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BLACAT1 is negatively associated with prognosis in patients with NSCLC and inhibits cell progression, metastasis and epithelial-mesenchymal transition through down-regulating Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: To evaluate the clinical significance and molecular mechanism of bladder cancer-associated transcript 1 (BLACAT1) in non-small-cell lung cancer (NSCLC).

PATIENTS AND METHODS: Overall, 156 NSCLC cancer patients were recruited and divided into high and low BLACAT1 level group by the median value of BLACAT1 expression. The associations of BLACAT1 expression with the clinicopathological features and prognosis were evaluated. A series of *in vitro* assays were performed to explore the role of BLACAT1 on NSCLC progression and metastasis.

RESULTS: Patients with high BLACAT1 expression had shorter overall survival and progression-free survival than those with low BLACAT1 expression. Multivariate analyses showed that BLACAT1 was an independent prognostic factor of survival in NSCLC patients. *In vitro* assays showed that the downregulation of BLACAT1 significantly suppressed cell progression, migration, and invasion. The epithelial-mesenchymal transition was also inhibited when BLACAT1 was silenced, indicated by an increase in E-cadherin expression and a decrease in vimentin expression by mediating Wnt/β-catenin signaling pathway.

CONCLUSIONS: BLACAT1 should be a potential prognostic biomarker and therapeutic target for NSCLC.

Key Words:
BLACAT1, NSCLC, Survival, Wnt/β-catenin.

Introduction

Lung cancer is the most frequently diagnosed malignancy and the leading cause of cancer-related death worldwide[1]. It is estimated that 1.8 million new cases were diagnosed in 2012, 58% of which occurred in less developed countries. The disease took the lives of approximately 1.59 million people globally, accounting for 19.4% of cancer caused deaths per year. According to the Global Burden of Disease study 2020[2], the healthcare burden and costs attributed to lung cancer was substantial. Despite treatment with multiple and newly developed therapeutic agents, the 5-year survival rate (17.8%) of lung cancer was still much lower than that of other leading cancers[3]. Effective biomarkers for individualized prediction and treatment of NSCLC are urgently warranted.

Long non-coding RNAs (lncRNAs) are RNA genomically transcribed noncoding transcripts longer than 200 nucleotides[4,5]. They have many functions in various pathophysiological processes, including cell differentiation, development, and carcinogenesis[5-8]. The lncRNAs circulating in serum/plasma are relatively stable because they are not degraded by RNase even in the complex environment *in vivo*[9]. Thus, they could act as potential diagnostic or prognostic markers in multiple types of cancer[10,11]. Bladder cancer-associated transcript 1 (BLACAT1) is a novel identified lncRNA that was firstly found in bladder cancer[12]. Recently, Chen et al[13] showed that BLACAT1 was associated with the malignant status and prognosis in patients with small-cell lung cancer (SCLC), and functioned as an oncogenic lncRNA in regulating cell proliferation and motility. Though the overexpression of BLACAT1 was reported to be associated with a poorer prognosis in patients

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with non-small-cell lung cancer (NSCLC)[14], their relationship needs to be further observed due to the limited sample size (totally 48 cases). Moreover, the functional role of BLACAT1 on the progression and metastasis of NSCLC cells should be explored.

The objectives of the present study were to evaluate the association of BLACAT1 with the clinicopathological parameters and prognosis in NSCLC patients following chemotherapy. The molecular mechanism of BLACAT1 in tumor progression, migration, and invasion were further investigated *in vitro*.

Patients and Methods

Tissue Specimens

Between January 2012 and June 2013, 156 fresh surgical NSCLC tumor tissue samples were collected at the Heze Municipal Hospital. All samples were snap-frozen in liquid nitrogen and stored at -80°C until analysis. All patients were Han Chinese and diagnosed pathologically or cytologically. None of the patients received preoperative chemotherapy, radiotherapy, or hormonal therapy at the time of the original biopsy. Patients with primary liver and kidney dysfunctions, hypertension, cardiovascular, cerebrovascular diseases, or other malignancies were excluded. The present study was approved by the Ethics Committee of the Heze Municipal Hospital. All procedures involving human participants were performed in accordance with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants.

The clinical and pathological data pertaining to the patients with NSCLC were retrieved from the patient charts and original pathology reports. The pathological diagnosis of NSCLC was established in accordance with the revised World Health Organization classification of lung tumors[15] and staged relying on the tumor, node, and metastasis (TNM)-7 staging system[16]. The response to treatment after 2 cycles of therapy was determined according to revised RECIST criteria version 1.1[17]. The patients who achieved a complete response (CR) or partial response (PR) were considered responders while those with stable disease (SD) or progressive disease (PD) were considered non-responders. The follow-up data were obtained by reviewing out-patient charts or by contacting patients. Overall survival (OS) was defined as the amount of time between the date of primary surgery and the date of death or the end of follow-up. The progression-free survival (PFS) was defined as the amount of time between the date of the primary surgery and the date of disease recurrence or progression or the end of follow-up.

Cell Lines and Transfection

The NSCLC cell lines (A549 and SK-MES-1) were maintained in RPMI-1640 medium (Gibco BRL, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin sulfate) and were cultured in a humidified 5% CO_2 incubator at 37°C.

A549 and SK-MES-1 cells were transfected with either 50 nM siRNA targeting BLACAT1 (si-BLACAT1) or scrambled negative control (si-NC) using Lipofectamine 2000 reagent (Invitrogen, Shanghai, China) according to the manufacturer's protocol. After 48 h, the efficiency of BLACAT1 knockdown was confirmed via quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RNA Extraction and qRT-PRC

The total RNA was isolated from cancerous specimens or cell lines using TRIzol Reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. BLACAT1 cDNA was generated using the Reverse Transcription System Kit. qRT-PCR reactions were performed using an ABI7500 System and SYBR Green PCR Master Mix (TaKaRa, Dalian, China). The primer sequences for BLACAT1 were 5'-CCTGCTTGGAAACTAATGACC-3' (forward) and 5'-AGGCTCAACTTCCCAGACTCA-3' (reverse). Each assay was performed in triplicate, and the average was calculated. The BLACAT1 expression levels were normalized to GAPDH.

MTT Assay

The cell proliferation was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetra-zoliumbromide (MTT) assay. The A549 and SK-MES-1 cells were seeded in 96-well plates at a density of 5×10^3 cells/well and allowed to attach overnight. After 24, 48, 72, and 96 h of incubation, 20 μl/well MTT (Sigma-Aldrich, St. Louis, MO, SA) were added to each well. The cells were incubated for an additional 4 h period, followed by an addition of 100 μL of solubilization solution (10% SDS in 0.01 M HCl). The plates stayed overnight at 37°C, and the spectrophotometric

absorbance of the samples was measured using a microplate (ELISA) reader at a wavelength of 570 nm. Each assay was performed in triplicates.

Transwell Invasion Assay

Invasion of A549 and SK-MES-1 cells was measured in Matrigel (BD, Franklin Lakes, NJ, USA)-coated Transwell inserts (Costar, Manassas, VA, USA) containing polycarbonate filters with 8 μm pores. The inserts were coated with 50 μl of 1 mg/ml Matrigel matrix according to the manufacturer's recommendations. 1×10^5 cells in 200 μl of serum-free medium were plated in the upper chamber, whereas 600 μl of medium with 10% fetal bovine serum were added to lower well. After incubation for 48 h at 37°C with 5% CO_2, the cells that did not penetrate through the membrane were removed with a cotton swab, while the invading cells were fixed with 0.1% crystal violet.

Wound Healing Assay

Each cell line (A549 and SK-MES-1) was plated into 6-well plates and cultured to confluence. The cells were rinsed with PBS and serum starved overnight in 0.5% FBS media at 37°C and 5% CO_2. Next day, the separate scratches were introduced through the monolayer of cells in each of the wells using sterile 200 μL plastic pipette tips. The cells were then gently rinsed with PBS to remove cellular debris and were replaced with fresh culture media supplemented with 0.5% FBS. The wounded cells were incubated at 37°C, and representative fields were photographed with an inverted-phase microscope at different time intervals.

Western Blot Assays

A549 cells were lysed with RIPA buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate) supplemented with protease inhibitors (Roche, Basel, Switzerland); 20-30 μg samples of the lysates were separated on 8%-12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies overnight at 4°C. The primary antibody incubation was followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The bound antibodies were detected using an enhanced chemiluminescence (ECL) kit (ComWin Biotech Co., Beijing, China). The primary antibodies were as follows: E-cadherin (cell signaling, Danvers, MA, USA), vimentin (cell signaling, Danvers, MA, USA), β-catenin (Abcam, Cambridge, UK), c-myc (Abcam, Cambridge, UK), cyclinD1 (Abcam, Cambridge, UK) and GAPDH (Millipore, Billerica, MA, USA).

Statistical Analysis

All quantitative data were expressed as mean ± standard deviation (SD) and compared by the Student's *t*-test or One-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc tests. The Chi-square test or Fisher's exact test were used to compare the categorical variables. The log-rank test was used as the primary test of an overall difference between the Kaplan-Meier curves for both OS and PFS. Cox's proportional hazard model was used to perform multivariate analysis. All tests were 2-sided, and a *p*-value of less than 0.05 was considered significant.

All statistical analyses were performed with the Statistical Product and Service Solution (SPSS) statistical software program package (SPSS version 20.0 for Windows, SPSS Inc., Chicago, IL, USA).

Results

BLACAT1 Expression Was Associated with Clinicopathological Features in Patients with NSCLC

The associations between the clinicopathological features and BLACAT1 expression in NSCLC tissues were described in Table I. The median value of BLACAT1 expression levels in tissues was chosen as a cutoff value and used to assign the 156 patients with NSCLC to the high (n=78) or the low BLACAT1 expression group (n=78). It is shown that the BLACAT1 expression was significantly associated with the TNM stage (p=0.006) and response to chemotherapy (p=0.016). There is no significant difference in age, gender distribution, smoking status, and histology between patients with high and low BLACAT1 expression (all p>0.05).

BLACAT1 Expression was Associated with Overall Survival and Progression-Free Survival

Univariate analyses showed that in elderly age (above 60 years), advanced TNM stages, non-responsive to chemotherapy, and high BLACAT1 level were negatively associate with OS (all p<0.001; Table II); while patients with advanced TNM stages non-responsive to chemotherapy or high BLACAT1 level had shorter PFS (all p<0.001; Table III). The survival analysis using

Table I. Baseline characteristics of patients with non-small cell lung cancer according to BLACAT1 expression levels.

		BLACAT1 expression		
	NSCLC patients	Low	High	*p*-value
No.	156	78	78	-
Age (years)				
<60	74 (47.4%)	42 (26.9%)	32 (20.5%)	0.149
≥60	82 (52.6%)	36 (23.1%)	46 (29.5%)	
Gender				
Male	114 (73.1%)	62 (39.7%)	52 (33.3%)	0.104
Female	42 (26.9%)	16 (10.3%)	26 (16.7%)	
Smoking status				
Smoking	98 (62.8%)	48 (30.8%)	50 (32.1%)	0.869
Non-smoking	58 (37.2%)	30 (19.2%)	28 (17.9%)	
Histology				
Adeno	66 (42.3%)	31 (19.9%)	35 (22.4%)	0.808
Squamous	59 (37.8%)	31 (19.9%)	28 (17.9%)	
Others (unclassified)	31 (19.9%)	16 (10.3%)	15 (9.6%)	
TNM stage				
I-IIIA	41 (26.3%)	28 (17.9%)	13 (8.3%)	0.006
IIIB-IV	115 (73.7%)	50 (32.1%)	65 (41.7%)	
Response to chemotherapy				
Responsive (PR and CR)	75 (48.1%)	45 (28.8%)	30 (19.2%)	0.016
Non-responsive (SD and PD)	81 (51.9%)	33 (21.2%)	48 (30.8%)	

Notes: CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

Table II. Univariate analysis and multivariate analysis for overall survival of all patients with non-small cell lung cancer (NSCLC).

	Univariate analyses			Multivariate analyses		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age, year (<60 vs. ≥60)	1.374	1.087-1.726	<0.01	1.221	0.853-1.407	0.126
Gender (male vs. female)	1.284	0.785-1.621	0.238			
Smoking status (yes vs. no)	0.893	0.634-1.187	0.375			
Histology (adeno vs. squamous)	1.267	0.754-1.593	0.349			
TNM stage (I-IIIA vs. IIIB-IV)	1.614	1.205-2.147	<0.001	1.375	0.905-1.788	0.098
Response to chemotherapy (responsive vs. non-responsive)	1.587	1.105-1.946	<0.001	1.275	0.842-1.607	0.205
BLACAT1 expression (low vs. high)	2.135	1.653-2.572	<0.001	2.076	1.586-2.476	<0.001

Table III. Univariate analysis and multivariate analysis for progression-free survival of all patients with non-small cell lung cancer (NSCLC).

	Univariate analyses			Multivariate analyses		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age, year (<60 *vs.* ≥60)	1.274	0.798-1.452	0.563			
Gender (male *vs.* female)	1.164	0.915-1.398	0.357			
Smoking status (yes *vs.* no)	0.954	0.874-1.156	0.744			
Histology (adeno *vs.* squamous)	1.202	0.946-1.532	0.473			
TNM stage (I-IIIA *vs.* IIIB-IV)	1.462	1.099-1.832	<0.001	1.475	1.255-1.621	<0.001
Response to chemotherapy (responsive vs. non-responsive)	1.518	1.312-1.844	<0.001	1.472	1.187-1.764	<0.001
BLACAT1 expression (low *vs.* high)	1.985	1.552-2.538	<0.001	2.365	1.754-2.966	<0.001

the Kaplan-Meier method revealed that the patients with high BLACAT1 level had significantly shorter OS (14 months *vs.* 21 months; HR=1.561, 95% CI: 1.073 to 2.272, p=0.020; Figure 1A) and PFS (7 months *vs.* 9 months; HR=1.492, 95% CI: 1.036 to 2.147, p=0.031; Figure 1B) than those with low BLACAT1 level.

The multivariate analysis revealed that the TNM stage and response to chemotherapy were independent factors for PFS (both p<0.001; Table III) but not for OS (both p>0.05; Table II). It is shown that low BLACAT1 level was significantly associated with longer OS (HR=2.076, 95% CI: 1.586-2.476, p=0.020; Table II) and PFS (HR=2.365, 95% CI: 1.754-2.966, p=0.031; Table III).

BLACAT1 Silencing Inhibited NSCLC Cell Proliferation, Migration, and Invasion

To explore the functional role of BLACAT1 on NSCLC tumorigenesis, A549 and SK-MES-1 cells were transfected with si-BLACAT1 or si-NC. Forty-eight hours after transfection, qRT-PCR was performed to assess the BLACAT1 expression. As shown in Figures 2A and 2B, the BLACAT1 expression significantly decreased in both A549 and SK-MES-1 cells transfected with si-BLACAT1 (p<0.001). The cell viability was assessed by MTT assay. As shown in Figure 2C and 2D, A549 and SK-MES-1 cells transfected with si-BLACAT1 resulted in significantly decreased viability compared with the si-NC group (p<0.001). The wound healing assay demonstrated that the knockdown of BLACAT1 significantly suppressed cell migration in A549 (Figure 2E) and SK-MES-1 (Figure 2F) cells (p<0.001). The Matrigel invasion assay indicated that the suppression of BLACAT1 reduced the invasive ability of A549 (Figure 2G) and SK-MES-1 (Figure 2H) cells (p<0.001). These findings demonstrated that BLACAT1 silencing could inhibit cell proliferation, migration, and invasion *in vitro*.

BLACAT1 Silencing Inhibited EMT and Wnt/β-Catenin Signaling Pathway

Epithelial-mesenchymal transition (EMT) is associated with tumorigenesis. To further explore the influence of BLACAT1 silencing on the EMT, the expression levels of two widely accepted biomarkers for the epithelial phenotype (E-cadherin) and mesenchymal phenotype (vimentin) were measured. It is shown that BLACAT1 knockdown significantly increased the E-cadherin expression and decreased vimentin expression (Figure 3A).

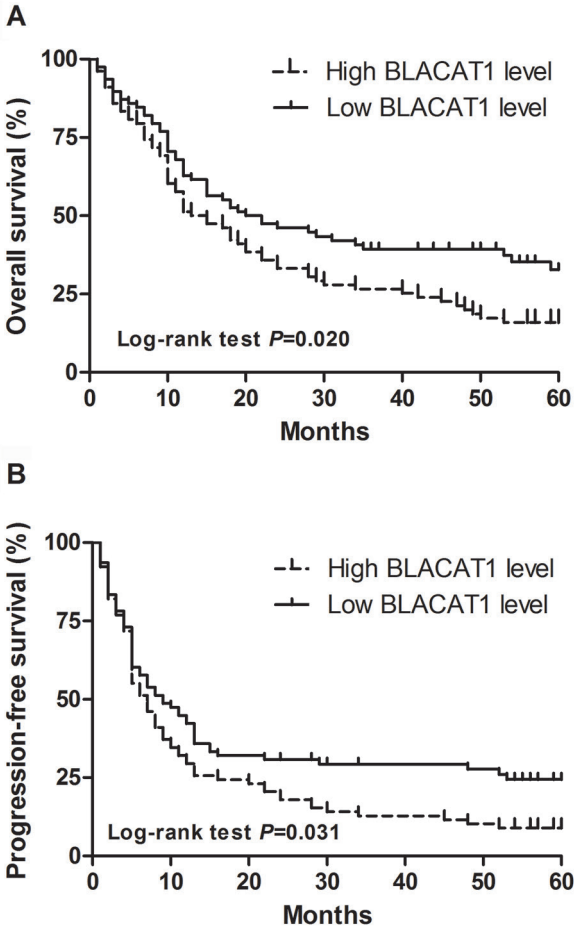

Figure 1. Kaplan-Meier survival curves for NSCLC according to BLACAT1 expression level. Both overall survival (**A**) and disease-free survival (**B**) of NSCLC patients with low BLACAT1 expression were significantly longer compared with those with high BLACAT1 level (p<0.05).

Due to the critical role of Wnt/β-catenin pathway on the tumorigenesis and EMT, the expression of the several key members in Wnt/β-catenin pathway was investigated, including β-catenin, and its two downstream signaling molecules (c-myc and cyclinD1). The Western blot results showed that the suppression of BLACAT1 significantly inhibited the expression of β-catenin, c-myc, and cyclinD1 (Figure 3B).

Discussion

The present study showed that patients with high BLACAT1 expression were associated with shorter OS and PFS. The multivariate analysis using the Cox proportional hazard model showed that BLACAT1 was an inde-

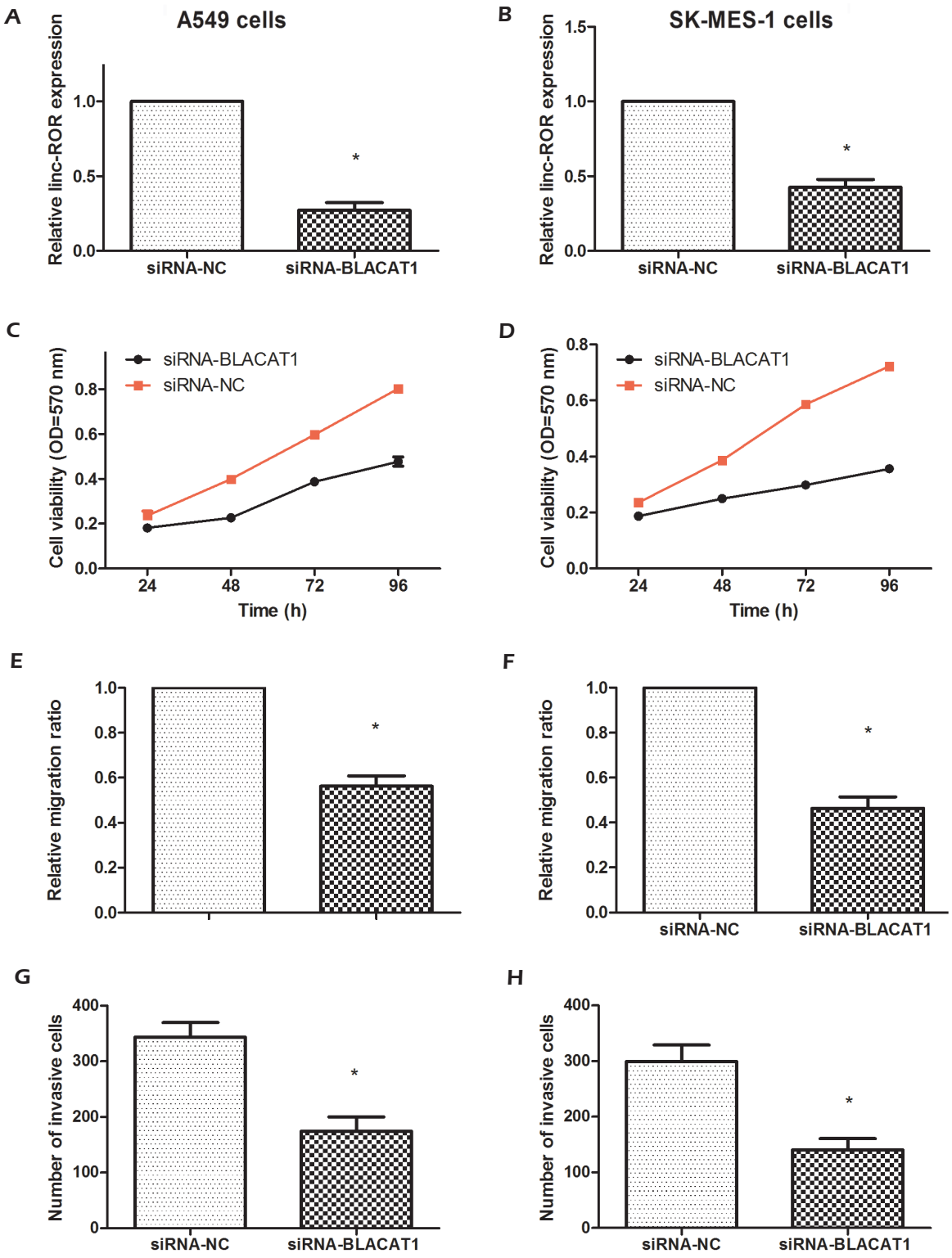

Figure 2. Knockdown of the BLACAT1 inhibits proliferation, migration, and invasion in A549 and SK-MES-1 cells. qRT-PCR revealed that BLACAT1 was efficiently knocked down by treatment with si-BLACAT1 in A549 (**A**) and SK-MES-1cells (**B**). A549 (**C**) and SK-MES-1 (**D**) cells transfected with si-BLACAT1 displayed significantly lower proliferation ability compared with those transfected with si-NC. A549 (**E**) and SK-MES-1 (**F**) cells transfected with si-BLACAT1 showed markedly lower migration ability compared with those transfected with si-NC. A549 (**G**) and SK-MES-1 (**H**) cells transfected with si-BLACAT1 displayed significantly lower invasion ability compared with those transfected with si-NC. *p<0.05.

pendent predictor of survival in patients with NSCLC. The suppression of BLACAT1 inhibited cell proliferation, migration, and invasion, which may be mediated through the Wnt/β-catenin pathway.

The dysregulation of BLACAT1 has been found to be associated with tumorigenesis and tumor progression in various cancers. It is indicated that the high BLACAT1 expression was associated with shorter OS and PFS in gastric cancer[18], bladder cancer[12], and cervical cancer[19]. Recently, the prognostic value of BLACAT1 has been investigated in lung cancers. Chen et al[13] suggested that BLACAT1 may be regarded as a potential target for SCLC prevention and treatment. Ye et al[14] revealed that patients with high BLACAT1 expression had lower OS and poorer prognosis than those with low BLACAT1 expression. However, their sample size is limited (48 cases), and the confounding factors were not controlled in the survival analysis. Our study, based on the Kaplan-Meier method and multivariate Cox's proportional hazard model, showed that BLACAT1 may serve as a prognostic factor in NSCLC patients.

Our subsequent *in vitro* assays, including the wound healing and Matrigel invasion assays, revealed that BLACAT1 silencing inhibited the ability of both A549 and SK-MES-1 cells to migrate and invade. Previous studies also demonstrated that BLACAT1 should be a tumor promoter by promoting cells proliferation and metastasis in cervical cancer[19], bladder cancer[12], SCLC[13], and NSCLC[14]. All these findings indicated that BLACAT1 contributed to the progression of tumors.

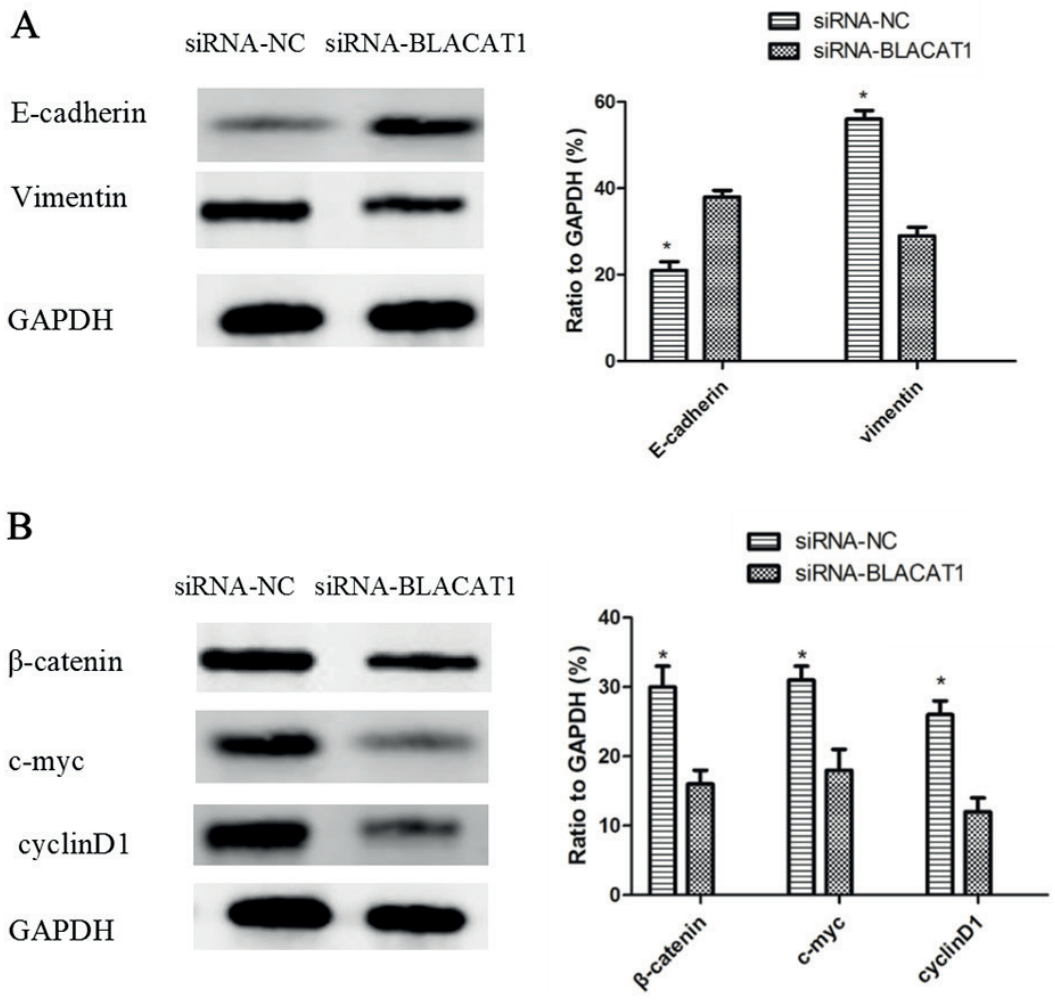

Figure 3. BLACAT1 silencing inhibits EMT and Wnt/β-catenin signaling pathway in A549 cells. (**A**) E-cadherin expression significantly increased, and vimentin decreased in the BLACAT1 knockdown group compared with controls. (**B**) When BLACAT1 was silenced, β-catenin, c-myc, and cyclinD1 significantly decreased. *$p<0.05$.

EMT is a hallmark of distant tumor metastasis[20]. It is involved in cell motility and invasiveness in cancer progression, and the suppression of EMT could significantly inhibit metastasis and growth of NSCLC. EMT is a conserved cellular process in which epithelial tumor cell lacks its polarity and transforms itself into a mesenchymal phenotype. The occurrence of EMT is characterized by the decreased epithelial marker E-cadherin and increased mesenchymal marker (e.g., vimentin). Our Western blot analysis revealed that BLACAT1 silencing inhibited EMT.

Previous studies[21,22] have shown that the activation of Wnt/β-catenin signaling pathway can regulate cells proliferation, migration, and invasion by inducing EMT. Activation of the Wnt/β-catenin pathway contributed to the tumor development and progression. Several lncRNAs have been demonstrated to be regulators in Wnt/β-catenin pathway[23,24]. Given the role of the Wnt/β-catenin pathway on carcinogenesis, we further explored whether BLACAT1 exerted its oncogenic by modulating Wnt/β-catenin pathway. Our results showed that β-catenin expression was down-regulated when BLACAT1 was silenced, together with decreased expression in downstream signaling factors, including cmyc and cyclinD1.

Conclusions

We indicated that the overexpression of BLACAT1 was found to be associated with a poor prognosis in NSCLC patients. The knockdown of BLACAT1 inhibited proliferation, migration, and invasion in NSCLC cells. Furthermore, we detected that BLACAT1 may act as a potential oncogene in NSCLC by mediating Wnt/β-catenin pathway. These findings suggest that BLACAT1 might function as an oncogene in NSCLC and could be served as a potential prognostic marker and therapeutic target for NSCLC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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