate the potential mechanisms of BLACAT1 function in CC progression.

PATIENTS AND METHODS: The expressions of BLACAT1 in CC tissues and cells were estimated by quantitative Real-time polymerase chain reaction (qRT-PCR). We compared the expression of BLACAT1 with the clinicopathological characteristics and survival of CC patients. MTT, colony formation, and transwell assay were performed to explore the effects of BLACAT1 expression on growth, migration, and invasion of CC cells. Protein levels of β-catenin and MMP-7 were evaluated by Western blotting.

RESULTS: We found that BLACAT1 expression was significantly increased in CC tissues and cell lines. In addition, the expression level of BLACAT1 was positively correlated with distant metastasis (p<0.001), FIGO stage (p=0.010), and histological grade (p=0.012). Moreover, patients with high BLACAT1 expression had shorter overall survival and progression-free survival time than those with low BLACAT1 expression, with the data provided by multivariate analysis suggesting that BLACAT1 expression could serve as an independent prognostic factor in CC patients. Functionally, in vitro assay indicated that down-regulation of BLACAT1 significantly suppressed CC cells proliferation, migration, and invasion. Mechanistically, the results of Western blot showed that the expression of β-catenin and MMP-7 was significantly down-regulated in CC cells transfected with si-BLACAT1.

CONCLUSIONS: These findings suggested that BLACAT1, as a novel prognostic biomarker, might be an oncogenic lncRNA which promoted proliferation, migration, and invasion by modulating Wnt/β-catenin signaling. Our results enlarged our knowledge in the molecular pathology of CC tumorigenesis.

Key Words
Long noncoding RNA, BLACAT1, Proliferation, Metastasis, Prognosis, Wnt/β-catenin signaling.

Introduction
Cervical cancer (CC) is the second most common malignant tumor worldwide and is the leading type of gynecologic malignancies in China1,2. With the development of diagnostic methods, CC can be diagnosed in younger patients and at an early stage1. However, CC metastasis negatively impacts the survival of affected CC patients directly4. Up to date, the molecular mechanism underlying progression of CC remains unknown fully. Thus, understanding the potential mechanism of CC metastasis is important for improving treatment and prevention.

Long non-coding RNA (lncRNA) is a type of RNA molecule with a length of more than 200 bp and lack an open reading frame of significant length5. Recently, growing evidence indicates that lncRNAs are widely expressed in human cells and serve crucial roles in various biological events, such as cell proliferation, differentiation, and apoptosis6,7. More importantly, dysregulation of lncRNAs is closely associated with human diseases, including cancers8,9. For instance, lncRNA 19 function as a tumor promoter in several tumors, including gastric cancer, bladder cancer, and glioma10-12. LncRNA CASC2 served as a tumor suppressor in some cancers, including colorectal cancer and lung adenocarcinoma13,14. Although more and more lncRNAs have been identified, most of them remain largely uncharacterized and little is known about their potential mechanism in cancer progression.
BLACAT1 (also known as linc-UBC1), a newly identified lncRNA, was reported to be dysregulated in several tumors. Previous studies\textsuperscript{15-17} showed that BLACAT1 could act as an oncogene in different cancers, including uterine carcinoma, gastric cancer, and colorectal cancer. Shan et al\textsuperscript{18} reported that BLACAT1 promoted cell proliferation, invasion, and EMT process in human CC. However, the prognostic value of BLACAT1 in CC patients was not determined in their study. In the present study, we firstly detected the clinical significance of BLACAT1 and further explored the potential mechanism by which BLACAT1 promoted CC proliferation and metastasis.

**Patients and Methods**

**Patient Tissues**
133 cases of CC tissue specimens (tumor tissues with corresponding normal adjacent tissues) were obtained from the Department of Gynecology, Weifang People’s Hospital between July 2010 and December 2013. All cancers were confirmed as cervical cancer by the medical examination of hematoxylin and eosin. None of the patients had received chemotherapy or radiotherapy before surgery. The disease stages of all the patients were classified according to the International Federation of Gynecology and Obstetrics (FIGO) guidelines for clinical staging. The clinicopathological information of the patients is shown in Table I. The study was approved by the hospitals’ Ethics Committees and all patients signed a written consent form.

**Cell Culture and Transfection**
CC cell lines, HeLa, CaSki, SiHa, ME-180 and normal human cervical epithelial cell line (H8), were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA, USA) including 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C and 5% CO\textsubscript{2}.

Non-specific siRNA (si-NC) and BLACAT1 small interfering RNA (si-BLACAT1) were purchased from Invitrogen (Carlsbad, CA, USA). The sequences of si-BLACAT1 were 5’-CCAGTCGTTGTTCAGATGCTTT-3’. CaSki and SiHa cells cultured on the six-well plate were trans-

<table>
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<th>Number of patients</th>
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<tr>
<td></td>
<td></td>
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sected with si-BLACAT1 or si-NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNAs were isolated from fresh CC tissues and cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Performance of the qRT-PCR was carried out on ABI 7500 Fast Real-Time PCR system (Biosystems, Xuhui, Shanghai, China) with the use of SYBR Green PCR Kit (TaKaRa, Dalian, China). The expression of GAPDH was detected as the endogenous control. Relative gene expression was normalized to GAPDH using the 2-ΔΔCT method. The primer sequences were as follows: BLACAT1: 5'-CCTGCTTGGAAACTAATGACC-3' (forward), 5'-AGGCTCAACTTCCAGACTCA-3' (reverse). GAPDH: 5'-GTCAACGATTTGGTCTGTATT-3' (forward), 5'-AGTCTTCTGGGTGGCAGTGAT-3' (reverse).

Cell Viability Assay

Cells were seeded in 96-well plates at a density of 2 × 10^3 cells per well and incubated for 1, 2, 3, 4, and 5 days. After transfection for indicated days, the cells were incubated with 20 mL 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 4 h. Then, the absorbance at 570 nm was measured by a microplate reader.

Colony Formation Assay

Cells (15 × 10^4/well) were plated in a 6-well plate and transfected with si-NC or si-BLACAT1 and allowed to grow in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS) for 14 days. The colonies were stained with 1.0% crystal violet for 5 min after a 15-min fixation with 10% formaldehyde. Visible colonies were manually counted.

Transwell Migration/Invasion Assay

Transwell assay was used to evaluate cell migration and invasion ability. After transfection for 48 h, CaSki, and SiHa in serum-free media were put into the upper chamber, and 400 mL media containing 10% FBS was added to the lower chamber. For invasion assay, the upper chamber was pre-coated with Matrigel (Sigma-Aldrich, St. Louis, MO, USA). After 48 h of incubation at 37°C, cells on the upper surface of the filter were removed using a cotton swab. The cells that migrated or invaded to the lower surface were counted in every five high power fields under a microscope.

Western Blot

Protein was extracted from the cells at 48 h after transfection using a protein extraction reagent (Beyotime, Xuhui, Shanghai, China) and protein concentration was measured using the BCA Protein Assay kit (Beyotime, Rockford, IL, USA) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Waltham, MA, USA). Then, the blots were incubated with the primary antibodies for β-catenin, MMP-7, and GAPDH overnight at 4°C. Subsequently, horseradish peroxidase (HRP)-linked IgG was used as the secondary antibody. The signals were visualized using ECL reagents (Pierce, Rockford, IL, USA).

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA, USA) were used for statistical analysis. Differences between two groups of BLACAT1 were analyzed by two-tailed Student’s t-test. The association of BLACAT1 expression with EOC clinicopathologic features was assessed by the x 2-test. The survival curves of the patients were calculated using the Kaplan-Meier method, and data were analyzed with the log-rank test. The significance of survival variables was analyzed using the Cox multivariate proportional hazards model. p-values <0.05 were considered statistically significant.

Results

BLACAT1 Was Significantly Up-Regulated in CC Tissues and Cells Lines

The expression of BLACAT1 in CC tissues and matched normal cervical tissues was examined by RT-qPCR. As shown in Figure 1A, in 133 pairs of CC tissues, the expression of BLACAT1 was significantly up-regulated in CC tissues versus adjacent non-tumor tissues (p < 0.01). Subse-
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The expression levels of BLACAT1 were also determined in four CC cell lines (HeLa, CaSki, SiHa, and ME-180) and H8. As shown in Figure 1B, we observed that BLACAT1 expression in a panel of CC cell lines was upregulated as compared with an H8 cell line. The expression levels of BLACAT1 in CaSki and SiHa cell line were the highest, and thus, they were used for further analysis.

Association Between BLACAT1 Expression and the Clinical Features of CC

To explore the role of BLACAT1 in the clinical stage of CC patients, CC samples were classified into high expression group (n = 68) and low expression group (n = 65) in accordance with the median BLACAT1 expression level in CC tissues. The correlation between BLACAT1 expression and the clinical features of CC was shown in Table I; our results showed that aberrant BLACAT1 expression was significantly associated with distant metastasis (p = 0.001), FIGO stage (p = 0.010) and histological grade (p = 0.012). However, BLACAT1 expression was not found to be associated with age, HPV, tumor histology, tumor size, lymphatic invasion (All p > 0.05).

Correlation Between BLACAT1 Expression and Patients’ Survival

To further determine the prognostic value of the BLACAT1 expression in CC, we performed Kaplan-Meier analysis and log-rank test. It was observed that the overall (p = 0.0049; Figure 2A) and progression-free survival (p = 0.0014; Figure 2B) of CC patients with high BLACAT1 expression were both significantly shorter than those with low BLACAT1 expression. Furthermore, in multivariate Cox model, we found that BLACAT1 expression was an independent prognostic indicator for OS (HR = 3.932, 95% CI, 1.663-7.455; p = 0.001) and PFS (HR=4.263, 95% CI, 1.993-8.788; p = 0.001) in patients with CC (Table II).

Down-Regulation of BLACAT1 Suppressed CC Cells Proliferation and Metastasis In Vitro

Next, we explored the functional effects of BLACAT1 on CC cells. CaSki and SiHa cells were transfected with si-NC or si-BLACAT1. The down-regulation of BLACAT1 expression in CaSki and SiHa was confirmed by RT-PCR (Figure 2A). The MTT assay showed that knock-down of BLACAT1 significantly suppressed proliferation of CaSki and SiHa cells compared with normal controls (Figure 2B and 2C). Moreover, BLACAT1 inhibition reduced the colony formation capacity of CaSki and SiHa cells (Figure 3D). Furthermore, the results of transwell assay indicated the down-regulation of BLACAT1 reduced cell migration and invasion in both CaSki and SiHa cell lines (Figure 3E and 3F). Taken together, BLACAT1 served as a tumor promoter in CC by suppressing the proliferation, migration, and invasion of CC.
Down-regulation of BLACAT1 Suppressed the Activation of Wnt/β-catenin Signaling Pathway

To elucidate the mechanism via which BLACAT1 promoted the proliferation and metastasis of CC cells, our attention focused on Wnt/β-catenin signaling pathway which has been confirmed to be involved in tumorigenesis and progression. Then, we detected the expression of β-catenin, and MMP-7, a major target gene of Wnt/β-catenin pathway related to migration and invasion in CaSki and SiHa transfected with si-BLACAT1. We found that suppression of BLACAT1 significantly inhibited the expression of β-catenin and MMP-7 (Figure 4A and 4B). Our data strongly supported that BLACAT1 contributes CC metastasis and it may be mediated through Wnt/β-catenin signaling pathway in CC patients.

Table II. Multivariable Cox’s regression analysis of prognostic factors for CC patients.

<table>
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<th>Variables</th>
<th>Overall survival</th>
<th>Progression-free survival</th>
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<tr>
<td></td>
<td>HR</td>
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<td>Age</td>
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<td>HPV</td>
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<td>Tumor histology</td>
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<td>Tumor Size</td>
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<td>0.774-2.564</td>
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<td>Lymphatic invasion</td>
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<tr>
<td>Distant metastasis</td>
<td>3.675</td>
<td>1.542-6.732</td>
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<td>FIGO stage</td>
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<td>1.287-5.583</td>
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<tr>
<td>Histological grade</td>
<td>2.894</td>
<td>1.442-4.392</td>
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<tr>
<td>BLACAT1 expression</td>
<td>3.932</td>
<td>1.663-7.455</td>
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</table>

Discussion

CC remains a problem in China due to poor prognosis for metastatic CC. Molecular biomarkers that could predicate the prognosis of CC patients is very important for determining an individualized treatment plan. However, current biomarkers are not satisfactory, and investigating ideal biomarkers is of great value\textsuperscript{19}. Recently, several studies\textsuperscript{20,21} indicated that lncRNAs may provide new insight for diagnostic and prognostic biomarkers of CC.

In the present work, we detected the expression of BLACAT1 in CC tissues and cell lines. Our results were in line with previous results. We also found that BLACAT1 expression was significantly up-regulated in both CC tissues and cell lines compared with normal cervical tissues and cervical cell lines. Then, we firstly analyzed the association between BLACAT1 and clini-
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We observed that high BLACAT1 expression level was significantly associated with advanced FIGO stage, distant metastasis, and poorer histological grade. In addition, our data suggested that patients with high BLACAT1 expression showed shorter OS and PFS than those with low BLACAT1 expression. Further multivariate survival analysis showed that BLACAT1 could be used as a potential prognostic biomarker for CC. In 2017, Gao et al. also found that overexpression of BLACAT1 was associated with poor prognosis of colorectal cancer patients. Hu et al. also showed that up-regulation of BLACAT1 correlates with lymph node metastasis, TNM stage, and poorer prognosis of patients with gastric cancer. Taken together, our observation in the current work is in agreement with previous studies revealing that BLACAT1 might serve as a promising biomarker for prognosis of tumors, including CC.

To further explore the biological function of BLACAT1 in CC, we performed the loss-function assay. Si-BLACAT1 was used to decrease the expression of BLACAT1. MTT and transwell assays indicated that knockdown of BLACAT1 significantly suppressed CC cells proliferation, migration, and invasion. Shan et al. also reported that the depletion of BLACAT1 suppressed cell proliferation and metastasis in ME180 and C33A cells. The cells used in our research are CaSki and SiHa cell lines.

![Figure 3](image-url)

**Figure 3.** Downregulation of BLACAT1 suppressed CC cell proliferation, migration and invasion. A, BLACAT1 was knocked-down in CaSki and SiHa cells by transfection of si-BLACAT1. Forty-eight hours later, BLACAT1 expression levels were tested by quantitative RT-PCR and normalized to GADPH. A-C, MTT assay showed the decreased proliferation of CaSki and SiHa cells by transfecting with si-BLACAT1. D, Colony formation capacity of CaSki and SiHa cells with manipulated BLACAT1 expressions. E, Transwell assay was used to evaluate the migration abilities of CaSki and SiHa cells transfected with si-BLACAT1 or si-NC. F, Transwell assay was used to evaluate the invasion abilities of CaSki and SiHa cells transfected with si-BLACAT1 or si-NC. **p < 0.01, *p < 0.05.
SiHa. Our data were in line with those results. In addition, previous researches also suggested BLACAT1 as a tumor promoter by promoting cells proliferation and metastasis in non-small cell lung cancer and bladder cancer. These findings, together with ours, indicated that BLACAT1 contributed to the progression of tumors.

The Wnt signaling pathways are a group of signal transduction pathways which involves many different proteins that are needed for cell proliferation and differentiation in various tissues. Previous studies have shown that Wnt/β-catenin pathway can regulate cells proliferation, migration, and invasion in certain types of cancers. Moreover, activation of Wnt/β-catenin pathway contributed to development and progression of various tumors, and several lncRNAs have been confirmed to be a regulator in Wnt/β-catenin pathway. Given the role of the Wnt/β-catenin pathway in carcinogenesis, we further explored whether BLACAT1 exerted its oncogenic by modulating Wnt/β-catenin pathway. In this investigation, we found that downregulated BLACAT1 contributed to a decreased expression of β-catenin, Furthermore, The MMP-7 expression, which is a major target gene of Wnt/β-catenin pathway, also exhibited a significantly down-regulated in CC cells transfected with si-BLACAT1. Taken together, we suggested BLACAT1 as a tumor promoter by modulating Wnt/β-catenin pathway.

Conclusions

The present report is the first to show that BLACAT1 might be a potential novel prognostic biomarker for CC. In addition, our findings suggested that BLACAT1 acts as a tumor promoter by modulating Wnt/β-catenin signaling pathway to regulate the proliferation and metastasis during CC development. However, more in-depth mechanistic studies are needed to further understand the mechanism of BLACAT1.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

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