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Original Article

Long non-coding RNA LINC00707, a prognostic marker, regulates cell proliferation, apoptosis, and EMT in esophageal squamous cell carcinoma

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Abstract: Background: Long intergenic non-protein coding RNA 707 (LINC00707) has been identified as a cancer-associated long non-coding RNA (lncRNA) in a variety of cancers. However, the functions and molecular mechanisms of LINC00707 in esophageal squamous cell carcinoma (ESCC) are still unclear. Methods: The expression of LINC00707 in esophageal cancer (ESCA) and ESCC tissues was determined by online tools, RNA-sequence (RNA-seq) dataset, and quantitative real time polymerase chain reaction (qRT-PCR). The associations between LINC00707 expression and clinicopathologic features and prognosis were investigated. Furthermore, the expression of LINC00707 in ESCC cell lines was determined by qRT-PCR. Then, using LncACTdb 2.0 database, combined with loss-of-function assay verification, we investigated the biologic role of LINC00707 in ESCC cell growth, apoptosis, invasion, and migration by CCK-8, colony formation, flow cytometry and transwell assays. Finally, western blot was used to evaluate the regulatory effect of LINC00707 on PI3K/Akt signaling pathway. Results: Increased LINC00707 expression was exhibited in ESCC tissues and cell lines. High expression of LINC00707 was positively associated with higher tumor-node-metastasis (TNM) stage and lymph node metastasis. Furthermore, LINC00707 expression was significantly higher in patients who drink alcohol, have lymph node metastasis, and harbor higher tumor stage. In addition, Kaplan-Meier survival analysis and receiver operating characteristic (ROC) curve confirmed the feasibility of LINC00707 as a prognostic signature or diagnostic marker. Functional experiments showed that LINC00707 downregulation suppressed ESCC cell proliferation, and metastasis, and induced ESCC cell apoptosis. Mechanistic investigation demonstrated that LINC00707 activated the PI3K/Akt signaling pathway in ESCC cells. Conclusions: Our findings suggest LINC00707 functions as an oncogenic lncRNA in ESCC, and imply that LINC00707 may be a promising prognostic biomarker and therapeutic target for ESCC patients.

Keywords: LINC00707, ESCC, prognosis, proliferation, metastasis

Introduction

Esophageal cancer (ESCA) is one of the most common malignant tumors in the digestive system globally [1] with a poor prognosis [2]. The 5-year survival rate after treatment for early ESCA is 90%, while the 5-year survival rate of patients for middle or late stage is only 6%~15% [3]. ESCA is mainly divided into esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (ECA) according to histologic type, of which more than 90% cases are ESCC [4, 5]. ECA is the predominant type of ESCA in North America and Europe whereas ESCC mainly occurs in Asia, especially in China [6]. Because the clinical symptoms of early ESCC patients are not obvious and can be easily ignored by patients, most ESCC patients in China are diagnosed at advanced stages [7]. As a result, the 5-year survival rate of ESCC patient is still quite poor [8, 9]. Therefore, it is urgent to investigate the molecular mechanisms underpinning the initiation and progression of ESCC and develop better therapeutic modalities for the treatment of ESCC patients.

With the widespread application of next-generation sequencing (NGS) technology, many non-coding genes, especially long non-coding RNAs (lncRNAs), have been identified [10] and found to be closely related to the development and progression of a variety of cancers [11]. LncRNAs, more than 200 nucleotides in length, lack protein coding ability [12, 13]. LncRNAs are abnormally expressed and are involved in cell survival, growth [14, 15], metastasis [16, 17], and maintenance of stemness [18] in several cancers, including ESCC. Therefore, it is essential to uncover the mechanism of cancer from the perspective of lncRNAs, which will be expected to become a new breakthrough in battling cancers including ESCC.

Long intergenic non-protein coding RNA 707 (LINC00707) with a length of 3097 bp is located on chromosome 10p14. Studies have demonstrated that LINC00707 is a promising cancer-associated lncRNA, mainly functions as an oncogene by promoting cell proliferation, invasion, migration, chemotherapy resistance and inhibiting cell apoptosis in hepatocellular carcinoma [19, 20], breast cancer [21, 22], colorectal cancer [23-25], gastric cancer [26], lung adenocarcinoma [27], clear cell renal cell carcinoma [28], and nonsmallcell lung cancer [29]. Notably, LINC00707 performs multiple biologic functions many different ways, including acting as a competitive endogenous RNA [20-25, 29], activating signaling pathway [19], and interacting with mRNA stabilizing protein Human antigen R (HuR) [26]. However, to date, the functions and molecular mechanisms of LINC00707 in ESCC are still unclear.

In the present study, we used the open-access website to explore the expression of LINC00707 in ESCA. RNA-sequence (RNA-seq) dataset (n = 68) and another independent 47 ESCC tissues for quantitative real time polymerase chain reaction (qRT-PCR) validation were used to determine the expression of LINC00707 in ESCC tissues. Furthermore, the associations of LINC00707 expression with clinicopathologic features, prognosis, and diagnosis were dissected. Then, we examined LINC00707 expression in a panel of ESCC cell lines. Using LncACTdb 2.0 database, combined with loss-of-function assays, we investigated the biological roles of LINC00707 in ESCC cell growth, apoptosis, invasion, and migration. Finally, the PI3K/Akt signaling pathway was verified by western blot. Collectively, our current data demonstrated that LINC00707 is an oncogenic lncRNA in ESCC through activating the PI3K/Akt signaling pathway, and may be a promising prognostic biomarker and therapeutic target for ESCC patients.

Materials and methods

Public databases

Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/) [30] and Starbase v3.0 (http://starbase.sysu.edu.cn/tutorialAPI.php) [31] were performed to investigate the expression of LINC00707 in ESCA samples. LncACTdb 2.0 (http://www.bio-bigdata.net/LncACTdb/) [32] was used to examine the possible biological functions of LINC00707.

Patients

68 cases of postoperative ESCC tissues and paired non-tumor tissues were collected from Anyang Tumor Hospital, a high-risk area of ESCC in China during 2014-2015, and organized relevant patient clinical information. Then, we examined the protein coding genes and lncRNA expression profiles of the 68 paired ESCC tissues by NGS [33, 34]. In addition, we collected another independent 47 ESCC tissues for qRT-PCR validation. Detailed clinicopathological characteristics of all ESCC patients in this study was shown in **Table 1**. Informed consent was obtained through the institutional review board. The study was approved by the Anyang Tumor Hospital Ethical Committee.

Cell culture

Seven human ESCC cell lines EC9706, KYSE70, KYSE150, KYSE450, Eca109, TE1 and KYSE30 were purchased from the Tumor Cell Bank of Chinese Academy of Sciences, and the normal human esophagus epithelial cell line Het-1A immortalized by introducing plasmid pRSV-T with RSV-LTR promoter and SV40 T antigen was purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were maintained in RPMI 1640 media (Biological Industries, Cromwell, CT, USA) supplemented with 10% Fetal bovine serum (FBS, Biological Industries, Cromwell, CT, USA) in humidified incubator with 5% CO_2 at 37°C.

Table 1. Clinicopathologic data in esophageal squamous cell carcinoma (ESCC) patients

	qRT-PCR		RNA-Seq	
	Numbers of patients	%	Numbers of patients	%
Total number of patients	47		68	
Gender				
Male	31	65.96	42	61.76
Female	16	34.04	26	38.24
Age (years)				
≤ 60	11	23.40	29	42.65
> 60	36	76.60	39	57.35
Smoking history				
Negative	23	48.94	36	52.94
Positive	24	51.06	32	47.06
Drinking history				
Negative	26	55.32	37	54.41
Positive	21	44.68	31	45.59
Tumor size				
≤ 4	27	57.44	35	51.47
> 4	20	42.55	33	48.53
Lymph node metastasis				
Non-metastasis	31	65.96	36	52.94
Metastasis	16	34.04	32	47.06
TNM stage				
I+II	30	63.83	39	57.35
III+IV	17	36.17	29	42.65
Tumor differentiation				
High or moderate	36	76.60	13	19.12
Low	11	23.40	55	80.88
LINC00707 expression				
Low	14	29.80	24	35.29
High	33	70.20	44	64.71

RNA isolation and qRT-PCR analyses

qRT-PCR assay was performed as described previously [35]. Briefly, total RNA was extracted from tissues or cultured cells using TRIZOL reagent (Tiangen, Beijing, China). RNA (1 μg) was reverse transcribed to cDNA in a final volume of 20 μl under standard conditions with the PrimeScript RT Reagent Kit (Takara, Dalian, China). Real-time PCR analyses were performed with SYBR Green PCR kit (Vazyme, Nanjing, China). The primer sequences used in the experiments were as follows: β-actin forward: 5'-GGAAATCGTGCGTGACATT-3', reverse: 5'-CAGGCAGCTCGTAGCTCTT-3'; LINC00707 forward: 5'-TCACATCTGTGAAAAGAGTGCT-3', reverse: 5'-CTGGACTGTGAGTACCAGGC-3'. qRT-PCR was carried out using a QuantStudio5 Sequence Detection System (Applied Biosystems). Relative fold-changes in the transcripts were calculated using the $2^{-\Delta\Delta CT}$ method. All samples were analyzed in triplicate.

RNA interference

LINC00707 small interfering RNAs (si-LINC00707#1 and si-LINC00707#2) and negative control siRNA (si-NC) were purchased from GenePharma (Shanghai, China). Cell transfection was performed using LipoRNAi (Beyotime, Shanghai, China) in EC9706 and Eca109 cells. The transfection efficiency was measured by qRT-PCR. si-LINC00707#1: 5'-CAUGACGUGAGAACUUACUAGAG-AU-3; si-LINC00707#2: 5'-UUCAGUGUUAGUCUUAUCCACCUGU-3'.

Cell counting kit-8 (CCK-8) assay

Cell proliferation was determined with the CCK-8 Cell Proliferation kit (APExBIO Technology LLC, Huston, USA) according to manufacturer's instructions. In brief, 3×10^3 cells were seeded in each well of a 96-well plate in 100 μl volume in triplicates. At 0, 24, 48, 72 and 96 hrs after transfection, 10 μl of the CCK-8 reagent was added into each well and the cells were incubated at 37°C and 5% CO_2 for 2 hrs. Absorbance at a 450 nm wavelength was measured on a microplate reader (ThermoFisher, Waltham, MA, USA).

Colony formation assay

For the colony formation assay, 5,000 transfected cells were seeded in each well of six-well plates and cultured at 37°C in an atmosphere with 5% CO_2 for 10 days, during which the medium was replaced every 3 days. After 10 days, the cells were treated with methanol to fix and stained with 0.1% crystal violet (Sigma-Aldrich). Visible colonies were then counted to evaluate the colony formation ability. A cell cluster con-

taining more than 50 cells is called a clone. Independent experiments were repeated three times.

Cell cycle and apoptosis assay

Cell cycle assay: Cells were seeded in six-well plates and synchronized at the G1/S boundary after starvation using the basal medium. After transfection, cells were fixed using 70% ethanol at 4°C overnight and stained using precooled phosphate-buffered saline (PBS) containing propidium iodide (PI, 50 mg/ml) and RNase A (20 mg/ml). A fluorescence-activated cell sorting (FACS) flow cytometer (Beckman Coulter, Danvers, MA) was used to analyze the changes in cycle distribution.

Apoptosis: After transfection, the cells were digested with EDTA-free trypsin. The cells were centrifuged at 1,000 rpm for 5 min, collected and rinsed three times with PBS. Then the cells were resuspended in 500 µl binding buffer and mixed with 5 µl of Annexin V-FITC and 5 µl of PI staining solution (KeyGEN, Nanjing, China). The cell apoptosis was examined by flow cytometry after incubation for 15 min.

Transwell migration and invasion assays

Migration and invasion were examined using the BD 24-well Transwell chamber (8 µm pores, Corning, Shanghai, China) without and with Matrigel (BD Biosciences, Bedford, MA, USA). Transfected cells (1×10^5) were seeded in serum-free medium in the top chamber of each well. The lower chamber was filled with 600 µl complete medium supplemented with 20% FBS. The cells were incubated in 37°C 5% CO_2 for 48 hrs. After incubation, the cells that migrated or invaded to the reverse side of chamber inserts were fixed with methanol for 30 min and stained with crystal violet for 30 min. The number of migrated or invasive cells was determined by counting the stained cells under a microscope. Independent experiments were repeated three times.

Western blot

Western blot assay was performed as our previous report [35]. Briefly, total proteins were lysed in buffer (Solarbio, Beijing, China). Protein concentration was determined using BCA Protein Assay Kit (Solarbio, Beijing, China) according to manufacturer's instruction. Equivalent amounts of denatured proteins (20 µg) were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with skimmed milk, the membranes were incubated at 4°C overnight with following primary antibodies: anti-β-actin (dilution 1:5,000, 20536-1-AP, Proteintech, Wuhan, China), anti-E-cadherin (dilution 1:10,000, ab40772, Abcam), anti-N-cadherin (dilution 1:5,000, ab76011, Abcam), Vimentin (dilution 1:1,000, ab92547, Abcam), Slug (dilution 1:1,000, ab51772, Abcam), anti-PI3K (dilution 1:1,000, ab32089, Abcam), anti-Akt (dilution 1:10,000, ab179463, Abcam), and anti-p-Akt (dilution 1:5,000, ab81283, Abcam). After washing with Tris-buffered saline containing Tween-20 (TBST), the membrane was incubated with a horseradish peroxidase (HRP)-labeled secondary antibody (dilution 1:5,000, SA00001-1 and SA00001-2, Proteintech, Wuhan, China) for 2 hrs at 37°C, followed by washing with TBST. The immunolabeled proteins were reacted with chemiluminescent HRP substrate (Sloarbio, Beijing, China). Quantification of western blot was performed using Image J to determine the relative protein level, and the results were from at least three independently repeated experiments.

Statistical analysis

All statistical analyses were performed using the SPSS 21.0 statistical software (SPSS, Chicago, IL, USA). For comparisons, Wilcoxon matched-pairs signed-rank sum test, Pearson Chi square test, log-rank test, unpaired Student's *t*-test, Mann-Whitney test, and one-way ANOVA test were carried as indicated. Results were considered significant when $P < 0.05$.

Results

LINC00707 expression is significantly upregulated in human ESCA and ESCC tissues

To investigate the expression pattern of LINC00707 in ESCA and ESCC tissues, first, the online tools GEPIA and Starbase v.3.0 were used to examine the expression of LINC00707 in ESCA tissues, and the results showed that the expression of LINC00707 in ESCA tissues

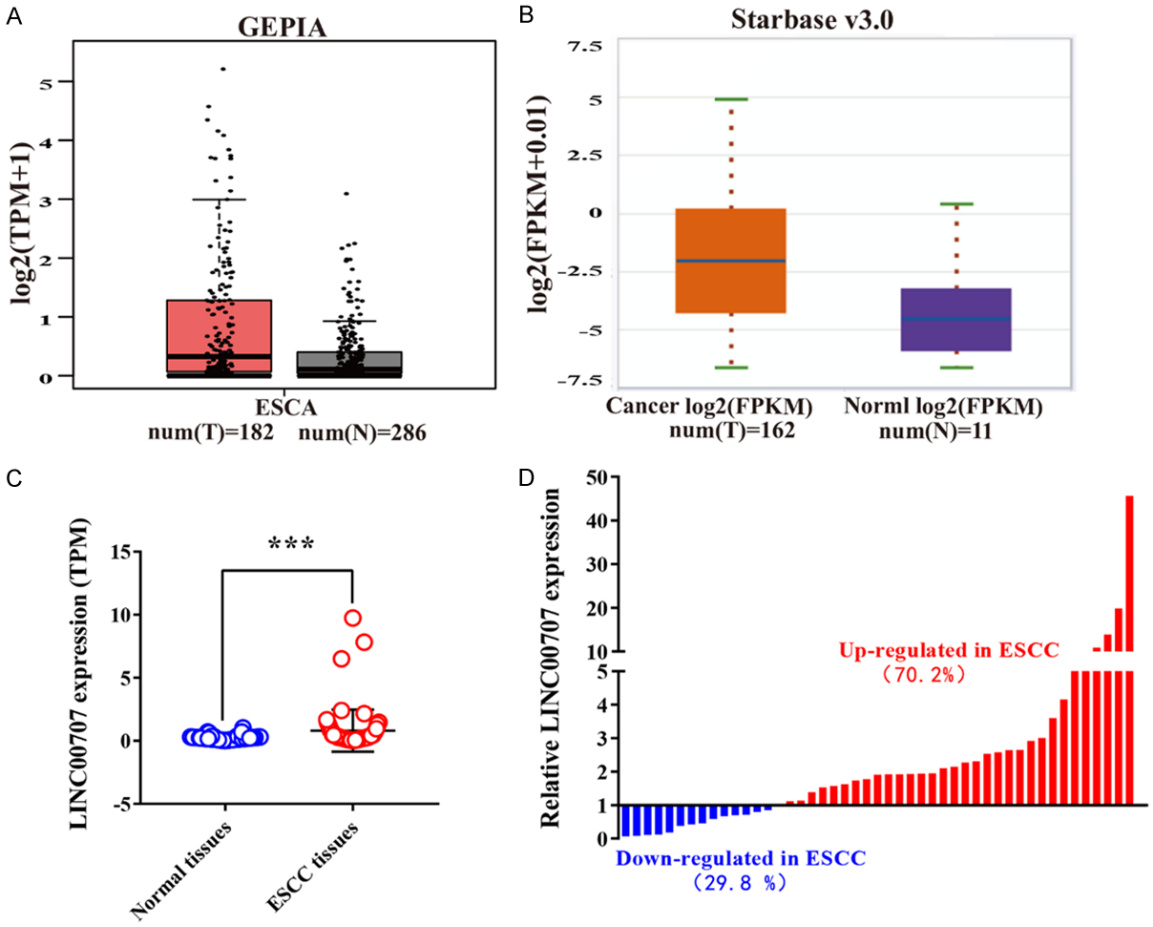

Figure 1. LINC00707 expression is significantly upregulated in human esophageal squamous cell carcinoma (ESCC) tissues. A. Upregulation of LINC00707 in esophageal cancer (ESCA) tissues (n = 182) compared to normal tissues (n = 286) from the online tool Gene Expression Profiling Interactive Analysis (GEPIA). B. Upregulation of LINC00707 in ESCA tissues (n = 162) compared to normal tissues (n = 11) from the online tool Starbase v3.0. C. LINC00707 expression level was analyzed in 68 paired human ESCC tissues and normal tissues from RNA sequencing (RNA-seq) data. Results are displayed as mean ± SD. $^{***}P < 0.001$ by Wilcoxon matched-pairs signed-rank sum test. D. LINC00707 expression was examined by quantitative real time polymerase chain reaction (qRT-PCR) and normalized to β-actin expression in 47 paired human ESCC tissues compared with corresponding non-tumor tissues.

was significantly higher than that in normal tissues (**Figure 1A** and **1B**). Subsequently, the expression of LINC00707 in ESCC was evaluated by RNA-seq (n = 68) dataset. The RNA-seq data normalized by Transcripts Per Million (TPM) revealed that LINC00707 expression was markedly increased in ESCC tissues compared with paired non-tumor tissues (**Figure 1C**), which was further validated by detecting 47 cases of human ESCC tissues and corresponding non-tumor tissues using qRT-PCR (**Figure 1D**). The data suggest that LINC00707 may function as an oncogene in ESCA and ESCC tissues.

Clinical signifcance of LINC00707 in ESCC tissues

There were a total of 115 samples with RNA-seq (n = 68) dataset and another independent 47 qRT-PCR data in this study. To determine the associations between LINC00707 expression and ESCC clinicopathologic features, ESCC patients were divided into low and high LINC0007 expression groups based on the relative expression of LINC0007 gene in tumoral tissues compared with its paired normal samples. In the RNA-seq dataset, higher LINC0007 expression was positively associated with higher TNM stage (P = 0.030) and lymph node

Table 2. Association between LINC00707 expression and clinicopathologic features in esophageal squamous cell carcinoma (ESCC) patients

	qRT-PCR				RNA-Seq			
	Low LINC00707 expression	High LINC00707 expression	X^2 value	*P* value	Low LINC00707 expression	High LINC00707 expression	X^2 value	*P* value
Gender			0.244	0.621			0.907	0.341
Male	8	23			13	29		
Female	6	10			11	15		
Age (years)			0.000	1.000			0.015	0.904
≤ 60	3	8			10	19		
> 60	11	25			14	25		
Smoking history			0.537	0.464			1.360	0.243
Negative	8	15			15	21		
Positive	6	18			9	23		
Drinking history			0.027	0.870			2.246	0.134
Negative	8	18			16	21		
Positive	6	15			8	23		
Tumor size			1.595	0.207			0.699	0.403
≤ 4	10	17			14	21		
> 4	4	16			10	23		
Lymph node metastasis			8.245	0.004			4.766	0.029
Non-metastasis	14	17			17	19		
Metastasis	0	16			7	25		
TNM stage			7.277	0.007			4.722	0.030
I+II	13	17			18	21		
III+IV	1	16			6	23		
Tumor differentiation stage			0.342	0.559			0.000	1.000
High or moderate	12	24			19	36		
Low	2	9			5	8		

We classified patients in down/up-regulation categories according to the relative expression of LINC00707 in tumoral tissue compared with its paired benign counterpart. Pearson Chi square tests were used to analyze the association between LINC-00707expression and clinical features.

metastasis (*P* = 0.029) (**Table 2**). In the 47 qRT-PCR data, higher LINC0007 expression was also positively associated with higher TNM stage (*P* = 0.007) and lymph node metastasis (*P* = 0.004) (**Table 2**). Next, we normalized LINC00707 expression using the (TPM_{tumor}/TPM_{paird}) formula and compared these values across clinicopathologic-based categories of ESCC patients in the RNA-seq dataset. LINC00707 expression in the RNA-seq dataset was significantly higher in patients with alcohol, lymph node metastasis, and higher tumor stages (**Figure 2**). In addition, LINC00707 expression in the 47 qRT-PCR data was significantly higher in patients with lymph node metastasis and higher tumor stage (**Figure 3**).

The prognostic power and predictive performance of LINC00707 in ESCC

We first evaluated the prognostic power and predictive ability of the LINC00707 in ESCC RNA-seq dataset. Kaplan-Meier survival analysis revealed that ESCC patients with higher LINC00707 expression had worse overall survival (OS) (*P* = 0.008) (**Figure 4A**). In addition, to analyze the predictive performance of the LINC0007, receiver operating characteristic (ROC) curve were performed. The Area Under Curve (AUC) of the LINC0007 was 0.645 in RNA-seq dataset (**Figure 4B**), which was validated in another independent 47 qRT-PCR data with 0.632 in AUC value (**Figure 4C**).

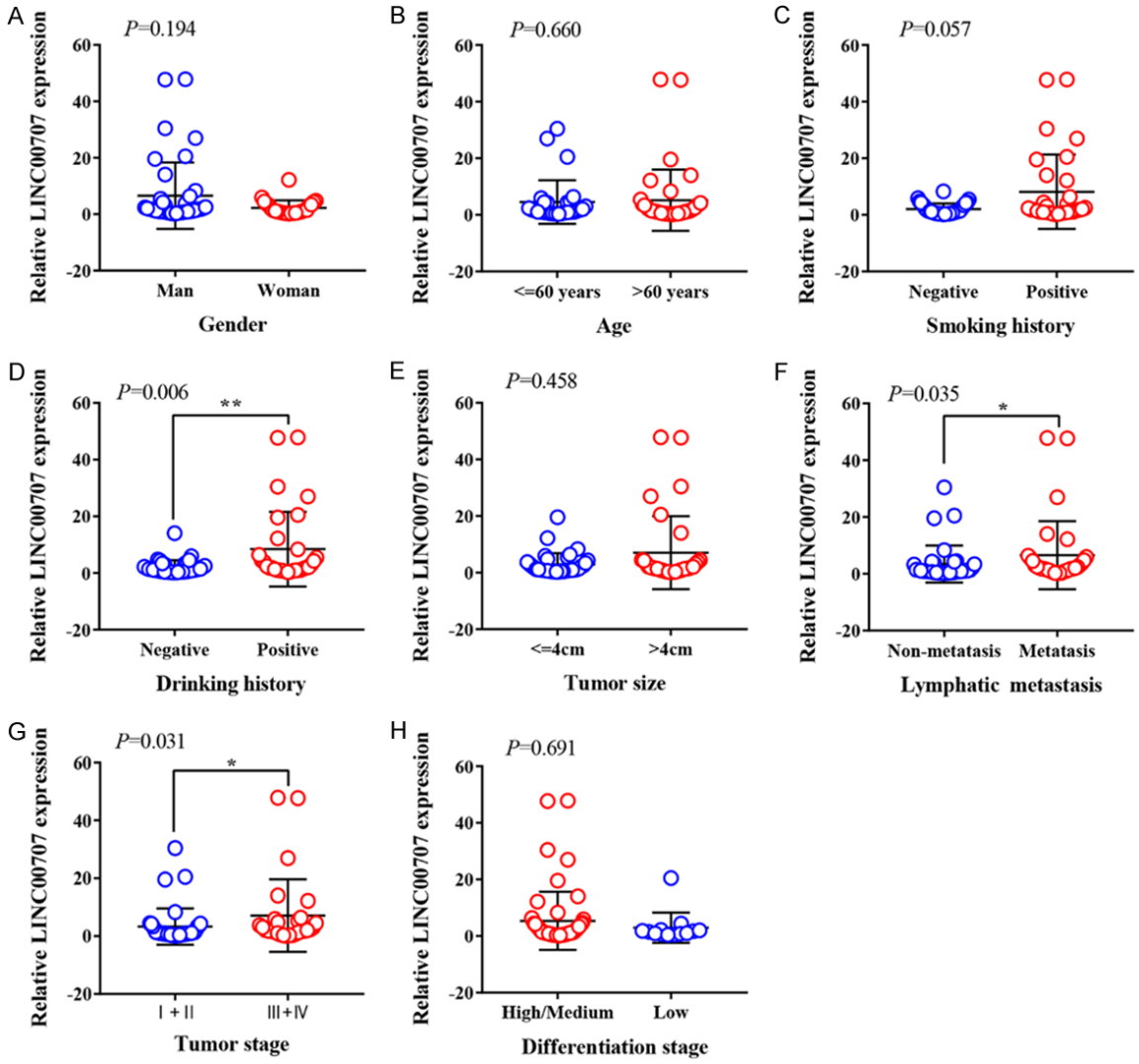

Figure 2. The associations between LINC00707 expression and clinicopathologic characteristics of patients with esophageal squamous cell carcinoma (ESCC) in the RNA-sequence (RNA-seq) data. LINC00707 expression was significantly correlated with drinking history (D), lymphatic metastasis (F), and tumor stage (G), but not significantly correlated with gender (A), age (B), smoking history (C), tumor size (E) or differentiation stage (H). All results are displayed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ by Mann-Whitney test.

Expression and function prediction of LINC00707 in ESCC cell lines

We measured LINC0007 expression in human esophagus epithelial cell line Het-1A and ESCC cell lines EC9706, KYSE70, KYSE150, KYSE450, Eca109, TE1 and KYSE30 via qRT-PCR. LINC0007 expression was increased in ESCC cell lines compared to normal esophagus epithelial cell line (**Figure 5A**). Next, to further determine LINC00707 function in ESCC, we investigated the biological roles of LINC0007 in ESCA via public available dataset. LncACTdb2.0 online database showed that LINC00707 may be related to the following genes: tumor necrosis factor associated death domain protein (TRADD) related to tumor cell proliferation and apoptosis [36], Cyclin E1 (CCNE1) that regulates ESCC cell cycle and proliferation [37], B-cell lymphoma 9 (BCL9) that promotes cancer cell stemness, invasion and metastasis [38], activating transcription factor 3 (ATF3) that regulates ESCC cell proliferation and invasion ability [39], and A Disintegrin And Metalloprotease 12 (ADAM12) closely related to ESCC invasion ability [40] (**Figure 5B**). In addition, we also predicted that the biologic characteristics of LINC00707 in ESCA may be implicated in the regulation of cancer cell proliferation, invasion, and metastasis by Lnc-

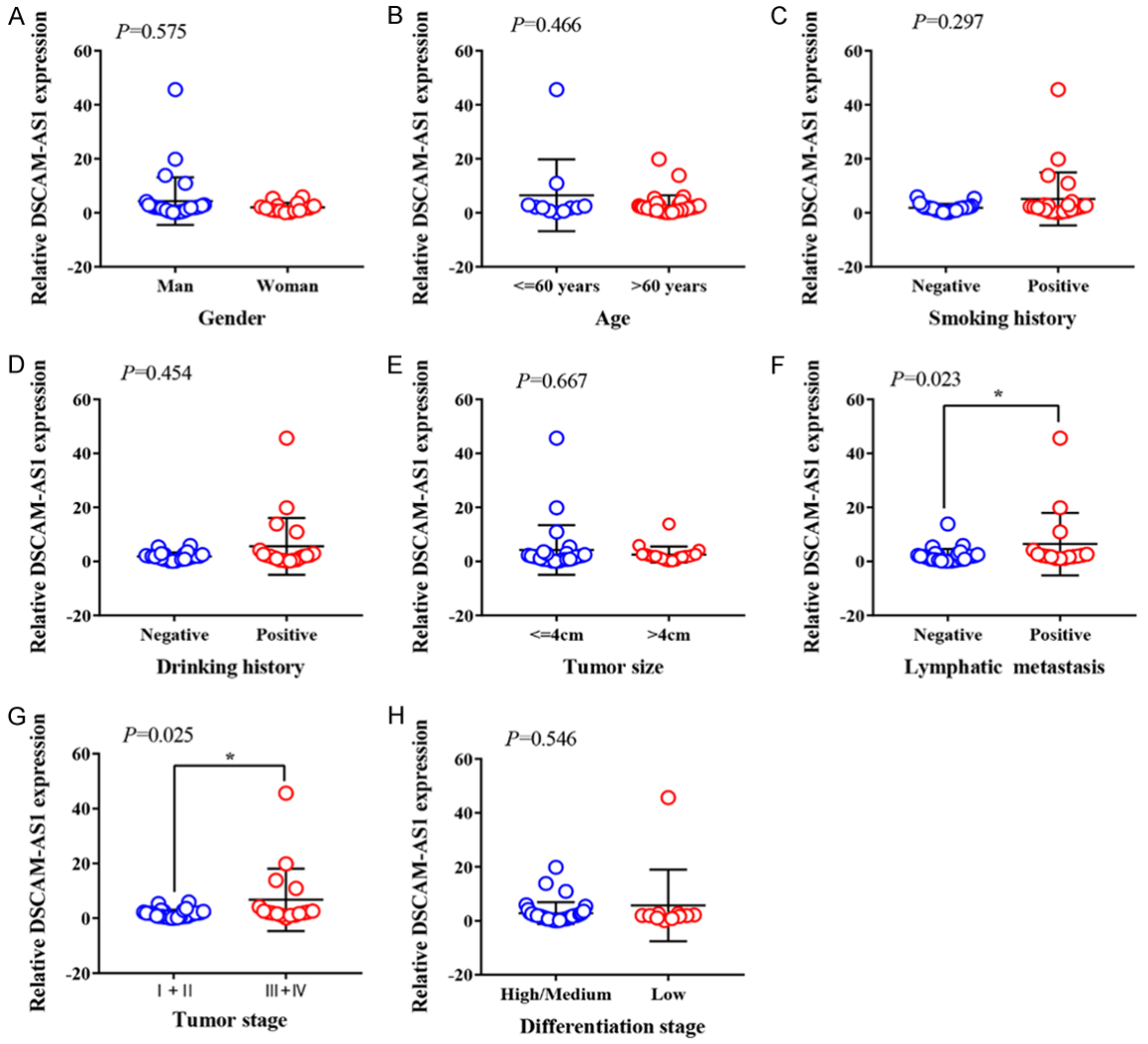

Figure 3. Correlation between LINC00707 expression and clinicopathological characteristics of patients with esophageal squamous cell carcinoma (ESCC) in the another independent 47 cases quantitative real time polymerase chain reaction (qRT-PCR) data. LINC00707 expression was significantly correlated with lymphatic metastasis (F), and tumor stage (G), but not significantly correlated with gender (A), age (B), smoking history (C), drinking history (D), tumor size (E) and differentiation stage (H). All results are displayed as mean ± SD. $^{*}P < 0.05$ by Mann-Whitney test.

ACTdb2.0 database (**Figure 5C**). Therefore, it was concluded that the poor clinical prognosis of LINC00707 in ESCC patients may be caused by regulting the proliferation, apoptosis, invasion, and metastasis of ESCC cells.

Knockdown of LINC00707 suppresses the growth of ESCC cells

To further verify LINC00707 as oncogene in ESCC, specific siRNA against LINC00707 was used to transfect to EC9706 and Eca109 cells, and LINC00707 expression was detected via qRT-PCR. We found that compared to si-LINC00707#1, si-LINC00707#2 has better knock-down efficiency in EC9706 (**Figure 6A**) and Eca109 cells (**Figure 6B**). Therefore, si-LINC00707#2 was selected for the subsequent experiments. Compared to the control group, 72 hours after knockdown of LINC00707, the ESCC cell lines EC9706 and Eca109 proliferated less (**Figure 6C** and **6D**). In addition, data from the CCK-8 assays revealed that knockdown of LINC0007 suppressed proliferation of EC9706 and Eca109 cells (**Figure 6E** and **6F**). Furthermore, results of the colony formation assay showed that the ability for colony formation was greatly attenuated following knockdown of LINC00707 in EC9706 and Eca109 cells (**Figure 6G** and **6H**). These findings suggest that LINC00707 may be a novel therapeutic target for ESCC patients.

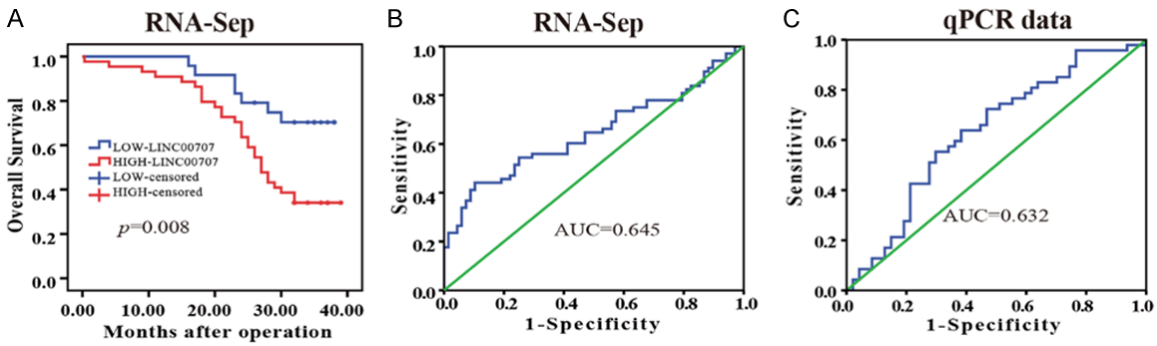

Figure 4. The prognostic power and predictive performance of LINC00707 in esophageal squamous cell carcinoma (ESCC). A. Kaplan-Meier survival analysis showed ESCC patients with higher LINC00707 expression had worse overall survival (OS) in the RNA-sequence (RNA-seq) dataset (n = 68) $P = 0.008$ by log-rank test. B. Receiver operating characteristic (ROC) curve showed that the Area Under Curve (AUC) of the LINC0007 was 0.645 in the RNA-seq dataset (n = 68). C. ROC analysis showed the AUC of the LINC0007 was 0.632 in another independent quantitative real time polymerase chain reaction (qRT-PCR) dataset (n = 47).

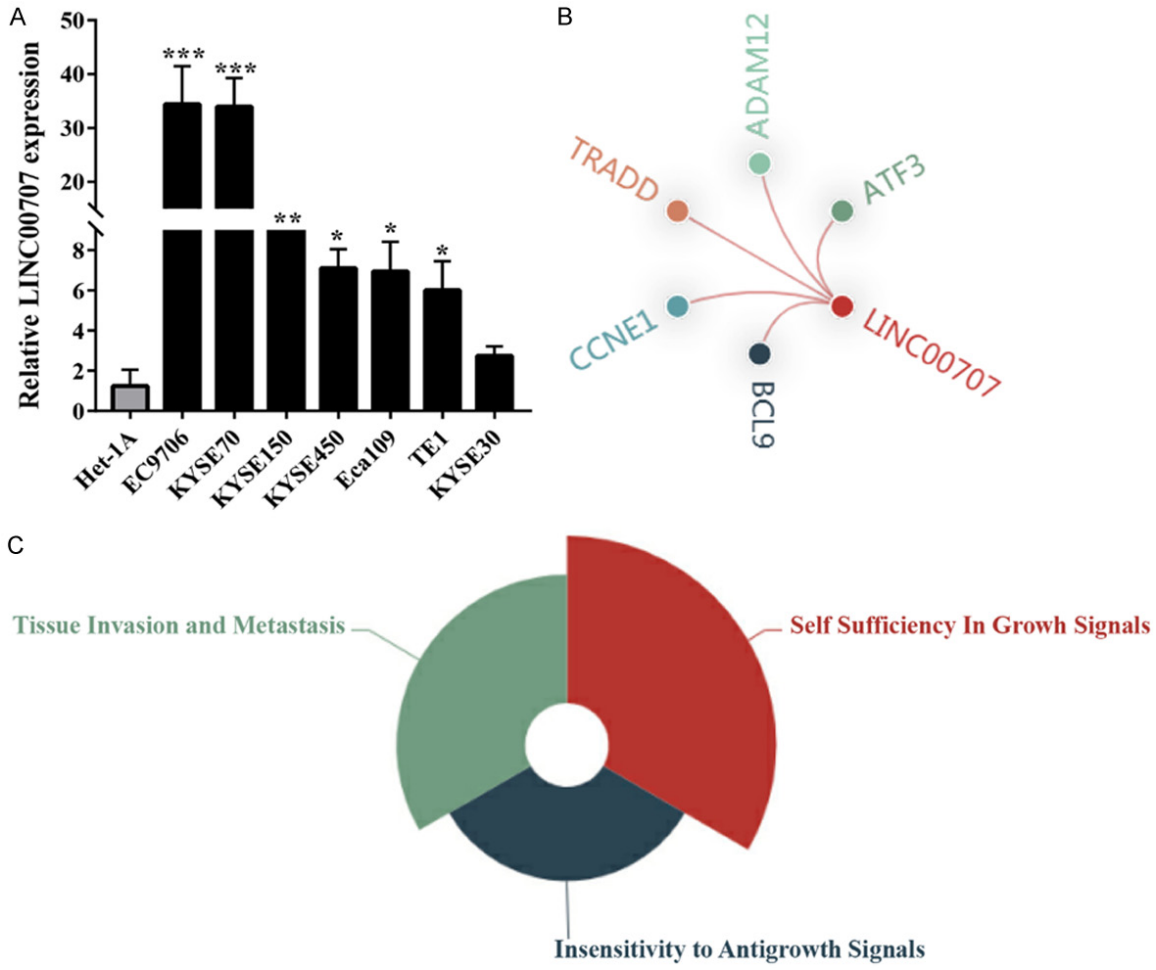

Figure 5. Expression of LINC00707 in esophageal squamous cell carcinoma (ESCC) cell lines and its function prediction. A. Quantitative real time polymerase chain reaction (qRT-PCR) assay was performed to examine LINC00707 expression in normal esophagus epithelial cell line and ESCC cell lines. Results expressed as mean ± SD are displayed from three independent repeated experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to normal esophagus epithelial cell line group by one-way ANOVA. B. The possible regulatory relationship of LINC00707 based on LncACTdb2.0 online database. C. Possible biological characteristics of LINC00707 based on LncACTdb2.0 online database.

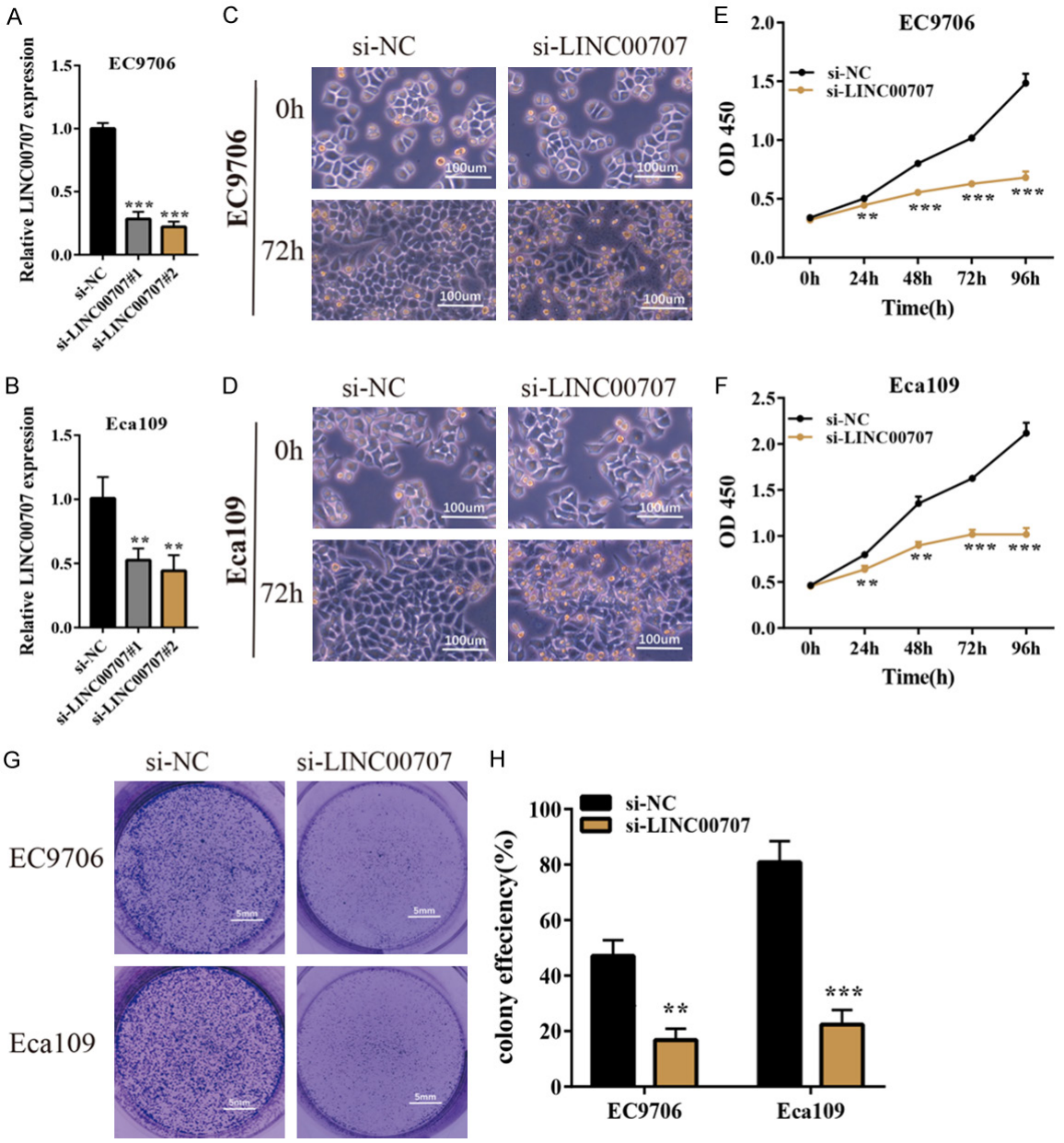

Figure 6. Knockdown of LINC0007 suppresses the growth of esophageal squamous cell carcinoma (ESCC) cells. A. The expression of LINC00707 in EC9706 cells transfected with si-NC, si-LINC00707#1, and si-LINC00707#2 was measured by quantitative real time polymerase chain reaction (qRT-PCR). B. The expression of LINC00707 in Eca109 cells transfected with si-NC, si-LINC00707#1, and si-LINC00707#2 was detected by qRT-PCR. A, B. Results are displayed as mean ± SD of n = 3 independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with si-NC group by one-way ANOVA. C. The effect of si-LINC00707 on the morphology of EC9706 cells was observed under an inverted microscope. D. The effect of si-LINC00707 on the morphology of Eca109 cells was inspected through an inverted microscope. E. After transfection with si-NC and si-LINC00707, cell proliferation of EC9706 cells was analyzed by CCK-8 assays. F. Following transfection with si-NC and si-LINC00707, cell proliferation of Eca109 cells was investigated by CCK-8 assays. G, H. After transfection with si-NC and si-LINC00707, colony formation assays were used to detect the colony formation ability of EC9706 and Eca109 cells. E, F, H. Results are displayed as mean ± SD of n = 3 independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to si-NC group by unpaired Student's *t*-test.

Downregulation of LINC00707 induces G0/G1 arrest and apoptosis of ESCC cells

The cell cycle and apoptosis are considered key factors regulating cell growth. Thus, flow cytometric analysis was performed to evaluate the changes in cell cycle distribution and apoptosis. EC9706 and Eca109 cells transfected with si-LINC00707 showed cell-cycle arrest at the G0/G1 phase compared to cells transfected

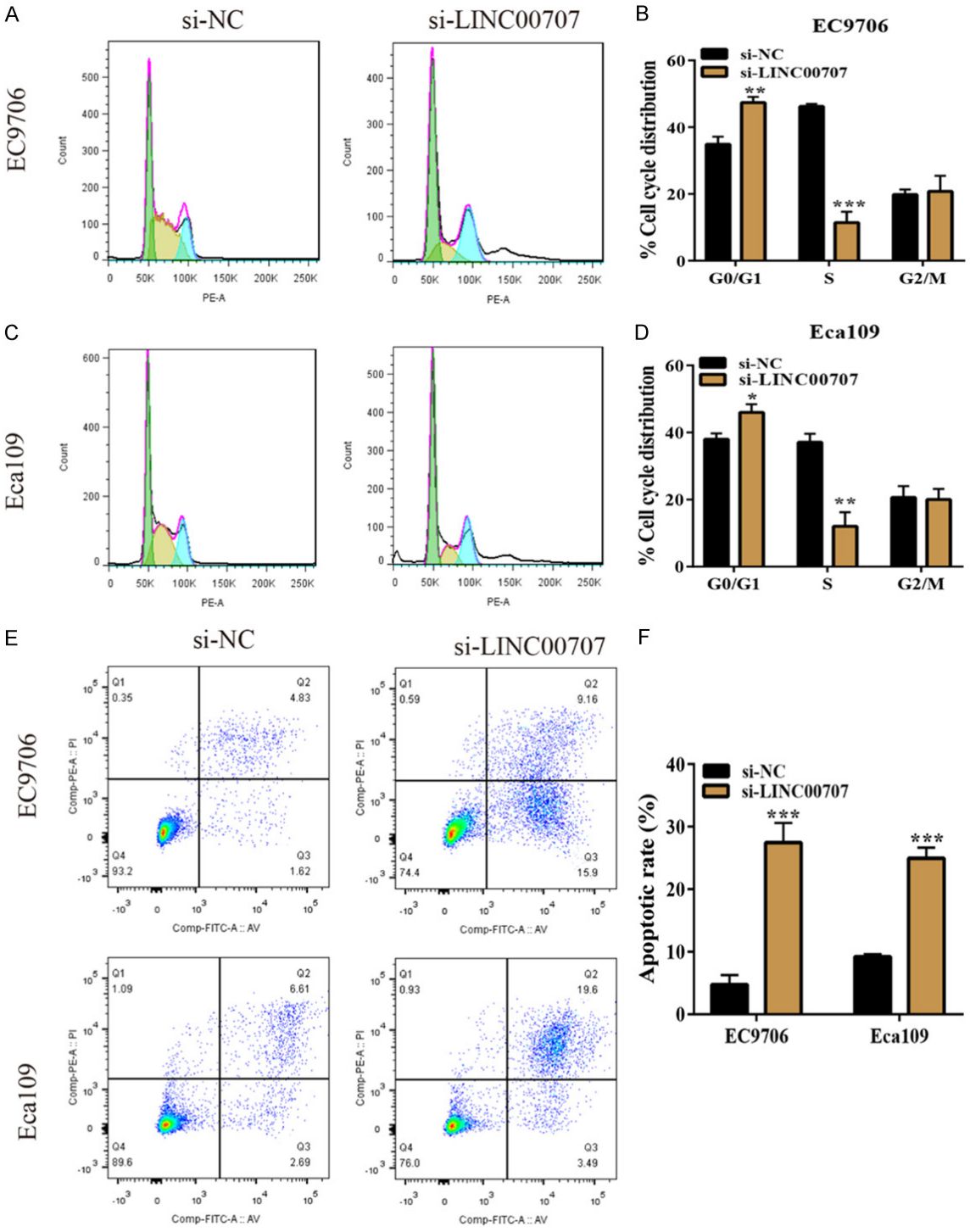

Figure 7. Downregulation of LINC00707 induces G0/G1 arrest and apoptosis of esophageal squamous cell carcinoma (ESCC) cells. A, B. Effects of si-LINC00707 on the cell cycle in EC9706 cells. C, D. Effects of si-LINC00707 on the cell cycle in Eca109 cells. E, F. Effects of si-LINC00707 on cell apoptosis in EC9706 and Eca109 cells. B, D, F. Results are displayed as mean ± SD of n = 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to si-NC group by unpaired Student's *t*-test.

with scrambled control (**Figure 7A-D**). The proportion of apoptotic cells treated with si-LINC00707 was markedly increased in EC9706 and Eca109 cells (**Figure 7E** and **7F**). These

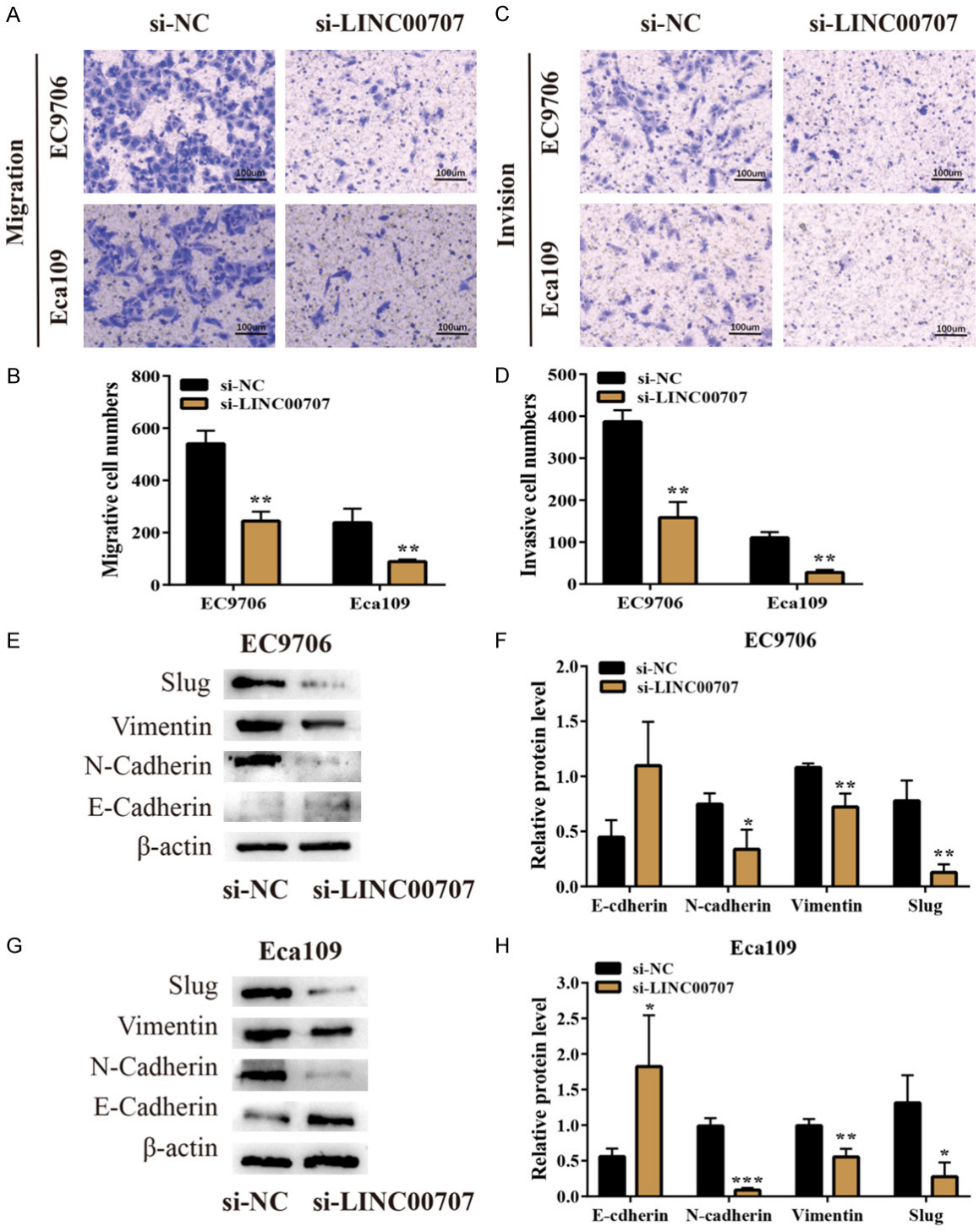

Figure 8. Knockdown of LINC00707 expression inhibits esophageal squamous cell carcinoma (ESCC) cell migration and invasion in vitro. A, B. Transwell migration and invasion assay was used to test the effects of si-LINC00707 on the cell migration of EC9706 and Eca109 cells. C, D. Transwell migration and invasion assay was used to test the effects of si-LINC00707 on the cell invasion of EC9706 cells and Eca109 cells. E, F. The protein expression levels of epithelial-mesenchymal transition (EMT)-related -markers in EC9706 cells treated with si-NC and si-LINC00707 were measured by western blot. G, H. The protein expression levels of EMT related-markers in Eca109 cells transfected with si-NC and si-LINC00707 were detected by western blot. B, D, F, H. Results are displayed as mean ± SD of n = 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the si-NC group by unpaired Student's *t*-test.

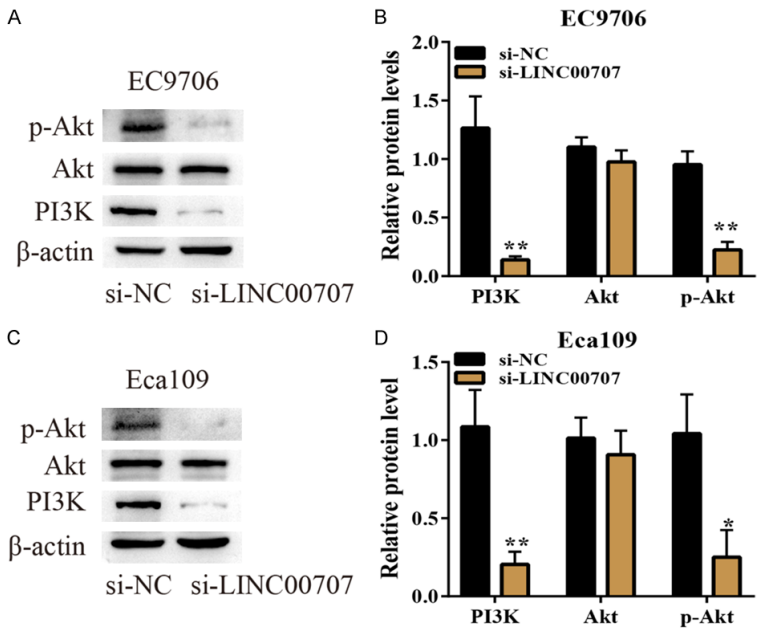

Figure 9. LINC00707 activates the PI3K/AKT signaling pathway in esophageal squamous cell carcinoma (ESCC) cells. A, B. The protein expression levels of PI3K/Akt/p-Akt in EC9706 cells transfected with si-NC and si-LINC00707 were measured by western blot. C, D. The protein expression levels of PI3K/Akt/p-Akt in Eca109 cells transfected with si-NC and si-LINC00707 were determined by western blot. B, D. Results are displayed as mean ± SD of n = 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to si-NC group by unpaired Student's *t*-test.

results confirmed that the suppressed proliferation of EC9706 and Eca109 cells transfected with LINC00707 siRNAs may be attributable to the induction of G0/G1 arrest and apoptosis.

Knockdown of LINC00707 expression inhibits ESCC cell migration and invasion in vitro

To further evaluate whether LINC00707 contributes to metastasis of ESCC, transwell assays were performed to detect cell migration and invasion abilities in ESCC cells. Our results demonstrated that the migration and invasion of EC9706 and Eca109 cells was inhibited following LINC00707 knockdown (**Figure 8A-D**). To further dissect the mechanism, the expressions of epithelial-mesenchymal transition (EMT)-related proteins such as E-cadherin, N-cadherin, Vimentin, Snail, and Slug were investigated by western blot [41]. Compared to the si-NC group, EC9706 and Eca109 cells transfected with si-LINC00707 displayed downregulation of the expressions of Snail, Slug, and mesenchymal marker N-Cadherin, and upregulation of E-Cadherin expression (**Figure 8E-H**). These data suggest that LIN00707 depletion-mediated suppression of cell migration and invasion may be achieved in part by affecting EMT progression in ESCC cells.

LINC00707 activates the PI3K/Akt signaling pathway

It is well documented that the PI3K/Akt signaling pathway is critical in tumorigenesis and plays an important role in cell proliferation, invasion, and migration. Therefore, we further investigated the effects of LINC00707 on the PI3K/Akt signaling pathway in ESCC cells. Total proteins from LINC00707 knockdown and control cells were extracted, and PI3K/Akt/p-Akt levels were determined using western blot. The results indicated that knockdown of LINC00707 markedly downregulated PI3K and p-Akt levels in EC9706 and Eca109 cells (**Figure 9A-D**). These findings imply that LINC00707 function is tightly involved in regulation of the PI3K/Akt signaling pathway.

Discussion

The molecular mechanism of ESCC is very complicated [42-45]. The main focus is still concentrated on a small part of the DNA sequence responsible for encoding proteins in ESCC. Recently, lncRNAs have gradually attracted the attention of researchers. For example, LncRNA TTN-AS1 promoted ESCC metastasis by competitively absorbing miR-133b [46]. LncRNA DUXAP10 modulated cell proliferation in ESCC by epigenetically silencing p21 [47]. Despite the discovery of a large number of lncRNAs, the clinical value and biologic functions of many lncRNAs in ESCC remains under investigation.

LINC00707, a discovered lncRNA, has been reported to be upregulated and plays an oncogenic role in several cancers. For example, LINC00707 promoted hepatocellular carcinoma proliferation and metastasis through sponging miR-206 to increase cyclin-dependent kinase 14 (CDK14) [20]. The LINC00707 pro-

moted proliferation and metastasis of gastric cancer by interacting with mRNA stabilizing protein HuR [26]. The LINC00707 promoted lung adenocarcinoma cell proliferation and migration by regulating cdc42 expression [27]. However, the function and clinical significance of LINC00707 in ESCC have not been investigated. In the present study, we mainly explored the expression and function of LINC00707 in ESCC. We identified that LINC00707 was upregulated in ESCC tissues and cell lines compared to adjacent noncancerous esophageal tissues and a normal esophageal epithelial cell line, respectively. The RNA-seq data and qRT-PCR validation data demonstrated that high expression of LINC00707 was positively associated with higher TNM stage. Lymph node metastasis and LINC00707 expression were significantly higher in patients with alcohol history, lymph node metastasis, and higher tumor stage. In addition, Kaplan-Meier survival analysis revealed that the prognosis for OS was significantly poorer in ESCC patients with high LINC00707 expression than in those with low expression, and the AUC value obtained by ROC curve analysis confirmed the feasibility of LINC00707 as a diagnostic signature. These findings highlight the clinical value of LINC00707 in ESCC patients, and the application of more clinical samples may accelerate the future clinical use of LINC00707. Then, using online tool LncACTdb 2.0, combined with loss-of-function assay verification, we identified that LINC00707 knockdown inhibits ESCC cell proliferation, apoptosis, invasion, and migration. Therefore, our data demonstrated that LINC00707 also functions as an oncogene in ESCC, further supporting LINC00707 as a cancer-associated lncRNA. Our findings implied that LINC00707 might be a promising prognostic biomarker and therapeutic target for ESCC.

Accumulating evidence has suggested that lncRNAs are implicated in multiple biologic processes through various regulatory mechanisms in cancer [48]. In this study, we identified a novel mechanism mediating the oncogenic roles of LINC00707 in ESCC, namely activation of the PI3K/Akt signaling pathway. Evidence has confirmed that serine/threonine kinase Akt, as a proto-oncogene, can regulate the growth, proliferation [49, 50], apoptosis [51], invasion, and metastasis [52, 53], of many different cancer types including ESCC. PI3K induces the activation of phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn activates the dominant effector Akt [54]. However, whether LINC00707 affects the PI3K/Akt signaling pathway in ESCC progression has still not been revealed. Given that knockdown of LINC00707 mediated the suppression of proliferation, invasion, and metastasis as well as induction of apoptosis in ESCC cells, we speculated that LINC00707 knockdown in ESCC cells would affect the PI3K/Akt signaling pathway. Therefore, western blot was performed to detect the alterations of PI3K, Akt, and phosphorylated Akt expressions in ESCC cells after LINC00707 depletion, and the current results were completely in line with our expectations. Knockdown of LINC00707 inhibited the expressions of PI3K, Akt, and phosphorylated Akt in ESCC cells. Therefore, we concluded that LINC00707 downregulation mediated the suppression of the proliferation, invasion, and metastasis as well as induction of cell apoptosis were tightly implicated in inactivation of the PI3K/Akt signaling pathway.

In conclusion, our findings demonstrate that LINC00707 expression was increased in ESCC tissues and cell lines. The RNA-seq data and qRT-PCR validation data revealed that high expression of LINC00707 was positively associated with higher TNM stage, and lymph node metastasis. LINC00707 expression was significantly higher in patients who drink alcohol, have lymph node metastasis, and have higher tumor stage. In addition, Kaplan-Meier survival analysis and ROC curve analysis confirmed the feasibility of LINC00707 as a prognostic or diagnostic signature. Functional experiments showed that enhanced expression of LINC00707 promoted ESCC cell proliferation, apoptosis, invasion, and migration. Mechanistic investigation demonstrated that LINC00707 activated the PI3K/Akt signaling pathway. Our results imply that LINC00707 may be a promising prognostic biomarker to evaluate ESCC progression and a useful therapeutic target for ESCC treatment.

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Disclosure of conflict of interest

None.

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