LncRNA SNHG14 promotes proliferation of endometrial cancer through regulating microRNA-655-3p

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Abstract. – OBJECTIVE: Previous studies have shown that long non-coding RNA (lncRNA) SNHG14 can act as a cancer-promoting gene, but the role of SNHG14 in the development of endometrial carcinoma (EC) has not been reported. This study was designed to investigate the expression characteristics of SNHG14 in EC tissues and cells and to specify whether SNHG14 promotes the malignant progression of EC by modulating microRNA-655-3P.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qPCR) was carried out to examine SNHG14 expression in tumor tissue specimens and paracancerous tissue specimens collected from 52 patients with EC, and the relationship between SNHG14 expression and clinical indicators or prognosis of these subjects was analyzed as well. Further, the expression level of SNHG14 in EC cell lines was also verified by qRT-PCR. In addition, SNHG14 knockdown and the overexpression models were constructed using lentivirus in EC cell lines, Ishikawa, and KLE, and the influence of SNHG14 on EC cell biological functions was evaluated by Cell Counting Kit-8 (CCK-8), plate cloning, 5-ethynyl-2'-deoxyuridine (EdU) and flow apoptosis assays. Finally, *in vitro* recovery experiments were conducted to explore the mechanism by which SNHG14 interacts with microRNA-655-3P to exert its effect on the progression of EC.

RESULTS: qPCR results indicated that SHHG14 expression in EC tumor tissues was remarkably higher than that in adjacent tissues. Compared with patients with low expression of SNHG14, patients with high expression of SNHG14 had larger tumor size, lower overall survival, and more advanced pathological stage. *In vitro*, compared with those in the control group, the overexpression of SNHG14 markedly enhanced EC cell proliferation while inhibited cell apoptosis, and the opposite result was observed in SNHG14 silencing group. Subsequently, qRT-PCR verified that microRNA-655-3P expression was significantly reduced in EC tissues and negatively correlated with SNHG14. In addition, recovery experiment revealed a mutual regulation between SNHG14 and microRNA-655-3P, the two of which may together modulate the malignant progression of EC.

CONCLUSIONS: EC tumor tissues contain a significantly high expression of LncRNA SNHG14, which has been confirmed to be remarkably associated with tumor size, pathological stage, and poor prognosis of EC patients. Additionally, lncRNA SNHG14 is capable of accelerating malignant progression of EC by regulating microRNA-655-3P expression.

Key Words:
LncRNA SNHG14, MicroRNA-655-3P, EC, Malignant progression.

Introduction

Endometrial carcinoma (EC) is a common epithelial malignant tumor of the female reproductive system[1,2]. In 2015, the number of new cases of EC in China was still on the rise, reaching about 63,400, with a mortality rate of about 21.8%. In 2016, there were about 60,050 EC new cases in the United States, becoming the second largest gynecological cancer after breast cancer[3,4]. The prognosis of patients with early EC is generally good, with 5-year survival rate reaching about 95%, but the prognosis of EC patients in advanced stage (stage III or IV) is poor, with 5-year survival rates about 47%-69% and 15%-17%, respectively[5]. Therefore, studying the pathogenesis of EC and exploring new early diagnosis and treatment targets are essential to improve prognosis and quality of life of patients with EC[6,7]. In recent years, the research on long non-coding RNA (lncRNA) has provided a new idea for exploring the pathogenesis and diagnosis and treatment of EC[8,9].

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The Human Genome Project shows that less than 2% of the 3 billion base pairs that make up the human genome encode proteins, and the remaining 98% of the sequences are non-protein coding sequences[10,11]. According to the length of transcripts, non-coding RNAs can be divided into small non-coding RNAs and lncRNAs. LncRNAs are a type of transcripts with a length of more than 200 bp, with low conservation and a lack of functional open reading framework, so they cannot encode stable peptides or proteins[12,13]. Besides, lncRNAs play an important regulatory role in gene transcription, post-transcriptional, and translation levels through interaction with other molecules, thus participating in cell proliferation, differentiation, survival, and other life activities[13]. Compared with its regulation of normal physiological activities, the function of lncRNAs in disease diagnosis and treatment has attracted more attention[14]. Scarfi et al and Jiang et al[15,16] have shown that some lncRNAs, with high tissue specificity, may be dysregulated in certain cancer tissues and are remarkably related to the proliferation, invasion, and prognosis of cancer patients, which are expected to become new targets for cancer diagnosis and treatment. At present, there are relatively few reports on lncRNA in endometrial carcinoma tissues. Of note, the upregulation of LncRNA H19 in endometrial tissues may predict a poor prognosis of EC patients[17]. Therefore, it is of great significance to further study EC-related lncRNAs to clarify the pathogenesis of EC and explore new diagnostic and therapeutic targets for EC. Deng et al[18] and Wang et al[19] have suggested that LncRNA SNHG14 can serve as an oncogene, but the specific role of SNHG14 in EC has not been reported.

According to the ceRNA (competitive endogenous RNA) hypothesis, SNHG14 was selected as a candidate gene with sequence correlation of microRNA-655-3P through bioinformatics analysis. Some reports[20,21] have suggested that microRNA-655-3P can play a role in inhibiting the occurrence and development of tumors, especially in tumor metastasis and proliferation. Therefore, in this study, the expression levels of LncRNA SNHG14 and microRNA-655-3P in 52 pairs of tumor tissues and adjacent tissues of EC patients were analyzed, and the effects of LncRNA SNHG14 and microRNA-655-3P on EC cell functions were explored. Meanwhile, the mechanism of LncRNA SNHG14 affecting clinical parameters, prognosis, and malignant progression of EC patients was further explored.

Patients and Methods

Patients and EC Samples

Tumor and paracancerous tissue samples of 52 patients with EC were collected. All specimens were obtained from oncology, gynecological surgery, and biopsy specimens. In addition, the paracancerous tissues of all specimens were more than 5 cm away from cancerous tissues, and no anti-tumor treatment, such as radiotherapy or chemotherapy was performed before surgery. The investigation was approved by the Ethics Committee of the hospital and all patients signed informed consent. All patients were followed up after discharge, including general conditions, clinical symptoms, and imaging examination.

Cell Lines and Reagents

Human EC cell lines (HEC-1A, HEC-1B, KLE, Ishikawa) and human endometrial stromal cell line (T-HESC) were provided by American Type Culture Collection (ATCC, Manassas, VA, USA), while Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from Life Technologies (Gaithersburg, MD, USA). All cells were cultured at 37°C in a 5% CO_2 incubator with DMEM medium containing 10% FBS.

Transfection

The control group (NC or Anti-NC) and the lentivirus (SNHG14 or Anti-SNHG14) containing the LncRNA SNHG14 overexpression and knockdown sequences were purchased from Shanghai GenePharma Company (Shanghai, China). The cells were seeded in a 6-well plate and grew to a cell density of 30-40%, and then, transfection was carried out according to the manufacturer's instructions, After 48 h, the cells were collected for quantitative Real Time-Polymerase Chain Reaction (qPCR) analysis and cell functional assays.

Cell Counting Kit-8 (CCK-8) Test

The cells after 48 h of transfection were harvested and seeded into 96-well plates at 2000 cells per well. After cultured for 24 h, 48 h, 72 h, and 96 h, respectively, Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) reagent was added. After incubation for 2 hours, the optical density (OD) value of each well was measured in the microplate reader at 490 nm absorption wavelength.

Colony Formation Assay

After 48 h of transfection, the cells were collected, and 200 cells were seeded in each well of a 6-well plate and cultured with complete medium for 2 weeks. Then, the medium was changed after one week and then twice a week, and the medium should not be replaced as much as possible in the previous week to avoid cell adhesion. 2 weeks later, the cells were cloned and then fixed in 2 mL of methanol for 20 minutes. After the methanol was aspirated, the cells were stained with 0.1% crystal violet staining solution for 20 minutes, washed 3 times with phosphate-buffered saline (PBS), photographed, and counted under a light-selective environment.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

EDU proliferation assay (RiboBio, Nanjing, China) was performed according to the manufacturer's requirements. After transfection for 24 h, the cells were incubated with 50 μM EDU for 2 h and stained with AdoLo and 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA), and the number of EDU-positive cells was observed by fluorescence microscopy. The display rate of EDU positive is shown as the ratio of the number of EDU positive cells to the total DAPI chromogenic cells (blue cells).

Flow Cytometry

The method of binding with Annexin V-fluorescein isothiocyanate (FITC; Merck, Billerica, MA, USA) and Propidium Iodide (PI) was used for detection by flow cytometry. The cell density was adjusted to about 1×10^6 cells/mL. After the medium was discarded, the cells were washed twice with PBS and gently resuspended with 0.5 mL of pre-cooled 1× binding buffer, and then, 1.25 Ul Annexin V-FITC was added for incubation at room temperature and light-proof reaction for 15 min. Subsequently, the cells were centrifuged at 1000 × g for 5 min at room temperature, and the supernatant was removed. After gently resuspending the cells with 0.5 mL of pre-cooled 1 × binding buffer, 10 Ul PI was added, and the sample was placed on ice and stored in the dark, and then, immediately analyzed by flow cytometry (BD, Franklin Lakes, NJ, USA).

QRT-PCR

The total RNA was extracted from tissue samples using TRIzol method (Invitrogen, Carlsbad, CA, USA). 2 μg of extracted total RNA was added to the 20 μL system for complementary deoxyribose nucleic acid (cDNA) synthesis. Next, qRT-PCR reaction was carried out using SYBR® Premix Ex Taq™ on a qRT-PCR reactor (TaKaRa, Otsu, Shiga, Japan). β-actin was used as an internal reference and the data obtained from three independent experiments were analyzed by the formula $RQ=2^{-\Delta\Delta Ct}$.

The primers are as follows: LncRNA SNHG14: F: 5'-GGGTGTTTACGTAGACCAGAACC-3', R: 5'-CTTCCAAAAGCCTTCTGCCTTAG-3'; β-actin: F: 5'-CCTGGCACCCAGCACAAT-3', R: 5'-TGCCGTAGGTGTCCCTTTG-3'; microRNA-655-3p: F: 5'-CAATCCTTACTCCAGCCAC-3', R: 5'-GTGTCTTAAGGCTAGGCCTA-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'.

Dual-Luciferase Reporting Assay

HEK293T cells were seeded in 24-well plates and co-transfected with microRNA-655-3P mimics/miR-NC and pMIR Luciferase reporter plasmids. The plasmid was then introduced into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 hours of transfection, the reporter Luciferase activity was normalized to control firefly Luciferase activity using a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 19.0 statistical software (SPSS Inc., Chicago, IL, USA). Statistical differences between the two groups and multiple groups were analyzed using Student's *t*-test and One-way analysis of variance (ANOVA) followed by post-hoc test (Least Significant Difference), respectively. Independent experiments were expressed as mean ± standard deviation. $p<0.05$ was considered statistically significant.

Results

SNHG14 was Highly Expressed in EC Tissues and Cell Lines

QRT-PCR detected a significant increase in SNHG14 expression in EC tissue samples collected from 52 EC patients when compared to their corresponding paracancerous tissues (Figure 1A and 1B). At the same time, *in vitro* experiments also revealed an elevation in SNHG14 level in EC cell lines than in T-HESC (Figure 1C).

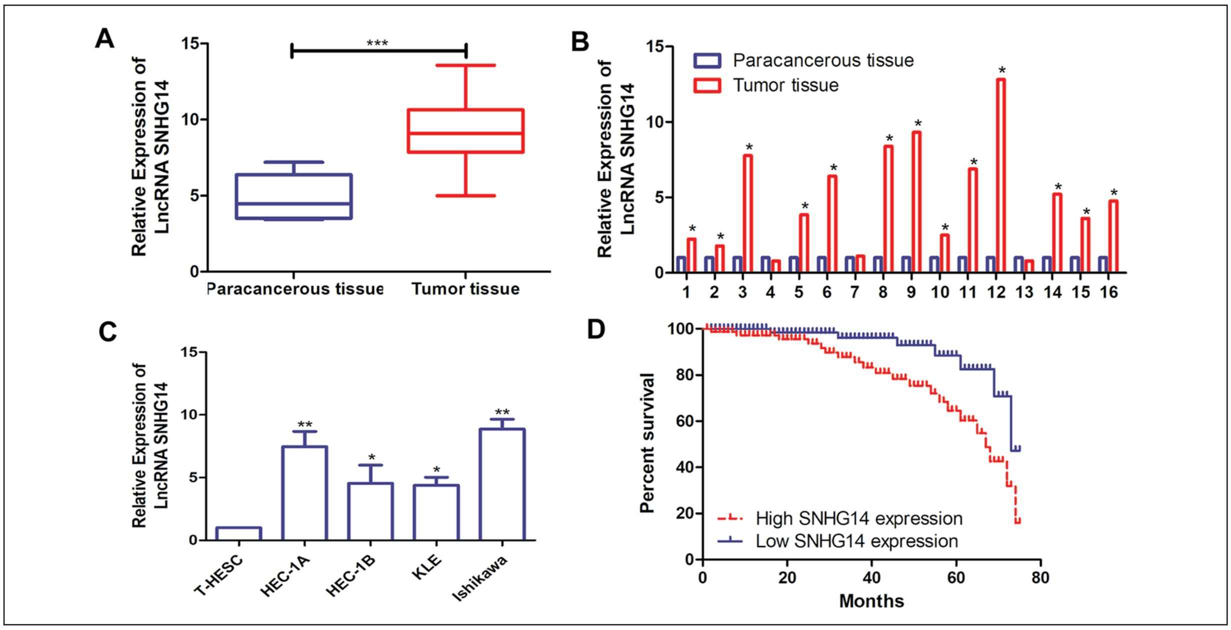

Figure 1. LncRNA SNHG14 is highly expressed in EC tissues and cell lines. **A, B,** qRT-PCR is used to detect the difference in expression of SNHG14 in EC tumor tissues and adjacent tissues. **C,** qRT-PCR is used to analyze the expression level of SNHG14 in EC cell lines. **D,** Kaplan Meier survival curve of patients with endometrial cancer based on SNHG14 expression, and the prognosis of patients in high expression group is significantly worse than that of patients in low expression group. Data are mean ± SD, **p*<0.05, ***p*<0.01, ****p*<0.001.

SNHG14 Expression was Correlated with Distance Metastasis Incidence and Overall Survival of EC Patients

Based on qRT-PCR results of SNHG14 expression, the above tissue samples were divided into two groups, namely, high expression group and low expression group, and then, the interplay between SNHG14 expression and some indicators of EC patients such as age, gender, pathological stage, incidence of lymph node or distant metastasis was analyzed through Chi-square test. As shown in Table I, high expression of SNHG14 was remarkably relevant to tumor size and pathological stage, but not with oth-

Table I. Association of LncRNA SNHG14 and miR-655-3p expression with clinicopathologic characteristics of endometrial carcinoma.

Parameters	No. of cases	SNHG14 expression			MiR-655-3p expression		
		Low (%)	High (%)	*p*-value	High (%)	Low (%)	*p*-value
Age (years)				0.397			0.782
< 60	21	12	9		8	13	
≥ 60	31	14	17		13	18	
Tumor size				0.027			0.048
< 4 cm	26	17	9		14	12	
≥ 4 cm	26	9	17		7	19	
T stage				0.048			0.043
T1-T2	31	19	12		9	22	
T3-T4	21	7	14		12	9	
Lymph node metastasis				0.244			0.105
No	34	19	15		11	23	
Yes	18	7	11		10	8	
Distance metastasis				0.158			0.147
No	31	18	13		10	21	
Yes	21	8	13		11	10	

er indicators. Meanwhile, the reduced microRNA-655-3P expression was also found to be correlated with tumor size, as well as pathological stage of EC patients. In addition, Kaplan-Meier survival curve indicated that high expression of SNHG14 may predict a poor prognosis of EC patients (p<0.05; Figure 1D).

SNHG14 Promoted Cell Proliferation while Inhibited Cell Apoptosis in EC

To explore the impacts of SNHG14 on EC cell proliferation and apoptosis, SNHG14 overexpression and the knockdown models were firstly constructed and the transfection efficiency was verified by qRT-PCR (Figure 2A). Subsequently, CCK-8,

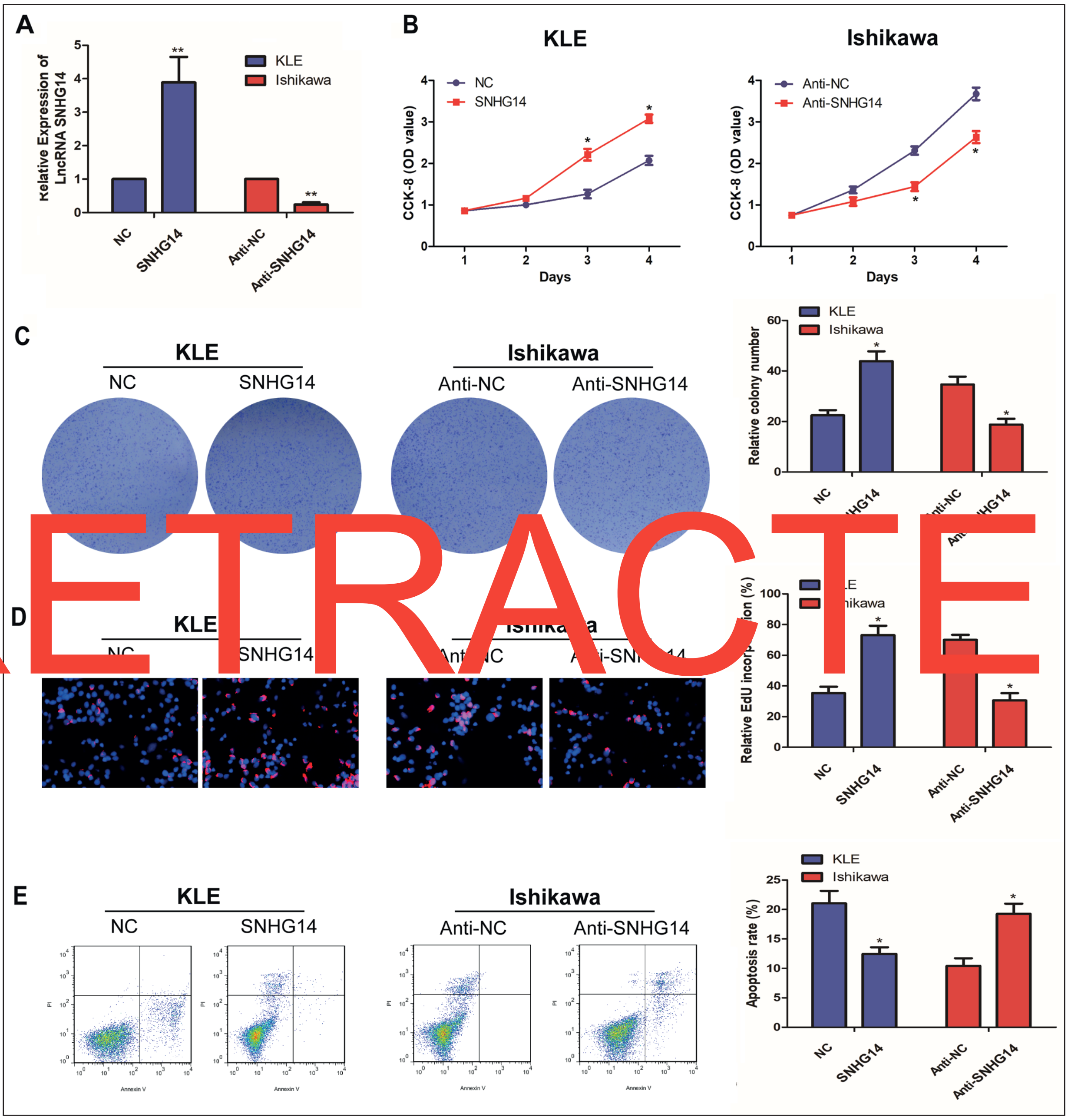

Figure 2. LncRNA SNHG14 promotes EC cell proliferation and inhibits cell apoptosis. **A,** qRT-PCR verifies the transfection efficiency of SNHG14 after transfection of the SNHG14 overexpression vector in the KLE cell line and transfection of the SNHG14 knockdown vector in the Ishikawa cell line. **B,** CCK-8 assay shows the ability of Ishikawa and KLE cell lines to proliferate after transfection. **C,** Plate cloning experiment is performed to detect the number of EC positive proliferating cells after transfection (magnification: 10×). **D,** EDU assay detects the cell proliferation of Ishikawa and KLE cell lines after transfection (magnification: 40×). **E,** Flow cytometry assay detects cell apoptosis of EC cells after transfection. Data are mean ± SD, *p<0.05.

plate cloning, and EDU experiments revealed an enhanced proliferation of EC cells after overexpression of SNHG14, but the knockdown of SNHG14 conversely inhibited cell proliferative ability (Figure 2B-2D). On the contrary, for the cell apoptosis detection, flow cytometry experiments revealed a completely opposite trend in EC cell lines with SNHG14 overexpression or knockdown (Figure 2E).

MicroRNA-655-3P was Lowly Expressed in EC Tissues and Cell Lines

Luciferase reporting assay demonstrated that SNHG14 could be targeted by microRNA-655-3P through a specific binding site (Figure 3A). In addition, it was found by qRT-PCR detection that, compared with the NC group, the overexpression of SNHG1 markedly elevated microRNA-655-3P expression while the knockdown of SNHG14 conversely decreased that (Figure 3B). Meanwhile, qRT-PCR also detected microRNA-655-3P expression in the above mentioned 52 pairs of tissue specimens, which, as a result, showed a significant reduction in EC tumor tissues when compared to the normal ones (Figure 3C). Similarly, microRNA-655-3P was also found to be remarkably lowly expressed in EC cell lines compared with T-HESC (Figure 3D). Kaplan-Meier survival curves showed that low

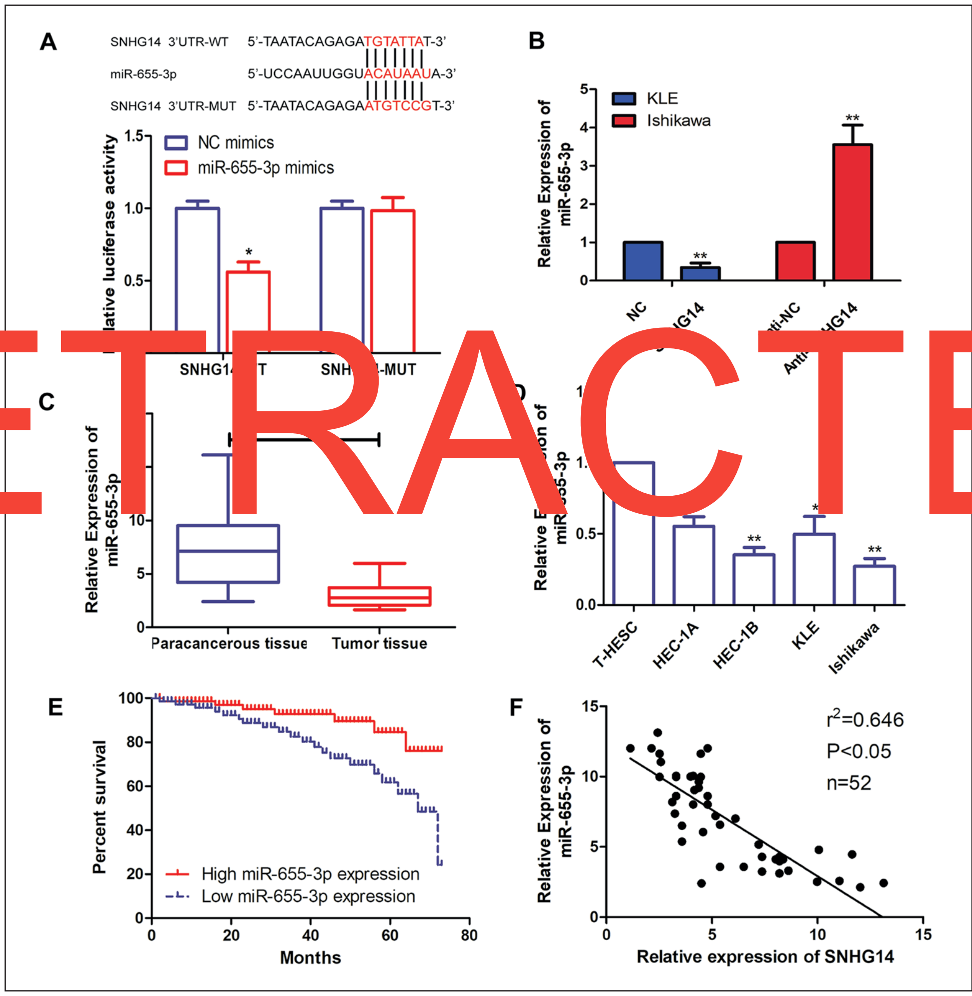

Figure 3. MiR-655-3p is under expressed in EC tissues and cell lines. **A,** The results of the Dual-Luciferase reporter assay in the HEK293T cell line indicates that LncRNA SNHG14 can be targeted by miR-655-3p *via* a specific binding site. **B,** qRT-PCR is used to examine the expression level of miR-655-3p after overexpression or knockdown of LncRNA SNHG14 in EC cell lines. **C,** qRT-PCR is used to detect the difference in the expression of miR-655-3p in EC tumor tissues and adjacent tissues. **D,** qRT-PCR is used to detect the expression level of miR-655-3p in EC cell lines. **E,** Kaplan-Meier survival curve of patients with endometrial cancer based on miR-655-3p expression, and the prognosis of patients in low expression group is significantly worse than that of patients in high expression group. **F,** There is a significant negative correlation between the expression of SOHG14 and miR-655-3p in EC tissues. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

expression of microRNA-655-3P could indicate a poor prognosis of EC patients ($p<0.05$, Figure 3E). In addition, it was discovered by qRT-PCR that the mRNA expression levels of SNHG14 and microRNA-655-3P showed a negative correlation in EC tissue samples (Figure 3F).

MicroRNA-655-3P Modulated SNHG14 Expression in EC

To further explore the ways in which SNHG14 regulates the malignant progression of EC, the co-transfection of microRNA-655-3P and SNHG14 overexpression or knockdown of vectors were performed. Consequently, qRT-PCR detection indicated that the overexpression of microRNA-655-3P increased SNHG14 level in EC cell lines while silencing microRNA-655-3P inhibited it (Figure 4A). Subsequently, it was found by CCK-8 and EDU experiments that microRNA-655-3P mimics reversed the enhanced proliferation capacity caused by upregulation of SNHG14, while microRNA-655-3P inhibitor reversed the inhibited proliferation induced by downregulation of SNHG14 (Figure 4B and 4C). In addition, the results of flow cytometry experiment suggested an opposite result in cell apoptosis (Figure 4D).

Discussion

EC is one of the three most common malignancies of the female reproductive tract and the most common gynecological tumor in developed countries[1-3]. More than 90% of EC cases occur in women over 50 years of age, with a median diagnosis age of 63 years[2,3]. According to the FIGO stage, 69% patients in I and II stage can be treated by surgery, while 28% of women need to accept radiation and/or chemotherapy[3-5]. The complications caused by surgery, such as loss of fertility caused by hysterectomy, menopausal symptoms

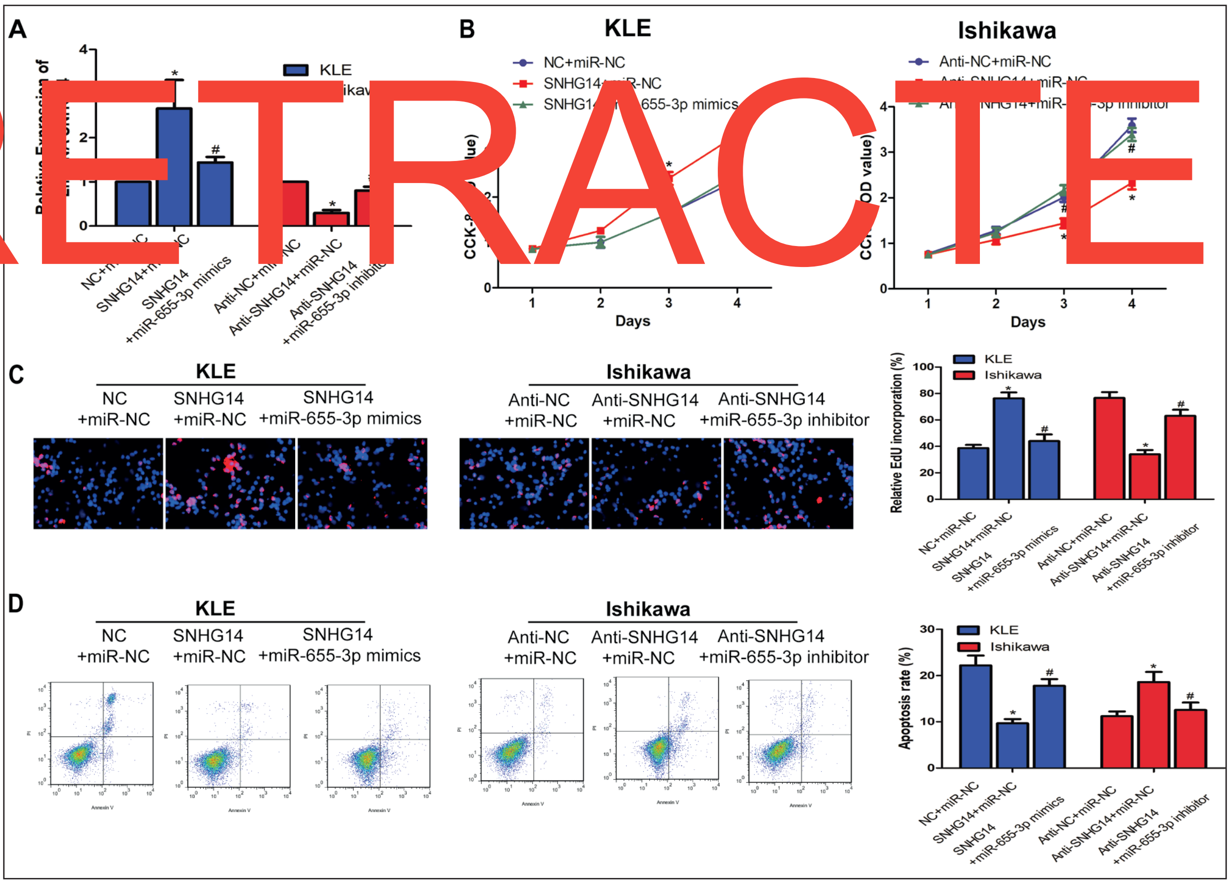

Figure 4. LncRNA SNHG14 can regulate the role of miR-655-3p in EC cell lines. **A,** qRT-PCR is used to reveal the expression level of LncRNA SNHG14 after co-transfection of SNHG14 and miR-655-3p. **B,** CCK-8 measures the proliferation of EC cells after co-transfection of SNHG14 and miR-655-3p. **C,** EDU assay is used to determine the proliferation of EC cells after co-transfection of SNHG14 and miR-655-3p (magnification: 40×). **D,** Flow cytometry assay is used to assess apoptosis of EC cells after co-transfection of SNHG14 and miR-655-3p. Data are mean ± SD, *#$p<0.05$.

caused by oophorectomy, lower extremity edema caused by lymph node dissection, gastrointestinal reactions caused by radiation therapy, chemotherapy resistance, etc., have a serious impact on the quality of life of patients, severely limiting the progress of diagnosis and treatment of endometrial cancer. Hence, exploring the pathogenesis of endometrial cancer and finding new diagnostic and therapeutic targets are crucial for improving the prognosis of endometrial cancer[5].

LncRNAs were originally thought to be "transcriptional noise", and as the research progressed, they were found to regulate gene expression at epigenetic, transcriptional, and post-transcriptional levels. LncRNAs not only play a pivotal role in physiological processes, but have also been proven to participate in the occurrence and development of a variety of tumors, so they are expected to become a new target for cancer diagnosis and treatment[8,9]. Recent reports have revealed that lncRNAs can be involved in the occurrence of EC. Of note, HOTAIR, one of the most well-studied lncRNAs, is found to be upregulated in endometrial [illegible] with poor prognosis of patients with endometrial cancer. However, there are still very few studies[22,23] on lncRNAs in EC, and the role of lncRNAs in EC is still worth exploring. The development of EC [illegible] regulated by multiple molecules and processes, including the activation of EC oncogenes and the silencing of cancer-related genes[24]. Therefore, exploring lncRNAs with the abnormal expression in EC and analyzing their functions will help improving the diagnosis and treatment and patients' prognosis. In this study, lncRNA SNHG14 was found to be remarkably upregulated in EC tumor tissues, while microRNA-655-3P was conversely downregulated. Meanwhile, it was discovered that the expression level of LncRNA SNHG14 was positively correlated with tumor size, pathological stage, and poor prognosis of EC patients. Therefore, it is believed that LncRNA SNHG14 can serve as an oncogene in EC. In order to further explore the effects of SNHG14 and microRNA-655-3P on EC cell functions, CCK8, plate cloning, EDU, and flow cytometry apoptosis experiments were performed to reveal that the overexpression of LncRNA SNHG14 could promote the proliferation of EC cells but inhibit the apoptosis of EC cells. However, the specific molecular mechanism still remains to be clearly determined.

Exploring the regulatory effect and mechanism of LncRNA SNHG14 on EC will contribute to discovering new methods for EC treatment. The results of this experiment showed that microRNA-655-3p was less expressed in tumor tissues of EC patients than in adjacent tissues, which inhibited the proliferation and promoted apoptosis of EC cells. In recent years, researches on the function of LncRNA have proposed a new ceRNA mechanism, which has been detected in some cancers to be able to explain the relationship between LncRNA and miRNA. In this study, the specific binding of LncRNA SNHG14 to microRNA-655-3p was verified through bioinformatics analysis and the Luciferase reporter gene assay. In addition, further experiments revealed that the knockdown of SNHG14 upregulated microRNA-655-3p, whereas the overexpression of microRNA-655-3p inhibited SNHG14 expression. The results of CCK-8 assay and flow cytometry experiment uncovered that LncRNA SNHG14 and microRNA-655-3p may form a mutually inhibiting feedback regulation in endometrial cancer cells, thereby jointly affecting the malignant progression of endometrial cancer.

Conclusions

This study revealed that LncRNA SNHG14 had a high expression in EC tumor tissues and was remarkably correlated with tumor size, pathological stage, and poor prognosis of EC patients. Meanwhile, LncRNA SNHG14 may promote the malignant progression of EC *via* regulating microRNA-655-3P.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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