

Original Article

Overexpressed lncRNA ZEB1-AS1 promotes cell invasion and angiogenesis through Wnt/ β -catenin signaling in non-small cell lung cancer

Li Li¹, Qing-Feng Wang², Ming-Lei Zou¹, Xin-Ai He¹, Jing-Jing Lv¹

Departments of ¹Medical Oncology I, ²Neurosurgery I, The First Affiliated Hospital of Henan Polytechnic University, The Second People's Hospital of Jiaozuo, Jiaozuo 454000, Henan China

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Abstract: Background: Increasing evidences showed that lncRNAs were aberrantly expressed and acted as key regulators in tumor progression. LncRNA ZEB1-AS1 has been found to be upregulated and acted as an oncogene in several cancers. However, the expression and detailed function of ZEB1-AS1 in non-small cell lung cancer (NSCLC) were still unclear. Methods: In the present study, the expression of ZEB1-AS1 in NSCLC tissues and cell lines were explored by quantitative real-time PCR (qRT-PCR), and its association with prognosis of NSCLC patients was analyzed by statistical analysis. In vitro assays were used to explore the function of ZEB1-AS1 on NSCLC cell. Furthermore, western blot was used to determine the effect of ZEB1-AS1 on Wnt/ β -catenin signaling pathway. Results: We found that ZEB1-AS1 was upregulated in NSCLC tissues and correlated with tumor stage, lymph node metastasis and poor overall survival of patients with NSCLC. In vitro assays, we showed that ZEB1-AS1 inhibition suppressed invasion and angiogenesis of NSCLC cells. In addition, western blot revealed that decreased expression of ZEB1-AS1 significantly inhibited the activity of Wnt/ β -catenin signaling pathway. Conclusion: Our study suggested the important roles and underlying molecular mechanisms of ZEB1-AS1 on NSCLC progression, and indicated that ZEB1-AS1 could be a potential therapeutic target for the treatment of NSCLC.

Keywords: lncRNA ZEB1-AS1, non-small cell lung cancer, progression, Wnt/ β -catenin

Introduction

Lung cancer is the second common malignant tumors and the leading cause of cancer death worldwide [1]. Non-small cell lung cancer (NSCLC) is the most common form of lung cancer, which accounts for approximately 85% of all lung cancer cases [2]. Although advances in surgical techniques, radiotherapy and chemotherapy have been made in the treatment of lung cancer over the past decades, the prognosis of patients with lung cancer remains poor [3]. The main reason of lung cancer-related deaths due to many patients were diagnosed at an advanced stage with extensive invasion and lymphatic metastasis [4]. Previous studies have found many genes closely associated with lung cancer progression, but the underlying complex molecular mechanisms were still obscure. Therefore, a better understanding of the molecular mechanisms underlying lung cancer will

improve the diagnosis and therapy of patients with lung cancer.

Long non-coding RNAs (lncRNAs) are a class of single stranded RNA molecules longer than 200 nucleotides without protein-coding capacity [5]. Increasing evidences demonstrated that lncRNAs played critical roles in multiple biological processes, including development, differentiation and carcinogenesis [6, 7]. Recent evidence showed that many lncRNAs were heavily involved in tumor oncogenic or suppressive pathways. For example, Zhao et al showed that upregulation of lncRNA HNF1A-AS1 promoted cell proliferation and metastasis in osteosarcoma through activation of the Wnt/ β -catenin signaling pathway [8]. Chen et al found that lncRNA CRNDE promoted hepatic carcinoma cell proliferation, migration and invasion by suppressing miR-384 [9]. Guo et al found that lncRNA MEG3 inhibited cell proliferation of endometrial carci-

noma by repressing Notch signaling [10]. Xue et al showed that lncRNA GAS5 inhibited proliferation and progression of prostate cancer by targeting miR-103 through AKT/mTOR signaling pathway [11].

LncRNA ZEB1 Antisense 1 (ZEB1-AS1) was first reported to be increased in hepatocellular carcinoma, promoted hepatocellular carcinoma metastasis and predicted poor prognosis of hepatocellular carcinoma patients [12]. ZEB1-AS1 orients in antisense direction with respect to ZEB1, a critical transcription factor functioning in many tumors. For example, Wang et al showed that ZEB1-AS1 was associated with tumor progression and could be an independent prognostic factor for esophageal squamous cell carcinoma patients [13]. Liu et al showed that ZEB1-AS1 was upregulated in osteosarcoma tissues and promoted osteosarcoma cells proliferation and migration via epigenetically activating ZEB1 [14]. However, its clinical significance to NSCLC and biological functions remains unclear.

In the present study, we firstly determined the expression of lncRNA ZEB1-AS1 was significantly upregulated in NSCLC tissues compared to adjacent non-tumor tissues. Correlation between ZEB1-AS1 expression and clinicopathological features of NSCLC patients was then evaluated. Furthermore, inhibition of ZEB1-AS1 significantly suppressed cell invasion and angiogenesis. In addition, western blot showed that ZEB1-AS1 inhibition suppressed the activity of Wnt/ β -catenin signaling pathway. Our data provided new insights into the role of ZEB1-AS1 in the progression of NSCLC.

Materials and methods

Patient samples

A total of 68 NSCLC tissues and adjacent non-tumor tissues were obtained from NSCLC patients receiving surgical treatment at The First Affiliated Hospital of Henan Polytechnic University, The Second People's Hospital of Jiaozuo between January 2011 and February 2012. None of the patients received preoperative therapy prior to surgical resection. The histologic diagnosis of tumors was made by at least two experienced pathologists based on World Health Organization (WHO) criteria. All samples were immediately frozen soon after resection in liquid nitrogen and stored at -80°C

until RNA extraction. The study was approved by the Research Ethics Committee of The First Affiliated Hospital of Henan Polytechnic University, The Second People's Hospital of Jiaozuo. Written informed consent was obtained from all of the patients. The clinicopathological information of the patients is shown in **Table 1**.

Cell culture and transfection

Four NSCLC cell lines (NCI-H1650, A549, NCI-H1975, SPC-A1) and a normal human bronchial epithelial cell line (16HBE) were purchased from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2 .

The siRNA specifically targeting ZEB1-AS1 (si-ZEB1-AS1) was commercially constructed by Shanghai GenePharma. The scrambled nucleotide was used as the negative control (si-NC). Cells were transfected with siRNAs using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Each experiment was performed in triplicate. The interfering efficiency was determined by qRT-PCR after transfection 48 h.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNAs of cells or tissue samples were isolated using TRIzol (Invitrogen) according to the manufacturers' instructions. First strand cDNA was generated using the Reverse Transcriptase (Transgene). QRT-PCR was performed in the ABI 7500 Real-Time PCR System using SYBR Green Mixture (Takara). GAPDH as an internal control was used to normalize the data to determine the relative abundance of the target RNA. Primers sequences for the target genes were as follows: ZEB1-AS1 forward 5'-AACCTTGTTGCTAGGGACCG-3' and reverse 5'-AGTCACTCCATCCCGGT-3'; GAPDH forward 5'-CCCATCACCATCTTCCAGGAG-3' and reverse 5'-GTTGCATGGATGACCTTGGC-3'.

Cell proliferation assay

Cell proliferation was assayed using a cell proliferation kit, Cell Counting Kit-8 (CCK-8; Dojindo) according to the manufacturer's

ZEB1-AS1 expression in NSCLC

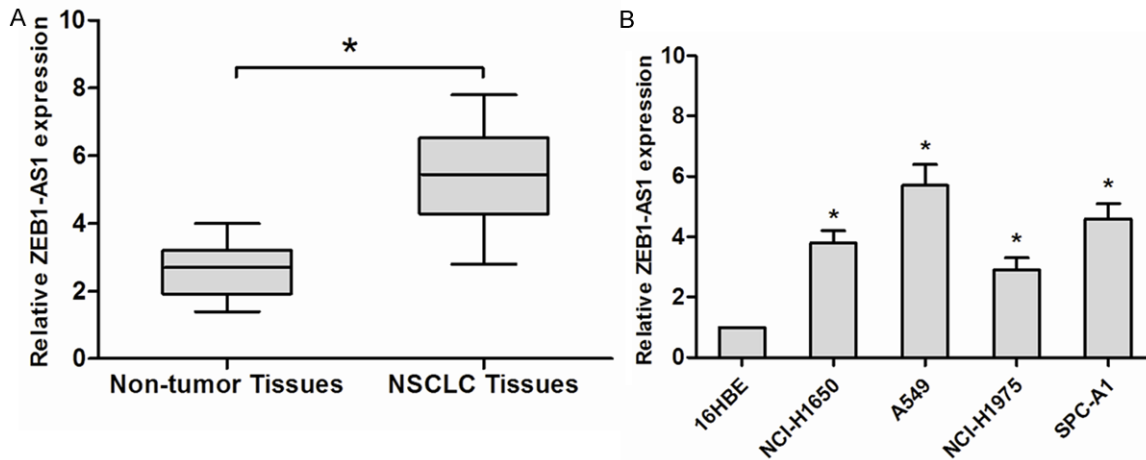


Figure 1. LncRNA ZEB1-AS1 was upregulated in NSCLC tissues and NSCLC cell lines. A. Relative expression of ZEB1-AS1 in human NSCLC tissues compared with adjacent non-tumor tissues (n=68). B. QRT-PCR analysis of ZEB1-AS1 expression levels in NSCLC cell lines (NCI-H1650, A549, NCI-H1975, SPC-A1) compared with normal human bronchial epithelial cell line (16HBE). GAPDH was used as the internal control. *P<0.05.

instructions. Cells were seeded into 96-well tissue culture plates at a density of 2×10^3 cells/well the day before transfection. At 24, 48, 72, 96 h after transfection, 20 μ l of CCK-8 reagent was added to each well and incubated at 37°C for 2 h. The absorbance value was detected at a wave length of 450 nm.

Cell apoptosis analysis

Cells were harvested 48 h after transfection for apoptosis analysis. Floating and adherent cells were collected using 0.1% trypsin, washed twice with cold PBS, and suspended in 1000 μ l binding buffer. The cells were then treated with FITC-Annexin V and propidium iodide (PI) in the dark at room temperature, according to the manufacturer's protocol. The cells were then examined by flow cytometry (BD Biosciences) on instrument equipped with CellQuest software (BD Biosciences).

Endothelial tube formation in vitro

NSCLC cells were transfected with si-ZEB1-AS1 or si-NC. 48 h after transfection, the supernatants were collected and centrifuged at 12000 rpm for 30 minutes to get rid of cell debris. The supernatant was harvested for further study. Tube formation assay was performed as described previously [15]. HUVECs were cultured in conditioned medium from the supernatant of NSCLC cells at a density of 20000 cells per well in a 96-well plate precoated with 150

μ l thick Matrigel. Then HUVECs were cultured at 37°C in a 5% CO₂ atmosphere for 6 h. The formation of HUVECs tubular structures was calculated and photographed under a light microscope (Olympus).

Cell invasion assay

Cell invasion was performed by transwell invasion assay and used with the Matrigel-coated (Costar) filters in 24-well plates according to the manufacturer's instruction. And 1×10^4 cells/well had been transfected to seed onto the upper chambers of the transwells in serum-free DMEM medium for an invasion assay. DMEM medium including 10% FBS was added to the lower chambers. The plates were incubated at 37°C with 5% CO₂ for 48 h, cells were viewed and photographed under a phase contrast microscope (Olympus) and counted in 5 randomly microscopic fields.

Western blot analysis

Cells were lysed in RIPA buffer with protease and phosphatase inhibitors (Roche). Equal amounts (30 μ g) of the protein were electrophoresed by SDS-PAGE, transferred to NC membranes and incubated with the following primary antibodies: anti-c-myc, anti-cyclin D1, anti- β -catenin, anti-GAPDH (Abcam). The primary antibody incubation for 12 h was followed by incubation with an HRP-conjugated secondary antibody for 2 h. The bound antibodies were

ZEB1-AS1 expression in NSCLC

Table 1. Correlation between lncRNA ZEB1-AS1 expression and clinicopathological features in NSCLC

Variable	Number	lncRNA ZEB1-AS1 expression		P value
		Low	High	
Age (years)				0.442
<60	23	13	10	
≥60	45	21	24	
Gender				0.462
Male	39	18	21	
Female	29	16	13	
Histologic type				0.331
Squamous	32	18	14	
Adenoma	36	16	20	
Tumor size(cm)				0.083
<3	41	17	24	
≥3	27	17	10	
Tumor stage				0.002
I-II	44	28	16	
III	24	6	18	
Lymph nodes metastasis				0.003
No	49	30	19	
Yes	19	4	15	

detected using enhanced chemiluminescence reagent (Thermo Fisher Scientific).

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software (IBM). Results are expressed as mean ± SD as indicated. Comparison of continuous data was analyzed using an independent t-test between the two groups, whereas categorical data was analyzed by the chi-square test. Kaplan-Meier analysis and the log-rank test were used to assess the survival plots. P<0.05 was considered statistically significant.

Results

lncRNA ZEB1-AS1 expression is up-regulated in NSCLC tissues and cell lines

To determine whether lncRNA ZEB1-AS1 was involved in the tumorigenesis of NSCLC, we determine the expression of ZEB1-AS1 in NSCLC tissues and cell lines using qRT-PCR. The results showed that the expression of ZEB1-AS1 in NSCLC tissues was significantly increased compared to adjacent non-tumor tis-

sues (**Figure 1A**, P<0.05). In parallel, ZEB1-AS1 expression was markedly upregulated in four NSCLC cell lines (NCI-H1650, A549, NCI-H1975, SPC-A1) compared with normal human bronchial epithelial cell line (16HBE) (**Figure 1B**, P<0.05). Since A549 and SPC-A1 cells exhibited relative high endogenous ZEB1-AS1 expression among all tested cell lines, then these two cell lines were selected for the following experiments.

lncRNA ZEB1-AS1 expression correlates with clinicopathological features and prognosis of NSCLC patients

In order to find out whether the expression of ZEB1-AS1 in NSCLC tissues had effect on patients' clinicopathological features, the correlation between ZEB1-AS1 expression and clinicopathological features was assessed. The 68 NSCLC patients were divided into two groups relative to the median ratio of ZEB1-AS1 expression in NSCLC tissues: high ZEB1-AS1 group (n=34, ZEB1-AS1 expression ratio > median) and low ZEB1-AS1 group (n=34, ZEB1-AS1 expression ratio ≤ median). The association between ZEB1-AS1 expression and the clinicopathological features were summarized in **Table 1**. Interestingly, high ZEB1-AS1 expression in NSCLC was significantly correlated with advanced tumor stage and lymph node metastasis (P<0.05). However, there was no association between ZEB1-AS1 expression and other features. Furthermore, to determine the relationship between ZEB1-AS1 expression and the prognosis of NSCLC patients, overall survival curves were plotted according to ZEB1-AS1 expression level and analyzed by the Kaplan-Meier method and log-rank test. Our results showed that high ZEB1-AS1 expression group had significantly shorter overall survival than the low ZEB1-AS1 expression group (**Figure 2**, P<0.05). These results suggested that ZEB1-AS1 might be a useful biomarker of the prognosis or progression of NSCLC.

lncRNA ZEB1-AS1 inhibition suppresses NSCLC cell proliferation in vitro

To explore the biological role of ZEB1-AS1 in NSCLC, we evaluated the effect of ZEB1-AS1 on NSCLC cell lines. ZEB1-AS1 was down-regulated by transfecting si-ZEB1-AS1 into NSCLC

ZEB1-AS1 expression in NSCLC

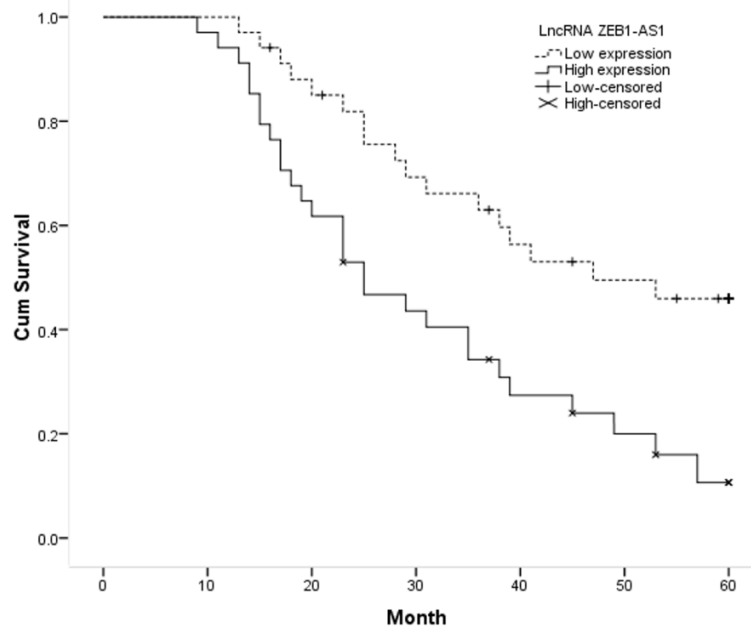


Figure 2. Kaplan-Meier survival curves of patients with NSCLC based on lncRNA ZEB1-AS1 expression. High ZEB1-AS1 expression group had significantly poorer prognosis than those in low ZEB1-AS1 expression group. * $P < 0.05$.

cells. QRT-PCR analysis was performed 48 h after post transfection and the results showed that si-ZEB1-AS1 significantly suppressed the expression of ZEB1-AS1 in A549 and SPC-A1 cell lines (**Figure 3A**, $P < 0.05$). To further identify the function of ZEB1-AS1 on cell proliferation, we did the CCK-8 assay. As shown in **Figure 3B**, the cell proliferation was significantly inhibited when ZEB1-AS1 was down-regulated in A549 and SPC-A1 cells ($P < 0.05$). Furthermore, compared with the si-NC group, ZEB1-AS1 inhibition markedly induced cell apoptosis in A549 and SPC-A1 cells (**Figure 3C**, $P < 0.05$). These data suggested that ZEB1-AS1 acted as an oncogene by promoting cell proliferation and inhibiting apoptosis of NSCLC cells.

lncRNA ZEB1-AS1 inhibition suppresses NSCLC cell invasion and angiogenesis

Since significant difference between ZEB1-AS1 expression and tumor stage, lymph node metastasis, it prompted us to explore whether ZEB1-AS1 involved in NSCLC cell invasion. Transwell invasion assay showed that ZEB1-AS1 inhibition suppressed A549 and SPC-A1 cells invasion compared to si-NC group (**Figure 4A**, $P < 0.05$). Moreover, our results showed that formation of capillary-like structure of HUVECs treated by si-ZEB1-AS1 conditioned medium

were significantly decreased compared to si-NC group (**Figure 4B**, $P < 0.05$). These findings suggested that ZEB1-AS1 acted as an oncogene via promoting cell invasion and angiogenesis.

lncRNA ZEB1-AS1 modulates Wnt/ β -catenin pathway in NSCLC cells

Wnt/ β -catenin signaling pathway is a well-recognized pathway involved in metastasis in tumor progression. Thus, we performed western blot analysis to explore whether suppressing ZEB1-AS1 affected the Wnt/ β -catenin signaling pathway. Western blot showed that ZEB1-AS1 inhibition significantly suppressed β -catenin expression compared with si-NC groups. In addition, our data reported that ZEB1-AS1 inhibition significantly suppressed expression of cyclin D1 and c-myc (classic downstream genes of the Wnt/ β -catenin signaling pathway) (**Figure 5**, $P < 0.05$). Thus, our data suggested that ZEB1-AS1 inhibition might suppress cell invasion and angiogenesis through Wnt/ β -catenin signaling in NSCLC.

Discussion

Increasing studies showed that over 10,000 lncRNAs were encoded by the human genome in integrative genomic [16]. Recent findings have suggested that many lncRNAs have acted as an oncogene or tumor suppressor and played important roles in cancers [17], including NSCLC. For example, Zhao et al showed that over-expression of lncRNA SBF2-AS1 was associated with advanced tumor progression and poor prognosis in patients with NSCLC [18]. Nie et al suggested that lncRNA UCA1 exerted oncogenic functions in NSCLC by targeting miR-193a-3p [19]. Han et al found that low expression of lncRNA PANDAR predicted a poor prognosis of NSCLC and affected cell apoptosis by regulating Bcl-2 [20].

In the present study, we focus on lncRNA ZEB1-AS1. Our data revealed that the expression levels of ZEB1-AS1 in NSCLC tissues and cell lines were significantly increased compared to adja-

ZEB1-AS1 expression in NSCLC

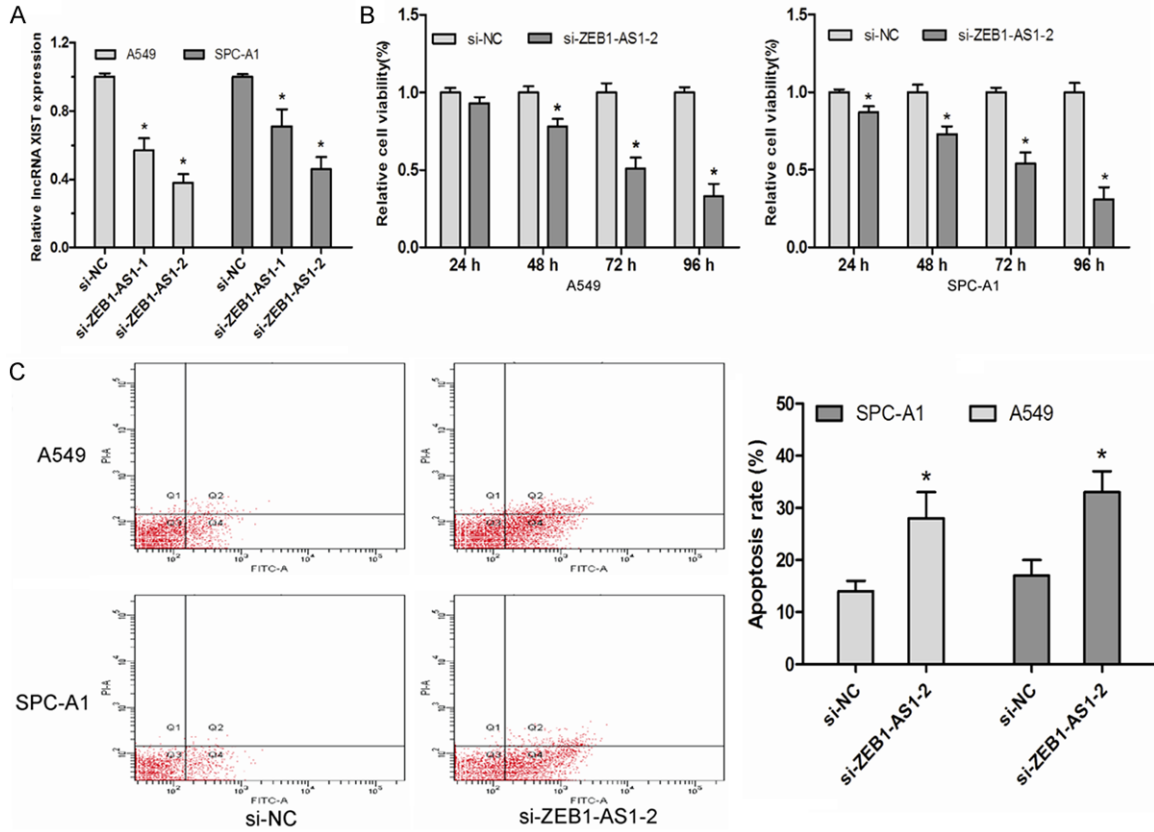
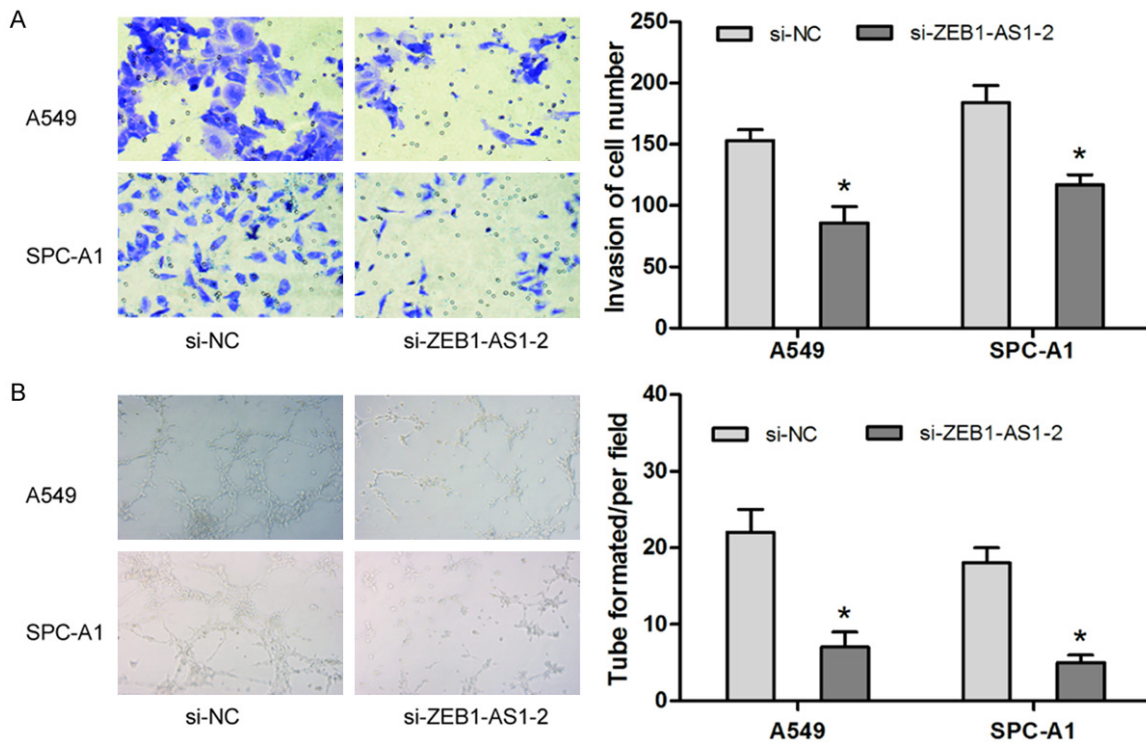


Figure 3. LncRNA ZEB1-AS1 inhibition suppressed NSCLC cell proliferation in vitro. A. QRT-PCR analysis of ZEB1-AS1 expression in A549 and SPC-A1 cells transfected with si-ZEB1-AS1 or si-NC. B. CCK-8 assay was used to detect the proliferation ability of A549 and SPC-A1 cells transfected with si-ZEB1-AS1 or si-NC. C. Apoptosis in A549 and SPC-A1 cells after transfected with si-ZEB1-AS1 or si-NC was detected by Flow cytometry. *P<0.05.



ZEB1-AS1 expression in NSCLC

Figure 4. LncRNA ZEB1-AS1 inhibition suppressed NSCLC cell invasion and angiogenesis. A. Transwell invasion assay was performed to determine the invasion ability of A549 and SPC-A1 cells transfected with si-ZEB1-AS1 or si-NC. B. Representative images of tube-like structures of HUVECs cultured with conditioned medium derived from si-ZEB1-AS1 or si-NC NSCLC cells were used for Matrigel tube formation assay. *P<0.05.

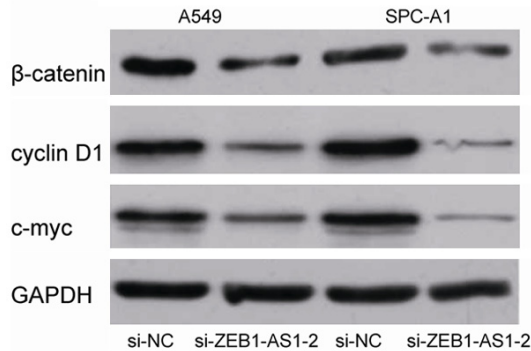


Figure 5. Knockdown of lncRNA ZEB1-AS1 inhibited the activity of Wnt/ β -catenin signaling pathway. Western blot showed that si-ZEB1-AS1 suppressed β -catenin, cyclin D1 and c-myc expression in A549 and SPC-A1 cells.

cent non-tumor tissues and normal human bronchial epithelial cell line 16HBE. Statistical analysis reported that high ZEB1-AS1 expression was associated with advanced tumor stage and lymph node metastasis. Furthermore, Kaplan-Meier analysis and the log-rank test showed that high ZEB1-AS1 expression was correlated with lower overall survival rates and could be an independent prognostic factor in NSCLC patients. These findings suggested that ZEB1-AS1 might function as an oncogene in NSCLC development and progression. In addition, we explored the function of ZEB1-AS1 in NSCLC in vitro. We found that ZEB1-AS1 inhibition suppressed NSCLC cell proliferation by inducing cell apoptosis. Transwell assay found that ZEB1-AS1 inhibition decreased NSCLC cell motility and angiogenesis.

In recent years, lncRNAs have attracted attention to understand the functional implications in various cell biology, particularly in cancers. The molecular mechanisms by which lncRNAs drive evolution, development and cancers are diverse. Wnt/ β -catenin signaling is known to regulate a tumor progression through regulating the ability of the multifunctional β -catenin protein, which is a crucial signaling molecule in the Wnt/ β -catenin pathway [21]. Mounting evidences suggested that aberrant activation of the Wnt/ β -catenin pathway was involved in NSCLC progression [22]. For example, Huang et

al showed that Wnt2 could promote NSCLC progression by activating Wnt/ β -catenin pathway [23]. Zhang et al found that miR-410 could act as oncogene in NSCLC through downregulating SLC34A2 via activating Wnt/ β -catenin pathway [24]. Fu et al suggested that lncRNA AK126698 inhibited proliferation and migration of NSCLC cells by targeting Frizzled-8 and suppressing Wnt/ β -catenin signaling pathway [25]. However, whether aberrant lncRNA ZEB1-AS1 expression modulates the Wnt/ β -catenin signaling pathway in NSCLC is still unclear. In the present study, we found that ZEB1-AS1 inhibition suppressed β -catenin expression compared with si-NC groups. In addition, our data showed that ZEB1-AS1 inhibition reduced expression of cyclin D1 and c-myc (classic downstream genes of the Wnt/ β -catenin signaling pathway) compared to si-NC group, indicating ZEB1-AS1 may suppress cell invasion and angiogenesis through Wnt/ β -catenin signaling in NSCLC. Thus, our present study found a NSCLC related lncRNA, ZEB1-AS1, and elucidated its functional roles in NSCLC development and progression.

In summary, the present study showed that ZEB1-AS1 acts as an oncogene by promoting malignant progression of NSCLC, notably, mechanistic analysis revealed a novel ZEB1-AS1-Wnt/ β -catenin signaling pathway regulatory network in NSCLC. Thus, these results suggest that ZEB1-AS1 may be a potential therapeutic target for the treatment of NSCLC patients.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Li Li, Department of Medical Oncology I, The First Affiliated Hospital of Henan Polytechnic University, The Second People's Hospital of Jiaozuo, South NO.17 Minzhu Road, Jiaozuo 454000, Henan, China. E-mail: yijiezhang1973@163.com

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