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Original Research Article

lncRNA ENST00000434223 exerts regulatory effects on NSCLC cells via Wnt/β-catenin signaling pathway

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Abstract

Purpose: *To identify the role and underlying mechanism of lncRNA ENST00000434223 (lncRNA ENST) in the occurrence of NSCLC.*
Methods: *Quantitative real time-polymerase chain reaction (qRT-PCR) was used to evaluate relative lncRNA ENST, β-catenin, Wnt1, and cyclin D1 expression levels in NSCLC tissues, cells, and sera. Proliferation of NSCLC cells was assessed using CCK-8 assay, while Transwell assay was utilized in evaluating changes in cell invasion/migration. Changes in Wnt1, cyclin D1, and β-catenin expressions were determined using qRT-PCR, while alterations in their protein expressions were determined by western blot (WB). In vivo experiments were conducted in nude mice to investigate the effects of lncRNA ENST on tumor growth.*
Results: *lncRNA ENST expression was low in sera, cells, and tissues of NSCLC ($p < 0.05$). Tumor metastasis and size, lymphatic metastasis, and clinical stage were correlated with lncRNA ENST expression ($p < 0.05$). It was found that ENST could be applied as independent prognostic factor in NSCLC patients. Upregulated expression of lncRNA ENST restrained cell migration, proliferation, and invasion ($p < 0.05$). Compared with t NC group, lncRNA ENST expression decreased expressions of Wnt1, cyclin D1, and β-catenin in SPC-A1 cells and A549 cells ($p < 0.05$).*
Conclusion: *lncRNA ENST inhibits the proliferation and apoptosis of NSCLC cells via Wnt/β-catenin pathway. Thus, it is a possible biomarker for NSCLC, and provides a novel insight into the clinical management of NSCLC.*

Keywords: *lncRNA ENST, Cell invasion, Cell migration, Wnt/β-catenin, NSCLC, Cell proliferation*

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INTRODUCTION

NSCLC is a common malignancy [1], with an incidence of approximately 80 – 85 % and a high mortality rate [2]. Evidence has shown that the high mortality rate of NSCLC is closely correlated with early metastasis [3]. Therefore, it is meaningful to investigate the mechanism connected with the pathogenesis of NSCLC to serve as a basis for the targeted therapy of NSCLC.

lncRNAs have a crucial role in the progression of diseases, by targeting the regulation of

microRNAs and target proteins [4,5]. It was found that lncRNAs regulate the pathogenesis of gastric cancer, colon cancer, lung cancer, and other tumors [6]. The newly identified lncRNA ENST00000434223 (lncRNA ENST) is involved in the proliferation and migration of various cancers. Over expressed lncRNA ENST was revealed as suppressing tumor cell migration, proliferation, as well as invasion [7, 8], and Wnt/β-catenin pathway participated in lung and gastric cancers [9, 10]. A study also revealed that lncRNAs suppress tumorigenesis through the suppression of Wnt/β-catenin signaling [11], while lncRNA ENST inhibits the proliferation and migration of gastric cancer cells through the suppression of Wnt/β-catenin signaling [8]. However, the mechanism of lncRNA ENST in the migration and proliferation of NSCLC tumor cells remains unclarified. Therefore, the current research intended to determine the function and underlying mechanism of lncRNA ENST in NSCLC.

EXPERIMENTAL

Tissue samples

In total, 84 NSCLC tumor and paracancerous tissues were obtained from NSCLC patients treated in Shanghai Jiaotong University Affiliated Sixth People's Hospital between January 2015 and January 2020. All tissue specimens were placed at -80 °C in liquid nitrogen. Peripheral blood sampling was conducted from entire NSCLC patients preoperatively, as well as from healthy subjects who served as controls. The blood specimens were centrifuged, followed by storing supernatant at -80 °C.

Ethical approval

This research received approval from the ethics committee of Shanghai Jiaotong University Affiliated Sixth People's Hospital (approval no. NCT02563124) and strictly followed the guidelines of Declaration of Helsinki [12]. All patients with NSCLC were diagnosed by pathological biopsy, and signed written informed consent prior to participation.

Cell cultures

SPC-A1, NCI-H157, NCI-H1299, and A549 cells (human NSCLC cell lines) were supplied by Typical Culture Collection Centre (Manassas, USA). 16HBE (human bronchial epithelial cell line, Shanghai, China) was utilized as a normal control. The cells were incubated in RPMI-1640 medium (Thermo Fisher, Waltham, USA) comprising streptomycin (50 μg/mL), fetal bovine serum (FBS, 10 %), and penicillin (50 U/mL) in 5 % CO_2 at 37 °C. The medium was altered every 24 h, and after incubating for 72 h, the cells were passaged.

Cell transfection

Empty pGMLV, transfected with overexpressed RNA pGMLV-ENST and overexpressed RNA pGMLV-WIF1 vector, was purchased from GenScript (Nanjing, China). Small interfering RNA si-lnc-ENST and negative control were supplied by GenePharm Co. Ltd (Shanghai, China). According to instructions of manufacturer, transfection was conducted, and transfection efficiency was evaluated by real-time polymerase chain reaction (PCR).

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

Cells and tissues were utilized for extractions of total RNA by means of TRIzol reagent (Invitrogen, Carlsbad, USA), followed by conducting reverse transcription by means of M-MLV RT kit (Promega, Madison, USA). Specific RT primers and PCR primers (GeneCopoeia) were established. PCR was carried out with ABI 7500 PCR instrument and SYBR PreMix Ex Taq kit supplied by TaKaRa (Tokyo, Japan). An internal reference of GAPDH was selected for lncRNA. Comparative cycle threshold method (2-ΔΔCT) was employed for data collection (Table 1) [13].

Table 1: The primers used in PCR

Primers	Sequence	
LncRNA ENST	F:	5′-AGGCCTCGTTCACCTTGACG-3′:
	R:	5′-CCCTTGCCACGTCCACTACC-3′
Wnt1	F:	5′-TTCTGAGGAAGAACAGCATGAA-3′
	R:	5′-CCTTTTGGAGTCTGACCATTTC-3′
β-catenin	F:	5′-ATGCGGCTGCTGTTCTATTC-3′
	R:	5′-ACCAATGTCCAGTCCGAGAT-3′
cyclinD1	F:	5'-GAGTAGTGCGAAGCATAGGTCT-3′
	R:	5'-CTAC,CACGAGTAGTCGAGCGC-3′
GADPH	F:	5'-TACCCACGGCAAGTTCAACG-3′
	R:	5'-CACCAGCATCACCCATTTG-3′

Western blot (WB) analysis

Radioimmunoprecipitation assay (RIPA) lysis buffer was employed for lysis of corresponding tissues or cells. Concentrations of related

proteins were determined utilizing BCA analysis kit (Pierce, Rockford, USA). An 8% SDS-PAGE gel was utilized to separate proteins, followed by transferring proteins to polyvinylidene difluoride membranes (Roche, Basel, Switzerland). Following blocked by skim milk (5%) at indoor temperature for 1.5 h, membranes were added with primary antibodies to perform overnight incubation at 4 °C. Next step, secondary antibodies were added for 1.5 h of incubation. ECL chemiluminescence reagent (Millipore, Billerica, USA) was adopted for blots detection. Image J (NIH, Bethesda, USA) was utilized for quantification of obtained bands.

Cell proliferation assay

Cell viabilities within 0 h, 24 h, 48 h, and 72 h were calculated by means of CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) complying with manufacturer's instructions. After collection and resuspension, transfected cells were adjusted to 3×10^3 cells/well and inoculated into 96-well plates. Then, α-MEM (100 μL) containing FBS (10%) was added in each well. The CCK-8 solution was supplemented to every well within designated observation time for 1.5 h of incubation at 37 °C. Next, absorbance was measured at 450 nm.

Determination of cell migration/invasion

The cells capacity to migrate and invade was assessed utilizing Transwell chambers (Corning, USA) in 24-well plates with the 8.0-μm-pore polycarbonate membrane insert. Migration assay was carried out, with upper chamber filling with serum-free medium and lower chamber supplementing with medium comprising FBS (10%). Twenty-four hours later, cell fixing was conducted utilizing paraformaldehyde (4%), followed by staining with crystal violet (0.1%). The number of cells traversing porous membrane was calculated in 6 random sights of each chamber, with mean value taken as the result. Except for pre-coating Matrigel in the Transwell chamber, Transwell invasion assay had the same experimental steps with Transwell migration assay.

Statistical method

The data are presented as mean ± standard deviations (SD). Data analysis was carried out using Statistical Package for the Social Sciences (SPSS) 20.0 (IBM Corp, Armonk, USA), and GraphPad 8 (La Jolla, USA). Two-group comparison was carried out by means of independent sample t-test, while multi-group comparison was analyzed by one-way ANOVA; subsequent pairwise comparisons were implemented with LSD t-test. The 2-year survival rate of NSCLC patients with low expression levels of lncRNA ENST was assessed by Kaplan–Meier method, while diagnostic values of lncRNAs in NSCLC were mapped utsing receiver operating characteristic (ROC) curve. A statistical difference was deemed to $P < 0.05$.

RESULTS

lncRNA ENST expression in NSCLC

The tumor tissues showed elevated lncRNA ENST expression in contrast to the paracancerous tissues ($p < 0.05$; Figure 1 A). Pearson's test displayed decreased lncRNA ENST expression in the serum of tumor patients ($p < 0.05$; Figure 1 B), which had positive correlation with that in tissues ($r = 0.03620$, $p = 0.9552$; Figure 1 C). In tumor cell lines, lncRNA ENST expression was down-regulated in contrast to that in 16HBE cell line ($p < 0.05$; Figure 1 D).

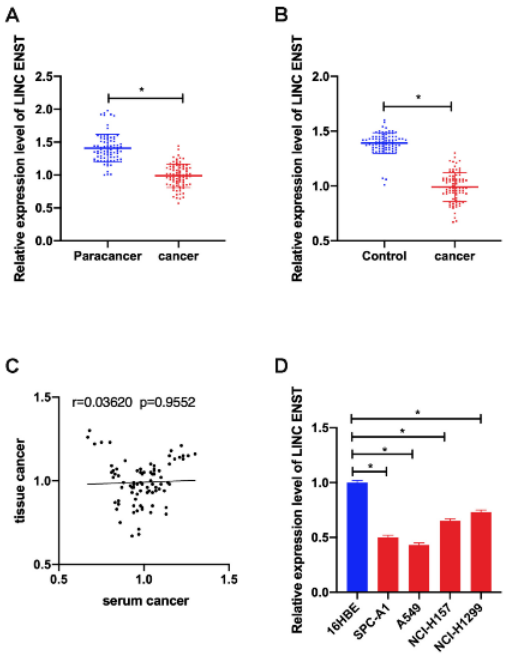

Figure 1: Expression of lncRNA ENST in NSCLC-related tissues, sera, and cells. (A) lncRNA ENST expression in tumor and adjacent tissues of NSCLC patients. (B) lncRNA ENST expression is decreased in the serum of patients. (C) Correlation analysis of lncRNA ENST expression in sera and tissues of NSCLC patients. (D) Expression of lncRNA ENST in various NSCLC cell lines (SPC-A1, A549, NCI-H157, and NCI-H1299) and a human bronchial epithelial cell line (16HBE). The NSCLC cell lines showed decreased lncRNA ENST expression compared with the 16HBE group

Diagnostic value of lncRNA ENST in NSCLC

Samples were categorized in groups with low and high expression levels of lncRNA ENST according to median expression values, so as to determine the value of lncRNA ENST in the diagnosis of NSCLC. Tumor size, tumor metastasis, clinical stages, and lymphatic metastasis showed correlation with lncRNA ENST expression ($p < 0.05$; Table 2). It was also found that lncRNA ENST expression can be applied to distinguish NSCLC patients from healthy subjects, and AUC was 0.947 (Figure 2 A). The diagnostic values of lncRNA ENST in tumor metastasis, size, clinical stages, and lymphatic metastasis were observed. AUC for lncRNA ENST in terms of distinguishing tumors ≥ 5 cm from those < 5 cm was 0.821 (Figure 2 B), that for distinguishing tumor metastasis was 0.639 (Figure 2 C), that for distinguishing stage I-IIA from stage IIB - III was 0.866 (Figure 2 D), and that for distinguishing lymph node metastasis was 0.903 (Figure 2 E). Further follow-up demonstrated that 2-year survival rate was lower in group with low expression of lncRNA ENST than in group with high expression of lncRNA ENST (Figure 2 F). According to Cox regression analysis, ENST may be the independent prognostic factor in NSCLC patients (Table 3). This suggests that lncRNA ENST may be a diagnostic indicator for NSCLC.

Table 2: Relationship between lncRNA ENST expression and clinical data of NSCLC patients

Variable		LINC ENST expression		*P*-value
		Low (n = 42)	*High (n = 42)*	
Sex				
	Male (n = 36)	15	21	0.2702
	Female (n = 48)	27	21	
Age (years)				
	≥ 60 (n = 58)	32	26	0.2377
	< 60 (n = 26)	10	16	
Tumor size				
	≥ 5 cm (n = 47)	29	18	0.0273*
	< 5 cm (n = 37)	13	24	
Tumor metastasis				
	yes (n = 51)	36	15	<0.0001*
	no (n = 33)	6	27	
Clinical stage				
	I-IIA (n = 38)	11	27	0.0009*
	IIB-III (n = 46)	31	15	
Lymph node metastasis				
	Absent (n = 32)	11	23	0.0077*
	Present (n = 52)	33	19	

*$P < 0.05$

Table 3: Cox regression analysis

Variable	Univariable analysis			Multivariable analysis		
	HR	*95%CI*	*P-value*	*HR*	*95%CI*	*P*-value
Sex	0.827	0.514 ~ 1.545	0.812			
Age	0.903	0.568 ~ 1.537	0.798			
Tumor size	2.346	1.367 ~ 4.642	0.285			
Tumor metastasis	0.562	0.688 ~ 0.901	0.006*	0.477	0.352 ~ 1.082	0.037*
Clinical stage	1.221	0.877 ~ 2.231	0.557			
Lymph node metastasis	0.487	0.368 ~ 0.812	0.042*	0.421	0.331 ~ 1.224	0.027*
LINC ENST	0.221	0.108 ~ 0.866	0.008*	0.252	0.117 ~ 0.638	0.012*

*$P < 0.05$

Down-regulated lncRNA ENST inhibits migration, invasion and proliferation of SPC-A1 and A549 cells

To further expound the role of lncRNA ENST in NSCLC, lncRNA ENST expression was up-regulated in NSCLC cells and was more pronounced in A549 cells and SPC-A1 cells. lncRNA ENST expression was up-regulated and down-regulated in SPC-A1 and A549 cells by transfection (Figure 3 A). Downregulation of lncRNA ENST expression in NSCLC cells promoted proliferations of both A549 and SPC-A1cells ($p < 0.05$) in contrast to in control group (Figure 3 B). Transwell invasion and migration assays demonstrated that down-regulated

lncRNA ENST expression in NSCLC cells facilitated migration and invasion of A549 cells and SPC-A1 cells ($p < 0.05$; Figure 3 C and D). This indicated that upregulation of lncRNA ENST expression could inhibit migration, invasion and proliferation.

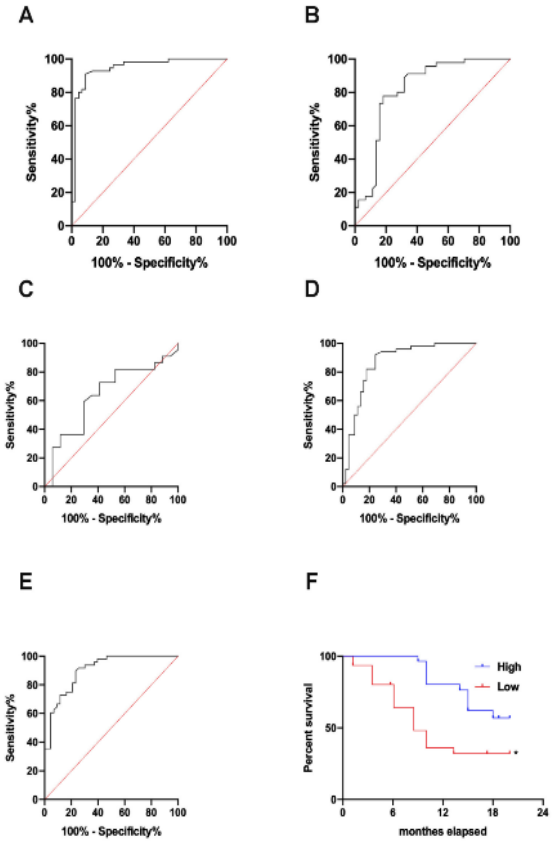

Figure 2: Diagnostic value of lncRNA ENST in NSCLC. (A) AUC (0.947) in distinguishing NSCLC patients from healthy controls. (B) AUC (0.821) in distinguishing tumors ≥ 5 cm from those < 5 cm. (C) AUC (0.639) in distinguishing tumor metastases. (D) AUC (0.866) in distinguishing stage I-IIA from stage IIB-III. (E) AUC (0.933) in distinguishing lymph node metastasis. (F) The 2-year survival rate of patients with low expression was lower than that of patients with high expression

lncRNA ENST functionss in SPC-A1 and A549 cells via Wnt/β-catenin pathway

ENST (Figure 4 A), Wnt1, β-catenin, and cyclin D1 expressions in NSCLC SPC-A1 and A549 cells were analyzed using qRT-PCR. In contrast to NC group, upregulation of lncRNA ENST expression reduced Wnt1, β-catenin, and cyclin D1 expressions in SPC-A1 cells and A549 cells ($p < 0.05$; Figure 4 B - D). mRNA expressions of Wnt1, β-catenin, and cyclin D1 were elevated in SPC-A1 cells and A549 cells after downregulation of lncRNA ENST expression ($p < 0.05$; Figure 4 B - D).

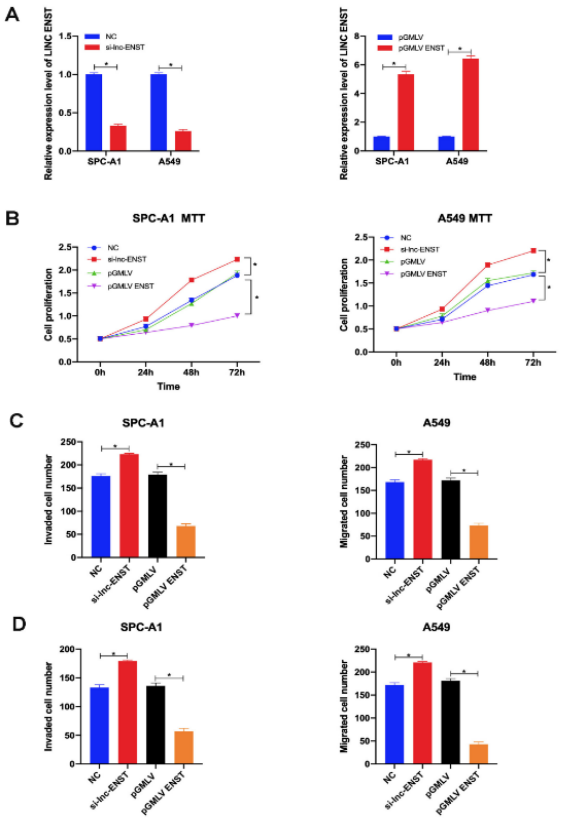

Figure 3: Knockdown of lncRNA ENST inhibits the proliferation, invasion, and migration of SPC-A1 and A549 cell lines. (A) WB assay: upregulation and knockdown of lncRNA ENST expression in SPC-A1 and A549 cells. (B) Downregulation of lncRNA ENST expression in NSCLC cells promotes proliferation of both SPC-A1 and A549 cells. (C) Down-regulated expression of lncRNA ENST in NSCLC cells contributes SPC-A1 and A549 cells invasion. (D) Down-regulated expression of lncRNA ENST in NSCLC cells contributes migration of SPC-A1 and A549 cells

lncRNA ENST participates in regulating proliferation of A549 and SPC-A1 cell lines via Wnt/β-catenin pathway

Wnt1 expression in cells after transfection of pGMLV-WIF1 increased significantly ($p < 0.05$; Figure 5 A). SPC-A1 cells' growth was accelerated in pGMLV-WIF1 group compared with NC group ($p < 0.05$; Figure 5 B). pGMLV-WIF1+pGMLV ENST group had minimized cell proliferation after upregulation of lncRNA ENST in contrast to NC group ($p < 0.05$; Figure 5 B). A549 cell growth was accelerated in pGMLV-WIF1 group compared with NC group ($p < 0.05$; Figure 5 C). Cell growth of pGMLV-ENST group was delayed compared to that of the pGMLV-WIF1+pGMLV ENST group after upregulation of lncRNA ENST (Figure 5 C).

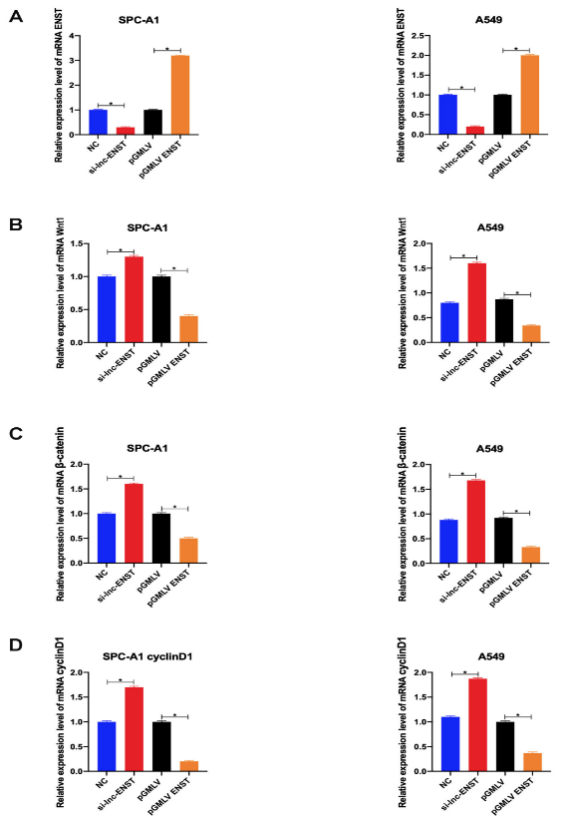

Figure 4: lncRNA ENST is involved in the regulation of Wnt/β-catenin signaling in SPC-A1 and A549 cell lines. (A) mRNA ENST expression. (B) mRNA Wnt1 expression. (C) mRNA β-catenin expression. (D) mRNA cyclin D1 expression

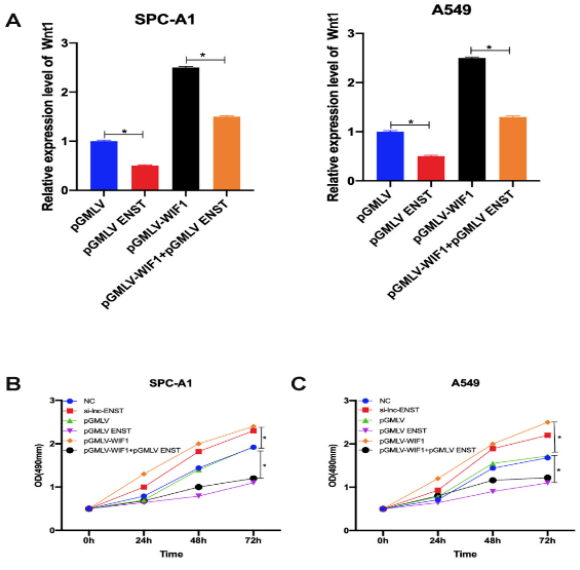

Figure 5: lncRNA ENST participates in regulating the proliferation of SPC-A1 and A549 cell lines through the Wnt/β-catenin pathway. (A) WB assay results. (B) lncRNA ENST reduces SPC-A1 cell proliferation via Wnt/β-catenin signaling. (C) lncRNA ENST reduces A549 cell proliferation via Wnt/β-catenin signaling

lncRNA ENST participates in regulating migration/invasion of A549 cells and SPC-A1 cells via Wnt/β-catenin pathway

In A549 and SPC-A1cell lines, invasion ability of pGMLV-WIF1 group increased and that of pGMLV-ENST group reduced ($p < 0.05$) compared with that in NC group. In contrast to pGMLV-WIF1+pGMLV ENST group after upregulation of lncRNA ENST, the cell invasion ability decreased ($p < 0.05$; Figure 6 A). pGMLV-WIF1 group exhibited increased migration in A549 cells and SPC-A1 cells in contrast to NC group ($p < 0.05$; Figure 6 B), and pGMLV-ENST group exhibited decreased migration compared to pGMLV-WIF1+pGMLV ENST group after upregulation of lncRNA ENST ($p < 0.05$, Figure 6 B).

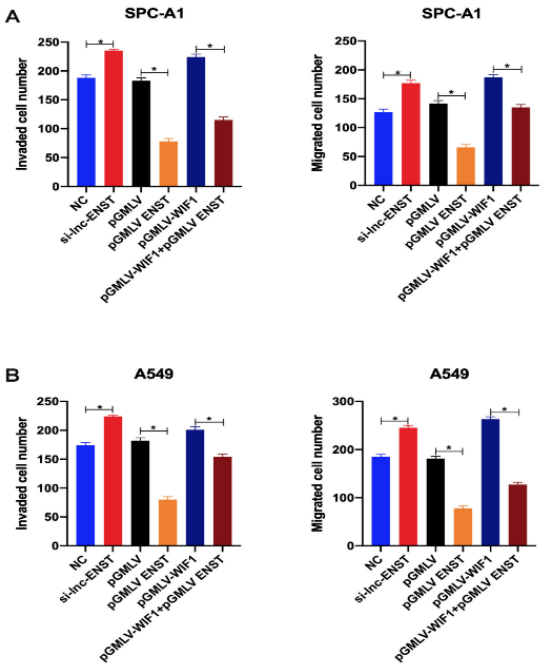

Figure 6: lncRNA ENST reduces the invasion and migration ability of SPC-A1 and A549 cell lines through Wnt/β-catenin signaling. (A) lncRNA ENST reduces the invasion ability of SPC-A1 and A549 cells through Wnt/β-catenin. (B) lncRNA ENST reduces the cell migration ability through Wnt/β-catenin signaling

lncRNA ENST participates in Wnt/β-catenin signal regulation *in vivo*

Through *in vivo* experiments, it was observed that nude mice injected with pGMLV-ENST showed decreased tumor volume and mass compared with those injected with si-NC, whereas nude mice injected with si-lnc-ENST showed an increasing trend in the tumor mass and volume ($p < 0.05$, Figure 7 A and B). si-lnc-ENST group showed elevated Wnt1, β-catenin, and cyclin D1 expression levels than si-NC group

(Figure 7 C - E). si-lnc-ENST group exhibited decreased Wnt1, β-catenin, and cyclin D1 expression levels than pGMLV-injected group (Figure 7 C - E). The outcomes indicated that ENST inactivated Wnt/β-catenin signaling *in vivo* as well as inhibited tumor growth.

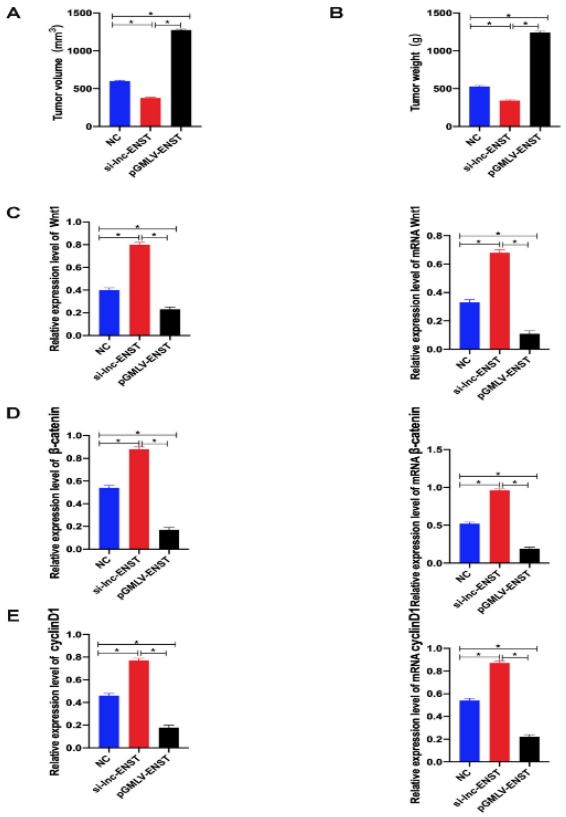

Figure 7: lncRNA ENST inhibits Wnt/β-catenin signaling in vivo. (A) Effect of lncRNA ENST on tumor volume in nude mice. (B) Effect of lncRNA ENST on tumor mass in nude mice. (C) Detection of Wnt1 expression in tumor tissues of NSCLC models by RT-qPCR and WB assay. (D) β-catenin Expression in tumor tissues of NSCLC models by RT-qPCR and WB assay. (E) Detection of cyclin D1 expression in tumor tissues of NSCLC models by RT-qPCR and WB assay

DISCUSSION

There is a strong correlation between the occurrence of NSCLC and lncRNAs. In this study, according to findings of in vivo and in vitro tests, lncRNA ENST was declined in NSCLC tissues, serum specimens, and cells. Decreased expression of lncRNA ENST had strong association with poor prognosis. Moreover, lncRNA ENST was found to be valuable in the diagnosis of NSCLC. Experiments and qRT-PCR assay displayed that ENST influenced invasion, metastasis, and proliferation of NSCLC through Wnt/β-catenin pathway. lncRNA ENST restrained tumor growth in NSCLC mouse model.

Studies have identified a close correlation between lncRNAs and malignancy [6,14,15]. Abnormal expression of lncRNAs can serve as an indicator in clinical diagnosis of cancer [16-19]. lncRNAs are abnormally expressed in cancer tissues than in paracancerous tissues [20]. Our results revealed that NSCLC tissues showed lower lncRNA ENST expression than paracancerous tissues.

lncRNA ENST expression was decreased in renal tumor tissues and had correlation with poor prognosis [8, 21]. NSCLC patients showed decreased lncRNA ENST expression in tumor tissues and serum, and low lncRNA ENST expression had strong association with adverse prognosis, which is consistent with the above results. In addition, *in vitro* experiments revealed that NSCLC cell proliferative ability was increased by lncRNA ENST knockdown, whereas these results were reversed after overexpression of lncRNA ENST, demonstrating that lncRNA ENST might be used as a therapeutic target for NSCLC.

lncRNAs are correlated to tumorigenesis through Wnt/β-catenin signaling pathway modulation [11, 22-24]. For example, it has been found that MIR100HG regulates colon cancer progression by modulating the miR-100 and miR-125b/Wnt/β-catenin axis [11]. It was demonstrated that the knockdown of lncRNA ENST contributed to increases in expression levels of Wnt/β-catenin signaling pathway-related proteins in NSCLC cells; and after the overexpression of lncRNA ENST, the expressions decreased. This suggests that lncRNA ENST contributes to the development of NSCLC through Wnt/β-catenin axis modulation.

The proliferation, invasion, and migration ability of cells significantly increased after transfection with pGMLV-WIF1, and decreased after transfection of pGMLV-WIF1 and pGMLV ENST0. Furthermore, in nude mice injected with si-lnc-ENST, tumor volume and mass were markedly as increased, and expressions of Wnt1, β-catenin, and cyclin D1 were marked as decreased. Whereas, the results were opposite in nude mice injected with pGMLV ENST00000434223, supporting our conclusions.

Nevertheless, this research also has some limitations. The included patients were single-center cases with regional limitations; further validation with large-scale, multicenter cases is needed. Moreover, the specific mechanism by which ENST affects Wnt/β-catenin pathway should be investigated further.

CONCLUSION

lncRNA ENST shows high expression in NSCLC and has close relation to adverse prognosis in NSCLC patients. ENST affects NSCLC cells through Wnt/β-catenin pathway and inhibits migration, invasion and proliferation of NSCLC cells, which may be a possible biomarker for NSCLC and provide a novel insight into the management of NSCLC.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jinge Shen and Song Yu contributed equally to this work and should be considered as equal first co-authors.

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