

Long non-coding RNA LINC00675 inhibits tumorigenesis and EMT via repressing Wnt/ β -catenin signaling in esophageal squamous cell carcinoma

Y.-B. ZHONG, A.-J. SHAN, W. LV, J. WANG, J.-Z. XU

Emergency Department, Shenzhen People's Hospital, the Second Medical College of Jinan University, Shenzhen, Guangdong, China

Abstract. – OBJECTIVE: Long noncoding RNA LINC00675 (LINC00675) seems to play an anti-oncogenic role in cancers, though its exact function remains unknown. Up to date, little is known about the role of LINC00675 in esophageal squamous cell carcinoma (ESCC). In this study, we aimed to explore the expression pattern, clinical significance and biological function of LINC00675 in ESCC.

PATIENTS AND METHODS: RT-PCR was performed to detect the expression levels of LINC00675 in both ESCC tissue and cell lines. The association of LINC00675 expression with clinicopathological factors and prognosis was statistically analyzed. Cell growth was detected by MTT assay and colony formation assay. Cell apoptosis was evaluated with flow cytometry. Migration and invasion ability of ESCC cells were detected wound healing assay and transwell assays. The expressions of EMT-related proteins and Wnt/ β -catenin-related proteins by Western blot were investigated.

RESULTS: LINC00675 expression was significantly downregulated in both ESCC tissues and cell lines. Decreased LINC00675 expression was correlated with histological grade, lymph nodes metastasis and advanced clinical stage. Furthermore, LINC00675 could serve as an independent predictor for overall survival in ESCC. Importantly, *in vitro* experiments indicated that that forced LINC00675 expression significantly suppressed inhibited ESCC cell proliferation, colony formation, migration, invasion and EMT, and promoted cell apoptosis through suppressing Wnt/ β -catenin signaling pathway.

CONCLUSIONS: We suggested that LINC00675 acted as a tumor suppressor in ESCC via regulation of Wnt/ β -catenin signaling pathway and may be a new prognostic biomarker and potential therapeutic target for ESCC intervention.

Key Words:

Long noncoding RNAs, LINC00675, Esophageal squamous cell carcinoma, Prognosis, Epithelial-mesenchymal transition, Wnt/ β -catenin signaling.

Introduction

Esophageal cancer, a common type of malignant tumor that forms in the upper digestive tract, is among the ten most incident and fatal malignancies in the world^{1,2}. According to its pathological characteristics, esophageal cancer has two main histological subtypes, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma³. In China, ESCC is the major histological subtype of esophageal cancer⁴. Although many antitumor therapies have been used in patients with ESCC, the prognosis of these patients is rather poor, and the long-term survival rate is only 10-30%^{5,6}. Patients are usually diagnosed at late stages, which has limited treatment options and the prognosis remains poor⁷. Thus, it is still necessary to explore the molecular pathways underlying ESCC progression and identify the novel biomarkers for predicting the prognosis of ESCC patients. Recent advancement on human genomes have confirmed that non-coding RNAs (ncRNAs), which do not encode proteins, account for more than 90% of the transcriptome⁸. Among the various classes of ncRNAs, long non-coding RNAs (lncRNAs) are more than 200 nucleotides in length with limited and serve as the primary regulatory ncRNA⁹. It has been showed that lncRNAs have emerged as important players in

the regulation of various biological and pathological processes, such as the immune response, differentiation and metabolism¹⁰⁻¹². In recent years, lncRNAs have been confirmed to be involved in development and progression of several cancers^{13,14}. Moreover, growing researches have shown that lncRNAs play important roles in tumor development, such as lncRNA hTERT¹⁵, lncRNA TUG1¹⁶, and lncRNA HO-TAIR¹⁷. Numerous studies^{18,19} have also showed that lncRNAs are novel type of biomarkers and therapeutic targets for cancer, including ESCC. LINC00675, also known as TMEM238L, is located on 17p13.1-p12. Its dysregulation has been reported in several tumors, including pancreatic ductal adenocarcinoma²⁰, colorectal cancer²¹ and glioma²². Interestingly, all previous investigations showed that LINC00675 served as a tumor suppressor in malignancy. However, the expression pattern and potential effect of LINC00675 in ESCC have not been reported. In this study, we firstly detected the expression levels of LINC00675 in both ESCC tissues and cell lines, and explored its prognostic value in ESCC patients. Consistent with previous study, we also found that LINC00675 acted as a tumor promoter and exerted its oncogenic effects partly through modulating Wnt/ β -catenin signaling.

Patients and Methods

Patients and Tissue Samples

ESCC tissues and corresponding adjacent normal tissues were obtained from ESCC patients undergoing surgery who had not had chemotherapy or radiotherapy, at Shenzhen People's Hospital between March 2010 and April 2013. All clinical tissues samples were immediately frozen in liquid nitrogen and stored at -80°C for future use. Written informed consent was obtained from each participant prior to the study. This study was approved by the Ethics Committee of Shenzhen People's Hospital. The clinical characteristics of the patients were listed in Table II.

Cell Lines and Cell Culture

Human ESCC cell lines (C-18, 108CA, Kys510, HKESC1, EC-1, EC9706) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Xuhui, Shanghai, China). Human epithelial cell line, Het-1A, was obtained from American Type Culture Collection (ATCC,

Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (HyClone, South-Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 50 $\mu\text{g}/\text{mL}$ streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 100 U/mL penicillin (Sigma-Aldrich, Pudong, Shanghai, China) at 37°C with 5% CO_2 .

Cell Transfection

Plasmid vector, pCDNA3.1-LINC00675, which caused a constitutive expression of LINC00675, and empty pCDNA3.1 plasmid (as negative control) were all purchased and constructed by Generay Biotech Co., Ltd. (Songjiang, Shanghai, China). The plasmids were prepared using DNA Midiprep Purification Kits (Qiagen, Hilden, Germany) and transfected into EC9706 or EC-1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's manuals. In short, EC9706 or EC-1 cells (2×10^5 cells per well) were seeded in a 6-well plate (Corning, Corning, NY, USA) to be about 70% confluence before transfection. Then, 4 μg plasmids and 10 μl Lipofectamine 2000 reagents were separated and added into 250 μl Opti-MEM (Invitrogen, Carlsbad, CA, USA). Thereafter, the complexes were mixed and added into each well. The cells were incubated at 37°C with 5% CO_2 for 4-5 h. Finally, the medium was replaced with fresh Roswell Park Memorial Institute-1640 (RPMI-1640) medium for the following experiments.

RNA Purification and Real-Time Quantitative RT-PCR (qRT-PCR)

Total RNA from esophageal cancer tissues and cell lines was isolated using GenElute Total RNA Purification Kit (Sigma Aldrich, St. Louis, MO, USA) according to the manufacture's protocols. The cDNA was reverse transcribed from the total RNAs using Golden RT MasterMix Kit (HaiGene, Haerbin, Heilongjiang, China). Then, RT-PCR was carried out using FastKing One Step RT-PCR Master Mix Kit (Tiangen, Haidian, Beijing, China) on a Quant Studio 6 Flex Real-time PCR instrument (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocols. Results were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative expression was analyzed using $2^{-\Delta\Delta\text{Ct}}$ method. The primers synthesized by Generay Biotech Co., Ltd. (Songjiang, Shanghai, China) were summarized in Table I.

Table I. The sequences of primers for RT-PCR.

Name	Sequences (5'-3')
LINC00675 (Sense)	GCCTACTGCTCTGGATCATCTGGTA
LINC00675 (Antisense)	ACCTGCG TCTCTTCTCTCTTCC
GAPDH (Sense)	CGAGCCACATCGCTCAGACA
GAPDH (Antisense)	GTGGTGAAGACGCCAGTGA

Western Blot Analysis

Total proteins were prepared using radio immunoprecipitation assay (RIPA) lysis buffer (Meilun Biotechnology, Dalian, Liaoning, China) and the concentrations of the proteins were qualified by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's manuals. Protein extracts (20 µg) were separated by 10% SDS polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were then incubated with primary antibodies after being blocked with 5% BSA in TBST. The vimentin antibody, GAPDH antibody and N-Cadherin antibody were purchased from ProteinTech Co., Ltd. (Wuhan, Hubei, China). The β-catenin antibody, Cyclin D1 antibody and c-Myc antibody were purchased from Cell Signaling Technology Co., Ltd. (Danvers, MA, USA). The signals were then revealed after incubation with the recommended secondary antibodies using ECL chromogenic substrate (Beyotime, Shanghai, China) and an Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, USA).

MTT Assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Proliferation Assay Kit (GeFan Bio, Pudong, Shanghai, China) in accordance with the manufacturer's protocols. Briefly, after resuspension of EC9706 or EC-1 cells, we diluted the cells to 1×10^4 cells/ml, and then added 100 µl the cell suspension into each well containing 1 ml medium. Then, we grow the cells in 96-well plates, followed by adding 20 µl MTT solution (0.5 mg/ml) after transfection of plasmids at 24 h, 48 h, 72 h and 96 h, and then incubated for an additional 4 h at 37°C. Thereafter, the medium was replaced by 100 µl dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). After incubation for 10-15 min, the absorbance at the wavelength of

490 nm were then detected by a microplate reader (Molecular Devices Spectra MAX 190, Sunnyvale, CA, USA).

Colony Formation Assay

EC9706 or EC-1 cells transfected with plasmids were seeded in 6-well plates (Corning, NY, USA) at a density of 500 cells per well and maintained for 10-14 days for the cell colony formation. Afterwards, the cell colonies were fixed with methanol and stained with 0.1% crystal violet (Yesen, Pudong, Shanghai, China). Formed colonies containing more than 50 cells were counted using a microscope (DMI6000B, Leica, Shenzhen, Guangdong, China) and recorded for statistical analysis.

Apoptosis Assay

Apoptosis assay was conducted using an Annexin-V/propidium iodide (PI) Apoptosis Assay Kit (KeyGEN BioTech, Jiangning, Nanjing, China) according to the manufacturer's instructions. In brief, EC9706 or EC-1 cells were harvested using trypsin and washed twice with ice-cold phosphate-buffered saline (PBS). Next, the cells were resuspended in 100 µl binding buffer. Subsequently, the cells were stained with 5 µl Annexin V/FITC followed by 5 µl PI in the dark for 15 min at the room temperature. Finally, the number of apoptotic cells was determined by FACS Calibur flow cytometry system (BD Biosciences, Franklin Lakes, NJ, USA) and the data were analyzed using FlowJo-V10 software (FlowJo, Ashland, OR, USA).

Wound-Healing Assay

The wound healing assays were carried out to assess the effects of LINC00675 on cell migration of EC9706 and EC-1 cells, using a 35 mm high culture µ-dish with culture insert. Cell suspensions (70 µl, 5×10^5 cells/ml) of EC9706 or EC-1 cells were then seeded into the chambers of the culture dish insert. After 24 h, the insert was gently removed and fresh culture medium was

added into the dish to start the migration process. The images of wounded areas were taken by use of a microscope (DMI6000B, Leica, Shenzhen, Guangdong, China) at 0 h and 18 h.

Transwell Invasion Assay

The invasive abilities of EC9706 and EC-1 cells were evaluated using matrigel-coated transwell chambers (8 μ m pore; Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Briefly, EC9706 or EC-1 cells were detached by trypsinization and resuspended in medium without FBS. Thereafter, 200 μ l EC9706 or EC-1 cells (2.5×10^5 cells) were seeded into the upper chamber, and the medium containing 15% FBS were added into the lower chamber. The cells were then incubated for 24 h. After that, the non-invaded cells were removed using a cotton swab from the upper surface of the membrane, the invasive cells on the lower surface of the membrane were fixed using 4% paraformaldehyde (Aladdin, Pudong, Shanghai, China) and stained with 0.3% crystal violet solution (Solarbio, Tongzhou, Beijing, China). The cells were then washed twice with PBS and the images were captured using a microscopy (DMI6000B, Leica, Shenzhen, Guangdong, China).

TOPFlash Luciferase Assay

The TOPFlash luciferase assays were utilized to detect the activity of Wnt/ β -catenin signaling. TOPFlash reporter plasmid and FOPFlash reporter plasmid were purchased from Addgene (Cambridge, MA, USA). In brief, EC9706 cells were seeded in 24-well plates and co-transfected with the TOPFlash reporter plasmid, FOPFlash

reporter plasmid as well as Renilla TK-luciferase vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Luciferase activity was evaluated using the dual luciferase reporter assay kit (Promega, Madison, WI, USA).

Statistical Analysis

All statistical analyses were performed using SPSS version 18.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (Graphpad Software Inc, La Jolla, CA, USA). Data were expressed as mean \pm the standard error of the mean (SEM). Comparison of continuous data was analyzed using an independent *t*-test between the two groups. The Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. Categorical data were analyzed by the chi-square test. Survival curves were obtained by using the Kaplan-Meier method and compared by using the log-rank test. Multivariate analysis of the prognostic factors was performed with Cox regression model. A *p*-value of less than 0.05 was considered statistically significant.

Results

LINC00675 Was Down-Regulated in ESCC Tissues and Correlated With Prognosis

In order to explore the potential role of LINC00675 in progression of ESCC, we firstly performed RT-PCR to investigate whether LINC00675 was abnormally expressed in ESCC. As shown in Figure 1A, we found that the expression levels of LINC00675 were significantly down-regulated

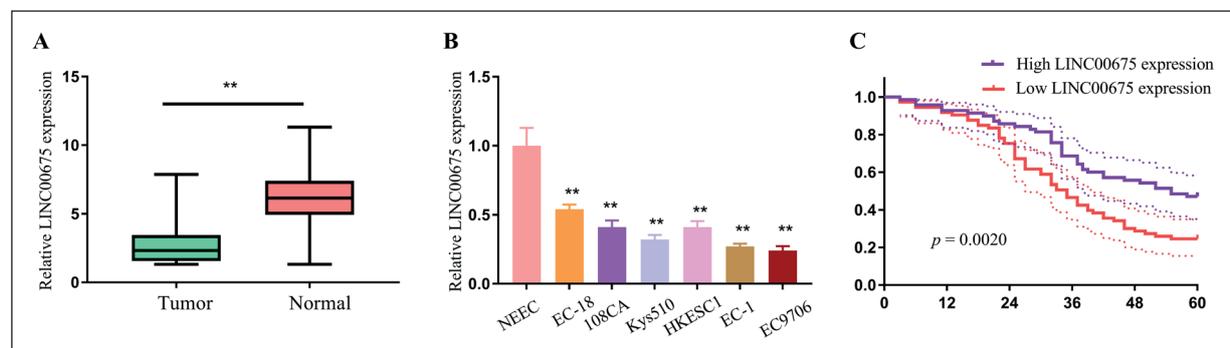


Figure 1. Expression levels of LINC00675 in ESCC and its prognostic value. (A) The relative levels of LINC00675 in 143 paired of ESCC samples were measured by real-time quantitative RT-PCR, and the GAPDH was used as an internal control. (B) Relative expression of LINC00675 between six ESCC cell lines (EC-18, 108CA, Kys510, HKESC1, EC-1, EC9706) and normal esophageal epithelial cells (NEEC). (C) Kaplan-Meier method with the log-rank test was used to analyze the overall survival curves of patients in high and low LINC00675 expression groups. **p* < 0.05, ***p* < 0.01.

Table II. Association of LINC00675 with clinicopathological characteristics of ESCC patients.

Parameters	Group	Total	LINC00675 expression		<i>p</i> -value
			High	Low	
Gender	Male	71	31	40	NS
	Female	72	39	33	
Age (years)	< 60	76	34	42	NS
	≥ 60	67	36	31	
Tumor size (cm)	< 4	81	44	37	NS
	≥ 4	52	26	36	
Tumor location	Upper	25	10	15	NS
	Middle	75	41	43	
	Lower	43	19	24	
Histological grade	G1	89	50	39	0.038
	G2+G3	54	20	34	
Lymph nodes metastasis	Absence	85	49	36	0.012
	Presence	58	21	37	
Clinical stage	I-II	81	48	33	0.007
	III-IV	62	22	40	

in ESCC tissues compared to matched normal tissues ($p < 0.01$, Figure 1B). In addition, we also confirmed that down-regulation of LINC00675 was significantly observed in six ESCC cell lines compared to NEEC. Then, we further explore the clinical significance of LINC00675 in ESCC patients. All ESCC samples were classified into low LINC00675 expression group ($n = 73$) and the high LINC00675 expression group ($n = 70$) according to the median LINC00675 expression level of all ESCC samples. As shown in Table II, the results of clinical assay indicated that low expression of LINC00675 was significantly associated with histological grade ($p = 0.038$), lymph nodes metastasis ($p = 0.012$) and clinical stage ($p = 0.007$). However, no significant difference was observed between LINC00675 expression and other clinical features such as patients' age, gender, tumor size and tumor location ($p > 0.05$). Furthermore, we performed Kaplan-Meier assay to explore the ef-

fect of LINC00675 on prognosis of ESCC patients. As shown in Figure 1C, the results showed that the 5-year overall survival of low LINC00675 expression group was significantly shorter than that of high LINC00675 expression group ($p = 0.002$). Of note, in a multivariate Cox model, low LINC00675 expression was confirmed to be an independent prognostic biomarker in ESCC patients ($p < 0.05$, Table III), indicating that low LINC00675 level was a promising biomarker for prognosis of patients with ESCC. Taken together, our results firstly revealed that LINC00675 may be involved in the clinical progression of ESCC patients.

Enhancing Expression of LINC00675 Inhibited ESCC Cell Proliferation and Accelerated Cell Apoptosis

To explore the effects of LINC00675 on the development and progression of ESCC *in vitro*, we first constructed the overexpression plasmid

Table III. Prognostic factors in Cox proportional hazards model.

Variable	Univariate analysis			Multivariate analysis		
	RR	95% CI	<i>p</i>	RR	95% CI	<i>p</i>
Gender	1.563	0.744-2.134	0.166	-	-	-
Age	1.375	0.846-2.316	0.236	-	-	-
Tumor size	1.778	0.549-2.348	0.115	-	-	-
Histological grade	3.548	1.452-5.437	0.005	2.895	1.123-4.354	0.017
Lymph nodes metastasis	3.784	1.895-6.448	0.001	3.016	1.137-4.774	0.009
Clinical stage	3.498	1.327-5.299	0.003	3.127	1.184-4.679	0.006
LINC00675 expression	4.213	1.958-7.449	0.001	3.264	1.253-5.572	0.005

vectors of LINC00675, which could lead to a constitutive expression of LINC00675 after transfecting it in ESCC cells. As showed in Figure 2A, transfection of LINC00675-overexpressing plasmids resulted significantly increase in the relative expression levels in EC9706 and EC-1 cells. We next performed the MTT assays to evaluate the effects of enhancing LINC00675 expression on the proliferative rates of ESCC cells. The data revealed that ectopic expression of LINC00675 remarkably depressed the proliferation of EC9706 and EC-1 cells compared with the control cells (Figure 2B and C). In addition, the colony formation assays demonstrated that forcing the expression of LINC00675 caused a notable decrease in the colony number of EC9706 and EC-1 cells, which was in accordance with the results of MTT assays (Figure 2D and E). Moreover, the apoptosis of ESCC cells was also detected using flow cytometry. The data showed that overexpression of LINC00675 led to a re-

markable increase of apoptotic EC9706 and EC-1 cells (Figure 2F and G). Overall, our data demonstrated that ectopic expression of LINC00675 suppressed the proliferation of ESCC cells and promoted cell apoptosis, which indicated that LINC00675 might serve as a tumor suppresser in the development of ESCC.

Ectopic Expression of LINC00675 Impaired the Migration and Invasion of ESCC Cells

We next investigated whether overexpression of LINC00675 could affect the migratory and invasive abilities of ESCC. Hence, the wound healing assays and transwell invasion assays were carried out in EC9706 and EC-1 cells. The results of wound healing assays uncovered that ectopic expression of LINC00675 resulted in a significant reduction of migratory capabilities of EC9706 and EC-1 cells, compared with the negative control (NC) cells transfected with empty plasmid

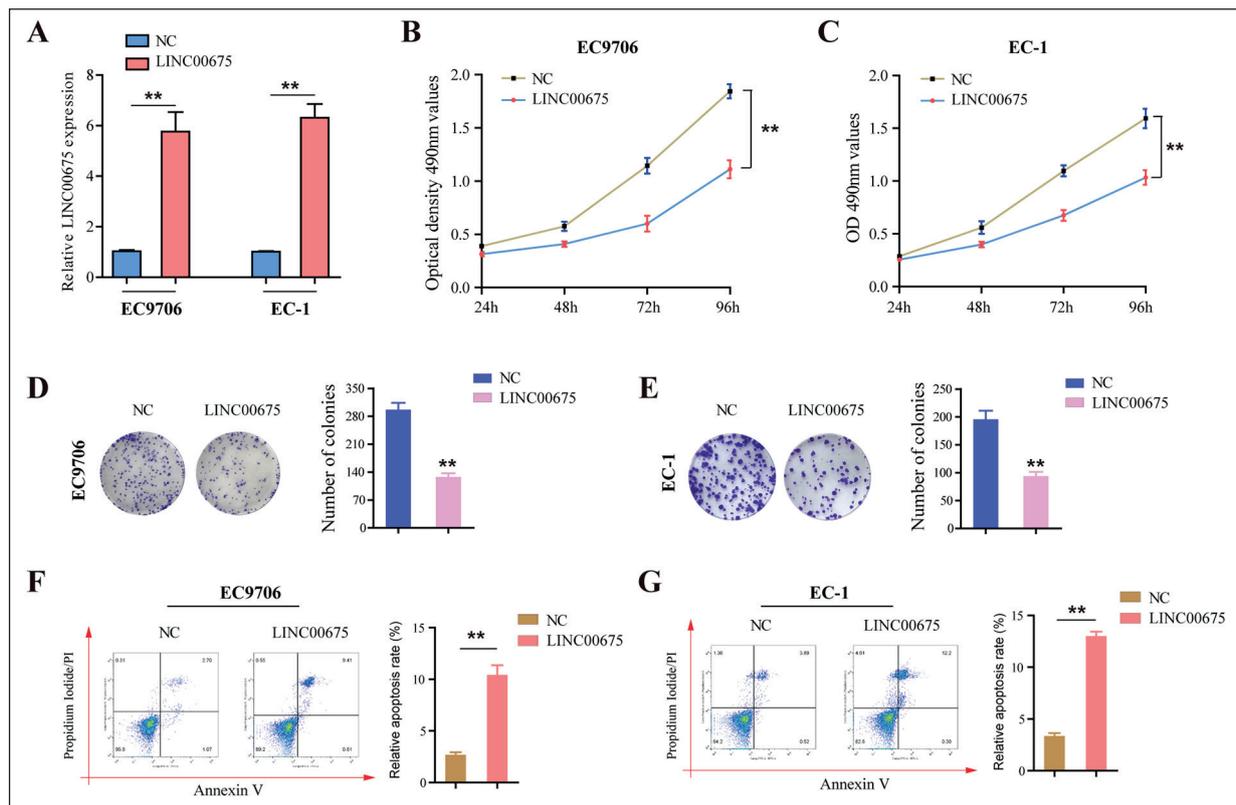


Figure 2. The effects of LINC00675 on the proliferation and apoptosis of EC9706 and EC-1 cells. (A) Relative expression levels of LINC00675 in EC9706 or EC-1 cells when transfected with negative control empty pCDNA3.1 plasmids (NC) or LINC00675 overexpression plasmids pCDNA3.1-LINC00675 (LINC00675). (B and C) Overexpression of LINC00675 inhibited the proliferation of EC9706 or EC-1 cells through MTT assays detection. (D and E) Colony formation assays indicated that LINC00675-overexpressing EC9706 or EC-1 cells declined the capacities of colony formation compared with the parallel control cells. (F and G) Flow cytometry analysis was carried out to detect the apoptotic rates of EC9706 or EC-1 cells transfected with NC plasmids or LINC00675-overexpressing plasmids. * $p < 0.05$, ** $p < 0.01$.

vectors (Figure 3A and B). Additionally, transwell invasion assays suggested that the invasive abilities of EC9706 and EC-1 cells were dramatically reduced when LINC00675 was overexpressed (Figure 3C and D). Besides, the molecular mechanisms underlying the effects of LINC00675 on migration and invasion of EC9706 and EC-1 cells were further investigated using Western blot assays. The results revealed that the protein expression levels of epithelial-mesenchymal transition (EMT) associated markers, N-cadherin and vimentin, were both markedly decreased in EC9706 and EC-1 cells (Figure 3E and F). Taken together, these data suggested that transfection

of LINC00675-overexpressing plasmids declined the migratory and invasive abilities of ESCC cells via modulating the EMT related molecules.

Overexpression of LINC00675 Abrogated the Wnt/ β -Catenin Signaling Pathway in ESCC Cells

Given that enhancing LINC00675 expression attenuated the development and metastasis of ESCC cells, we next focused on the alterations of Wnt/ β -catenin signaling pathway after EC9706 and EC-1 cells were transfected with LINC00675-overexpressing plasmids. Firstly, we performed TOP/FOP flash reporter assays

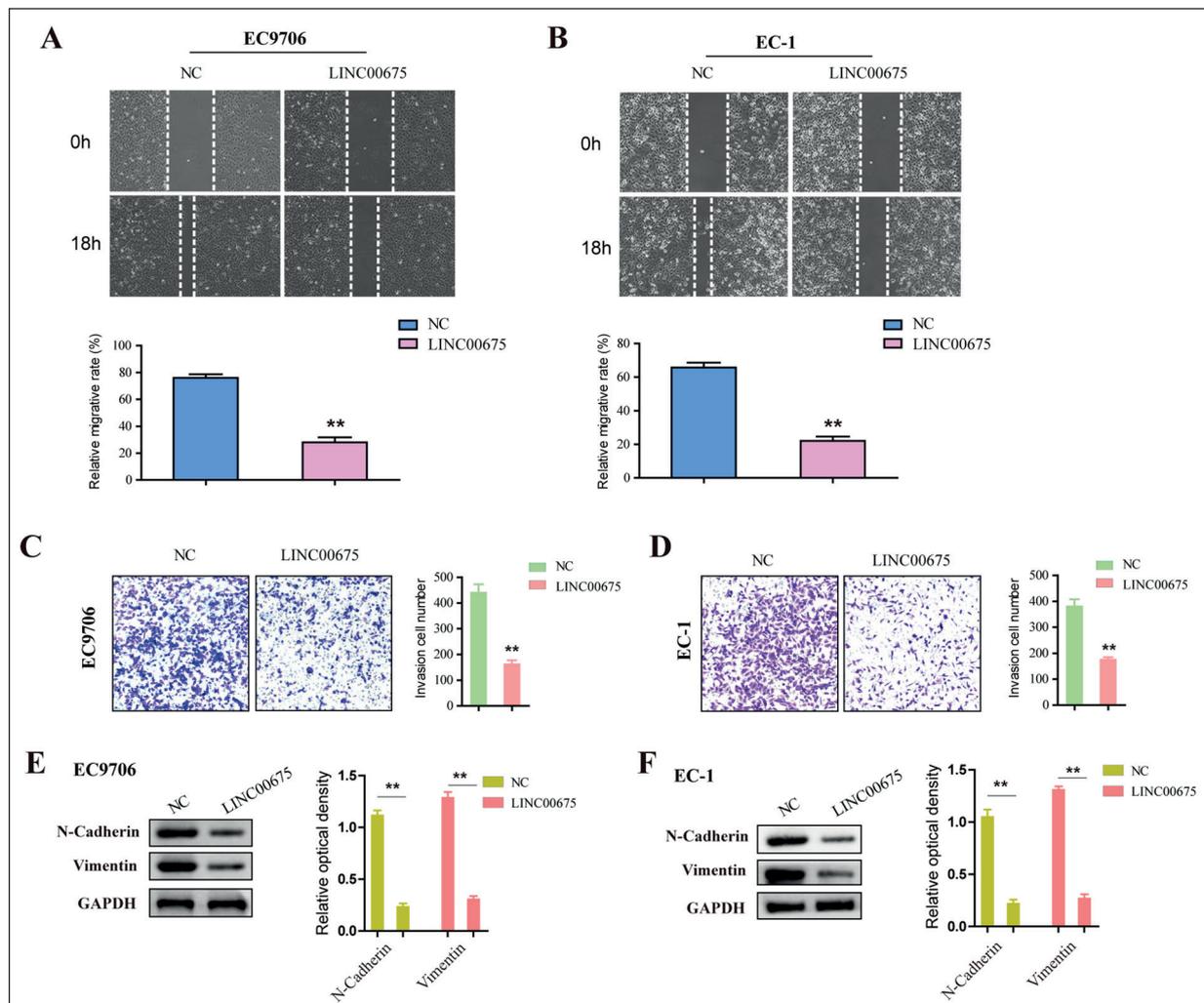


Figure 3. Overexpression of LINC00675 suppressed the migration and invasion of EC9706 and EC-1 cells. (A and B) Representative images of the wound healing assays suggested that overexpression of LINC00675 inhibited the migratory abilities of EC9706 and EC-1 cells. (C and D) Transwell assays were conducted to assess the invasive abilities of EC9706 and EC-1 cells. (E and F) Western blot assays detected the protein levels of N-cadherin and vimentin in EC9706 and EC-1 cells. * $p < 0.05$, ** $p < 0.01$.

using EC9706 cells. The results suggested that ectopic expression of LINC00675 caused a significant reduction of the relative luciferase activities of EC9706 cells compared with that of the control (Figure 4A). Furthermore, Western blot assays revealed overexpression of LINC00675 led to a notably decrease of the protein levels of β -catenin and a few of the downstream genes involved in the Wnt/ β -catenin signaling such as, cyclin D1 and c-myc, whereas the expression of E-cadherin was increased in ESCC cells (Figure 4B). Thus, our data provided evidence that enhancing the expression of LINC00675 inhibited the activities of Wnt/ β -catenin signaling pathway in ESCC cells.

Discussion

ESCC is one of the major challenges in clinical practice and is usually determined by clinical diagnosis in the middle-late period, delaying timely treatment and leading to poor outcomes^{23,24}. It is necessary for us to find a useful biomarker of ESCC to predict prognosis. In this study, we firstly reported that LINC00675 expression was significantly downregulated in both ESCC tissues and cell lines, indicating that LINC00675 may be involved in the progression of ESCC. Then, we evaluate the correlations between LINC00675 expression and clinicopathologic features, finding that low LINC00675 expression was significantly associated with histological grade, lymph nodes metastasis and clinical stage. Moreover, the results of Kaplan-Meier method confirmed that down-regulation of LINC00675 was significantly associated with five-year overall survival of ESCC patients, indicating that dysregulation of

LINC00675 may affect the prognosis of ESCC patients. Of note, to further explore whether LINC00675 serves as an independent molecular indicator for prognosis of ESCC patients, we performed the Cox proportional hazards regression model and the results confirmed our hypothesis. To our best knowledge, this is the first time the expression pattern and clinical significance of LINC00675 in ESCC have been reported. Dysregulation of LINC00675 was involved in development and progression of various tumors. Shan et al²¹ reported that LINC00675 was highly expressed in colorectal cancer and its overexpression could suppresses cell proliferation and metastasis in colorectal cancer via acting on miR-942 and Wnt/ β -catenin signaling. Zeng et al²⁵ found that up-regulation of LINC00675 was significant in gastric cancer and associated with the poor survival of gastric cancer patients. Furthermore, they observed that forced LINC00675 expression represses gastric cancer cell proliferation, migration and invasion by enhances phosphorylation of vimentin on Ser83. Li et al²⁰ also showed that down-regulation of LINC00675 was associated with poor prognosis of pancreatic ductal adenocarcinoma patients and attenuated pancreatic cancer cell proliferation and invasion. Interestingly, all those findings indicated that LINC00675 was a down-regulated lncRNA and served as a tumor suppressor in cancers *in vitro* and *in vivo*. In this study, we found that LINC00675 was lowly expressed in ESCC. Thus, we wondered whether LINC00675 may act as a positive regulator in progression of ESCC. Next, we up-regulated LINC00675 levels by pCDNA3.1-LINC00675 and performed *in vitro* experiment, finding that its LINC00675 significantly suppressed ESCC cells proliferation and

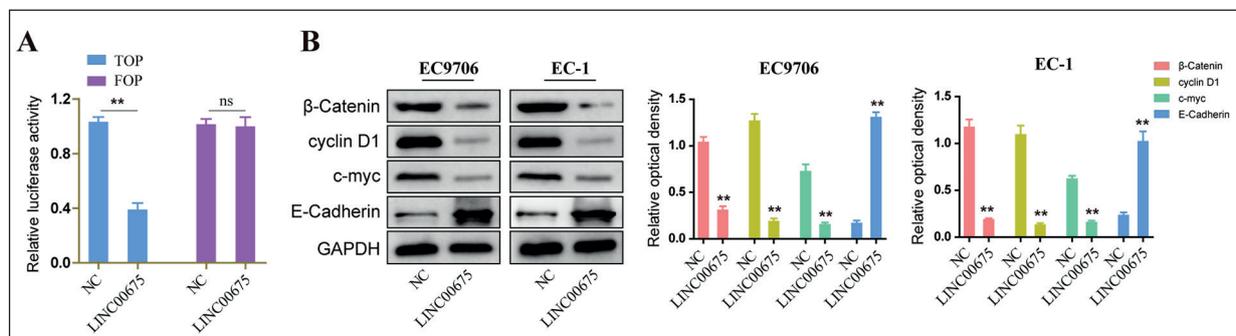


Figure 4. The effects of LINC00675 on the activities of Wnt/ β -catenin signaling pathway in EC9706 and EC-1 cells. (A) Dual luciferase reporter assays revealed that ectopic expression of LINC00675 significantly inhibited Wnt/ β -catenin signaling in EC9706 cells. (B) Western blot assays were performed to determine the protein levels of β -catenin, cyclin D1, c-myc and E-cadherin in EC9706 and EC-1 cells. * $p < 0.05$, ** $p < 0.01$.

induce apoptosis. Moreover, it was also found that forced LINC00675 expression suppressed ESCC cells migration and invasion. More importantly, in order to explore the potential mechanism by which LINC00675 exerted anti-oncogenic effects in ESCC, we further detected the expression levels of EMT-related proteins, finding that overexpression of LINC00675 suppressed EMT progression. Taken together, our study firstly revealed that LINC00675 served as a tumor suppressor in ESCC. Wnt/ β -catenin pathway, also called canonical Wnt pathway, is crucial to embryo development and adult tissue homeostasis²⁶. Evidence indicates that Wnt/ β -catenin signaling pathway regulates the crucial aspects of cellular processes, such as, cell migration, cell polarity and primary axis formation^{27,28}. In cancer research, it is showed that constitutive activation of the Wnt/ β -catenin signaling pathway is observed in almost various human malignancy, and is thus an important target for tumor treatment^{29,30}. Recently, more and more lncRNAs were reported to exerted anti-oncogenic or oncogenic effects by modulating Wnt/ β -catenin signaling pathway³¹⁻³³. To investigate the correlation between LINC00675 and Wnt/ β -catenin signaling pathway, we detected the proteins level of β -catenin, Cyclin D1, and c-Myc in ESCC cells were transfected with pCDNA3.1-LINC00675 and found that protein level of above factors remarkably reduced, indicating LINC00675 may suppress progression of Wnt/ β -catenin signaling pathway. Taken together, these experiments showed that LINC00675 served as a suppressor lncRNA by inhibiting Wnt/ β -catenin pathway in ESCC.

Conclusions

We revealed that LINC00675 was an anti-metastatic factor in ESCC. LINC00675 was lowly expressed in ESCC and acted as an independent prognostic factor for patients with ESCC. Moreover, we found that LINC00675 may suppress ESCC proliferation, migration and invasion by suppressing Wnt/ β -catenin pathway. Our results provide further insight into the role of lncRNAs in ESCC. Further investigations on the underlying mechanism are needed.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

This study was funded by Medical Scientific Research Foundation of Shenzhen Health and Family Planning Commission (No. 201601012).

References

- 1) SIEGEL RL, MILLER KD, JEMAL A. Cancer statistics, 2015. *CA Cancer J Clin* 2015; 65: 5-29.
- 2) TORRE LA, BRAY F, SIEGEL RL, FERLAY J, LORTET-TIEULENT J, JEMAL A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; 65: 87-108.
- 3) DOMPER ARNAL MJ, FERRANDEZ ARENAS A, LANAS ARBELLOA A. Esophageal cancer: Risk factors, screening and endoscopic treatment in Western and Eastern countries. *World J Gastroenterol* 2015; 21: 7933-7943.
- 4) CHEN W, ZHENG R, BAADE PD, ZHANG S, ZENG H, BRAY F, JEMAL A, YU XQ, HE J. Cancer statistics in China, 2015. *CA Cancer J Clin* 2016; 66: 115-132.
- 5) KATO H, NAKAJIMA M. Treatments for esophageal cancer: a review. *Gen Thorac Cardiovasc Surg* 2013; 61: 330-335.
- 6) PAUL S, ALTORKI N. Outcomes in the management of esophageal cancer. *J Surg Oncol* 2014; 110: 599-610.
- 7) BELKHIRI A, EL-RIFAI W. Advances in targeted therapies and new promising targets in esophageal cancer. *Oncotarget* 2015; 6: 1348-1358.
- 8) MILLER AD. Delivering the promise of small ncRNA therapeutics. *Ther Deliv* 2014; 5: 569-589.
- 9) BALLANTYNE MD, McDONALD RA, BAKER AH. lncRNA/MicroRNA interactions in the vasculature. *Clin Pharmacol Ther* 2016; 99: 494-501.
- 10) BASSETT AR, AKHTAR A, BARLOW DP, BIRD AP, BROCKDORFF N, DUBOULE D, EPHRUSSI A, FERGUSON-SMITH AC, GINGERAS TR, HAERTY W, HIGGS DR, MISKA EA, PONTING CP. Considerations when investigating lncRNA function in vivo. *Elife* 2014; 3: e03058.
- 11) JOHANSSON P, LIPOVICH L, GRANDER D, MORRIS KV. Evolutionary conservation of long non-coding RNAs; sequence, structure, function. *Biochim Biophys Acta* 2014; 1840: 1063-1071.
- 12) LI X, WU Z, FU X, HAN W. lncRNAs: insights into their function and mechanics in underlying disorders. *Mutat Res Rev Mutat Res* 2014; 762: 1-21.
- 13) KHORKOVA O, HSIAO J, WAHLESTEDT C. Basic biology and therapeutic implications of lncRNA. *Adv Drug Deliv Rev* 2015; 87: 15-24.
- 14) YANG X, ZHANG W, CHENG SQ, YANG RL. High expression of lncRNA GACAT3 inhibits invasion and metastasis of non-small cell lung cancer to enhance the effect of radiotherapy. *Eur Rev Med Pharmacol Sci* 2018; 22: 1315-1322.
- 15) MALHOTRA S, FREEBERG MA, WINANS SJ, TAYLOR J, BEEMON KL. A novel long non-coding RNA in the hTERT promoter region regulates hTERT expression. *Noncoding RNA* 2017; 4(1). pii: E1. doi: 10.3390/ncrna4010001.

- 16) GRADIA DF, MATHIAS C, COUTINHO R, CAVALLI IJ, RIBEIRO E, DE OLIVEIRA JC. Long non-coding RNA TUG1 expression is associated with different subtypes in human breast cancer. *Noncoding RNA* 2017; 3: E26.
- 17) MA DD, YUAN LL, LIN LQ. LncRNA HOTAIR contributes to the tumorigenesis of nasopharyngeal carcinoma via up-regulating FASN. *Eur Rev Med Pharmacol Sci* 2017; 21: 5143-5152.
- 18) ZHANG A, XU M, MO YY. Role of the lncRNA-p53 regulatory network in cancer. *J Mol Cell Biol* 2014; 6: 181-191.
- 19) REN ZP, CHU XY, XUE ZQ, ZHANG LB, WEN JX, DENG JQ, HOU XB. Down-regulation of lncRNA MIR31HG correlated with aggressive clinicopathological features and unfavorable prognosis in esophageal squamous cell carcinoma. *Eur Rev Med Pharmacol Sci* 2017; 21: 3866-3870.
- 20) LI DD, FU ZQ, LIN Q, ZHOU Y, ZHOU QB, LI ZH, TAN LP, CHEN RF, LIU YM. Linc00675 is a novel marker of short survival and recurrence in patients with pancreatic ductal adenocarcinoma. *World J Gastroenterol* 2015; 21: 9348-9357.
- 21) SHAN Z, AN N, QIN J, YANG J, SUN H, YANG W. Long non-coding RNA Linc00675 suppresses cell proliferation and metastasis in colorectal cancer via acting on miR-942 and Wnt/ β -catenin signaling. *Biomed Pharmacother* 2018; 101: 769-776.
- 22) LI Z, LI Y, WANG Q. LINC00675 is a prognostic factor and regulates cell proliferation, migration and invasion in glioma. *Biosci Rep* 2018; 38(5). pii: BSR20181039.
- 23) WON E, ILSON DH. Management of localized esophageal cancer in the older patient. *Oncologist* 2014; 19: 367-374.
- 24) LIN D, LEICHMAN L. The current status of neoadjuvant therapy for esophageal cancer. *Semin Thorac Cardiovasc Surg* 2014; 26: 102-109.
- 25) ZENG S, XIE X, XIAO YF, TANG B, HU CJ, WANG SM, WU YY, DONG H, LI BS, YANG SM. Long noncoding RNA LINC00675 enhances phosphorylation of vimentin on Ser83 to suppress gastric cancer progression. *Cancer Lett* 2018; 412: 179-187.
- 26) RING A, KIM YM, KAHN M. Wnt/ β -catenin signaling in adult stem cell physiology and disease. *Stem Cell Rev* 2014; 10: 512-525.
- 27) LIU L, WAN W, XIA S, KALIONIS B, LI Y. Dysfunctional Wnt/ β -catenin signaling contributes to blood-brain barrier breakdown in Alzheimer's disease. *Neurochem Int* 2014; 75: 19-25.
- 28) ZWEZDARYK KJ, COMBS JA, MORRIS CA, SULLIVAN DE. Regulation of Wnt/ β -catenin signaling by herpesviruses. *World J Virol* 2016; 5: 144-154.
- 29) SEBIO A, KAHN M, LENZ HJ. The potential of targeting Wnt/ β -catenin in colon cancer. *Expert Opin Ther Targets* 2014; 18: 611-615.
- 30) MACDONALD BT, TAMAI K, HE X. Wnt/ β -catenin signaling: components, mechanisms, and diseases. *Dev Cell* 2009; 17: 9-26.
- 31) LUO M, LI Z, WANG W, ZENG Y, LIU Z, QIU J. Long non-coding RNA H19 increases bladder cancer metastasis by associating with EZH2 and inhibiting E-cadherin expression. *Cancer Lett* 2013; 333: 213-221.
- 32) HAN GH, LU KJ, WANG P, YE J, YE YY, HUANG JX. LncRNA SNHG16 predicts poor prognosis in ESCC and promotes cell proliferation and invasion by regulating Wnt/ β -catenin signaling pathway. *Eur Rev Med Pharmacol Sci* 2018; 22: 3795-3803.
- 33) WANG X, GAO Z, LIAO J, SHANG M, LI X, YIN L, PU Y, LIU R. lncRNA UCA1 inhibits esophageal squamous-cell carcinoma growth by regulating the Wnt signaling pathway. *J Toxicol Environ Health A* 2016; 79: 407-418.