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Long-chain non-coding RNA Linc00888 promotes the proliferation and migration of esophageal cancer cells by downregulating miR-34a expression

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Abstract. – OBJECTIVE: To explore the role and potential mechanism of long-chain non-coding RNA 00888 in esophageal cancer (EC).

PATIENTS AND METHODS: The expression level of Linc00888 in esophageal cancer tissues and adjacent ones, as well as corresponding cell lines, was measured by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Survival prognosis information of patients was collected, and KM survival analysis was performed to determine the prognostic value of Linc00888. To better understand the effect of Linc00888 on the proliferative and migration ability of EC cells, Cell Counting Kit-8 (CCK-8), clone formation, and transwell assays were performed after Linc00888 was knocked down in EC cell lines. Furthermore, bioinformatics prediction website was used to discover the potential target of Linc00888. Then, Dual-Luciferase reporter gene assay was performed to verify the binding relationship between Linc00888 and the downstream gene miR-34a. Then, the expression relationship between the two was measured both in cell lines and tissues. Finally, to clarify the regulation between Linc00888 and miR-34a, a recovery experiment was performed using co-transfection technology.

RESULTS: Linc00888 was aberrantly upregulated in esophageal cancer tissues. The survival analysis showed that the higher expression of Linc00888 was significantly correlated with shorter overall survival. Cell functional experiment results suggested that Linc00888 played a role in promoting tumor proliferative and migration ability in EC cells. Besides, Dual-Luciferase reporter genes assay indicated that miR-34a and Linc00888 had binding sites. Meanwhile, we confirmed that there was a negative correlation between the expression levels of miR-34a and Linc00888 in cells and tissues. Cellular functional recovery experiments revealed that Linc00888 could modulate the progression of EC by miR-34a.

CONCLUSIONS: Linc00888 promotes the proliferative and migration ability of EC through miR-34a.

Key Words:
Esophageal cancer, Linc00888, MiR-24a, Cell proliferation, Cell migration.

Introduction

Esophageal cancer (EC) is the ninth most malignant tumor in the world[1], which has a poor prognosis and a high mortality rate. It is a malignant tumor with a high degree of malignancy[2]. Patients are often in the advanced stage at the time of initial diagnosis, accompanied by metastasis, and the 5-year survival rate is much lower than 10%[3]. Platinum-based chemotherapy is often used to treat advanced EC, but the overall effect is unsatisfactory[4]. In recent years, the emergence of large-scale sequencing technology has gradually discovered that heterogeneity at the gene level is the main problem that hinders the progress of effective target therapy for EC[5]. Thus, the specific discovery and elucidation of the role of gene mutations in tumorigenesis and continuous progress can provide new ideas for the further diagnosis and treatment of EC.

At present, more large-scale research data show that 90% of the RNA in the human genome is non-coding RNA (non-coding RNA). Among them, the long non-coding RNAs (lncRNA) have been shown to play an important part in cell differentiation, metabolism, proliferation, and metasta-

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sis[6,7]. Moreover, lncRNA dysregulation takes part in the progression of tumors, which could regulate intracellular signaling networks and maintain tumor cell proliferation and anti-apoptotic capabilities, etc[8]. In fact, lncRNA PEG10 can regulate the proliferative and invasion progress of EC tumor cells, thereby promoting tumor growth[9]; while lncRNA NEAT1 can induce malignant progression of bladder cancer by combining with miR-410, thus affecting HMGB1 gene expression[10]. In addition, lnc RNA CASC21 has been found to function as an oncogene in colorectal cancer, affecting tumor cell proliferation, migration, invasion, and EMT[11]. In previous studies, we found that Linc00888 could function as a potential oncogene in melanoma. Linc00888 can regulate the expression of miR-126/CRK signal axis, thereby inducing the continuous proliferation and invasion of melanoma cells[12]. However, the biological function and mechanism of Linc00888 in esophageal cancer are still unknown.

In the present study, we showed that Linc00888 was upregulated in esophageal cancer, and we demonstrated that Linc00888 could promote the malignant progress of esophageal cancer cells. Our results suggested that Linc00888 could promote the development of lncRNA-directed diagnostics and therapeutics of human esophageal cancer.

Patients and Methods

Specimen Collection

Human esophageal cancer tissue samples were collected from 23 esophageal cancer patients who underwent radical surgery at China-Japan Union Hospital of Jilin University from July 2016 to December 2018. All patients did not receive any treatment, such as radiotherapy and chemotherapy before the surgery and all collected tumor samples were showed as lung cancer by postoperative pathology. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). The patients included 15 male patients and 8 female patients, with a median age of 68 years old. The diagnosis of patients was based on the report of Pathology Department of China-Japan Union Hospital of Jilin University. All patients provided signed informed consent to use the specimens for this trial, which was approved by the Ethics Review Board of China-Japan Union Hospital of Jilin University. This study complies with the Declaration of Helsinki. All patient specimens were stored in -80°C refrigerators within 30 minutes of collection.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using the RNAiso Plus kit (TaKaRA, Otsu, Shiga, Japan). The extracted RNA was then subjected to complementary deoxyribose nucleic acid (cDNA) synthesis by HiScript II reverse transcription kit (Vazyme, Nanjing, China), and the products were analyzed by qRT-PCR by using the AceQ real-time (RT) qPCR reagent (Vazyme, Nanjing, China). The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6. The primer sequences are shown as follows: Linc00888: Forward: 5'-TGGCAATTTTTCTGGGATTT-3', Reverse: 5'-TTAGCGCTAGGTGTGAACCC-3'; miR-34a: Forward: 5'-CCCAGAACATAGACACGCTGGA-3', Reverse: 5'-ATCAGCTGGGCACCTAGGACA-3'; GAPDH: Forward: 5'-CGGAGTCAACGGATTTGGTCGTAT-3', Reverse: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'; U6: Forward: 5'-GCTGAAAAACTG-3', Reverse: 5'-GCCTCCCAGTTTCATGGACA-3'.

Cell Culture and Transfection

The esophageal cancer cell lines EC1 and KYSE190 were purchased from the National Cell Line Resource Bank (Beijing, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) added with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 100 U/mL of penicillin and 100 μg/mL of streptomycin (Invitrogen, Carlsbad, CA, USA). Cell incubator conditions were constant at 37°C and 5% CO_2. All cells were passaged less than 30 times. Small interfering plasmids, miRNA mimics, and inhibitors were purchased from GenePharma (Shanghai, China). At the same time, cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the product instructions. 24-48 hours after transfection, the cells were used for functional tests, and the remaining cells were collected for qRT-PCR analysis.

Dual-Luciferase Reporter Assay

Based on the prediction of the binding site on the promoter, the amplified Linc00888 wild-type and mutant sequence information were constructed into Pinc3 vector respectively, and plasmids, as

well as miR-34a mimics and corresponding negative controls were co-transfected into EC cells using Lipofectamine 2000. Finally, the Luciferase activity was detected using the Dual-Luciferase reporter detection system (Promega, Madison, WI, USA).

Cell Counting Kit-8 (CCK-8) Cell Proliferation Experiment

EC1 and KYSE190 cell lines were seeded into 96-well plates at approximately 2000 density levels per well. At 12, 24, 48, and 72 hours, the cell proliferation ability was measured using the CCK-8 kit (Dojindo Molecular Technologies, Kumamoto, Japan) according to the product instructions. The absorbance at 450 nm was measured using a Varioskan Flash Multimode reader (Thermo Fisher Scientific, Waltham, MA, USA).

Clone Formation Experiment

The colony formation experiment was performed 24 hours after transfection. The EC1 and KYSE190 cell lines were resuspended and planted in 6-well plates with a density of about 2000 cells/well. The culture medium was changed every 4 days and the cells were cultured under standard conditions for 10 days. Then, colonies formed by the clones were collected, and the cells were washed twice with phosphate-buffered saline (PBS) and fixed with a formaldehyde solution, followed by staining with 0.1% crystal violet dye. The stained colonies were photographed and compared.

Transwell Experiment

Cell invasion was measured using a transwell cell (Corning, Corning, NY, USA). About 2×10^5 cells were seeded in the upper chamber (8 mm pore size, Corning, Corning, NY, USA) in 100 uL of serum-free medium. Then, bottom of the chamber was added with a medium containing 10% FBS. The upper and lower culture fluids were separated by a polycarbonate membrane. After culturing the cells in a standard environment for 48 hours, the cells passing through the lower layer were collected and stained with 0.5% crystal violet dye, and finally photographed with a microscope and counted.

Statistical Analysis

Data analysis was performed using mean and standard deviation. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Rank sum test was used to calculate survival analysis to evaluate the overall survival of EC patients. Correlation analysis of the binary variables was performed by using Pearson's correlation analysis. A two-tailed *p*-value of less than 0.05 was considered as statistically significant. All experimental data analysis was performed in GraphPad Prism 7.0 software (San Diego, CA, USA).

Results

Linc00888 is Abnormally Highly Expressed in Esophageal Cancer and is Associated with Poor Prognosis

We used the GEPIA website to analyze esophageal cancer data in the TCGA database. The results showed that the expression level of Linc00888 in esophageal cancer was remarkably higher than that of normal tissues (Figure 1A). Then, the level of Linc00888 was detected in 23 pairs of clinical EC tissues and adjacent normal ones. qRT-PCR results showed that the expression level of Linc00888 was aberrantly higher in EC tissues than that of normal counterparts (Figure 1B). We also measured the expression of Linc00888 in EC cell lines at the same time. As it was shown in Figure 1C, Linc00888 expression was uniformly elevated in EC cells (EC9706, EC1, EC109, and KYSE150) than in the normal Het-1A cell (Figure 1C). To better understand the role of Linc00888 upregulation in EC, we performed survival analysis to analyze the relationship between clinical features of the 23 esophageal cancer patients and high expression of Linc00888. Notably, the poor survival prognosis was in correlation with the high expression of Linc00888 (Figure 1D). The above results showed that Linc00888 was markedly overexpressed in EC, and its expression was associated with the patient's poor prognosis.

Knockdown of Linc00888 Inhibits Proliferation and Migration of Esophageal Cancer In Vitro

To further explore the function of LINC00888 in EC, si-Linc00888 and corresponding negative controls were transfected into EC1 and KYSE190 cell lines. Then, the transfection efficiency was confirmed by qRT-PCR (Figure 2A). The CCK-8 assay revealed that the knockdown of Linc00888 in EC cells remarkably inhibited cell viability

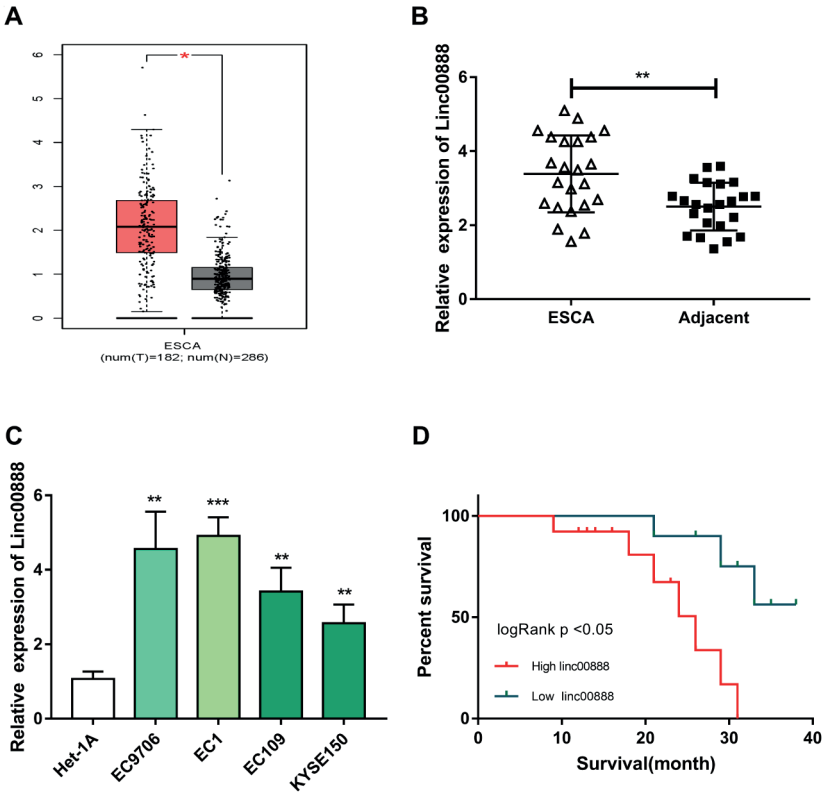

Figure 1. A, Bioinformatics website mining TCGA esophageal cancer specimens showed that the overall expression level of Linc00888 in esophageal cancer was slightly higher than that of normal control esophageal epithelium. **B,** The expression level of Linc00888 was determined by qRT-PCR in 23 pairs of clinical samples. **C,** qRT-PCR was used to determine the expression level of Linc00888 in different esophageal cancer cell lines. **D,** In patients with esophageal cancer, the total tumor survival time was shorter in the group of patients with high expression of Linc00888 than in the group of patients with low expression of Linc00888. (**p<0.01, ***p<0.001).

(Figure 2B-2C). Next, we considered the transwell assay and found that after knocking down Linc00888 in esophageal cancer cells, the cell line's migration ability was markedly inhibited (Figure 2D). Similarly, plate cloning assay suggested that silencing of Linc00888 could markedly reduce the clonogenic survival of EC cells (Figure 2E). The above experimental results disclosed that Linc00888 was essential for maintaining the proliferative and migration ability of esophageal cancer cells.

Linc00888 Negatively Correlates with MiR-34a

Furthermore, we explored the specific mechanism of Linc00888 in esophageal cancer, using the website LHGRNA to predict the potential possible binding of downstream genes to Linc00888. We found that miR-34a may be a potential downstream target in EC cells. The 3'UTR region of miR-34a had a binding site with Linc00888 (Figure 3A). The Dual-Luciferase reporter gene experiment confirmed that there was a binding between Linc00888 and miR-34a, which was markedly reduced after the binding site was mutated (Figure 3B-3C). At the same time, we verified the expression relationship between the two in EC cell lines and tissues. The knockdown of Linc00888 could lead to an increase in the expression level of miR-34a (Figure 3D, 3E). The correlation analysis suggested that Linc00888 had a negative correlation with miR-34a with the correlation p-value less than 0.05 using the Pearson algorithm (Figure 3E). These results indicated that Linc00888 may modulate the tumor phenotype by binding to miR-34a and affecting its expression.

Knockdown of MiR-34a can Reverse Linc00888's Inhibition on Esophageal Cancer Cell Proliferation and Migration

To further understand the role of miR-34a in EC, we co-transfected si-Linc00888 and miR-34a

inhibitors in EC cells. The results of CCK-8 experi-ments showed that knockdown of miR-34a could reverse the inhibitory effect of silenced Linc00888 on cell viability (Figure 4A-4B). Similarly, transwell re-sults showed that knockdown of of miR-34a restored the inhibited migration ability of EC cells caused by Linc00888 silencing (Figure 4C-4D). In addition, plate cloning results showed that the co-transfection of miR-34a inhibitors could restore the inhibitory effect of silenced Linc00888 on the proliferative ability of EC cells (Figure 4E). Collectively, these above experimental results indicated that Linc00888 played a role in promoting tumor progression by inhibiting the expression of miR-34a in esophageal cancer.

Discussion

The identifications of new biomarkers and therapeutic targets for EC will help improve diagnosis and treatment of esophageal cancer[13]. Thanks to the advent and popularity of high-throughput sequencing technology, many studies show that lncRNAs play an increasingly important role in the development of tumors[6]. LncRNAs are widely involved in regulating a variety of tumor signaling pathways, including the wnt pathway[14], the MAPK signaling pathway[15], the p53 pathway[16], etc, suggesting that they might function as a large regulatory component in tumorigenesis[17]. Research and exploration of newly discovered lncRNA have become a hot area of tumor research. So, Linc01638 could promote tumor cell migration and invasion through activation of TGF-β signaling pathway in pancreatic bile duct cancer[18]; CERS6-AS1 can maintain the continuous progress of breast cancer by binding to IGF2BP3 to improve the stability of its target genes[19]. In addition, ZEB2-AS1 is also considered to act as a tumor driver in colorectal cancer[20].

To discover new molecular targets in EC, we performed TCGA database mining and found that Linc00888 was abnormally expressed in esophageal cancer. Lu et al[21] have found that Linc00888 has a great impact on the prognosis in melanoma, suggesting that lnc00888 can serve as an oncogene and one of the risk indicators for tumor prediction. We subsequently verified the expression level of Linc00888 in clinical esophageal cancer tissue samples, and the results con-

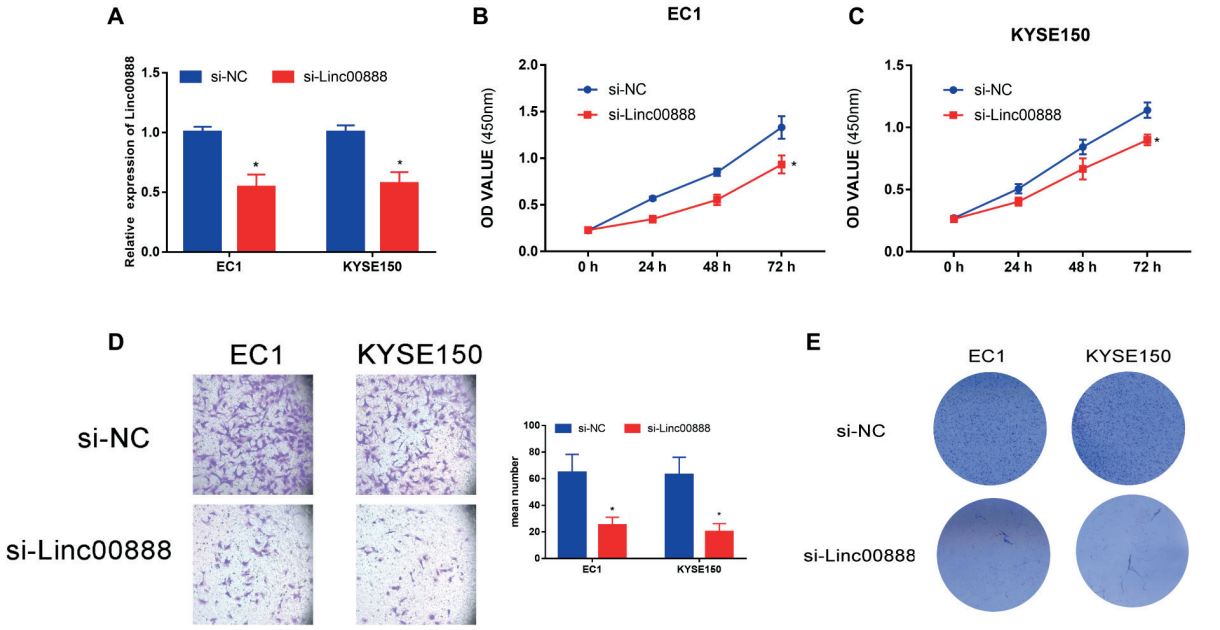

Figure 2. A, Transfection of si-Linc00888 in esophageal cancer cell lines EC1 and KYSE150 can reduce the expression level of Linc00888 gene. **B-C,** CCK8 cell proliferation experiment results showed that knockdown of Linc00888 expression could inhibit the proliferation of esophageal cancer cell lines EC1 and KYSE150. **D,** Transwell cell migration experiments showed that knockdown of Linc00888 could inhibit the migration of esophageal cancer cell lines EC1 and KYSE150; (magnification: 400×). **E,** The results of plate cloning experiments showed that knockdown of Linc00888 could inhibit the cell proliferation and proliferation of esophageal cancer cell lines H520 and SK-MES-1; (magnification: 10×) (*p<0.05).

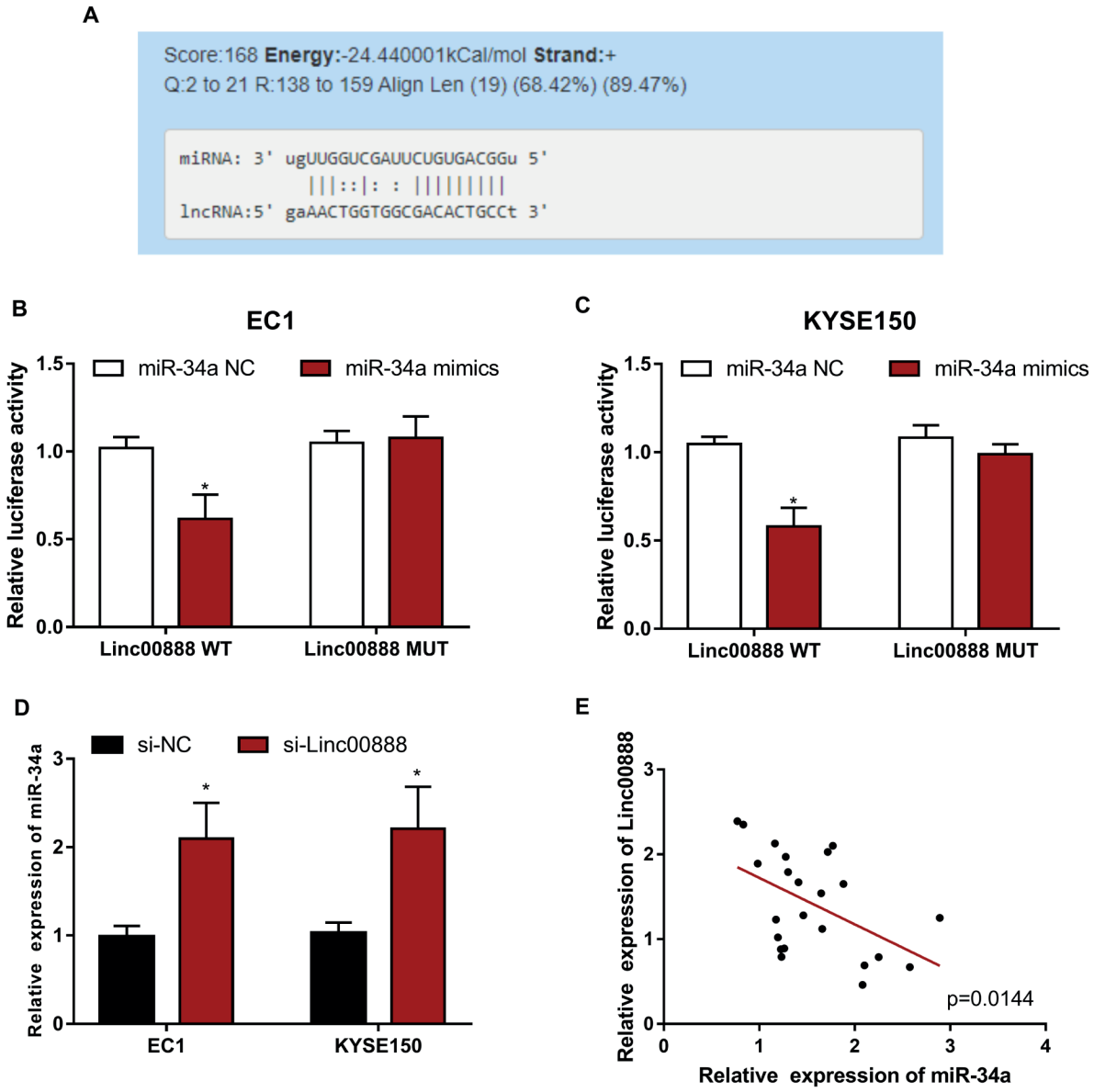

Figure 3. A, Bioinformatics prediction results found that miRNA-34a had binding sites to Linc00888. **B-C,** The results of the Luciferase reporter experiment proved that miRNA-34a and Linc00888 had a binding relationship in esophageal cancer. **D,** In the EC1 and KYSE150 cell lines, after knocking down the Linc00888 gene, the miRNA-34a expression levels in the cell lines EC1 and KYSE150 were measured. **E,** qRT-PCR was performed in 23 pairs of clinical samples to determine the correlation between the expression levels of Linc00888 and miRNA-34a. (*p<0.05).

sistently found that Linc00888 was abnormally highly expressed in esophageal cancer. Thus, we suggested that Linc00888 may play a role as an oncogene in EC. CCK-8, plate cloning, and transwell experiments revealed that the ability of EC cells to proliferate, migrate, and clone was significantly reduced after Linc00888 was silenced. According to the prediction results, we found that miR-34a was a downstream molecular target of Linc00888. It has been shown that lncRNAs can function as a 'sponge' to titrate microRNAs. So, we adopted a Dual-Luciferase reporter gene experiment to detect their binding relationship, and verified their expression relationship, which was found to be negatively related. MiR-34a has been widely concerned as a tumor suppressor gene in a variety of tumors, including liver cancer[22] and prostate cancer[23]. At the same time, it

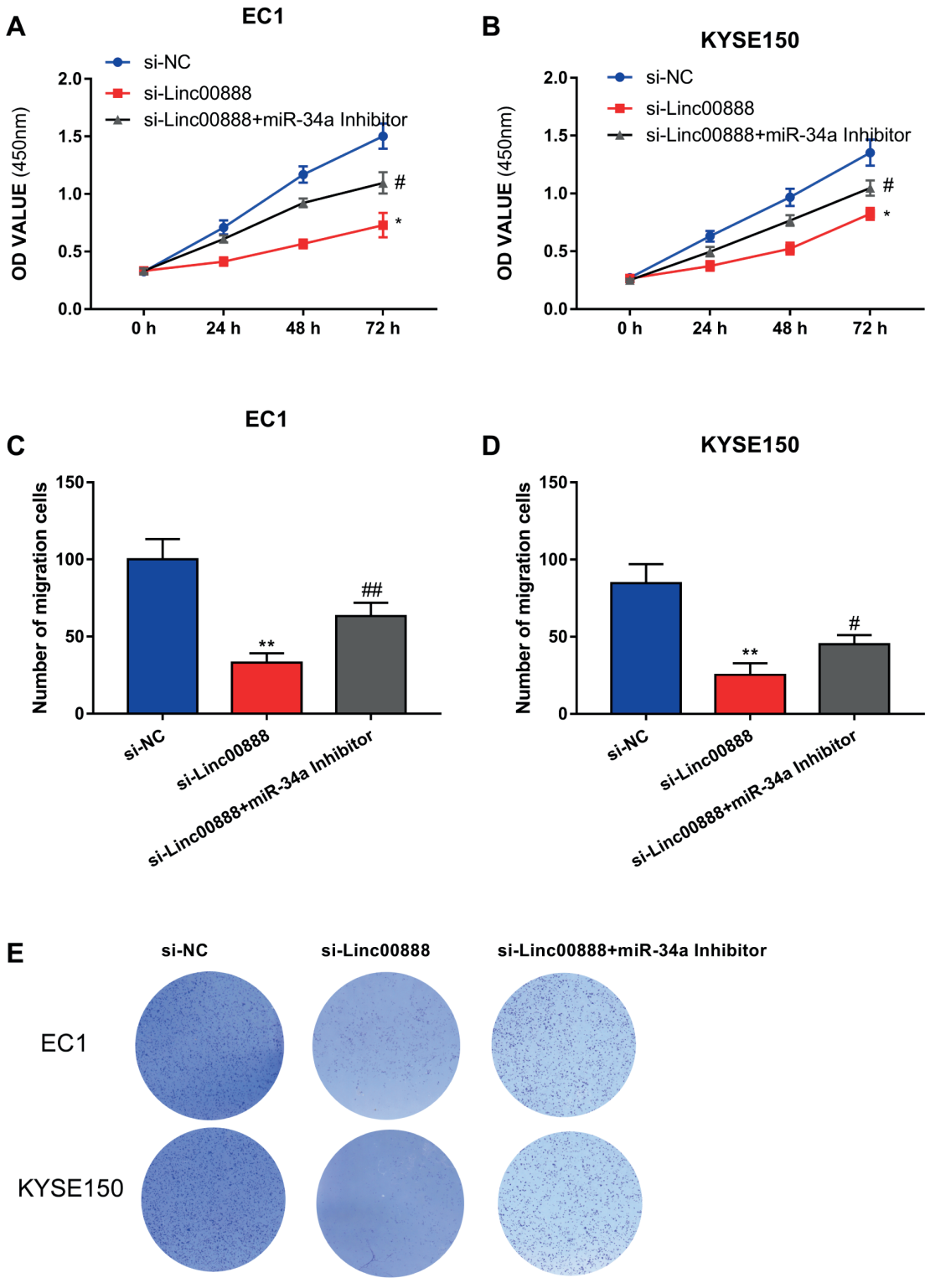

Figure 4. A-B, The results of CCK8 cell proliferation experiments showed that co-transfection of si-Linc00888 and miRNA-34a inhibitors can reverse the inhibition of the proliferation ability of Linc00888 esophageal cancer cell lines EC1 and KYSE150. **C-D,** Transwell cell migration experiment results show that co-transfection of si-Linc00888 and miRNA-34a inhibitors can reverse the inhibition of migration ability of Linc00888 esophageal cancer cell lines EC1 and KYSE150. **E,** The results of plate cell cloning activity test showed that after co-transfection of si-Linc00888 and miRNA-34a inhibitors could reverse the inhibition of the proliferation ability of Linc00888 esophageal cancer cell lines EC1 and KYSE150; (magnification: 10×) (*p<0.05 **p<0.01, #p<0.05, ##p<0.01).

is also involved in regulating EMT or p53 signal pathway, etc.[24]. Besides, miR-34a can participate in the regulation of the MAGE-A signaling pathway and can inhibit the occurrence and development of tumors[25]. However, the specific role of miR-34a in EC has not been studied so far. Further experiments showed that miR-34a can reverse the inhibitory effect of Linc00888 knockdown on the proliferative and migration ability of EC cells. These experimental results reveal that Linc00888 may play a role in EC by modulating miR-34a. The novelty of this study was that we firstly reported that Linc00888 could cat as an oncogene in EC and uncovered the possible mechanism in the development of EC, which could provide a new therapeutic target for EC.

Conclusions

Altogether, the above reported data showed that Linc00888 has abnormally high expression in EC, which can play a role in promoting tumor progression and lead to a poor prognosis of the tumor.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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