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MicroRNA-130-5p promotes invasion as well as migration of lung adenocarcinoma cells by targeting the EZH2 signaling pathway

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Abstract. – OBJECTIVE: This study was designed to investigate whether microRNA-130-5p could promote the progression and metastasis of lung cancer by targeting enhancer of zeste homolog 2 (EZH2).

PATIENTS AND METHODS: The level of microRNA-130-5p was examined in the tumor tissues and paracancerous tissues of lung adenocarcinoma patients. Meanwhile, quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the level of microRNA-130-5p in different lung adenocarcinoma cell lines, which was found markedly decreased in two lung cancer cell lines. Moreover, microRNA-130-5p mimic and inhibitor were transfected in cells to achieve microRNA-130-5p overexpression and knockdown model to observe its effect on cell biological function. Cell Counting Kit-8 (CCK-8) assay was performed to detect cell viability, while transwell assay was used to detect cell invasion as well as migration. Subsequently, EZH2 was predicted as the target gene of microRNA-130-5p using the Bioscan prediction site Targetscan, which was confirmed by Luciferase reporter gene assay. Besides, the level of EZH2 was detected by qRT-PCR and Western blot after overexpression and knockdown of microRNA-130-5p. Finally, the combination of si-EZH2 and inhibitor was used to further verify whether microRNA-130-5p could promote cell metastasis and invasion of lung cancer by targeting EZH2.

RESULTS: MicroRNA-130-5p expression in tumor tissues of patients with lung adenocarcinoma was markedly lower than that in normal tissues. Besides, microRNA-130-5p expression in tumor tissues with large diameter was lower than that with a small diameter. In addition, microRNA-130-5p was also lowly expressed in lung cancer cell lines. High expression of microRNA-130-5p reduced the cell viability and inhibited cancer cell metastasis and invasion. At the same time, microRNA-130-5p knockdown enhanced the activity of lung cancer cells and promoted cancer cell invasion as well as migration. Dicated confirmed that microRNA-130-5p could bind to EZH2. Additionally, the overexpression or knockdown of microRNA-130-5p could decrease or increase the levels of EZH2 mRNA, respectively. Finally, EZH2 silencing reduced lung cancer cell activity and inhibited cancer cells invasion as well as migration, which could be reversed by the microRNA-130-5p inhibitor.

CONCLUSIONS: The reduced microRNA-130-5p expression in lung cancer tissues and cells promoted metastasis and invasion of this tumor by targeting EZH2.

Key Words:
MicroRNA, EZH2, Lung cancer, Metastasis and invasion, Cell proliferation.

Introduction

As the leading cause of cancer-related deaths worldwide, lung cancer poses a serious threat to human life and quality[1]. Lung cancer is widely divided into small cell lung cancer and non-small cell lung cancer (NSCLC). The main histological subtypes of NSCLC are adenocarcinoma and squamous cell carcinoma, which could be further classified according to specific DNA mutations. If found at the early stage, surgical resection of NSCLC can bring a desirable prognosis for patients[2]. However, despite advances in surgery and medical treatment, the 5-year survival rate of lung cancer patients remains in a low level[3]. Local control of early NSCLC has significantly helped patients getting through the past few decades, but the molecular mechanisms by which NSCLC leads to regional and distant lesions remain unexplained[4]. In recent years, studies have revealed that non-coding RNA plays a vital regulatory role in the development of tumors. Therefore, in this

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work, we discuss the role of microRNAs with carcinogenic and anticancer effects in the pathogenesis of lung cancer.

MicroRNA (microRNA) is a small non-coding RNA of 19-25 nucleotides in length that has been proved to have a negative regulatory effect on mRNA stability by inhibiting mRNA translation[5,6]. Small molecule ribonucleic acids (microRNAs) interfere with the expression of a target gene by binding to the 3'-untranslated regions 3'-UTR of mRNA to degrade the mRNA or inhibit its translation. In plants, complete complementarity between microRNAs and 3'UTR mRNA results in degradation of the target mRNA, while in mammals, partial complementation results in inhibition of mRNA translation[7]. Numerous studies have shown that a single microRNA can simultaneously target multiple mRNAs, and different microRNAs can synergistically target a single mRNA. The ability of microRNAs to target multiple transcripts suggests that microRNAs could regulate disease processes through a complex regulatory network to coordinate gene expression[8]. MicroRNAs have been shown to participate in the development of several cancers including lung cancer and breast cancer[9-11]. It has also been shown that microRNA could serve as a biomarker for disease diagnosis and prognosis. For instance, microRNA-155 and let-7a are associated with poor lung cancer prognosis[10]. In addition, microRNA-135b up-regulation is in association with advanced tumor stage and poor clinical outcome in colorectal cancer (CRC)[12].

Enhancer of zeste homolog 2 (EZH2) is highly expressed in many malignant tumors including prostate cancer and breast cancer, and participates in the occurrence and development of tumors by promoting cell proliferation, cell cycle arrest, cell migration as well as invasion ability[13]. Many investigations have emphasized the function of the PRC2 catalytic component EZH2 in tumor development, and meanwhile, EZH2 mutations have been found in many malignancies. EZH2 actively participates in the basic processes of cell cycle progression, differentiation, apoptosis, etc. by regulating the expression of key genes. In addition, EZH2 is also involved in the differentiation of T effector cells and T regulatory cells[14]. EZH2 mutations were found in various malignant tumors and were in correlation with the prognosis of human tumors, suggesting that EZH2 may be involved in tumor development[15]. The development of new drugs that inhibit the activity of EZH2 has helped us with a deeper understanding of the function of EZH2 in the tumor. Strong evidence also shows that the activation of carcinogenic signaling pathways in a variety of human tumors can result in altered transcriptional regulation of EZH2, which will promote cell proliferation, invasion, migration and cancer progression[16].

Here, we explored the function of microRNA-130-5p in lung cancer and verified its binding with EZH2, hoping to find new biological evidence for its prognosis and treatment.

Patients and Methods

Patients

The 33 specimens selected were from the tumor tissues and adjacent tissues of lung cancer patients excised from The Seventh People's Hospital of Jinan from 2016 to December 2017 years. None of the patients had received any adjuvant chemotherapy such as radiotherapy and chemotherapy. Importantly, all tumor specimens in our experiment had been confirmed by histopathological analysis. The control group specimens were from paracancerous tissues of the same patient (at least 3 cm away from the surgical margin), and no cancer cells were found after pathological examination. All specimens were frozen in liquid nitrogen and then stored at -80°C. This experimental study was approved by the Ethics Committee of The Seventh People's Hospital of Jinan.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then stored at -80°C after the concentration was measured by a micronuclear quantifier. The complementary deoxyribose nucleic acid (cDNA) was obtained by reverse transcription, and the SYBR Green method was used for quantitative Real Time-Polymerase Chain Reaction (qPCR) detection (TaKaRa, Otsu, Shiga, Japan).

Cell Culture and Transfection

The cell lines including BEAS-2, NCI-H1975, NCI-H441, AsPC-1, and CFPAC-1 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose complete medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA)

in a 37°C, 5% CO_2 incubator. Cells were seeded in a six-well plate at a density of about 10^4/well. When the density reached about 75-85%, transfection was performed in cells according to the instructions of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). After 6 h, the medium was replaced, and the cells were further cultured for 48 h at 37°C. Then, the cells were collected for subsequent experiments.

Cell Counting Kit-8 (CCK-8) Assay

Cells transfected with microRNA mimics or inhibitors, as well as their negative controls, were digested with trypsin and seeded (2×10^3/well) into 96-well plates. Cell proliferative activity at a different time point (24 h, 48 h, 72 h, 96 h) was detected by measuring the absorbance at 450 nm with the Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan). Cells were added with 10 μL/well CCK-8 reagent and incubated for 2 h. The absorbance was measured using a microplate reader.

Cell Migration

Cells (3.0×10^4/mL) were seeded in the upper chamber of the transwell in 24-well plates. The serum-free medium was added into the lower chamber while 500 μL of complete medium was added into the upper chamber. After cells were placed in a 37°C incubator and cultured for 12 h, they were taken out and fixed with 4% paraformaldehyde for 30 min, followed by staining with crystal violet for 15 min. After cleaning the inner surface of the cell basement membrane and removing the inner cells carefully, stained cells in the outer base were observed and counted using a microscope.

Cell Invasion

The fibronectin FN was diluted to the concentration of 100 μg/mL. Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was diluted 1:9 with the serum-free, non-antibiotics medium. The bottom of each chamber was coated with 50 μL of FN and placed in a clean bench for 2 h to air dry. The inside of the chamber was coated with 100 μL of Matrigel and placed in a cell culture incubator overnight. 100 μL of cells (1×10^6 cells/mL) were seeded in the upper layer of the transwell chamber. Then, 600 μl of 10% FBS medium was added to the outside of the chamber. After incubating for 24 h in the incubator, cells were fixed with methanol, stained with trypan blue and washed three times with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA). Then, the cells in the upper chamber were wiped with a cotton swab and photographed under a microscope.

Western Blot

Cells were digested and bicinchoninic acid (BCA) protein quantification kit (Pierce, Waltham, MA, USA) was used to detect the protein concentration. Then, the total protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), which was then blocked in 5% skim milk blocking solution and incubated with primary antibodies. Protein bands were detected using enhanced chemiluminescence machine (ECL; Thermo Fisher Scientific, Waltham, MA, USA).

Luciferase Reporter Gene Assay

Cells (3×10^5) were seeded into a 24-well plate. After that, microRNA-130-5p mimics and wild-type or mutant psiCHECK-2 vector were co-transfected into the cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). 24 hours after transfection, cells were collected for the measurement of Luciferase activity using a Dual-Luciferase reporter assay kit (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) and measurement data were expressed as mean ± standard deviation. The *t*-test was used to compare the data between the two groups, while Chi-square test was used to analyze the correlation between clinical information and NKILA expression. The difference was statistically significant at $p<0.05$.

Results

MicroRNA-130-5p Was Lowly Expressed in Lung Cancer Tissues and Cells

To investigate microRNA-130-5p expression in lung cancer patients, we analyzed the levels of microRNA-130-5p in 33 tumor tissues and normal paracancerous tissues by qRT-PCR and found that microRNA-130-5p was lowly expressed in lung cancer patients relative to normal

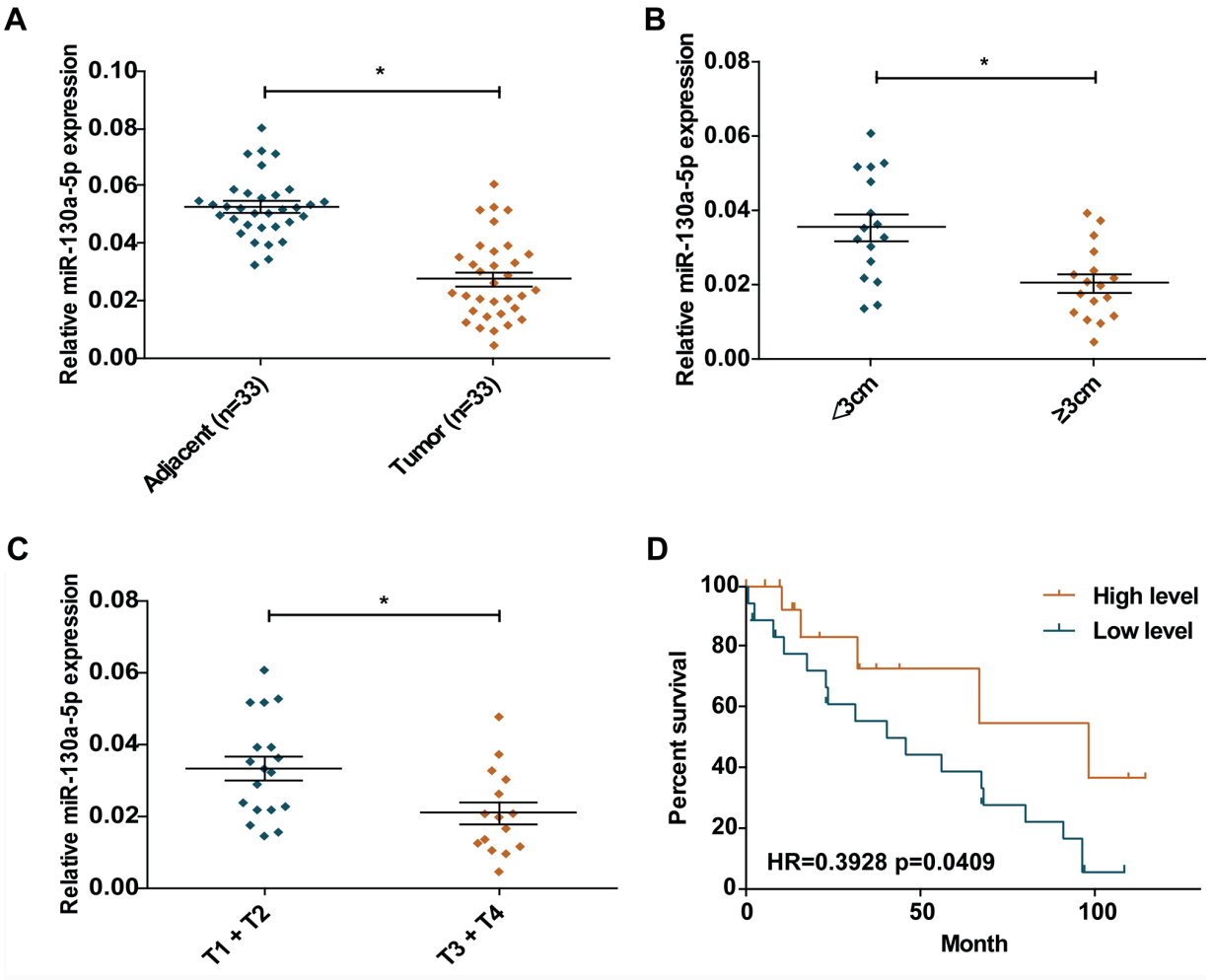

Figure 1. MicroRNA-130-5p is lowly expressed in lung cancer tumor tissues of patients and cell lines. **A,** qRT-PCR was performed to detect the level of microRNA-130-5p in lung cancer tumor tissues and adjacent tissues. **B,** MicroRNA-130-5p expression was significantly lower in tumor tissues with a diameter greater than or equal to 3 cm. **C,** The level of microRNA-130-5p in lung cancer tissues with distant metastasis (T3+T4) was markedly lower than that in lung cancer tissues with near metastasis (T1+T2). **D,** Population data analysis showed that the survival rate of people with high microRNA-130-5p level was remarkably higher than that of people with low microRNA-130-5p level.

ones (p<0.001; Figure 1A). Besides, microRNA-130-5p expression was negatively correlated with lung cancer tissue diameters (Figure 1B). Furthermore, the level of microRNA-130-5p in lung cancer tumor tissues with distant metastasis (T3+T4) was markedly lower than that in the proximal metastasis (T1+T2; Figure 1C). At the same time, the analysis showed that patients in the microRNA-130-5p high expression group had a longer survival time relative to those in microRNA-130-5p low expression group (Figure 1D). These results indicated that microRNA-130-5p was lowly expressed in lung cancer and closely correlated with tumor metastasis and prognosis of patients.

MicroRNA-130-5p Promoted Cell Proliferation, Invasion and Migration of Lung Cancer Cells

To further investigate the function of microRNA-130-5p on the progression of lung cancer, microRNA-130-5p expression was detected using qRT-PCR in different cell lung cancer lines including AsPC-1 and CFPAC-1. It was found that microRNA-130-5p was also lowly expressed in lung cancer cells (Figure 2A). Subsequently, AsPC-1 and CFPAC-1 were transfected with microRNA-130-5p mimic or inhibitor to achieve microRNA-130-5p overexpression or knockdown model, and the transfection efficiency was then confirmed by qRT-PCR assay. (Figure 2B, 2C).

Afterward, the CCK-8 assay was performed to detect lung cancer cell proliferation at 6, 24, 48, 72, and 96 hours, respectively. The results showed that microRNA-130-5p overexpression promoted the proliferation of lung cancer cells while microRNA-130-5p knockdown inhibited that (Figure 2D, 2E). The transwell assay revealed that microRNA-130-5p overexpression could promote cell invasion as well as migration, whereas the opposite result was observed after knockdown of microRNA-130-5p (Figure 2F, 2G). These results demonstrated that microRNA-130-5p could promote cell proliferation, invasion and migration of lung cancer cells.

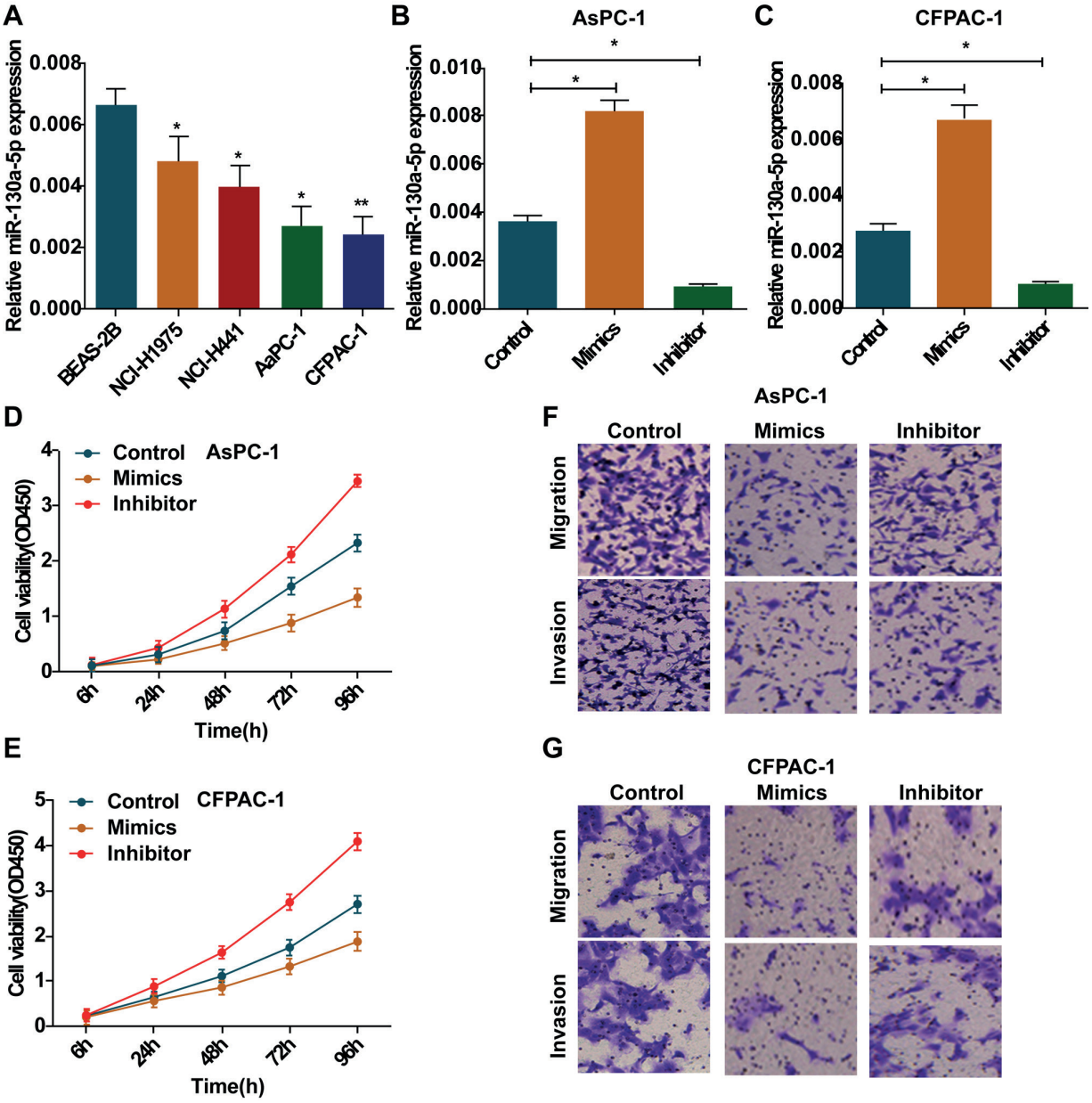

Figure 2. MicroRNA-130-5p promotes proliferation, invasion as well as migration of lung cancer cells. **A,** qRT-PCR assay was used to detect the microRNA-130-5p levels in two different lung cancer cell lines, AsPC-1 and CFPAC-1. **B,** qRT-PCR was used to detect transfection efficiency of microRNA-130-5p mimic and inhibitor, respectively in AsPC-1. **C,** qRT-PCR was used to detect transfection efficiency of microRNA-130-5p mimic and inhibitor, respectively in CFPAC-1. **D,** and **E,** CCK-8 assay was performed in AsPC-1 and CFPAC-1 to investigate the effect of microRNA-130-5p on cell activity. **F,** and **G,** transwell experiments were used to demonstrate the effect of microRNA-130 -5p on metastasis and invasion of lung cancer cells (magnification: 40×).

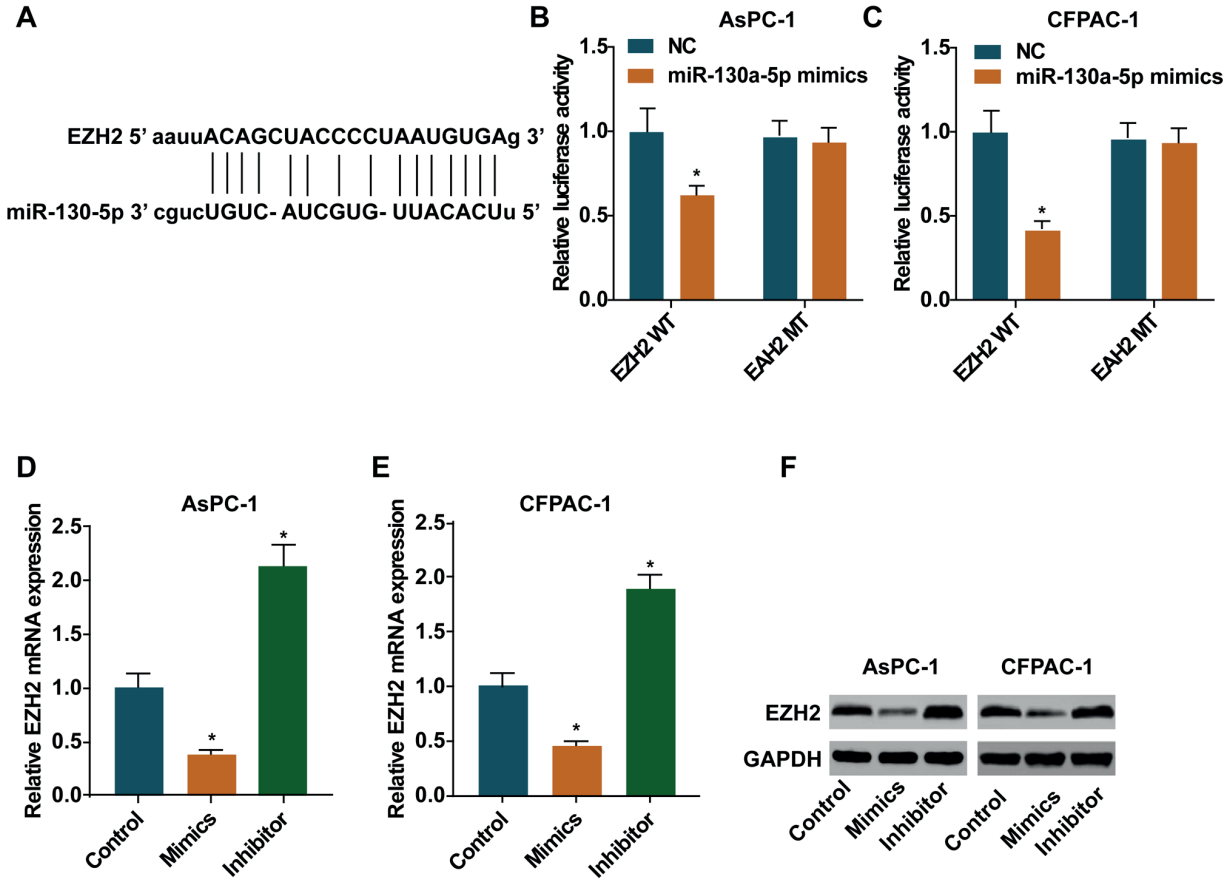

Figure 3. MicroRNA-130-5p could target EZH2. **A,** Bioinformatics prediction site Targetscan was used to predict the target gene of microRNA-130-5p and that EZH2 may be its targeted binding sites. **B,** and **C,** Luciferase reporter gene assay was performed in two lung cancer cell lines, AsPC-1 and CFPAC-1, respectively, to demonstrated that microRNA-130-5p and EZH2 have binding sites. **D,** and **E,** EZH2 mRNA levels in AsPC-1 and CFPAC-1 were detected after microRNA-130-5p overexpression and knockdown. **F,** Western blot was used to detect the protein level of EZH2 in AsPC-1 and CFPAC-1 after microRNA-130-5p overexpression and knockdown.

MicroRNA-130-5p Could Target EZH2 and Combine With It.

To investigate the specific mechanism by which microRNA-130-5p promoted the proliferative activity and invasion and migration of lung cancer cells, we predicted and discovered a targeted binding site between microRNA-130-5p and EZH2 by Bioscan prediction site Targetscan (Figure 3A). Luciferase reporter gene assay was subsequently performed in AsPC-1 and CFPAC-1 cells, respectively, which directly demonstrated that microRNA-130-5p could target EZH2 (Figure 3B, 3C). Further experiments showed that microRNA-130-5p overexpression could decrease the level of EZH2, while knockdown of microRNA-130-5p conversely increased EZH2 level (Figure 3D, 3E). These results suggested that microRNA-130-5p could bind to EZH2 and regulate its expression.

MicroRNA-130-5p Promoted Lung Cancer Cells Metastasis and Invasion by Targeting to EZH2

To further explore the regulatory network of microRNA-130-5p and EZH2, we transfected siEZH2 along with microRNA-130-5p inhibitors into AsPC-1 and CFPAC-1 cells. CCK-8 assay revealed that EZH2 knockdown reduced lung cancer cell activity, whereas microRNA-130-5p inhibitor reversed the inhibitory effect of si-EZH2 on cell proliferation (Figure 4A, 4B). Transwell experiment also confirmed that transfection of si-EZH2 reduced the cell invasion as well as migration ability, whereas transfection of mi-

croRNA-130-5p inhibitor could reverse the inhibitory effect of si-EZH2 on cell invasion and migration ability (Figure 4C, 4D). These results suggested that microRNA-130-5p promoted lung cancer metastasis and invasion by targeted binding to EZH2.

Discussion

As one of the most common causes of cancer-related deaths worldwide, lung cancer is characterized by high incidence and high mortality. It has become a serious health problem and has caused enormous social and economic burdens[2]. Worldwide, the most common cause of cancer death in men is lung cancer, and even in women, the mortality rate of lung cancer has exceeded that of breast cancer[17]. The overall mortality rate induced by lung cancer is the highest among all cancers[18]. The occurrence of lung cancer is the result of a combination of genetic, environmental, food and lifestyle factors (especially related to smoking). Among all types of lung cancer, NSCLS accounted for approximately 85% and the 5-year survival rate was 15%[19]. Many investigations have shown that the invasion, metastasis and proliferation of lung cancer cells are the most important factors affecting the lung cancer prognosis. Therefore, looking for targets that affect metastasis and invasion is particularly important to improve lung cancer treatment and prognosis[20]. Non-coding RNA plays an important regulatory role in the development of tumors. Here, we dis-

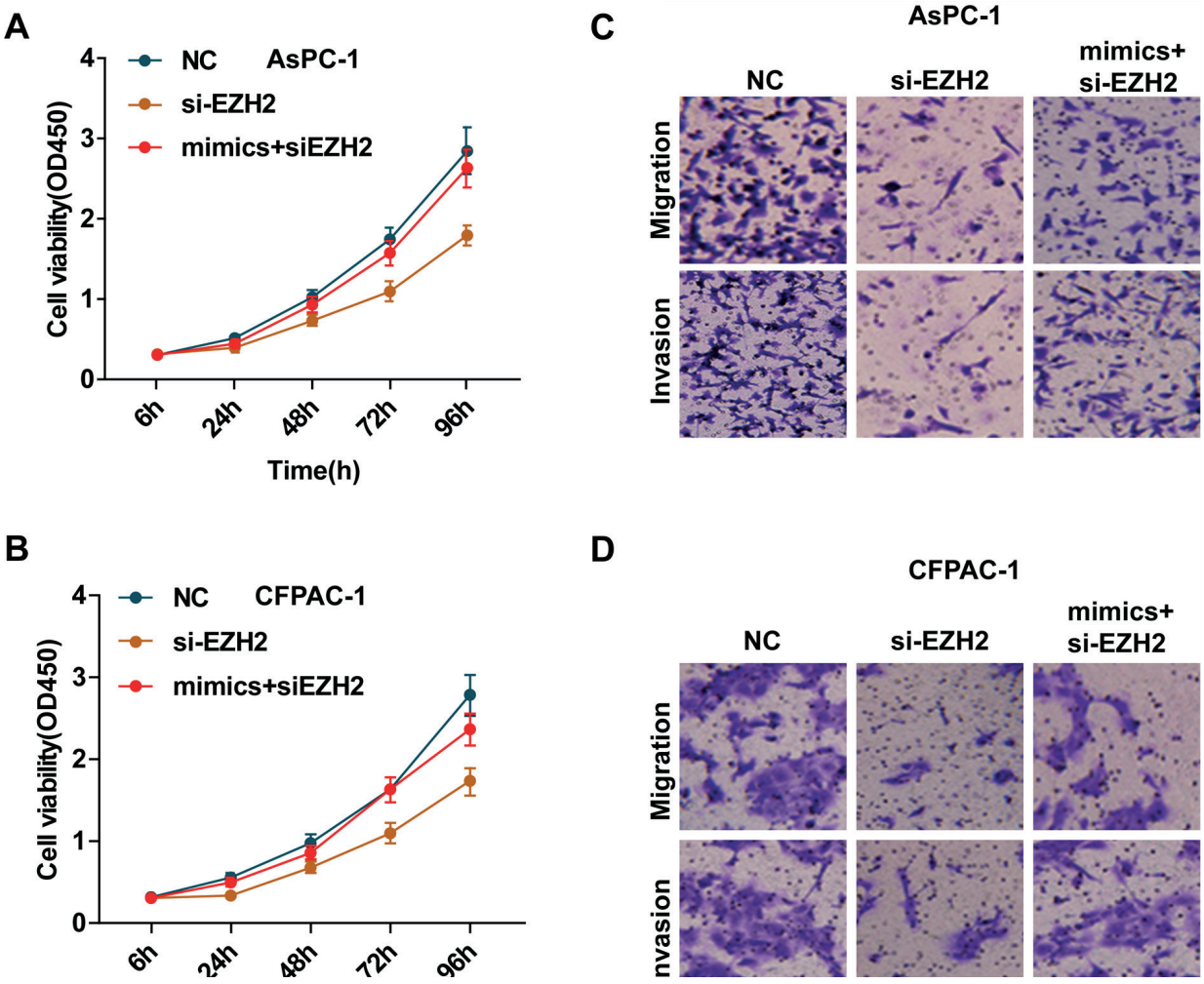

Figure 4. MicroRNA-130-5p promotes metastasis and invasion of lung cancer cells by targeting to EZH2. **A,** and **B,** CCK-8 assay was performed in AsPC-1 and CFPAC-1 to investigate the effect of EZH2 silencing as well as co-transfection of microRNA-130-5p inhibitor in cell activity. **C,** and **D,** Transwell experiments were used to demonstrate the effect of si-EZH2 and co-transfection of microRNA-130-5p inhibitor in lung cancer cell metastasis and invasion (magnification: 40×).

cussed the role of microRNAs with carcinogenic and tumor suppressor effects in the pathogenesis of lung cancer.

MicroRNAs regulate gene expression by binding to the 3'UTR region of the target gene mRNA through base complementation, and thus inducing targeted mRNA degradation or inhibition of target gene translation[21,22]. Each microRNA can specifically bind to hundreds of gene targets and regulate a variety of biological processes such as a variety of human cancers. MicroRNAs can function as tumor suppressor genes or oncogenes by negatively regulating the expression of oncogenic-related genes or increasing the translation of oncogenic proteins, thereby inhibiting or promoting tumor development[23,24]. Studies have shown that has-microRNA-155 is up-regulated in lung cancer tissues; meanwhile, microRNA-21 is overexpressed in both hepatocarcinoma and hepatoma cells. In addition, many aberrantly expressed microRNAs have been found to target PTEN to regulate cancer cell proliferation, migration as well as invasion during its occurrence and development. Therefore, it is suggested that some microRNAs contribute to the malignant biological phenotype of lung cancer.

In this work, we found that microRNA-130-5p was markedly down-regulated in 33 selected samples from hospitals (tumor tissue and adjacent tissues of lung cancer patients excised by lung cancer resection) by q-PCR analysis, and that the expression of microRNA-299-5p was inversely proportional to the tumor's diameter and metastasis. Through bioinformatics prediction, we found that microRNA-130-5p can targeted bind to the 3'-UTR region of the oncogene EZH2. Therefore, we hypothesized that microRNA-130-5p may be involved in the proliferation, metastasis and invasion of lung cancer cells by specific binding to EZH2 in lung cancer patients[25].

Drosophila Zeste gene enhancer human homolog (EZH2) is a catalytic subunit of core protein complex-2 with histone methyltransferase activity, which could silence target gene by methylation modification of histone H3K27 lysine site, leading to the occurrence of tumors[15]. Wang et al[26] have shown that EZH2 is widely expressed in many malignant tumors and participates in the occurrence and development of tumors by promoting tumor cell proliferation, cell cycle arrest, cell migration as well as invasion. Clinically abnormal expression of EZH2 is in association with poor prognosis; therefore, it is a potential target for tumor therapy[27]. As a candidate oncogene, EZH2 is located downstream of the pRb-E2F cell cycle regulatory pathway and is the target gene of the transcription factor E2F. E2F can bind to EZH2 promoter to promote its expression, and thereby shorten the cell cycle G1 phase and increase the S phase to promote cell proliferation. As a transcriptional repressor, EZH2 gene mainly inhibits tumor suppressor genes and tumor metastasis suppressor genes during tumor progression, suggesting that EZH2 promotes invasion as well as the migration of tumor cells. Our study demonstrated that microRNA-130-5p could promote invasion as well as the migration of lung cancer cells by targeting the EZH2 signaling pathway, which might provide a potential target for gene therapy of this disease[28].

Conclusions

These results showed that the reduced microRNA-130-5p expression in lung cancer tissues and cells promoted metastasis and invasion of this tumor by targeting EZH2.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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