

Original Article

miR-143 inhibits migration and invasion through regulating **LASP1** in human esophageal cancer

Huifeng Liu, Mengli Zheng, Yachao Zhao, Shuxin Zhang

Department of Thoracic Surgery, PLA 309 Hospital, Haidian, Beijing 100091, China

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Abstract: Background: Esophageal cancer (EC) is one of the common cancers in China with high incidence and poor prognosis. Increasing evidence has emphasized the important roles of differentially expressed miRNAs in esophageal squamous cell carcinoma (ESCC) progression. Previous studies indicated that miR-143-3p and **LASP1** influence cell growth in ESCC and other cancer types. However, the function and molecular mechanism of action of miR-143 and **LASP1** in ESCC have not been fully explored. Methods: miR-143 and **LASP1** expression were detected by quantitative real-time PCR. The protein level of **LASP1** was measured by western blot. Cell proliferation was evaluated by MTT assay. Cell migration and invasion capacity was measured by transwell assay. Targeting of **LASP1** mRNA by miR-143 was verified by luciferase reporter assay. Overall survival of ESCC patients with different miR-143 expression level was evaluated by Kaplan-Meier survival analysis. Results: miR-143 expression was down-regulated, while **LASP1** expression was up-regulated in ESCC tissues and cells compared to non-malignant counterparts. **LASP1** mRNA was identified as a target of miR-143. Low miR-143 expression or high **LASP1** expression significantly associated with ESCC patients' decreased survival. miR-143 mimic transfection inhibited ESCC cell proliferation, migration and invasion in vitro, which was impaired by **LASP1** overexpression. Conclusion: miR-143 suppressed cell proliferation, migration, and invasion by down-regulating **LASP1**.

Keywords: Esophageal squamous cell carcinoma, miR-143, **LASP1**, cell progression

Introduction

Esophageal cancer (EC) is the eighth most common cancer type worldwide with poor prognosis [1-3]. The two main subtypes of EC are esophageal squamous cell carcinoma (ESCC), which composed of 60-70% of all EC cases, and esophageal adenocarcinoma (EAC) [4]. ESCC is more prevalent than EAC in Asia, especially in China where more than half of global ESCC cases occurs [5, 6].

MicroRNAs (miRNAs) are endogenous small non-coding RNAs of 18-22 nt in length, inducing gene silencing by binding to their target sequences on mRNAs (most frequently in the 3' untranslated region, or 3' UTR) that results in translational repression or mRNA degradation [7]. As important post-transcriptional regulators, microRNAs regulate their target gene expression in many diseases, including ESCC [8-10]. Recent studies revealed that miRNAs might function as either tumor promotor or sup-

pressor based on their target genes, and their aberrant expression associates with ESCC progression and development [11-14]. For example, miR-9 was found up-regulated in ESCC, and high miR-9 expression promoted cell proliferation and invasion by down-regulating E-cadherin and FOXO1 expression [15], whereas miR-133a is down-regulated in ESCC, whose overexpression could significantly attenuate the aggressive phenotype of ESCC cells in vitro and in vivo by inhibiting SOX4 gene expression and the epithelial-to-mesenchymal transition (EMT) process [16].

LIM and SH3 protein 1 (**LASP1**) is a member of LIM and SH3 domain-containing protein family associated with tumor growth [17, 18]. **LASP1** expression was reported to be up-regulated in various cancers [19]. **LASP1** acts as an oncogene to promote tumorigenesis, cell proliferation, as well as chemoresistance, whose high expression significantly associated with poor prognosis of patients suffering from breast can-

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cer [20], colorectal cancer [21], gastric cancer [22], and EC [23]. Zhong *et al.* suggested that high LASP1 expression influenced poor overall survival. Meanwhile, knockdown of LASP1 decreased cell proliferation and increased chemosensitivity of glioblastoma cells to temozolomide by reducing the activation PI3K/AKT pathway [24]. However, the function and mechanism of action of LASP1 in ESCC have not been fully explored.

In the present study, our data suggested that miR-143 was down-regulated in ESCC tissues and cells compared to non-malignant counterparts, and miR-143 functioned as a tumor suppressor in ESCC cells *in vitro*. Our data further revealed LASP1 mRNA as a target of miR-143, and miR-143 suppressed ESCC cell proliferation, migration, and invasion *in vitro* by targeting LASP1.

Materials and methods

Patients and tissues samples

44 pairs of ESCC and matched adjacent tissue specimens were obtained from ESCC patients in PLA 309 hospital during 2010-2015. None of the patients underwent radiotherapy, chemotherapy, immunotherapy or targeted therapy before surgery. The research protocol was approved by the Institutional Review Committee of PLA 309 hospital, and informed consent was obtained from each patient or patient's guardian before enrollment.

Cell culture

The human esophageal cancer cell lines (KYSE510, Eca109, EC9701, EC1), human normal esophageal epithelial cell line (SHEE) and HEK293T cell line were purchased from RiboBio Co. (Guangzhou, China) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin-streptomycin solution (Thermo Fisher Scientific) at 37°C in a 5% CO₂ humidified incubator. Medium was changed every 2 days.

Transient transfection

KYSE510 and Eca109 cells were selected for this part of experiment based on their miR-143 expression level. MiR-143 mimics (miR-143 mimics) and miR-143 inhibitors (miR-143 inhib-

itors) and their corresponding controls (miR-NC and miR-NC inhibitors) were purchased from GenePharma (Shanghai, China). The induction of LASP1 expression (LASP1) and matched control (Vector) plasmids were also purchased from GenePharma (Shanghai, China). All plasmids and oligos were transfected into KYSE510 and Eca109 cells using Lipofectamine 3000 (ThermoFisher Scientific, San Jose, CA, USA) when the confluence reach up to 70-80% according to manufacturers' protocols.

Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from tissue homogenate samples or cell lysate samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For miRNA reverse transcription, cDNA was synthesized using TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). For mRNA reverse transcription, cDNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen). U6 and GAPDH were used as reference gene for miRNA and mRNA, respectively. Real-time quantitative PCR was performed with SYBR[®] Green (Promega, Madison, WI, USA) according to the manufacturer's instructions. Primer sequences were as follows: miR-143 forward, 5'-AGC GTG TGT CGT GGA GTC-3'; miR-143 reverse, 5'-TCG TGA GAT GAA GCA CTG TAG-3'; U6 forward, 5'-CTCGCT-TCGGCAGCACA-3'; U6 reverse, 5'-AACGCTTCA-CGAATTTGCGT-3'; LASP1 forward, 5'-TGCGGC-AAGATCGTGATCC-3'; LASP1 reverse, 5'-GCAG-TAGGGCTTC TTCTCGTAG-3'; GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAAT-3'; GAPDH reverse, 5'-GGCTGTTGTCATACT TCTCATGG-3'. Fluorescence was detected in an ABI 7300 System (Applied Biosystems, Foster City, CA, USA). Relative gene expression levels were calculated using the 2^{-ΔΔCt} method.

Western blot

Cells and tissue samples were gently rinsed with phosphate-buffered saline and lysed with ice-cold RIPA buffer (Thermo Fisher Scientific). Total protein concentration of each lysate sample was measured using Nanodrop2000 (Thermo Fisher Scientific). Twenty micrograms of total protein were loaded into each well on a sodium dodecyl sulfate-polyacrylamide gel for electrophoresis. The proteins were then transferred onto PVDF membrane, which was blocked by 3% BSA in TBST at room temperature for

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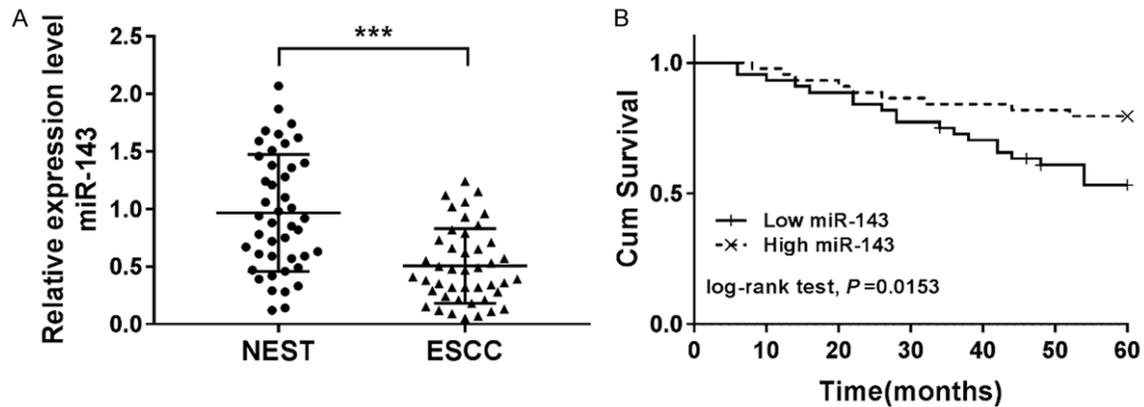


Figure 1. miR-143 expression in ESCC tissue (A) The miR-143 expression level in 44 ESCC tissues and normal esophageal squamous tissue (NEST) was detected by qRT-PCR. (B) Kaplan-Meier survival analysis showing correlation between miR-143 expression levels and overall survival rate of patients with esophageal cancer. *** $P < 0.001$.

1 h. The membrane was incubated with the primary antibody at 4°C overnight and secondary antibody for 1 h at room temperature. Immunoblot was detected with ECL substrate (Pierce, Thermo Fisher Scientific) and ChemiDoc XRS System (Bio-Rad) following manufacturers' instructions. Primary and secondary antibodies used in this research were as follows: Rabbit anti-human LASP1 (Bethyl, USA, catalog No. A303-289A); Rabbit anti-human GAPDH (Cell signaling, USA, catalog No. 5174); HRP-conjugated Goat anti-Rabbit secondary antibody (Abcam, USA, catalog No. ab97051).

Cell migration and invasion assays

For the transwell migration assay, 1×10^5 cells were seeded to the upper chambers of the transwell compartment (24-well format with 8 μ m pore size insert, Sigma, St. Louis, MO, USA). For the Matrigel-coated transwell invasion assay, 1×10^5 cells were seeded into the upper chambers of a 24-well BioCoat Matrigel invasion chamber (BD Bioscience, Bedford, MA, USA). The transwell plates were incubated at 37°C in a 5% CO₂ humidified incubator for 12 h. Cells migrated to the underside of the chamber were stained by 1% crystal violet (Sigma) in 2% ethanol for 20 min. The number of migrated and invaded cells was counted under a fluorescence microscope in five randomly picked views.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

miR-NC mimics or miR-143 mimics were transfected and co-transfected with empty vector or

LASP1 overexpressing vector into KYSE510 and Eca109 cells. These cells were seeded into 96-well plates (Corning Costar, Corning, NY, USA) at a density of 2×10^3 cells per well. 30 μ l of serum-free media supplemented with MTT solution (MTT assay kit, Sigma-Aldrich, St. Louis, MO) was added into each well, followed by incubation for 4 h at 37°C with 5% of CO₂. After discarding the media, 150 μ l of MTT solvent (4 mM HCl, 0.1% NP40 in isopropanol) was added into each well. Then the plates were incubated at 37°C with 5% of CO₂ for 3 h. The optical densities of the samples were measured using a spectrophotometric microplate reader (Beyotime Institute of Biotechnology, Haimen, China) at OD = 450 nm.

Luciferase reporter assay

Bioinformatic analysis using Targetscan database revealed LASP1 as a potential target gene of miR-143. Luciferase reporters were constructed based on the pMIR-REPORT™ (ThermoFisher Scientific). 3' UTR of LASP1 mRNA was amplified by PCR and inserted into the pMIR-REPORT™ to construct LASP1 wild-type reporter vectors (LASP1-WT). 3' UTR of LASP1 was changed with GeneArt™ Site-Directed Mutagenesis PLUS System (ThermoFisher Scientific) and inserted into the pMIR-REPORT™ to construct the mutant type reporter vectors (LASP1-MUT). Then LASP1-WT or LASP1-MUT and miR-NC mimics or miR-143 were transfected into HEK293T cells by Lipofectamine 3000. At 48 h post-transfection, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

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Table 1. Relationship between miR-143 expression and patients' characteristics in ESCC

Variables	No. of cases (n = 44)	miR-143 expression		P value
		Low (n = 26)	High (n = 18)	
Age (years)				P = 0.803
< 50	19	11	8	
≥ 50	25	15	10	
Gender				P = 0.854
Man	30	18	12	
Woman	14	8	6	
TNM stage				P = 0.000 ^a
II	19	6	13	
III	17	12	5	
IV	8	8	0	
Tumor size				P = 0.546
< 5 cm	23	12	11	
≥ 5 cm	21	14	7	
Vessel invasion				P = 0.023 ^b
Negative	34	16	18	
Positive	10	10	0	

^ap-value < 0.001, ^bP < 0.01.

Statistical analysis

All statistical analyses in this study were performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). All data are listed as mean value ± SD (standard deviation). The relationship between two variables and numerical values were analyzed using Student's t-test and Pearson's correlation analysis. Survival analysis using the Kaplan-Meier method was performed using the log-rank test. *P*-value < 0.5 was considered significant.

Results

miR-143 expression in ESCC tissue

To investigate the role that miR-143 plays in ESCC development, we first evaluated the clinical significance of miR-143 by analyzing its expression level in 44 pairs of ESCC and adjacent tissue specimens. As shown in **Figure 1A**, miR-143 expression was significantly down-regulated in ESCC tissues compared with non-malignant counterparts (**Figure 1A**), implying it has a tumor-suppressive role. Based on the average miR-143 expression level, the 44 patients were divided into miR-143 high expression (n = 18) and low expression group (n = 26).

The patients' clinical data showed that low expression of miR-143 significantly correlated with TNM stage progression and vessel invasion (**Table 1**). K-M curve analysis revealed that low expression of miR-143 significantly associated with ESCC patients worsened overall survival (**Figure 1B**). These results suggested that down-regulation of miR-143 was involved in tumorigenesis and development of ESCC.

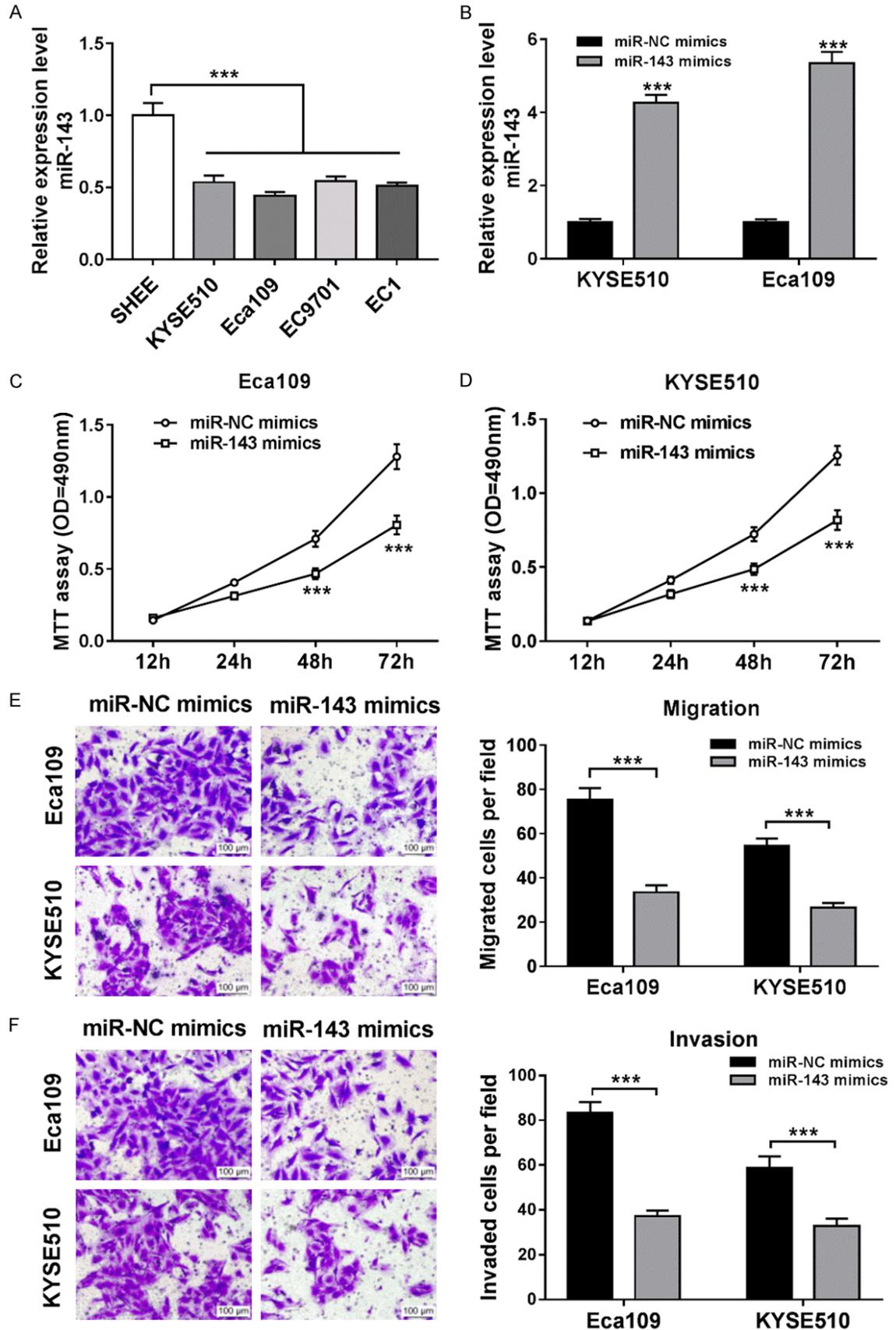
Up-regulation of miR-143 inhibited cell proliferation, migration, and invasion in ESCC

To further verify the function of miR-143 in ESCC, we evaluated the expression of this miRNA in several ESCC cell lines and a non-malignant, immortalized esophageal epithelial cell line SHEE by qRT-PCR. As shown in **Figure 2A**, miR-143 expression was significantly lower in ESCC cell lines compared to SHEE cells. By transfecting miR-143 mimic into KYSE510 and Eca109 cells, miR-143 level in the two cell lines was significantly increased compared to those transfected with miRNA mimic control (miR-NC mimic) (**Figure 2B**). As revealed by MTT assay results, increase in miR-143 level significantly inhibited cell growth in the two ESCC cell lines (**Figure 2C and 2D**), while the transwell assay results showed that cell migration and invasion capacity of the two ESCC cell lines was significantly reduced by miR-143 upregulation (**Figure 2E and 2F**). These results suggested that up-regulation of miR-143 could attenuate the malignancy of ESCC cells in vitro.

LASP1 was a target of miR-143

To explore the mechanism of action of miR-143 in ESCC, we performed bioinformatic analysis and identified LASP1 mRNA as a potential target of miR-143 (**Figure 3A**). To confirm this predicted result, a luciferase reporter vector containing the full-length LASP1 mRNA 3' UTR (LASP1-WT) and a luciferase reporter vector containing the mutated LASP1 mRNA 3' UTR (LASP1-MUT) was constructed and transfected into HEK293T cells. Co-transfection with miR-143 mimics or miR-143 inhibitor significantly decreased or increased luciferase activity in HEK293T cells transfected with LASP1-WT

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Figure 2. Upregulation of miR-143 inhibited cell proliferation, migration, and invasion in ESCC cells. (A) The miR-143 expression level in SHEE and four esophageal cancer cell lines; GAPDH was used as an internal control (B) The expression of miR-143 was increased in KYSE510, Eca109 cells transfected with miR-143 mimics. (C, D) MTT assay was used to evaluate the effect of miR-143 mimic transfection on Eca109 and KYSE510 cells proliferation. (E) Transwell migration assay was used to evaluate the effect of miR-143 mimic transfection on Eca109 and KYSE510 cells migratory capacity. (F) Transwell invasion assay was used to evaluate the effect of miR-143 mimic transfection on Eca109 and KYSE510 cells invasive capacity. ***P < 0.001.

A Position 1683-1689 of LASP1 3' UTR

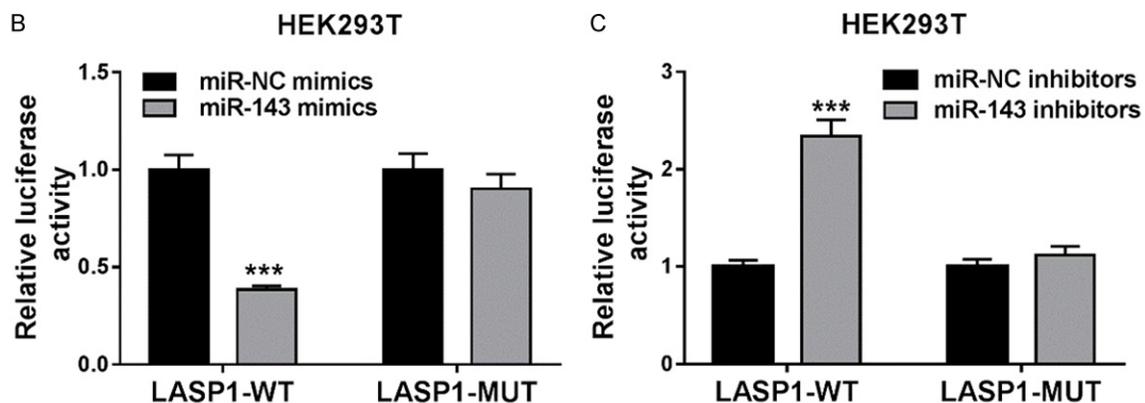
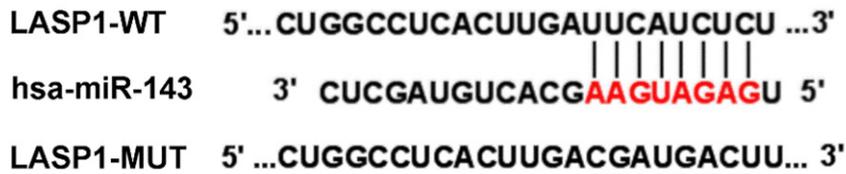


Figure 3. LASP1 is a target of miR-143. A. Binding and mutant sites between LASP1 and miR-143. B. Luciferase activity was detected in HEK 293T cells after co-transfection with miR-143 mimics or miR-NC mimics and LASP1-WT or LASP1-MUT reported plasmid. C. Luciferase activity was detected in HEK 293T cells after co-transfection with miR-143 inhibitors/miR-NC inhibitors and LASP1-WT or LASP1-MUT reporter plasmid. ***P < 0.001.

reporter plasmids but not in those with LASP1-MUT ones (Figure 3B and 3C). These results suggested that 3' UTR of LASP1 mRNA is a direct target of miR-143.

miR-143 directly regulated LASP1 expression in ESCC

Previous studies have demonstrated that LASP1 can promote ESCC cell proliferation, migration and invasion in vitro, but the clinical significance of this gene's expression was not evaluated. We therefore measured the expression of LASP1 in ESCC and adjacent tissue specimens by qRT-PCR, and analyzed the influence of LASP1 expression on ESCC patients' overall survival. The results showed that LASP1 expression was significantly higher in ESCC tissues compared to non-malignant counterparts (Figure 4A), and survival of ESCC patients with high

LASP1 expression was significantly lower compared to those with low LASP1 expression (Figure 4B). We also found that LASP1 expression was significantly increased in YSE510 and Eca109 cells compared to SHEE cells (Figure 4C). Pearson's correlation analysis revealed that miR-143 expression significantly correlated with that of LASP1 (Figure 4D). Transfection of miR-143 mimic significantly down-regulated LASP1 protein level in Eca109 and KYSE510 cells, while miR-143 inhibitor transfection showed opposite effects (Figure 4E and 4F). These results further confirmed that miR-143 negatively regulates LASP1 expression in ESCC.

Overexpression of LASP1 reversed the effects of miR-143 mimic transfection on ESCC

To verify whether miR-143 inhibits ESCC cell proliferation, migration, and invasion by target-

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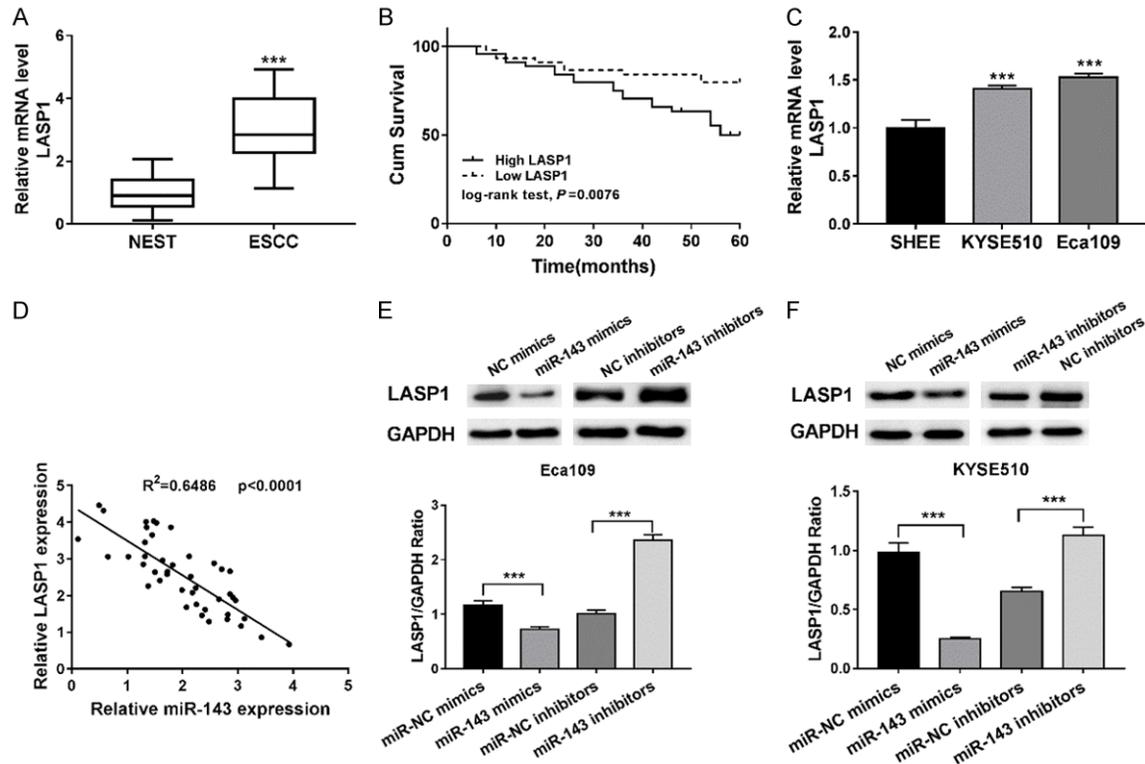


Figure 4. miR-143 regulated LSAP1 expression directly in ESCC. (A) The LASP1 expression level in 44 ESCC tissues and normal esophageal squamous tissue (NEST) was detected by qRT-PCR. (B) Kaplan-Meier survival analysis showing correlation between LASP1 expression levels and overall survival rate of patients with esophageal cancer. (C) The LASP1 expression level in SHEE and four esophageal cancer cell lines; GAPDH was used as an internal control (D) The correlation analysis of miR-143 and LASP1 by Pearson's correlation analysis. (E, F) The protein level of LASP1 were measured in Eca109 (E) and KYSE510 (F) cells with transfection of miR-NC mimics, miR-143 mimics, miR-NC inhibitors, and miR-143 inhibitors by western blot. *** $P < 0.001$.

ing LASP1, KYSE510 and Eca109 cells transfected with miR-143 mimic were co-transfected with LASP1 overexpressing vector or control vector. Western blot results showed that down-regulation of LASP1 protein expression by miR-143 mimic transfection could be significantly rescued by LASP1 overexpression in the two ESCC cell lines (Figure 5A), while MTT assay and transwell assay results showed that ESCC cell growth, migration, and invasion impaired by miR-143 mimic transfection were significantly recovered by LASP1 overexpression (Figure 5B-E). These results suggested that miR-143 could reduce ESCC cell malignancy in vitro by targeting LASP1.

Discussion

As one of the most common cancers in the world, esophageal cancer has a high incidence and poor prognosis, which has around 15-25% in five-year survival rates [3]. Esophageal can-

cer threatens human life and health, especially in China [5]. Therefore, finding an effective targeted therapy for esophageal cancer treatment is still urgently needed.

In esophageal adenocarcinoma, miRNAs influence cell growth and tumor development by regulating their target gene expressed. For example, miR-503 reduces ESCC cell growth by targeting the expression of cyclin D1, and decreases in this miRNA contributes to ESCC tumor development down-regulation of miR-503 contributed to ESCC Cell growth by liberating Cyclin D1 [25]. Wang *et al.* suggested that miR-221 contributes to esophageal adenocarcinoma cell proliferation and chemoresistance by targeting DKK2 [26]. miR-577 was found directly regulating TSGA10 expression in ESCC cells, and miR-577 increased ESCC cell malignancy by targeting TSGA10 [27]. In addition, miR-143 levels were down-regulated in ESCC, and reinforced miR-143 expression suppressed

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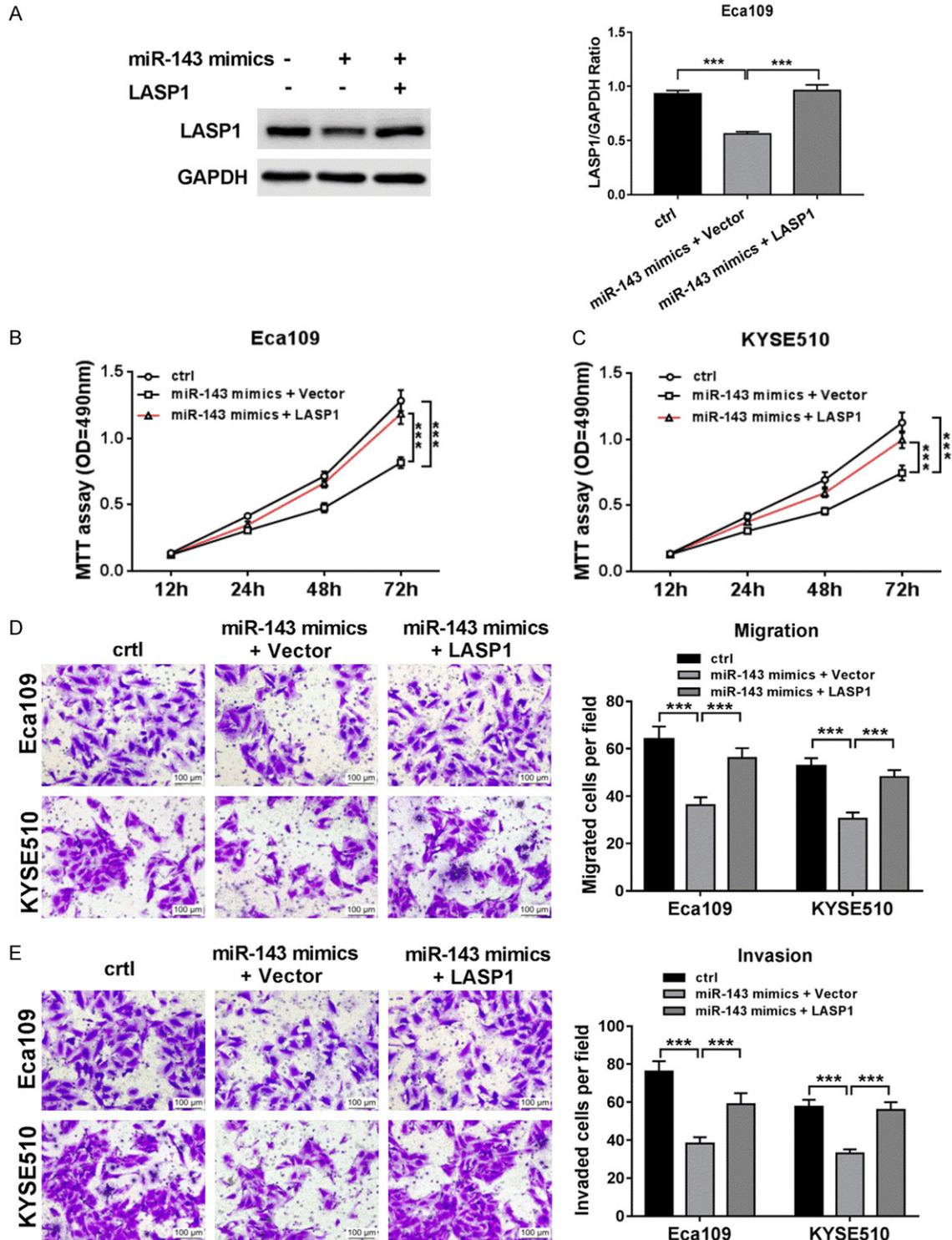


Figure 5. Overexpression of LASP1 reversed the effects of miR-143 on ESCC. (A) The protein level of LASP1 was detected in Eca109 cells with transfection of ctrl, miR-143 mimics + vector and miR-143 mimics + LASP1. (B, C) MTT assay was used to evaluate cells proliferation in Eca109 (B) and KYSE510 (C) cells with transfection of ctrl, miR-143 mimics + vector and miR-143 mimics + LASP1. (D) Transwell migration assay was used to evaluate cells migratory capacity in Eca109 and KYSE510 cells with transfection of ctrl, miR-143 mimics + vector and miR-143 mimics + LASP1. (E) Transwell invasion assay was used to evaluate cells invasive capacity in Eca109 and KYSE510 cells with transfection of ctrl, miR-143 mimics + vector and miR-143 mimics + LASP1. ***P < 0.001.

proliferation and induced apoptosis in ESCC cells by down-regulating QKI-5 expression [28]. In the present research, we found that miR-143 was significantly down-regulated in ESCC tissues and cells compared to non-malignant counterparts, and low expression of miR-143 significantly associated with ESCC progression and patients worsened survival. Functional assays demonstrated that up-regulation of miR-143 attenuated ESCC cell proliferation, migration, and invasion in vitro. These results suggested miR-143 as a tumor-suppressive miRNA in ESCC.

To further investigate the underlying mechanisms of miR-143's action, we identified LASP1 as a downstream target of miR-143 by bioinformatic analysis and luciferase reporter assay. Recent studies reported that LASP1 was up-regulated in various types of cancer and was implicated in cancer cell proliferation, associating with patients' poor prognosis. For example, overexpression of LASP1 in SW480 colorectal cancer cells promoted cell proliferation and metastasis, while LASP1 silencing inhibited cell growth. High expression of LASP1 was also significantly associated with impaired overall survival of CRC patients [21]. A study of Zhao *et al.* showed that LASP1 is up-regulated in pancreatic ductal adenocarcinoma (PDAC), whose overexpression promoted cell metastasis and tumor progression [29]. On the other hand, He *et al.* reported that silencing of LASP1 significantly inhibited ESCC cell growth [30]. As expected, in the present investigation we found that LASP1 was up-regulated in ESCC tissue specimens and cells compared to non-malignant counterparts, inversely correlating with that of miR-143. Moreover, the survival rates of patients with high LASP1 expression were significantly decreased compared to those with low LASP1 expression.

Except for miR-143-3a, LASP was also found a target of other miRNAs. Sui *et al.* demonstrated that miR-133a/LASP1 axis decreased breast cancer cell proliferation [31]. miR-29b inhibited gastric cancer cell growth and metastasis by down-regulating LASP1 [22]. miR-218 repressed trophoblast invasion of preeclampsia as well as GC cell growth and survival by down-regulating LASP1 [32, 33]. Our experimental results showed that overexpression of LASP1 abolished the suppressive effects of miR-143 mimic transfection on ESCC cell proliferation, migra-

tion, and invasion, suggesting that miR-143 could suppress ESCC cell growth by targeting LASP1.

Although the function of miR-143/LASP1 axis has been verified in ESCC cells in vitro, it is necessary to establish an animal model to verify these influences of miR-143 and LASP1 on ESCC tumor growth in vivo. Other tumor-promoting genes, such as KRAS and Bcl-2, were also found as targets of miR-143 in colorectal and cervical cancer [34, 35]. Therefore, miR-143 might suppress ESCC development by targeting the expression of other genes except for LASP1.

In summary, our data identified LASP1 as a novel target of miR-143. miR-143 acted a tumor suppressor by targeting LASP1 in ESCC. Based on our findings, miR-143/LASP1 axis could be a novel therapeutic target in ESCC treatment.

Disclosure of conflict of interest

None.

Address correspondence to: Huifeng Liu, Department of Thoracic Surgery, PLA 309 Hospital, Heishanhu Road, Haidian, Beijing 100091, China. Tel: +86-138-1182-9502; E-mail: asdklsakdlis@sohu.com

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