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LINC00963 predicts poor prognosis and promotes esophageal cancer cells invasion *via* targeting miR-214-5p/RAB14 axis

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Abstract. – OBJECTIVE: To explore the roles and underlying mechanism of LINC00963 in esophageal squamous cell carcinoma (ESCC) progression.

PATIENTS AND METHODS: Quantitative Real Time-PCR (qRT-PCR) was used to detect LINC00963 expression in ESCC tissues. EdU, colony formation, and transwell invasion assays were used to detect the proliferation and metastasis ability of ESCC, respectively. The correlation between LINC00963 and miR-214-5p in ESCC was confirmed by a Luciferase reporter and RIP assays.

RESULTS: LINC00963 expression was significantly increased in ESCC tissues and correlated with advanced TNM stage, metastasis, and poor prognosis. The knockdown of LINC00963 expression reduced ESCC cells proliferation, invasion *in vitro*, and reduced tumor growth *in vivo*. In mechanism, LINC00963 served as a sponge for miR-214-5p in ESCC progression. In addition, miR-214-5p could bind to RAB14 and regulate its expression.

CONCLUSIONS: LINC00963 might promote ESCC cells proliferation and invasion *via* regulating the miR-214-5p/RAB14 axis and it might serve as a therapeutic target for ESCC treatment.

Key Words:
LINC00963, MiR-214-5p, RAB14, Esophageal squamous cell carcinoma.

Introduction

Esophageal cancer (EC) is one of the most common cancers worldwide[1]. According to its biological characteristics, EC categorized into esophageal adenocarcinoma and esophageal squamous cell carcinoma (ESCC)[2,3]. Although the great advanced treatments in ESCC, the 5-year overall survival rates remain poor[4,5]. Therefore, it is imperative to explore the molecular mechanisms of ESCC and develop novel therapeutic targets for tumor treatment.

Long non-coding RNAs (lncRNAs) are RNA molecules over 200 nucleotides without capacity to encode protein[6]. Recently, accumulating evidence[7-9] suggested that lncRNAs play important roles in tumor procession, including ESCC progression. Wu et al[10] showed that CASC9 promoted ESCC growth *via* suppressing PDCD4 expression by regulating EZH2. Chen et al[11] suggested that lncRNA FAM201A mediated the radio-sensitivity of ESCC *via* regulating the miR-101/ATM/mTOR axis. Yang et al[12] revealed that lncRNA FTH1P3 promoted ESCC cells proliferation and metastasis by regulating SP1/NF-kB axis.

LINC00963 is located in chromosome 9, which has been reported to play critical roles in tumor progression. Wang et al[13] showed that LINC00963 was involved in prostate cancer metastasis by regulating EGFR expression. Wu et al[14] suggested that LINC00963 promoted hepatocellular carcinoma cells proliferation by activating the PI3K/AKT axis. Yu et al[15] found that LINC00963 promoted lung cancer metastasis by regulating the PGK1/AKT/mTOR axis. However, the role and underlying mechanism of LINC00963 in ESCC progression remain unclear.

In the present study, we found that LINC00963 could act as an oncogenic lncRNA in ESCC progression. In mechanism, LINC00963 promoted ESCC progression by sponging miR-214-5p and upregulating RAB14 expression. These data suggested that LINC00963 might act as a potential therapeutic treatment in ESCC.

Patients and Methods

Tissue Specimens

A total of 54 ESCC tissues were collected from patients who underwent surgical resection at the First Hospital of Shijiazhuang City. No patients received chemotherapy or radiotherapy before

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surgery. All ESCC specimens were confirmed by experienced pathologists. The investigation was approved by the Ethics Committee of The First Hospital of Shijiazhuang City. Written informed consents were provided by all participants.

Cell Culture and Transfection

Human ESCC cell lines (TE-1, ECA-109, TE-13, EC9706, and KYSE-150) and normal esophageal epithelial cell line (HET-1A) were obtained from Shanghai Institutes for Biological Science (Shanghai, China). All cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37°C in a humidified 5% CO_2 incubator.

MiR-214-5p mimics, miR-214-5p inhibitors, and sh-LINC00963 were obtained from GenePharma (Shanghai, China). All plasmids were transfected into cells using Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA, USA).

RNA Isolation and QRT-PCR

The total RNAs from tissues or cells were extracted using TRIzol reagents (Invitrogen, Carlsbad, CA, USA). The reverse transcription was performed with 25 μL system based on the instructions of the cDNA reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA was diluted to 50 ng/μL for the following Real time-PCR, which was carried out on the fluorescence quantitative polymerase chain reaction instrument. The fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were severed as the internal controls, respectively.

Cell Proliferation Assay

EdU cell proliferation assay was determined as previously described[10]. For colony formation assay, cells were plated into 24-well plates (2×10^4 cells/well) and incubated at 37°C for 10 days. Cells were then fixed with 4% paraformaldehyde and stained with 0.05% crystal violet. The visible colonies were counted.

Transwell Assay

Transfected cells were plated into the upper chamber of 24-well plates with Matrigel-coated membrane. The bottom chamber was filled with DMEM medium supplemented with 20% FBS. Cells were incubated for 48 h. Next, the invaded cells were fixed with 4 % paraformaldehyde and stained with crystal violet dye.

Western Blot

Cells were lysed using Radio Immunoprecipitation Assay (RIPA) lysis (Beyotime, Shanghai, China) containing protease inhibitors. The cell lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were blocked by blocking solution containing 5% skim milk powder at room temperature for 2 h. The PVDF membranes were incubated with primary antibodies overnight, which were then incubated with secondary antibodies at room temperature for 2 h.

Luciferase Reporter Assay

Based on pmirGLO vectors (Promega, Madison, WI, USA), the 3' UTR of LINC00963 or RAB14 with miR-214-5p binding sites or mutant sites was used to generate wild type (WT) or mutant (MUT) Luciferase reporter vectors, named as LINC00963-Wt, LINC00963-Mut, RAB14-Wt or RAB14-Mut, respectively. MiR-214-5p mimics or miR-NC was co-transfected with LINC00963-Wt, LINC00963-Mut, RAB14-Wt or RAB14-Mut into HEK293T cells using Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA, USA). Cells were collected at 48 h after the transfection and the relative luciferase activity was detected using a luciferase reporter assay kit (Promega, Madison, WI, USA).

RNA Immunoprecipitation (RIP) Assay

RIP analysis was described as the previous study[16]. Briefly, cells were lysed with RIP-lysis buffer. The cell lysate was collected and incubated with the magnetic beads that were conjugated with an anti-Argonaute 2 (AGO2) or negative control IgG antibodies (Millipore, Billerica, MA, USA), treated with proteinase K to digest the proteins, and was subjected to RNA extraction using the TRIzol® reagent. The immunoprecipitated RNA was analyzed by RT-qPCR.

Xenograft Mouse Model

5 weeks old BALB/c nude mice were obtained from Shanghai SLAC Laboratory Animal (Shanghai, China). Transfected KYSE-150 cells (1×10^6) were subcutaneously inoculated into the right flank of the nude mice. Tumor volume was measured every 7 days for 42 days and calculated according to the equation: $V = (\text{length} \times \text{width}^2)/2$. All mice were sacrificed at day 42 of inoculation, and the tumor weight was measured. Mice were bred in the

Animal Core Facility by following procedures approved by Animal Ethics Committee in The First Hospital of Shijiazhuang City.

Statistical Analysis

Data were displayed as mean ± standard deviation (SD) from three separate experiments. SPSS 21.0 (IBM, Armonk, NY, USA) was selected for statistical analysis. The comparisons of the two groups or multiple groups were performed using the Student's *t*-test or One-way ANOVA. $p < 0.05$ was considered as statistically significant.

Results

LINC00963 was upregulated in ESCC

We first determined LINC00963 expression in ESCC tissues and adjacent non-tumor tissues (NC). Results showed that LINC00963 expression was upregulated in ESCC tissues (Figure 1A). Subsequently, high expression of LINC00963 was associated with advanced TNM stage and lymph-node metastasis in ESCC patients (Figure 1B and 1C). GEPIA database showed that LINC00963 was upregulated in ESCC tissues (Figure 1D).

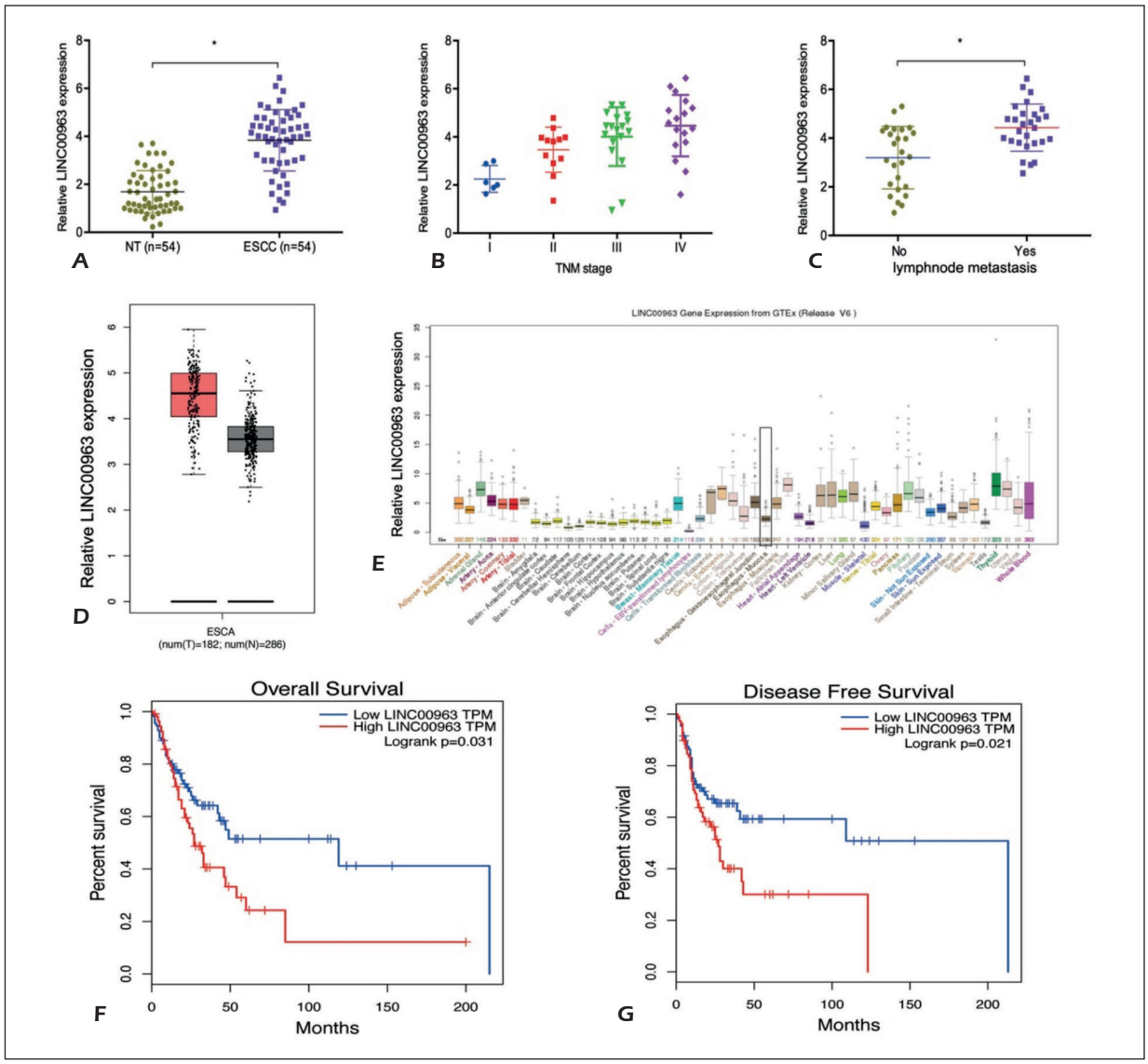

Figure 1. LINC00963 was highly expressed in ESCC. **A**, LINC00963 expression was upregulated in ESCC tissues compared to adjacent non-tumor tissues (NC). **B**-**C**, High LINC00963 expression was associated with advanced TNM stage, and lymph-node metastasis in ESCC patients. **D**, GEPIA showed that LINC00963 was upregulated in ESCC tissues. **E**, LINC00963 expression was decreased in esophageal tissues by GTEx database. F, G, High LINC00963 expression was associated with poor overall survival and disease-free survival in ESCC patients. *$p<0.05$

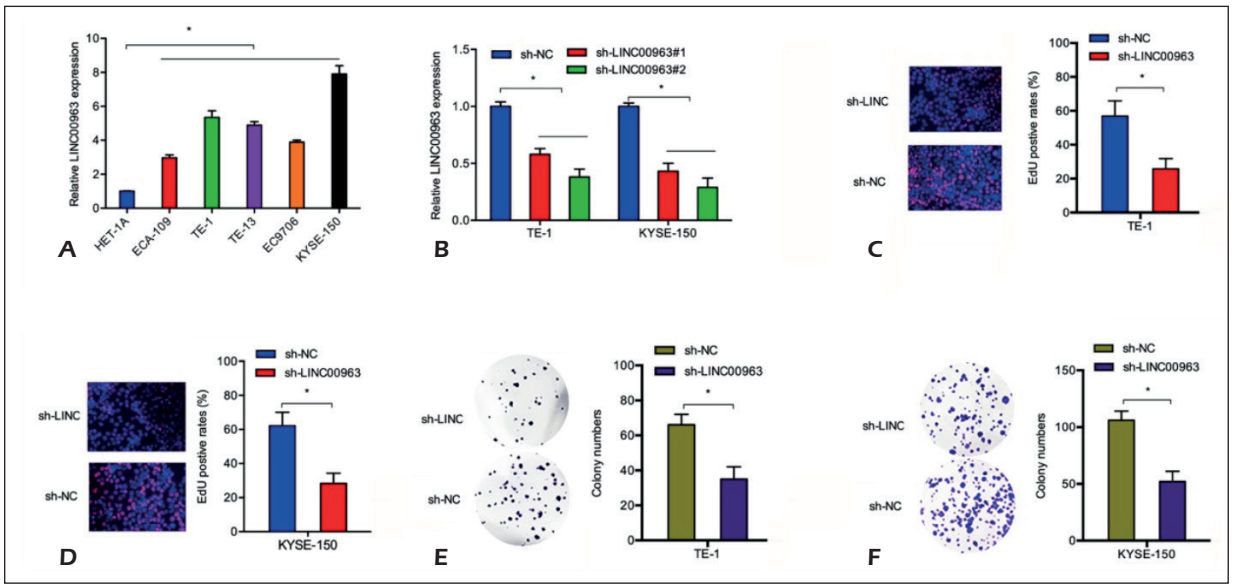

Figure 2. LINC00963 inhibition reduced ESCC cells proliferation abilities. **A**, LINC00963 expression was upregulated in ESCC cell lines. **B**, Sh-LINC00963 decreased LINC00963 expression in KYSE-150 and TE-1 cells. **C-D**, LINC00963 suppression reduced KYSE-150 and TE-1 cells proliferation abilities (magnification, ×200). E, F, LINC00963 inhibition decreased KYSE-150 and TE-1 cells colony numbers (magnification, ×200). *p<0.05

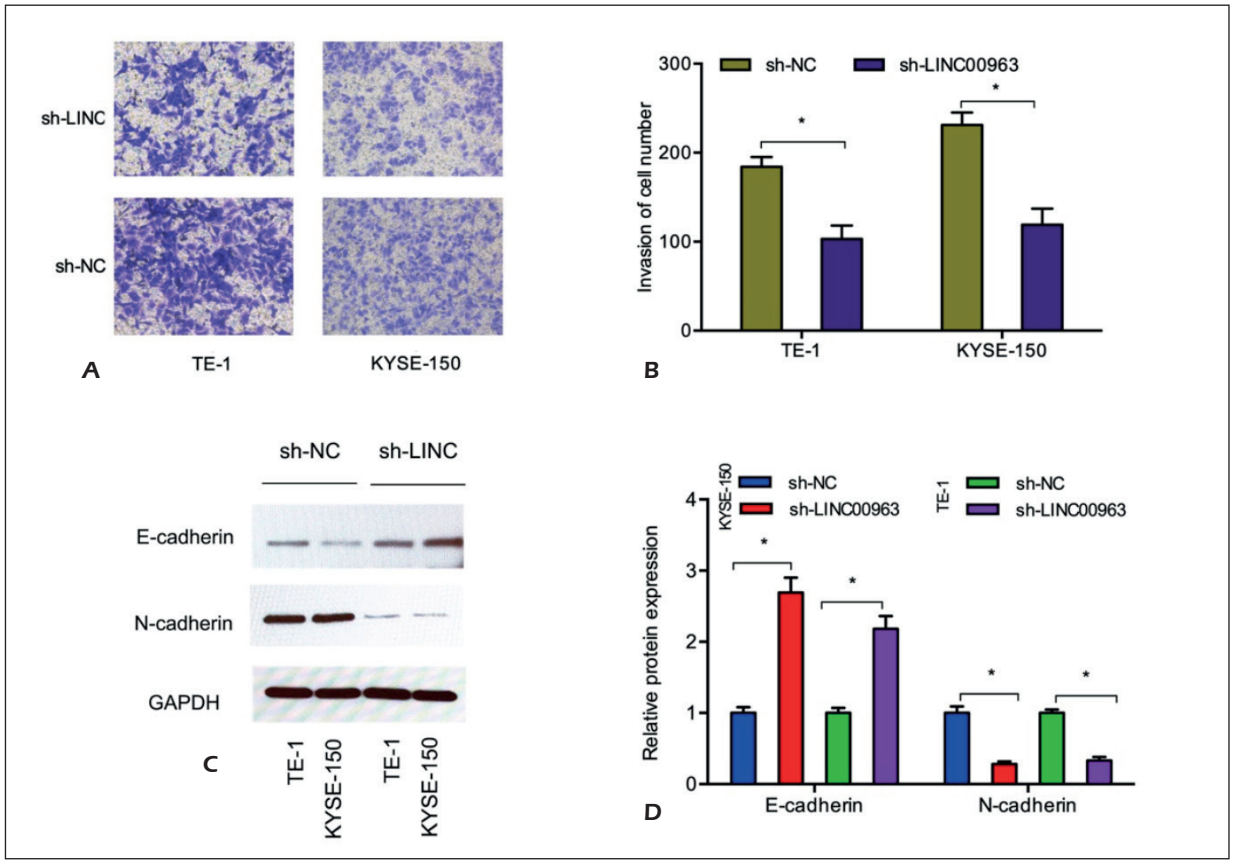

Figure 3. LINC00963 inhibition reduced EMT process. **A-B**, LINC00963 inhibition suppressed KYSE-150 and TE-1 cells invasion abilities (magnification, ×200). **C-D**, Effects of LINC00963 inhibition on N-cadherin and E-cadherin expression was explored by Western blot. *p<0.05.

GETx database showed that LINC00963 was significantly reduced in esophageal tissues (Figure 1E). Furthermore, high LINC00963 expression was associated with poor overall survival (OS) and disease-free survival (DFS) in ESCC patients (Figure 1F and 1G).

Downregulation of LINC00963 Reduced ESCC Cells Proliferation and Invasion

QRT-PCR showed that LINC00963 expression was upregulated in ESCC cell lines (Figure 2A), and the reduction efficiency was determined by qRT-PCR (Figure 2B). EdU and colony formation assays showed that LINC00963 suppression reduced ESCC cells proliferation *in vitro* (Figure 2C-2F). Transwell assay showed that the knockdown of LINC00963 reduced suppressed ESCC cells invasion *in vitro* (Figure 3A and 3B). Subsequently, the effects of LINC00963 on epithelial-mesenchymal transition (EMT) processes were explored, results showed that LINC00963 inhibition decreased N-cadherin expression (mesenchymal markers), while increased E-cadherin expression (epithelial marker) in ESCC cells (Figure 3C and 3D).

LINC00963 Promoted Tumor Growth In Vivo

We explored the roles of LINC00963 *in vivo*. Results showed that LINC00963 suppression significantly reduced tumor growth and weight *in vivo* (Figure 4A-4C). Moreover, IHC showed that LINC00963 inhibition significantly decreased Ki-67 expression in nude mice transfected with sh-LINC00963 compared to sh-NC group (Figure 4D).

LINC00963 Served as a Sponge for MiR-214-5p

To further explore the underlying mechanism of LINC00963, we determined the location of LINC00963 by lncLocator. Results showed that LINC00963 was mainly located in the cytoplasm (Figure 5A). Subsequently, we suggested that LINC00963 possessed a complementary sequence to miR-214-5p seed region (Figure 5B). Luciferase reporter assay showed that miR-214-5p mimics significantly reduced LINC00963-Wt group luciferase activity (Figure 5C).

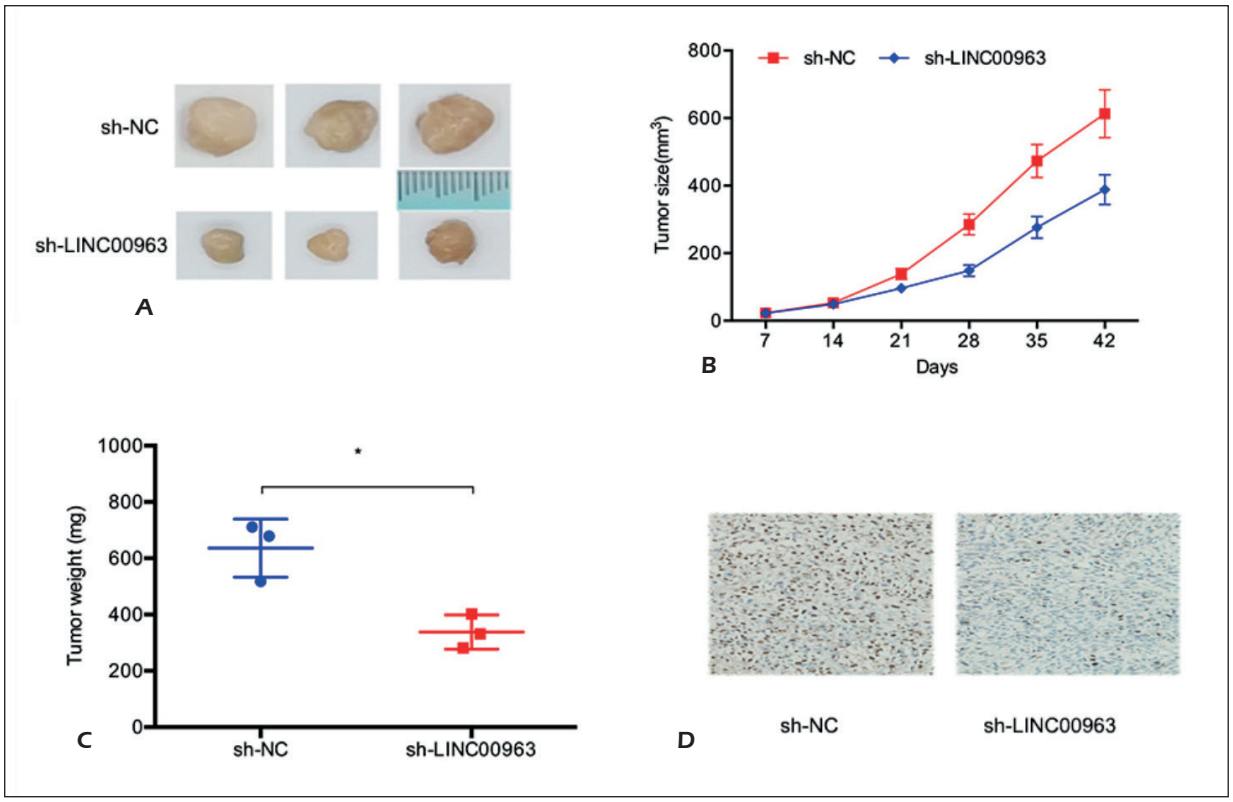

Figure 4. LINC00963 inhibition reduced tumor growth *in vivo*. **A**, Pictures of tumors. **B-C**, LINC00963 suppression remarkably reduced tumor growth in vivo. **D**, LINC00963 suppression reduced Ki-67 expression in nude mice (magnification, ×200). **p*<0.05.

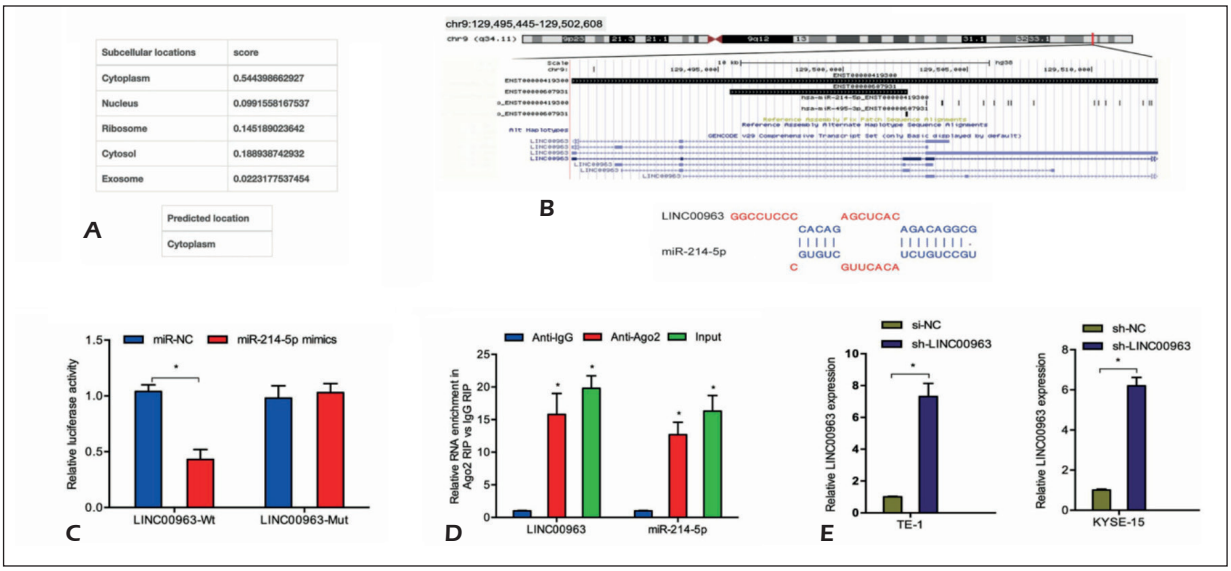

Figure 5. LINC00963 served as a sponge for miR-214-5p. **A**, lncLocator was used to explore the location of LINC00963. **B**, Putative binding sites of miR-214-5p on LINC00963. **C**, MiR-214-5p mimics reduced the Luciferase activity of LINC00963-Wt group. **D**, RIP assay showed that LINC00963 and miR-214-5p expression were enriched in ESCC cells. **E**, LINC00963 suppression increased miR-214-5p expression in KYSE-150 and TE-1 cells. **p*<0.05

RIP assay showed that both LINC00963 and miR-214-5p were enriched in ESCC cells (Figure 5D). In addition, qRT-PCR showed that LINC00963 suppression enhanced miR-214-5p expression in ESCC cells (Figure 5E).

In addition, we examined miR-214-5p roles in ESCC. Results proved that miR-214-5p expression was significantly decreased in ESCC patients and correlated with poor prognosis (Figure 6A-6C). Moreover, rescue assay showed that miR-214-

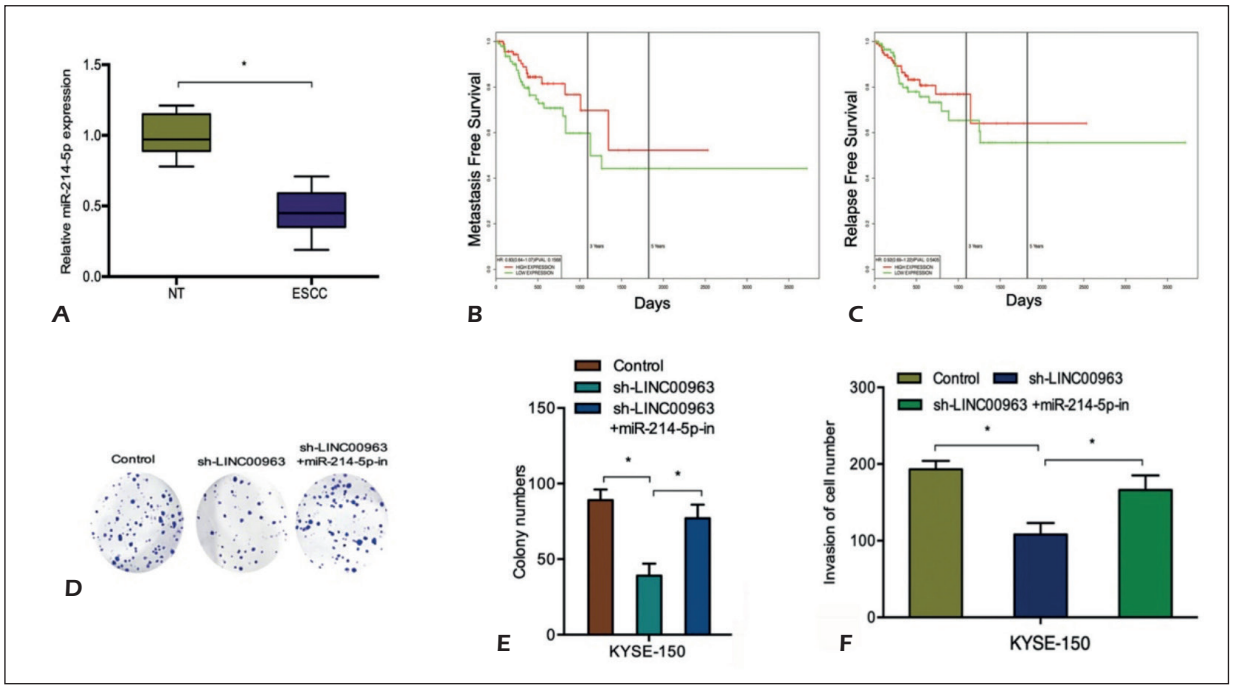

Figure 6. The roles of miR-214-5p in ESCC. **A**, Relative miR-214-5p expression was decreased in ESCC tissues. **B**-**C**, Low miR-214-5p expression was associated with poor prognosis in ESCC patients. **D**-**F**, MiR-214-5p inhibitors reversed colony formation and invasion abilities induced by LINC00963 suppression (magnification, ×200). **p*<0.05

5p inhibitors reversed the effects of LINC00963 inhibition on KYSE-150 cells proliferation and invasion *in vitro* (Figure 6D-6F). These findings suggested that LINC00963 might serve a sponge for miR-214-5p in ESCC.

RAB14 is Directly Targeted by MiR-214-5p

Bioinformatics analysis identified that RAB14 might act as a target of miR-214-5p (Figure 7A and 7B). Luciferase reporter assay revealed that miR-214-5p mimics reduced the luciferase activity of RAB14-Wt group (Figure 7C). Western blot disclosed that miR-214-5p mimics significantly reduced RAB14 protein in ESCC cells (Figure 7D and 7E). Next, we explored RAB14 expression in ESCC. Immunohistochemistry (IHC) showed that RAB14 expression was significantly increased and correlated with advanced TNM stage (Figure 7F). The results were further confirmed by The Cancer Genome Atlas (TCGA) database (Figure 7G). In addition, Kaplan-Meier analysis indicated that high RAB14 expression was correlated with poor overall survival in ESCC patients (Figure 7H).

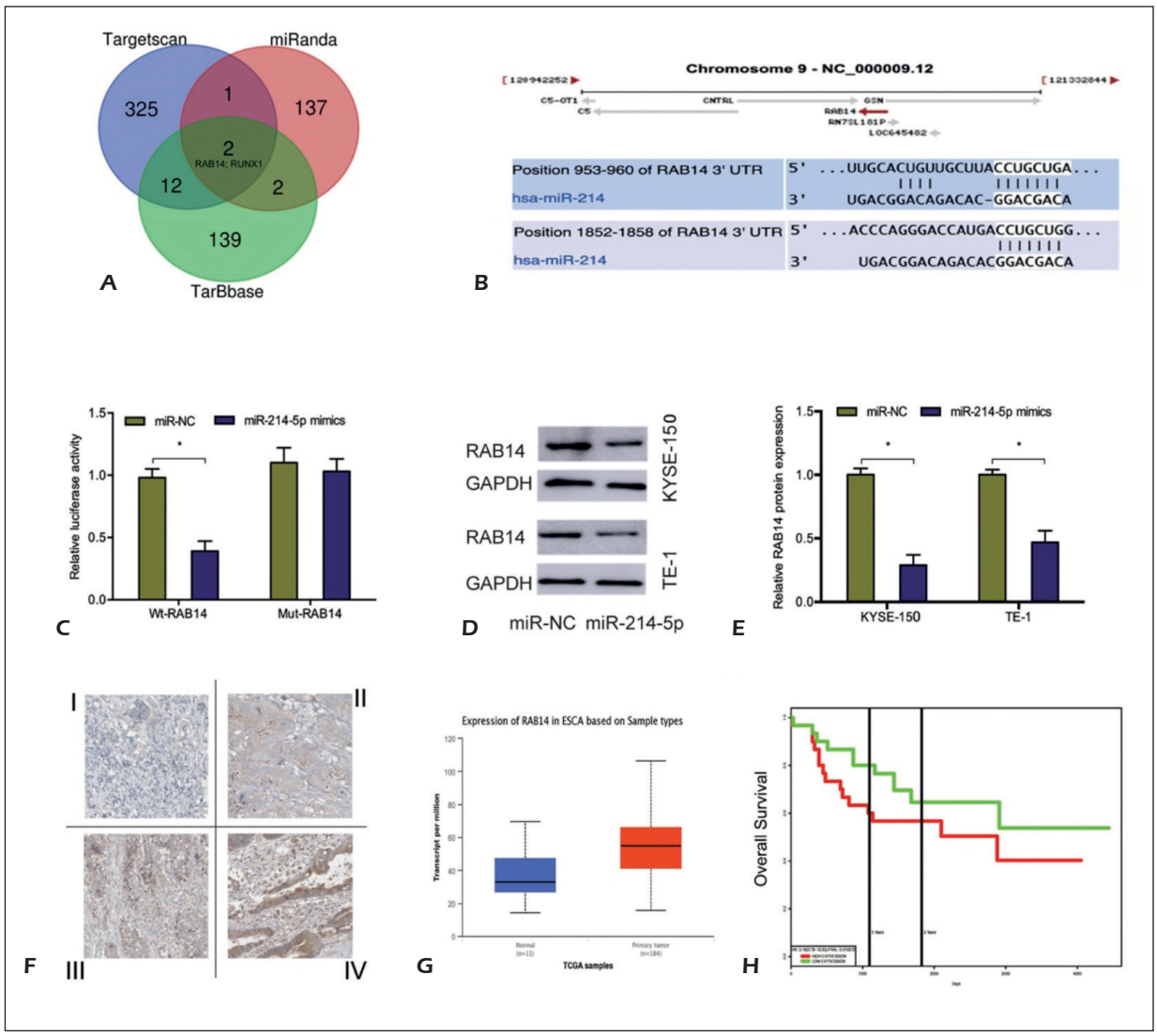

Figure 7. RAB14 acted as a target of miR-214-5p. **A-B**, Schematic illustration showed the potential binding sites between miR-214-5p and RAB14. **C**, MiR-214-5p mimics reduced the luciferase activity of RAB14-Wt group. **D-E**, MiR-214-5p mimics reduced RAB14 protein expression in KYSE-150 and TE-1 cells. **F**, RAB14 expression was increased and correlated with advanced TNM stage in ESCC (magnification, ×200). **G**, TCGA data showed that RAB14 was upregulated in ESCC tissues. **H**, High RAB14 expression was associated with poor overall survival in ESCC patients. *p<0.05

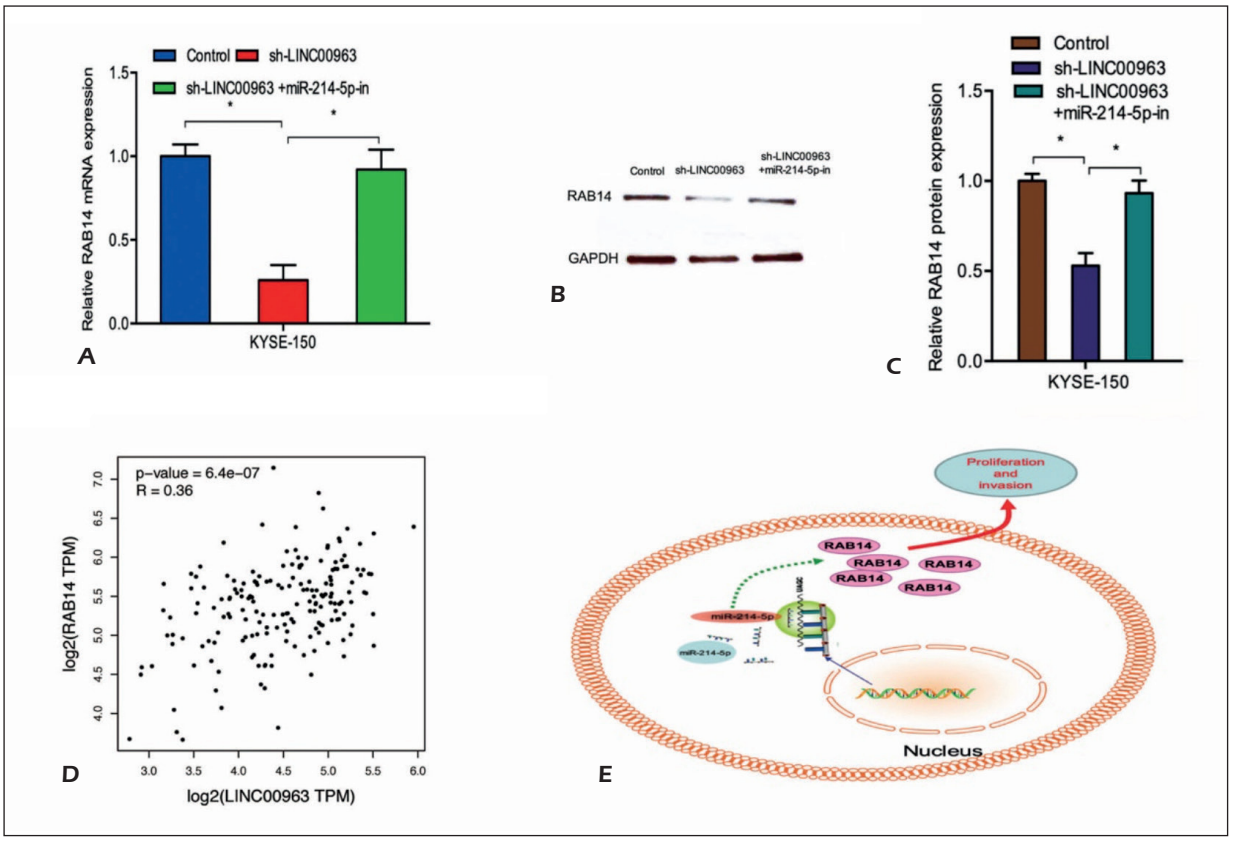

Figure 8. LINC00963/miR-214-5p/RAB14 axis in ESCC. **A-C**, MiR-214-5p inhibitors abolished the effects of sh-LINC00963 on RAB14 mRNA and protein expression. **D**, RAB14 expression was positively correlated with LINC00963 expression in ESCC tissues. **E**, Schematic diagram of LINC00963/miR-214-5p/RAB14 axis in ESCC. *p<0.05.

Then, we explored the effects of LINC00963 on RAB14 expression in ESCC cells. Results showed that sh-LINC00963 significantly reduced RAB14 expression in KYSE-150 cells, while miR-214-5p inhibitors (miR-214-5p-in) rescued the effects (Figure 8A-8C). In addition, the Gene Expression Profiling Interactive Analysis (GEPIA) database revealed that RAB14 expression was positively correlated with LINC00963 expression in ESCC (Figure 8D). Taken together, these results indicated that LINC00963 might promote ESCC cells progression via miR-214-5p/RAB14 axis (Figure 8E).

Discussion

Recently, increasing evidence[17] showed that lncRNAs play critical roles in malignant cancers progression. For example, Zhang et al[18] detected that MALAT1 was upregulated in renal cancer and associated with advanced tumor progression and poor overall survival. Zhang et al[19] found that H19 was overexpressed in glioma tissue and promoted tumor growth by regulated miR-675 expression. Song et al[20] revealed that HOTAIR mediated the switching of H3K27me3 acetylation to methylation to promote EMT in gastric cancer. In the present study, LINC00963 expression was significantly increased in ESCC tissues and cell lines. High LINC00963 expression was associated with advanced TNM stage, lymph-node metastasis, and poor prognosis of ESCC patients. Subsequently, function assays showed that LINC00963 inhibition suppressed ESCC cells viabilities both *in vitro* and *in vivo*. Thus, we demonstrated that LINC00963 might act as an oncogenic lncRNA in ESCC progression.

Recently, numerous studies[21] showed that LINC00963 could regulate tumor progression by serving as a ceRNA for miRNAs. Jiao et al[22] observed that LINC00963/miR-608/NACC1 axis could act as a therapeutic target for melanoma treatment. Zhou et al[23] found that LINC00963 served as a ceRNA in thyroid cancer progression via sponging miR-204-3p. In the present research, we found that LINC00963 possessed a complementary sequence to miR-214-5p seed

region. LINC00963 suppression enhanced the expression of miR-214-5p in ESCC cells. Subsequently, Luciferase reporter and RIP assays confirmed the correlation between miR-214-5p and LINC00963. In addition, rescue assays showed that miR-214-5p inhibitors abolished the effects of sh-LINC00963 on ESCC cells proliferation and invasion abilities *in vitro*.

RAB14 belongs to the large RAB family which plays key roles in the regulation of intracellular membrane trafficking[24]. RAB14 might play important functions in tumor progression. Lian et al[25] showed that RAB14 inhibition decreased the proliferation and migration abilities in oral squamous cell carcinoma. Chao et al[26] found that RAB14 acted as a tumor promoter in bladder cancer progression via activating the MAPK pathway. In the current study, RAB14 expression was increased ESCC and associated with advanced TNM stage and poor overall survival. Subsequently, we verified that RAB14 acted as a target of miR-214-5p in ESCC. Meanwhile, sh-LINC00963 reduced RAB14 expression in KYSE-150 cells, while miR-214-5p inhibitors abolished the effects. All these data suggested that LINC00963 might promote ESCC progression via regulating the miR-214-5p/RAB14 axis.

Conclusions

LINC00963 was highly expressed in ESCC, and closely related with clinical features and prognosis of ESCC patients. In mechanism, LINC00963 knockdown suppressed ESCC progression by regulating miR-214-5p/RAB14 axis, which provided a novel therapeutic target for ESCC treatment.

Conflict of Interests

The authors declare that they have no conflict of interests.

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