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Original Article

Increased LincRNA ROR is association with poor prognosis for esophageal squamous cell carcinoma patients

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Abstract: Identification of novel biological markers may be beneficial for diagnosis and prognosis for esophageal squamous cell cancer (ESCC) patients. Recent findings have highlighted that long noncoding RNAs (lncRNAs) were involved in the tumor progression including ESCC. However, the clinical significance and function role of LincRNA ROR in ESCC remains unknown. In our study, QRT-PCR assays results proved that LincRNA ROR expression levels were aberrantly higher in 120 cases of ESCC tissues compared to adjacent normal tissues. Furthermore, LincRNA ROR expression levels were significantly associated with lymph node invasion, distant metastasis and TNM stage in ESCC patients. The survival curves were plotted using the Kaplan-Meier methods showed that increased LincRNA ROR expression levels predicted poor disease-free survival (DFS) and overall survival (OS). Multivariate Cox analyses showed that LincRNA ROR expression was an independent risk factor of prognosis in ESCC patients. In vitro, we showed that Knockdown of LincRNA ROR inhibited the cell proliferation and invasion, and suppressed the epithelial-to-mesenchymal transition (EMT) process by decreasing the E-cadherin, but increasing the ZEB1, ZEB2 and Vimentin expression. Hence, these results indicated that lincRNA ROR may be a predicted marker for prognosis and target of therapy in ESCC patients.

Keywords: Esophageal squamous cell carcinoma, LincRNA ROR, prognosis, cell proliferation, epithelial-to-mesenchymal transition

Introduction

Esophageal cancer is the sixth lethal cancer worldwide and esophageal squamous cell carcinoma (ESCC) is the major pathological type [1, 2]. Although the development of diagnosis and therapeutic strategies, due to its aggressive nature, the over survival rates for esophageal cancer patients remains unsatisfactory [3, 4]. To investigate underlying molecular biological mechanisms may improve the prognosis and reduce the risk of tumor recurrence in ESCC patients.

The biological role of long noncoding RNAs in human esophageal squamous cell carcinoma (ESCC) has been reported in previous findings. For example, Down-regulation of MALAT1 expression inhibited cell proliferation, migration and tumor sphere formation, while increasing cell apoptosis of esophageal cancer [5]. High expression of PCAT-1 was specifically correlated with invasion of cancer tissues, metastasis of lymph node, and advanced tumor stage of ESCC [6]. Knockdown of long non-coding RNA TP73-AS1 inhibits cell proliferation and induces apoptosis in esophageal squamous cell carcinoma [7]. Enhanced expression of NEAT1 stimulated the proliferation of ESCC cells, and promoted their ability of forming foci, migration, and invasion [8].

The lncRNA linc-ROR (LincRNA ROR) has been shown to contribute to cancer progression. Hou *et al* reported that Linc-ROR functions as an important regulator of EMT and can promote breast cancer progression and metastasis through regulation of mir-205 [9]. Zhan *et al* found that Linc-ROR up-regulates ZEB1 and then induces epithelial-mesenchymal transition (EMT), which promotes the aggressive biological behaviors of PC [10]. Yang *et al* revealed that knockdown of lincRNA-ROR enhanced the sensitivity to radiotherapy for CRC by inhibiting cell viability and promoting apoptosis [11]. Zhou *et al* showed that Linc-RNA-RoR acts as a

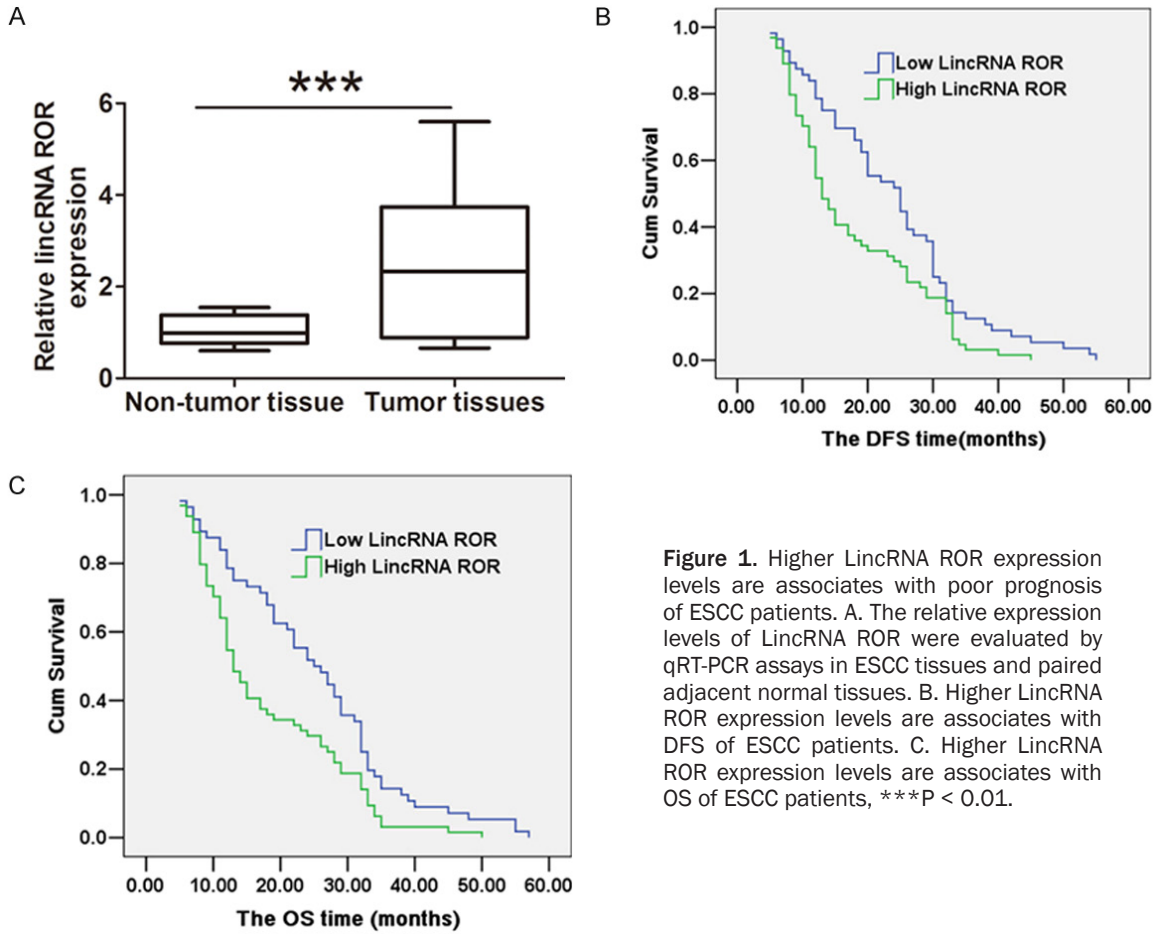

Figure 1. Higher LincRNA ROR expression levels are associates with poor prognosis of ESCC patients. A. The relative expression levels of LincRNA ROR were evaluated by qRT-PCR assays in ESCC tissues and paired adjacent normal tissues. B. Higher LincRNA ROR expression levels are associates with DFS of ESCC patients. C. Higher LincRNA ROR expression levels are associates with OS of ESCC patients, ***P < 0.01.

"sponge" against mediation of the differentiation of endometrial cancer stem cells by microRNA-145 [12]. Sahebi R *et al* found that Linc-ROR and its spliced variants 2 and 4 are significantly up-regulated in esophageal squamous cell carcinoma [13]. However, the clinical role and biological function are not been investigated in the previous studies.

In our report, LincRNA ROR was aberrantly higher in ESCC tissues and higher LincRNA ROR expression levels predicted poor prognosis. In vitro, LincRNA ROR promoted the cell proliferation and invasion. Hence, these findings offer novel potential biomarkers for prognosis evaluation and tumor therapy for ESCC patients.

Materials and methods

Clinical tissue samples

The 120 cases of ESCC tissues and the adjacent normal tissues samples were obtained at the Oncology department of The Second People's Hospital of Taizhou from March 2008 to June 2014. No patient received radio- or chemo-therapy before surgery. The clinical information and follow-ups were carried out in all patients via telephone or mails. The tumor tissues were confirmed diagnosis by two pathological professors. The tissues were snap-frozen in liquid nitrogen and stored at -80°C immediately after resection. The protocol was approved by the Ethics Committee of The Second People's Hospital of Taizhou and written informed consent from all patients in this study.

Cell culture and transfection

The ESCC cell lines (EC9706 and Eca109) were purchased from the Cell Bank of Shanghai Institute of Cell Biology (Chinese Academy of Medical Sciences, Shanghai, China) and were used in the study. Cells were cultured using the RPMI 1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), 100 μg/ml streptomycin and 100 units/ml penicillin (Sigma-Aldrich, USA), in a humidified tissue culture chamber with 5%

Table 1. Correlation between LincRNA ROR expression and clinicopathological features

Clinicopathologic factors	Patients number	LincRNA ROR expression		*P*-value
		Low	High	
Gender				0.250
Female	64	33	31	
Male	56	23	33	
Age (years)				0.941
≤ 60	69	32	37	
> 60	51	24	27	
Tumor size				0.903
< 5 cm	55	26	29	
> 5 cm	65	30	35	
Histological grade				0.109
High	45	26	19	
middle	40	14	26	
Low	35	16	19	
Lymph node invasion				0.001**
Negative	78	46	32	
Positive	42	10	32	
Infiltration depth				0.722
T1, T2	77	35	42	
T3, T4	43	21	22	
Distant metastasis				0.017**
No	72	40	32	
Yes	48	16	32	
Tumor location				0.860
Upper	43	21	22	
Middle	42	20	22	
Lower	35	15	20	
TNM stage				0.006**
I-II	70	40	30	
III-IV	50	16	34	

***P*-value < 0.05 was considered statistically significant.

CO_2 at 37°C. EC9706 or Eca109 cells were transfected with sh-negative control, shRNA LincRNA ROR (the sequence was shRNA LincRNA: CCTGAGAGTTGGCATGAAT). Transient transfections were performed using lipofectamine 2000 according to manufacturer's instructions (Invitrogen).

RNA extraction and quantitative real-time PCR

The tissues and cells RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's information. RNA was reversed into cDNA using the Prime-Script one-step RT-PCR kit (TAKARA, Dalian, China). The mRNA expression levels of LincRNA ROR were determined by using Prime Script RT-PCR kit (TAKARA, Dalian, China). The GAPDH was performed as an internal control. The PCR reactions were performed using the ABI 7500 Sequence Detection System (Applied Biosystems). Gene expressions were measured as fold-changes and compared using the $2^{-\Delta\Delta Ct}$ method. The primers for GAPDH were F: GAAGGTGAAGGTCGGAGTC, R: GAAGATGGTGATGGGATTTC. LincRNA ROR, F: CTCAGTGGGGAAGACTCCAG, R: AGGAAGCCTGAGAGTTGGC.

Cell proliferation assays

EC9706 or Eca109 (5×10^3/well) were seeded in 96-well plates and were transfected with sh-LincRNA ROR or sh-NC. After transfection, the cells were detected at 0, 24, 48, 72 h, and 96 h. MTT reagent was added and incubated for 4 h at 37°C. The absorbance was measured at 490 nm using a spectrophotometric plate reader (UV-200; Beckman Coulter, Inc.).

Western blotting assays

Total cell proteins were isolated and concentrations were detected using the BCA protein assay kit (Invitrogen, USA). Proteins were separated using 10% SDS-PAGE gel and transferred to PVDF membranes (Millipore). The membrane was blocked with 5% non-fat milk and was incubated with the E-cadherin (1:1000, CST, USA), Vimentin (1:1000, CST, USA), ZEB1 (1:1000, CST, USA), ZEB2 (1:1000, CST, USA) and GAPDH (1:1000, CST, USA). The blot was incubated with horseradish peroxidase (HRP) for 1 hour and then was detected using ECL (enhanced chemiluminescence) system (Pierce Biotechnology, IL).

Statistical analysis

The experiments were performed to repeat for at least three times. Data are shown as mean ± SD. Statistical comparisons were made by Student's t-tests. An unpaired two-tailed, the *P* value < 0.05 was identified to be significantly different.

Results

Linc RNA ROR was upregulated in ESCC tissues

QRT-PCR was applied to determine the LincRNA ROR expression levels in the 120 case of ESCC tissue samples and adjacent normal tissue specimens. As was presented in **Figure 1A**, the

Table 2. Multivariate analysis of disease-free survival (DFS) and the over survival (OS) time in 120 cases of ESCC patients

	DFS			OS		
Variables	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Gender	1.015	0.622-1.696	0.538	1.215	0.822-1.878	0.438
Age (years)	0.901	0.442-1.712	0.588	0.901	0.442-1.712	0.588
Tumor size	1.312	0.786-1.996	0.328	1.389	0.886-2.386	0.215
Histological grade	1.346	0.965-1.984	0.251	1.455	1.224-2.123	0.189
Lymph node invasion	2.856	1.118-4.935	0.001**	2.988	1.345-5.425	0.001**
Infiltration depth	0.866	0.322-1.446	0.796	0.959	0.556-1.768	0.566
Distant metastasis	2.669	0.972-4.767	0.001**	2.899	1.033-6.445	0.001**
Tumorlocation	1.221	0.994-1.992	0.203	1.366	1.122-2.116	0.178
TNM stage	2.584	1.011-4.433	0.001**	2.785	1.336-5.122	0.001**
LincRNA ROR	2.926	1.588-5.144	0.001**	3.221	1.446-6.228	0.001**

***P*-value < 0.05 was considered statistically significant.

results revealed that the expression levels of LincRNA ROR were significantly higher in the ESCC specimens compared to the adjacent normal tissue specimens. Subsequently, we examined the relationship between LincRNA ROR expression levels and clinical factors of patients. As shown in **Table 1**, the results revealed that LincRNA ROR expression were correlated with lymph node invasion (P = 0.001), distant metastasis (P = 0.017) and TNM stage (P = 0.006), but not with gender (P = 0.178), age (P = 0.941), tumor size (P = 0.903), and so on.

LincRNA ROR expression levels are associates with poor prognosis of ESCC patients

Furthermore, the survival curves were plotted using the Kaplan-Meier methods showed that patients with higher LincRNA ROR predicted poor disease-free survival (DFS) (P = 0.009, log-rank = 6.761) and overall survival (OS) (P = 0.003, log-rank = 8.816). To analyze the possibility of LincRNA ROR as an independent risk factor for predicting prognosis, both clinicopathological feathers and the LincRNA ROR expression were assessed by multivariate Cox regression analysis. As shown in **Table 2**, the data results from the multivariate analysis demonstrated that lymph node invasion (HR = 2.856, 95% CI: 1.118-4.935, P = 0.001), distant metastasis (HR = 2.669, 95% CI: 0.972-4.767, P = 0.001) and TNM stage (HR = 2.584, 95% CI: 1.011-4.433, P = 0.001) and LincRNR ROR expression (HR = 2.926, 95% CI: 1.588-5.144, P = 0.001) were independent risk factor for disease-free survival (DFS).

In addition, as shown in **Table 2**, we also demonstrated that lymph node invasion (HR = 2.988, 95% CI: 1.345-5.425, P = 0.001), distant metastasis (HR = 2.899, 95% CI: 1.033-6.445, P = 0.001) and TNM stage (HR = 2.785, 95% CI: 1.336-5.122, P = 0.001) and LincRNR ROR expression (HR = 3.221, 95% CI: 1.446-6.228, P = 0.001) were independent risk factor for overall survival (OS) for ESCC patients. Thus, these results showed that LincRNA ROR expression levels are associates with poor prognosis of ESCC patients.

Knockdown of Linc RNA ROR inhibited the cell proliferation and invasion by inhibiting the cell EMT in ESCC

We then analyzed the association between Linc RNA ROR expression and cell proliferation and invasion ability by knockdown of LincRNA ROR in EC9706 or Eca109 (**Figure 2A** and **2B**). The results of CCK8 cell proliferation assays showed that cell proliferation ability was significantly inhibited when LincRNA ROR was knocked down in EC9706 or Eca109 cells (**Figure 2C** and **2D**). In the other hand, after transfection with sh-LincRNA ROR, compared with the sh-NC group, the results of transwell cell assays results showed that cell invasion was significantly decreased and the invasive cell number was reducing in the sh-lincRNA ROR group (**Figure 3A** and **3D**). Thus, our data indicate that LincRNA ROR promoted ESCC cell proliferation and invasion in ESCC cells.

LincRNA-ROR was found to induce epithelial-to-mesenchymal transition and contributes to

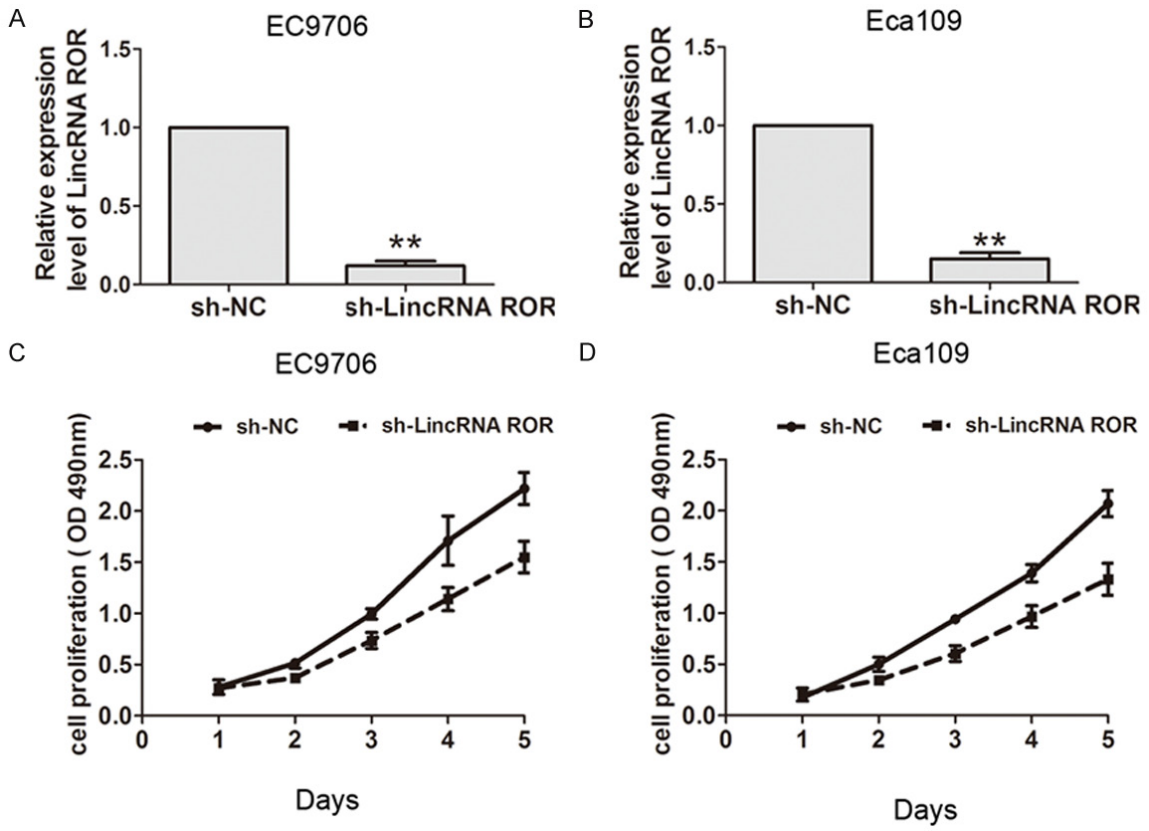

Figure 2. Knockdown of LincRNA ROR inhibited the cell proliferation in ESCC cells. A. B. The relative expression levels of LincRNA ROR were evaluated by qRT-PCR assays after LincRNA ROR was knocked down in EC9706 or Eca109. C, D. CCK8 cell proliferation was performed to evaluate cell proliferation after LincRNA ROR was knocked down in EC9706 or Eca109, Data are shown as mean ± SD from at least three independent experiments, **P < 0.05.

breast cancer tumorigenesis and metastasis [9]. Overexpression of LncRNA-ROR promotes the tumor cells proliferation, migration, and invasion and mediates the EMT in gallbladder cancer [14]. Furthermore, we investigated the effect of LincRNA ROR on the epithelial-to-mesenchymal transition (EMT) process of ESCC cells. After transfection with sh-LincRNA ROR, the results of western-blot assays showed that the EMT marker Ecadherin expression level was significantly upregulated, but the transcription factors ZEB1, ZEB2 and EMT marker Vimentin expression levels were decreased in EC9706 or Eca109 cells (**Figure 3E** and **3F**). Thus, our results suggested that inhibition of Linc RNA ROR expression could suppress cell epithelial-to-mesenchymal transition process in ESCC.

Discussion

Recent studies had have previously shown that LncRNAs was involved in the progression of esophageal squamous cell carcinoma (ESCC). CASC9 is significantly upregulated in ESCC tissues and may represent a new marker of poor prognosis [15]. Knockdown of H19 not only exerts inhibitory effect on tumor proliferation in vitro and in vivo, but also represses the migratory and invasive capacity in esophageal squamous cell carcinoma [16]. LncRNA ZEB1-AS1 is found to be up-regulated in ESCC tissues, increased lncRNA ZEB1-AS1 expression is significantly associated with tumor grade, depth of invasion, and lymph node metastasis [17]. In present study our results demonstrated that lincRNA ROR was aberrantly higher in ESCC tissues compared with adjacent normal tissues. Besides, LincRNA ROR expression was significantly associated with lymph node invasion, distant metastasis and TNM stage in ESCC patients. The survival curves were plotted using the Kaplan-Meier methods showed that increased LincRNA ROR expression levels predicted poor disease-free survival (DFS) and overall survival (OS).

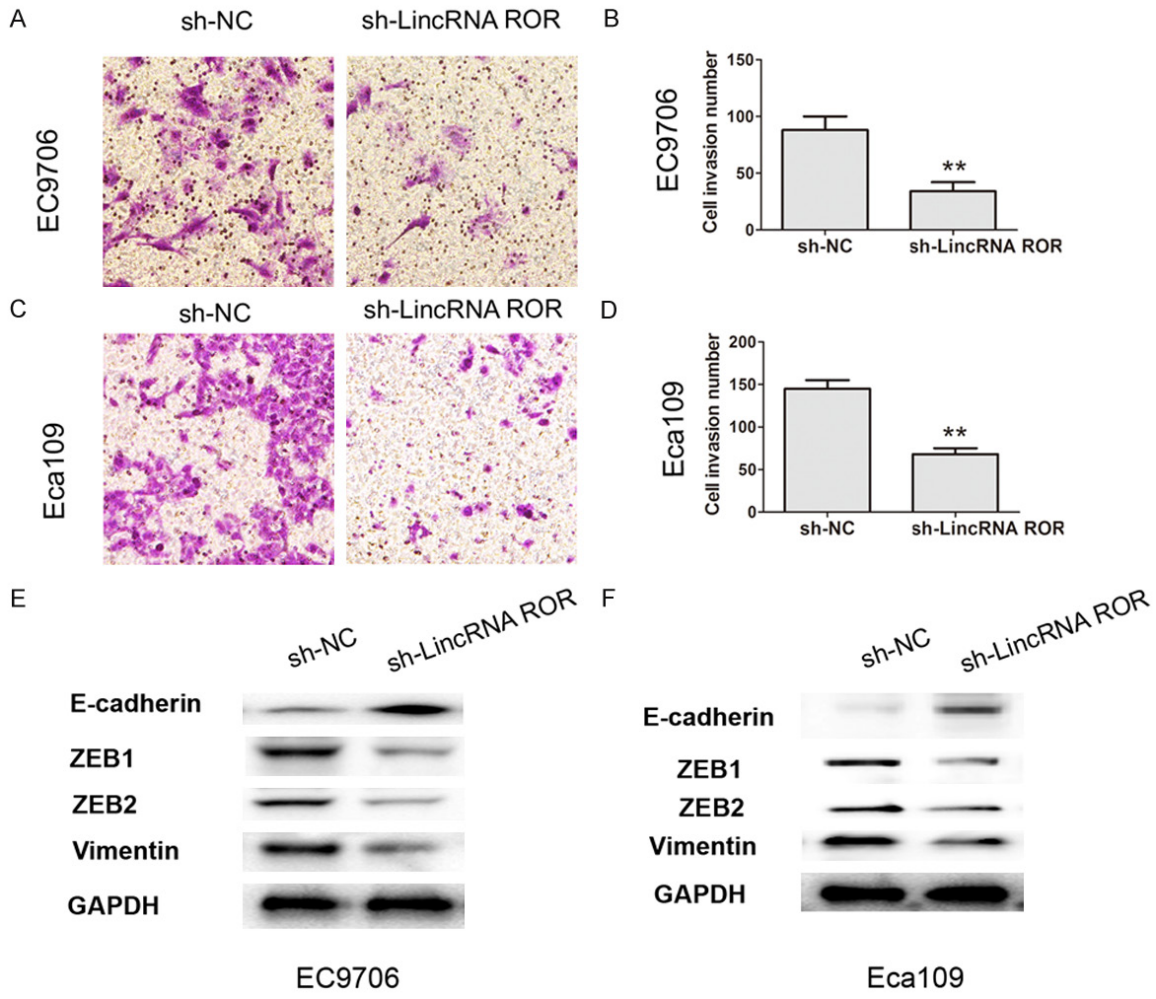

Figure 3. Knockdown of LincRNA ROR inhibited the cell invasion and EMT in ESCC cells. A-D. The cell invasion ability and cell invasive number was evaluated by transwell assays after LincRNA ROR was knocked down in EC9706 or Eca109. E, F. The relative protein expression levels of E-cadherin, ZEB1, ZEB2, and Vimentin were evaluated by western-blot assays after LincRNA ROR was knocked down in EC9706 or Eca109. Data are shown as mean ± SD from at least three independent experiments, **P < 0.05.

Some studies have reported that lncRNAs was participated in tumor invasion and metastasis. Up-regulation of long noncoding RNA MALAT1 contributes to proliferation and metastasis in esophageal squamous cell carcinoma [18]. Long noncoding RNA SPRY4-IT1 promotes esophageal squamous cell carcinoma cell proliferation, invasion, and epithelial-mesenchymal transition [19]. In the study, we demonstrated that LincRNA ROR promoted the cell proliferation and invasion and enhanced the EMT process by inhibiting the E-cadherin expression and upregulating the ZEB1, ZEB2 and Vimentin expression in ESCC cells. In previous study, lincRNA-ROR was negatively correlated with stem cell factor KLF4 and the up- and down-regulation f lincRNA-ROR resulted in inverse modulation of KLF4 messenger RNA (mRNA) expression [20]. LincRNA-ROR promotes invasion, metastasis and tumor growth in pancreatic cancer through activating ZEB1 pathway [10]. Consistent with these results, we found that LincRNA ROR promoted cell proliferation and invasion in ESCC cells.

In conclusion, in the study, we found that lncRNA ROR was aberrantly higher in ESCC and higher LincRNA ROR had a poor prognosis in ESCC patients. In vitro, LincRNA ROR promoted the cell proliferation, cell invasion and EMT process in ESCC cells. Thus, these finds indicated that LincRNA ROR may be a potential predictor for prognosis and target of treatment in ESCC.

Disclosure of conflict of interest

None.

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