

LncRNA SNHG6 Inhibits Apoptosis by Regulating EZH2 Expression via the Sponging of MiR-101-3p in Esophageal Squamous-Cell Carcinoma

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Background: The long non-coding RNA (lncRNA) SNHG6 was significantly upregulated in esophageal squamous-cell carcinoma (ESCC), and it promoted ESCC cell proliferation, invasion, and migration. However, the effects of SNHG6 on cell apoptosis and the corresponding underlying mechanisms have not yet reported.

Methods: Apoptosis was detected by flow cytometric analysis. Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting were used for mRNA and protein quantification, respectively. A luciferase reporter assay was performed to verify downstream target genes for SNHG6 and miR-101-3p.

Results: Dysregulation of SNHG6 inhibited apoptosis in ESCC cells and regulated the expression of apoptosis-related proteins such as Bcl-2, Mcl-1, Bax and Caspase-3. Functionally, miR-101-3p could compete binding with 3'-untranslated region of SNHG6 and downregulation of miR-101-3p reversed its effect on cell apoptosis in SNHG6 knock-down cells. *EZH2* was confirmed as a downstream target gene of miR-101-3p, silencing *EZH2* expression had the same effect on apoptosis and protein expression as knocking down SNHG6. Overexpression of *EZH2* reversed the effects of miR-101-3p overexpression on cell apoptosis in ESCC cells.

Conclusion: In this study, we found that upregulation of the lncRNA SNHG6 inhibited apoptosis via miR-101-3p/*EZH2* axis in ESCC. These findings may contribute to the diagnosis and treatment of ESCC.

Keywords: lncRNA SNHG6, miR-101-3p, *EZH2*, esophageal squamous-cell carcinoma, apoptosis

Introduction

Esophageal cancer (EC) is one of the most common malignant tumors of the digestive system.¹ EC is classified into two pathological subtypes, esophageal adenocarcinoma and esophageal squamous-cell carcinoma (ESCC), ESCC is the predominant pathological subtype prevalent in the Chinese population.^{2,3} Although the treatment of EC has progressed significantly, the overall and 5-year survival rates of patients with EC are still low.² In 2019, the immunotherapy drug pembrolizumab was approved by the United States Food and Drug Administration for the treatment of EC, but only in patients with tumors expressing programmed-death ligand 1.⁴ Thus, it is imperative to conduct more studies on targeted molecular therapeutic for EC.

Long non-coding RNAs (lncRNAs) are a class of RNA molecules spanning over 200 nucleotides in length. Increasing evidence has shown that lncRNAs do not encode

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proteins, but have a variety of biological functions affecting the occurrence and development of disease.⁵ The effects of lncRNAs on the occurrence and development of cancer is a research hotspot, and many studies have shown that lncRNAs can affect multiple stages of tumor progression such as invasion, metastasis, prognosis.^{6–8} lncRNAs also play an essential role in EC by affecting its development, diagnosis and treatment.^{9,10} MicroRNAs (miRNAs) are another class of non-coding RNAs. A number of studies have confirmed that the biological effects of many lncRNAs on tumors are mediated by miRNAs.^{11–13} For example, lncRNA MCM3AP-AS1 promotes the growth of hepatocellular carcinoma by directly binding to miR-194-5p and subsequently inducing FOXA1 expression.¹⁴ Similarly, lncRNA PVT1 promotes gallbladder cancer progression by regulated HK2 expression through its competing endogenous RNA activity on miR-143 in vitro.¹⁵

SnoRNA host gene 6 (SNHG6), a novel lncRNA, affects the progression of a variety of tumors.¹⁶ Studies have also shown that SNHG6 can affect the biological function of tumor cells by sponging miRNAs.^{17–19} For example, SNHG6 enhances the chemoresistance of colorectal cancer cells to 5-fluorouracil via regulating ULK1 expression by targeting miR-26a-5p.¹⁹ SNHG6 also affects the progression of EC. Our previous study confirmed that upregulating SNHG6 promotes cell proliferation, invasion, and migration in ESCC.²⁰ However, studies of whether SNHG6 affects cell apoptosis and the corresponding mechanism have been rarely reported. To explore whether SNHG6 can influence on cell apoptosis through targeting miRNAs, a web-based lncRNA prediction tool was used for screening target miRNAs. MiR-101-3p was recognized as a target miRNA of SNHG6 and *EZH2* was confirmed as a downstream target gene of miR-101-3p. SNHG6/miR-101-3p/*EZH2* signaling axis may be a novel regulatory mechanism of cell apoptosis in ESCC and may thus, aid ESCC treatment and diagnosis.

Materials and Methods

Patient Tissues Collection

A total of 55 ESCC tumor and paracancerous tissue pairs were collected from the Zhengzhou central hospital affiliated Zhengzhou university and the Shaanxi Provincial cancer Hospital. Informed consent was obtained from all patients before the collection and use of specimens. The acquisition and separation of tissues are performed by specialized surgeons, and the histopathological type were diagnosed by three clinical pathologists. The protocol was approved by

the Committee for the Protection of Human Subjects of the Zhengzhou Central Hospital.

Cell Culture and Transfection

All cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Five Human ESCC cell lines (EC109, EC9706, EC1, KYSE30, and KYSE150) and a normal human esophageal epithelial cell line (HET-1A) were used in this study. The cells were cultured in RPMI 1640 medium (GIBCO, HyClone, USA) containing with 100 units/mL of penicillin and streptomycin, and 10% fetal bovine serum at 37°C under 5% CO₂ conditions. All small interfering RNAs (si-RNAs), miRNAs and plasma-cloning DNAs (pcDNAs) were transfected into cell using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA). Two siRNAs were designed for silencing SNHG6, and the sequence of si-SNHG6 and the nonsense control (si-NC) were used according to a previous report.²⁰ To silence *EZH2*, si-*EZH2* (sense: 5'-AAGACTCTGAATGCAGTTGCT-3') and si-NC were transfected into ESCC cells. To evaluate the role of miR-101-3p in ESCC, cells were divided into three groups: miR-NC, miR-101-3p mimics, and miR-101-3p inhibitor. To evaluate the effect of miR-101-3p on SNHG6-depletion, si-SNHG6 was co-transfected with a miR-101-3p inhibitor. To evaluate the effect of overexpression of *EZH2* on miR-101-3p, pcDNA-*EZH2* was co-transfected with miR-101-3p mimics.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA extraction and reverse transcription were performed as described previously.²⁰ The qRT-PCR primer sequences of SNHG6 and GAPDH were also described previously.²⁰ Other primer sequences were as follows: *EZH2* forward: 5'-AGGACGGCTCCTCTAACCAT-3', reverse: 5'-CTTGGTGTTGCACTGTGCTT-3'. MiR-101-3p forward: 5'-AAAGATTCCAAGAAGCTAAGGGTG-3'; reverse: 5'-CCTAACTGGTTTCCTGTGCCTA-3'. U6 forward: 5'-CTCGCTTCGGCAGCACA-3'; reverse: 5'-AACGCTTCACGAATTTGCGT-3'. Relative expression levels were calculated by using the 2^{-ΔΔCt} method.

Cell Apoptosis

Apoptosis was detected using the Annexin V/PI double staining assay. Briefly, cells were harvested after different treatments and washed with PBS. The cells were resuspended with 400 µl 1× binding buffer, mixed with 5 µl Annexin V-FITC, and incubate at room temperature in dark for 15 minutes, then, 10 µl of PI were added and the cells were incubated in ice bath

and dark for 5 minutes. Cells were then analyzed with flow cytometry (Beckman Coulter, Brea, CA, USA).

Luciferase Reporter Assay

The SNHG6 and EZH2 sequences were amplified by PCR and cloned into pmirGLO Dual-luciferase miRNA target expression vectors. EC-1 cells were seeded in a 96-well plate. The next day, the cells were co-transfected with miR-101-3p mimics or miR-NC, and pmirGLO-SNHG6-3'-untranslated region (UTR)-wild-type (WT) (SNHG6-WT), pmirGLO-SNHG6-3'-UTR-mutant type (MUT) (SNHG6-MUT), pmirGLO-EZH2-3'-UTR-WT (EZH2-WT), or pmirGLO-EZH2-3'-UTR-MUT (EZH2-MUT). After 48 h, the luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega, Madison WI, USA).

Western Blotting

Total protein was extracted using RIPA buffer containing protease K inhibitor and quantified using a NanoDrop 2000 spectrophotometer. Proteins separated were by using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% skim milk and incubated samples at 4°C overnight after additions of anti-EZH2 (1:500 dilution, Bioworld, MN, USA), anti-Bcl-2 (1:200 dilution, Bioworld, MN, USA), anti-Bax (1:500 dilution, Bioworld, MN, USA), anti-Mcl-1 (1:200 dilution, Bioworld, MN, USA), or anti-Caspase3 (1:300 dilution, Bioworld, MN, USA) antibodies. The membranes were then incubated with corresponding secondary antibody (1:2000 dilution, Abcam, Cambridge, MA, USA). Blots were visualized by using chemiluminescence kits (Beyotime Biotechnology, Haimen, China).

Statistical Analysis

Statistical analyses were performed using SPSS 19.0 software (IBM, Chicago, IL, USA). All data are reported as mean \pm standard deviations. Statistical significance of the results was determined using Student's *t*-test or a one-way analysis of variance. Differences were considered statistically significant at $P < 0.05$.

Results

Upregulation of SNHG6 Inhibited Apoptosis

To evaluate the role of SNHG6 in ESCC cell apoptosis, we first assessed the expression of SNHG6 in ESCC and

paired paracancerous tissues. The results showed that SNHG6 expression was significantly higher in tumor compared with paracancerous tissue (Figure 1A). SNHG6 expression was also significantly higher in the five ESCC cell lines examined (EC109, EC9706, EC1, KYSE30, and KYSE150) than in HET-1A (Figure 1B). We selected the two ESCC cell lines with the highest differential expression of SNHG6 for the functional assays (EC1 and KYSE30). Cells transfected with si-SNHG6-1 or si-SNHG6-2 showed significantly reduced SNHG6 expression compared with cells transfected with si-NC (Figure 1C). Flow cytometry showed that knockdown of SNHG6 significantly promoted apoptosis (Figure 1D). We also examined the expression of apoptosis-related proteins by Western blots. Knockdown of SNHG6 decreased the expression levels of protein Bcl-2 and Mcl-1 but promoted the expression levels of Bax and cleaved-Caspase-3 (Figure 1E).

Role of MiR-101-3p in ESCC

The qRT-PCR analysis showed that the expression level of miR-101-3p was lower in tumor compared with paracancerous tissue (Figure 2A). We then analyzed the relationship between miR-101-3p and SNHG6 and found that their expression levels were negatively correlated in ESCC tissue (Figure 2B). Figure 2C shows the complementary binding site between miR-101-3p and SNHG6. Co-transfection with WT-SNHG6 and miR-101-3p mimics significantly decreased the luciferase activity in EC-1 cells (Figure 2D). As in tumor tissue, miR-101-3p expression was significantly lower in the five ESCC cell lines than in HET-1A (Figure 2E). Cells transfected with miR-101-3p mimics showed significantly increased miR-101-3p expression compared with miR-NC group (Figure 2F). Flow cytometry showed that overexpression of miR-101-3p significantly promoted apoptosis, but transfected with miR-101-3p inhibitor resulted in no significant difference in the extent of apoptosis compared with miR-NC group (Figure 2G). The Western blot results showed that overexpression of miR-101-3p significantly decreased the expression levels of protein Bcl-2 and Mcl-1, but that promoted the expression levels of Bax and cleaved-Caspase-3 (Figure 2H).

Role of EZH2 in ESCC

EZH2 was predicted to be a downstream target gene of miR-101-3p. *EZH2* was higher expression in tumor tissues and the expression was negatively correlated to miR-101-3p

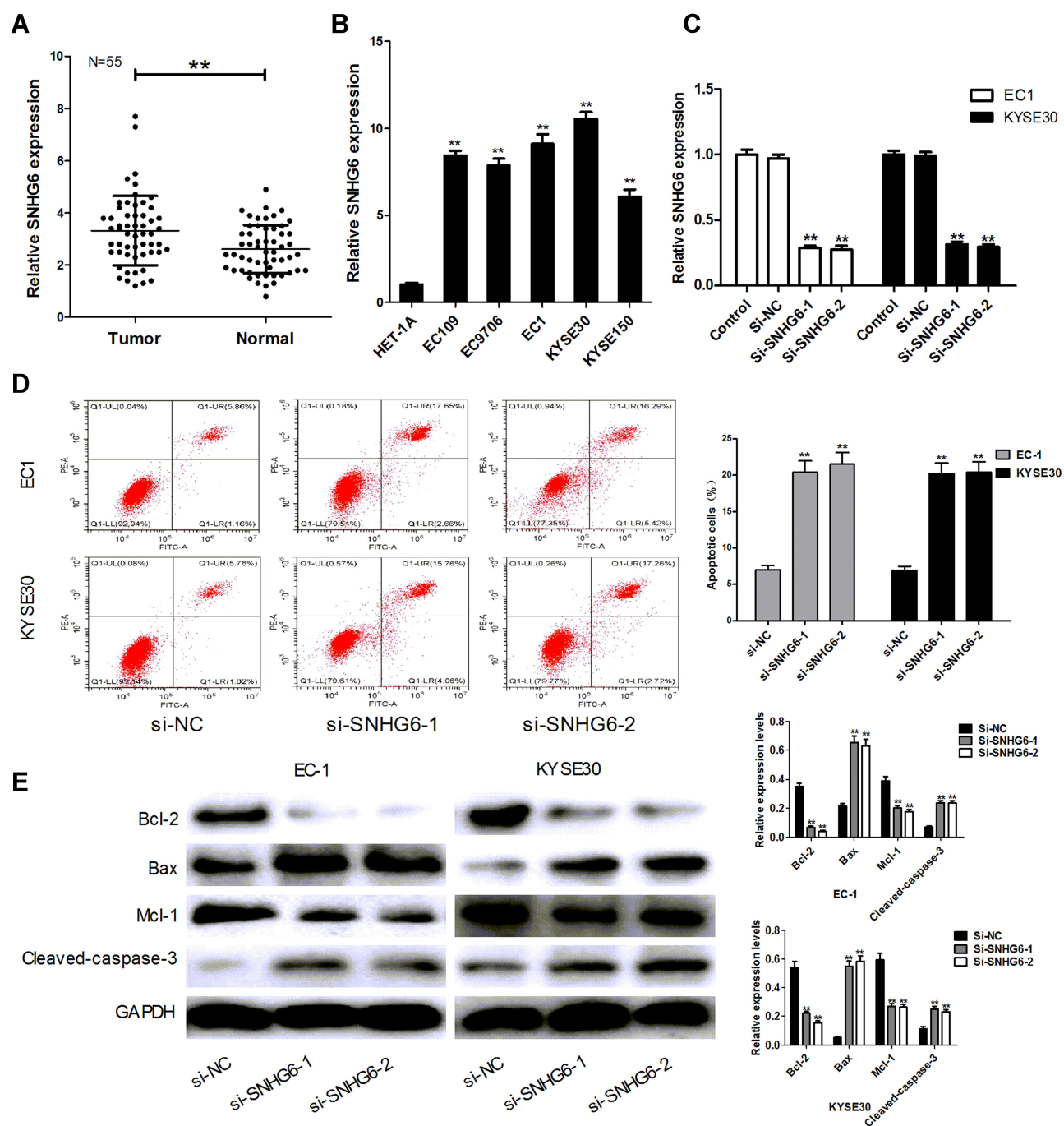


Figure 1 Upregulated expression of SNHG6 inhibits apoptosis. **(A)** SNHG6 expression is significantly higher in tumor compared with paracancerous tissue. **(B)** The expression levels of SNHG6 are significantly higher in five human ESCC cell lines tested compared with the HET-1A cell line. **(C)** Effect of transfection with si-RNAs on SNHG6 expression. **(D and E)** Effects of SNHG6 knockdown on cell apoptosis and the expression levels of apoptosis-related proteins. $^{**}P < 0.01$.

expression (Figure 3A and B). Figure 3C shows the complementary binding site between miR-101-3p and *EZH2*. Co-transfection with WT-*EZH2* and miR-101-3p mimics significantly decreased the luciferase activity in EC-1 cells (Figure 3D). *EZH2* expression was significantly higher in the five ESCC cell lines than in HET-1A (Figure 3E). Transfection of cells with miR-101-3p mimics significantly

decreased the expression of *EZH2* in mRNA and protein levels (Figure 3F and G). To observe the role of *EZH2* in ESCC, si-*EZH2*-1 was transfected into cells. This resulted in a significant decrease in *EZH2* expression (Figure 3H). *EZH2* silencing had the same effect on apoptosis and protein expression as SNHG6 knockdown and miR-101-3p overexpression (Figure 3I and J).

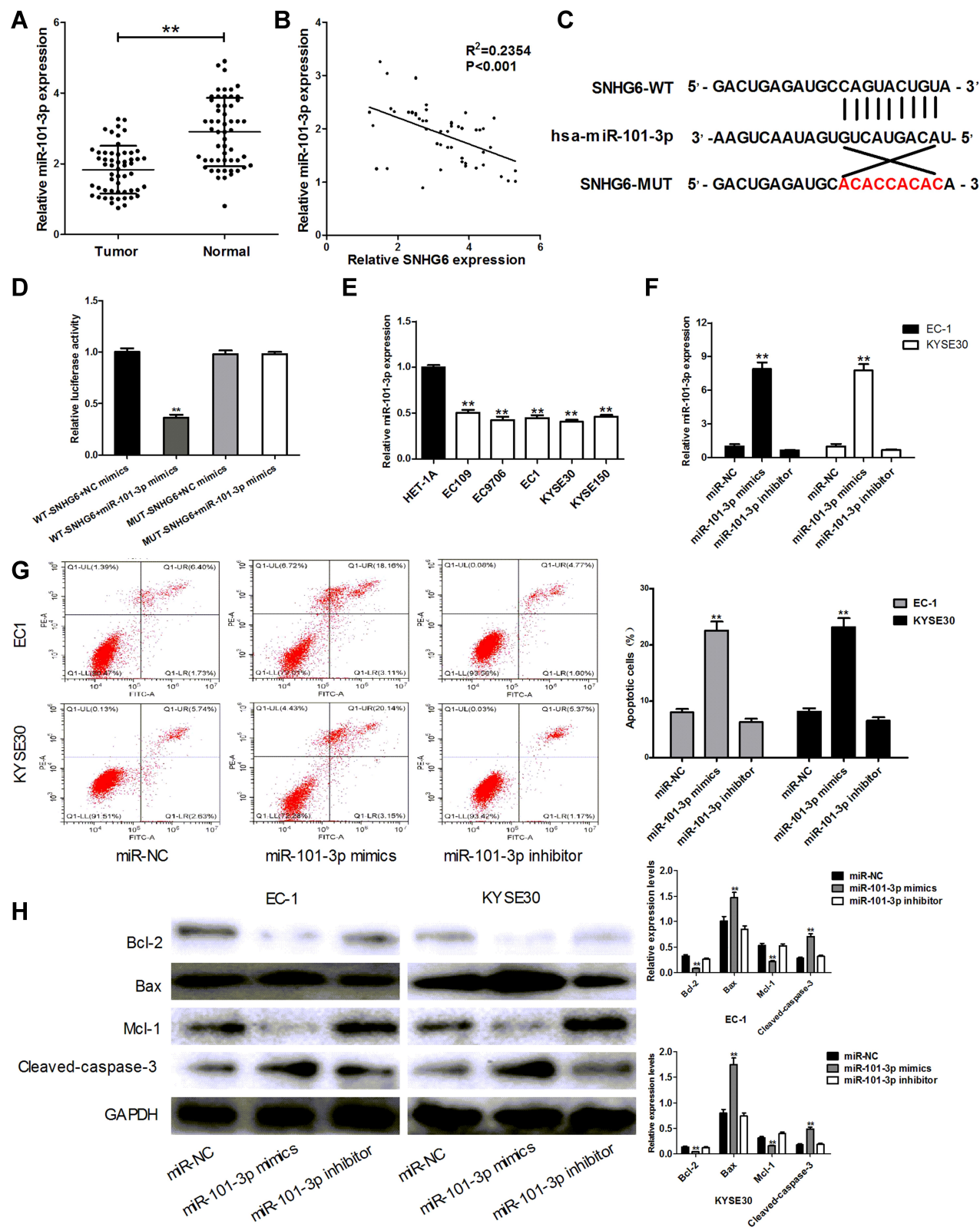


Figure 2 Role of miR-101-3p in ESCC. (A) MiR-101-3p expression levels are lower in tumor compared with paracancerous tissue. (B) MiR-101-3p expression is negatively correlated with SNHG6 expression in ESCC tissues. (C and D) A complementary binding site between miR-101-3p and the 3'-UTR of SNHG6 was confirmed by a luciferase reporter assay. (E) Expression levels of miR-101-3p in ESCC cells. (F) Effect of transfection with miR-101-3p mimics or its inhibitor on miR-101-3p expression. (G and H) Effects of transfection with miR-101-3p mimics or its inhibitor on cell apoptosis and the expression levels of apoptosis-related proteins. ** $P < 0.01$.

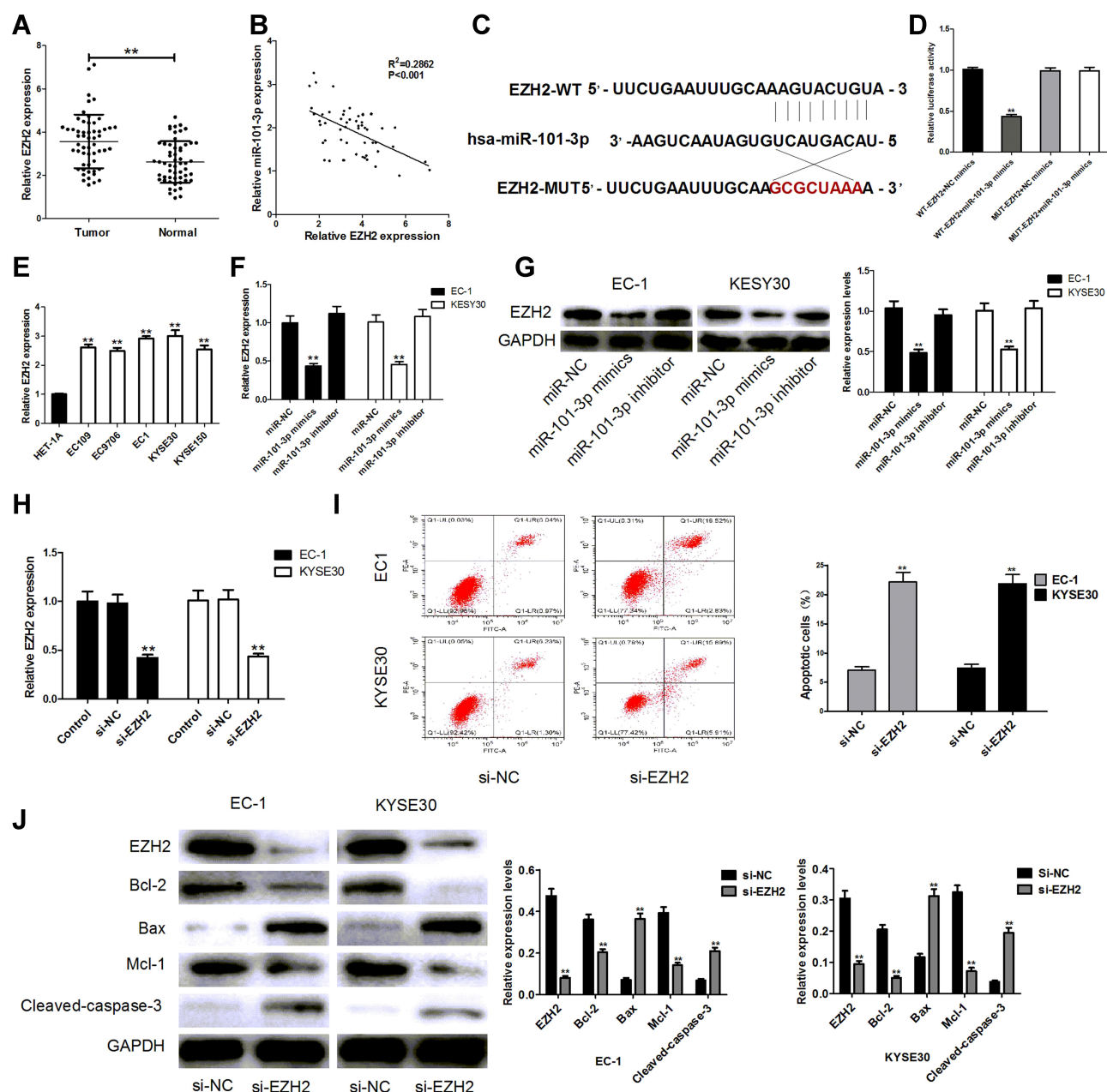


Figure 3 Role of EZH2 in ESCC. (A) EZH2 is highly expressed in tumor compared with paracancerous tissue. (B) EZH2 expression is negatively correlated with miR-101-3p expression in ESCC tissues. (C and D) A complementary binding site between miR-101-3p and the 3'-UTR of EZH2 was confirmed by a luciferase reporter assay. (E) Expression levels of EZH2 in ESCC cells. (F and G) Effect of transfection with miR-101-3p mimics or its inhibitor on EZH2 expression. (H) Effect of transfection with si-RNA on EZH2 expression. (I and J) Effects of EZH2 knockdown on cell apoptosis and the expression levels of apoptosis-related proteins. ** $P < 0.01$.

Inhibiting MiR-101-3p Reversed the Effects of SNHG6 Knockdown

To confirm that SNHG6 affected apoptosis of ESCC cells by regulating miR-101-3p, we co-transfection with si-SNHG6 and miR-NC or miR-101-3p inhibitor. Flow cytometry showed that co-transfection with si-SNHG6 and the miR-101-3p inhibitor significantly decreased the extent of apoptosis in EC-1 and KYSE30 cells compared with that in the si-SNHG6 + miR-NC group (Figure 4A). Meanwhile,

co-transfection with si-SNHG6 and the miR-101-3p inhibitor promoted the expression levels of protein EZH2, Bcl-2 and Mcl-1, but that inhibited the expression levels of Bax and cleaved-Caspase-3 (Figure 4B).

MiR-101-3p Affected Apoptosis by Regulating EZH2 Expression

These results showed that overexpression of miR-101-3p significantly inhibited the expression of *EZH2* in mRNA

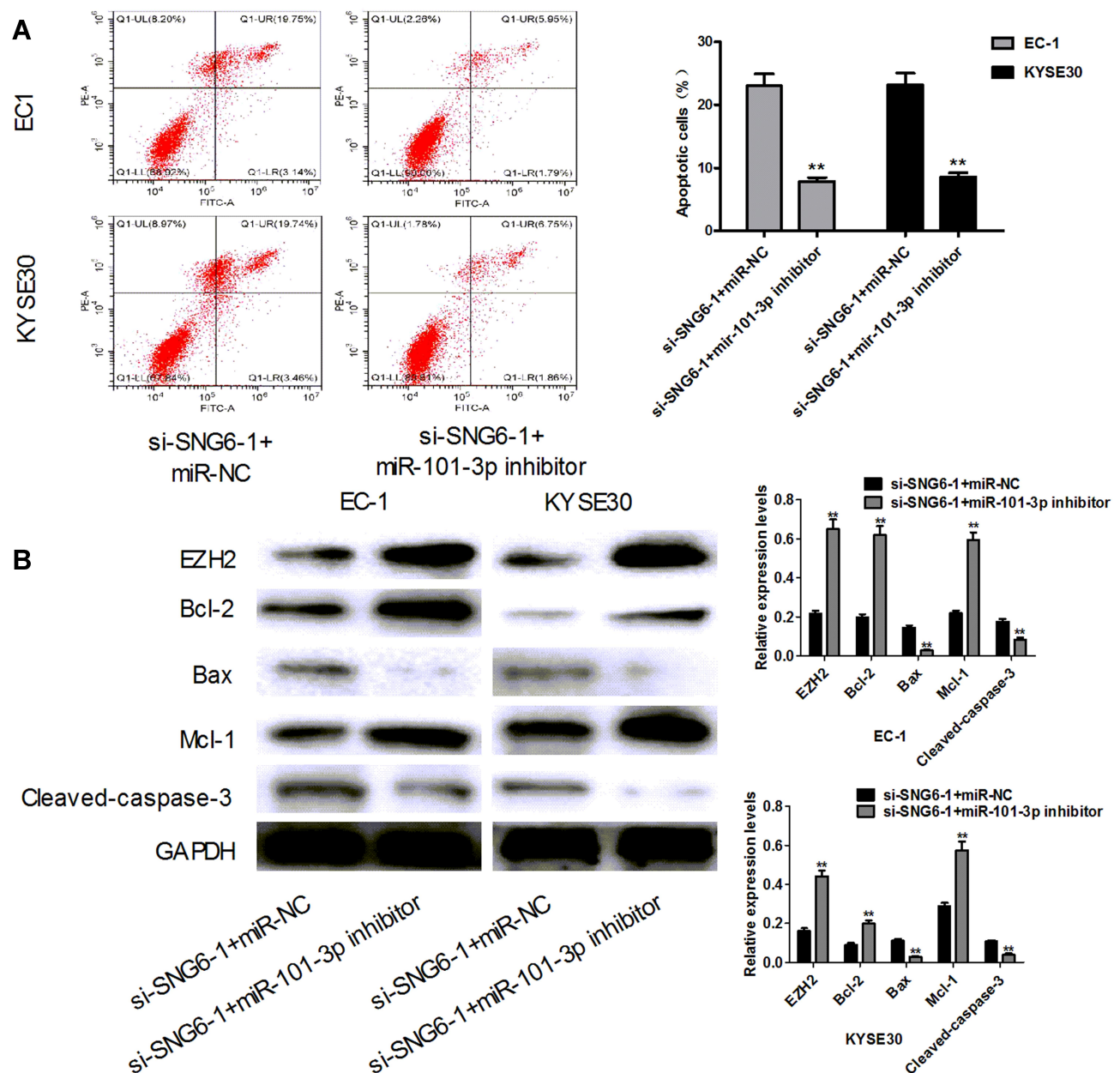


Figure 4 Inhibiting miR-101-3p reverses the effects of SNHG6 knockdown in ESCC. **(A and B)** Effects of co-transfection with si-SNHG6 and the miR-101-3p inhibitor on cell apoptosis and the expression levels of apoptosis-related proteins. $^{**}p < 0.01$.

and protein levels. To confirm whether miR-101-3p affected apoptosis by regulating *EZH2* expression, we co-transfection with miR-101-3p mimics and pcDNA-NC or pcDNA-EZH2. Flow cytometry showed that co-transfection with miR-101-3p mimics and pcDNA-EZH2 significantly inhibited apoptosis in EC-1 and KYSE30 cells compared with cells where miR-101-3p mimics were co-transfected with pcDNA-NC group (Figure 5A). Consistent with this finding, co-transfection with miR-101-3p mimics and pcDNA-EZH2 promoted the expression levels of protein *EZH2*, *Bcl-2* and

Mcl-1, but that inhibited that of *Bax* and cleaved-Caspase-3 (Figure 5B).

Discussion

The lncRNA SNHG6, affects various cellular processes in EC such as proliferation, migration, and invasion.²¹ Silencing SNHG6 inhibits the proliferation, migration, and invasion of ESCC cells through regulating miR-186-5p/HIF1 α signaling pathway.²² Our previous study showed that SNHG6 expression was significantly upregulated in

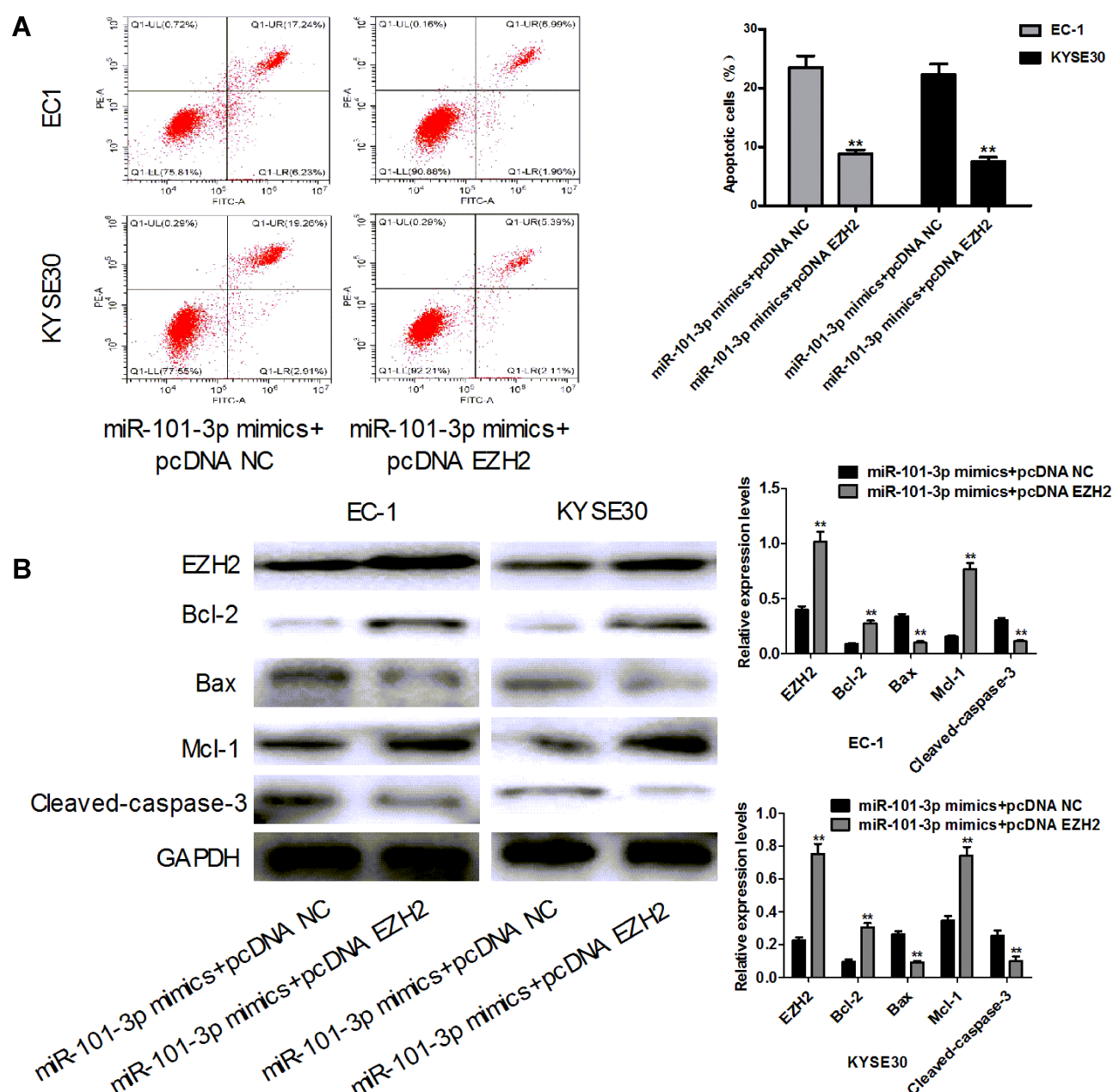


Figure 5 MiR-101-3p affects cell apoptosis by regulating EZH2 expression. **(A and B)** Effects of co-transfection with miR-101-3p mimics and pcDNA-EZH2 on cell apoptosis and the expression levels of apoptosis-related proteins. $^{**}P < 0.01$.

ESCC tissue and that upregulated SNHG6 promoted cell proliferation, invasion, and migration.²⁰ In the current study, we explored whether SNHG6 affected apoptosis and elucidated the mechanism underlying this phenomenon.

Increasing evidence shows that upregulation of SNHG6 inhibits cell apoptosis in ESCC,²¹ colorectal cancer,²³ osteosarcoma,²⁴ and breast cancer.²⁵ A previous study has confirmed that silencing SNHG6 induced apoptosis in EC-109 cell.²¹ Thus, we first evaluated the relationship between high SNHG6 expression and apoptosis in ESCC. For this

purpose, two siRNAs were designed for silencing SNHG6 expression. We found that knockdown of SNHG6 significantly promoted cell apoptosis and led to downregulation of protein Bcl-2 and Mcl-1, but upregulation of protein Bax and cleaved-Caspase-3 in EC-1 and KYSE30 cells. SNHG6 can affect a variety of cell cellular function by targeting miRNAs. For example, SNHG6 promoted cell apoptosis by competing binding with miR-181a-5p to regulate E2F5 expression in colorectal cancer.²³ Similarly, downregulation of SNHG6 significantly inhibited proliferation, invasion and induced

cell autophagy by competitively sponging miR-26a-5p in human osteosarcoma.²⁴ Therefore, we want to explore whether or not SNHG6 could influence cell apoptosis through targeting miRNAs in ESCC. Specifically, we focused on miR-101-3p, which was predicted as a target miRNA of SNHG6 through a web-based lncRNA prediction tool. The binding of miR-101-3p with SNHG6 was confirmed by a luciferase reporter assay. A previous study also proved that SNHG6 could compete with miR-101-3p and thereby promote the progression of colorectal cancer.¹⁸

These results demonstrated that SNHG6 may inhibit apoptosis by acting as a competing endogenous RNA for miR-101-3p. MiR-101-3p serves as a tumor suppressor gene in a variety of tumors including colorectal cancer,¹⁸ gastric cancer,²⁶ and cholangiocarcinoma.²⁷ In the current study, we showed that miR-101-3p function as a tumor suppressor gene in ESCC. To confirm that SNHG6 affected apoptosis in ESCC cells by inhibiting miR-101-3p expression, we co-transfection si-SNHG6 and a miR-101-3p inhibitor and found that down-regulation of miR-101-3p reversed its effects on apoptosis in SNHG6 knockdown cells. A previous study also showed that SNHG6 promoted the proliferation and angiogenesis of cholangiocarcinoma cells by sponging miR-101-3p.²⁷ These results confirmed that SNHG6 affects apoptosis in ESCC cells by regulating miR-101-3p expression.

Previous studies showed that miR-101-3p affected the progression of cancer by regulating *EZH2* expression.^{26,28} To explore the mechanism by which SNHG6 modulated ESCC cell apoptosis, *EZH2* was predicted to be a downstream target gene of miR-101-3p. A previous study also showed that miR-101-3p inhibits proliferation of retinoblastoma cells by targeting *EZH2* and HDAC9.²⁸ In the current study, *EZH2* was significantly upregulated in ESCC cells and tumor tissues, which was consistent with previous research.²⁹ Knockdown of *EZH2* had the same effect on apoptosis and protein expression as overexpression of miR-101-3p. Thus, we hypothesized that miR-101-3 affects apoptosis in ESCC cells by regulating *EZH2* expression. The results showing that overexpression of *EZH2* reversed its effects on apoptosis in miR-101-3p overexpressing cells confirmed our hypothesis. These results show that SNHG6/miR-101-3p affects apoptosis in ESCC cells by regulating *EZH2* expression.

Conclusion

We found that upregulation of the lncRNA SNHG6 inhibited apoptosis via miR-101-3p/*EZH2* axis in ESCC. These findings provide further evidence for SNHG6 as a biomarker in

ESCC, which may contribute to the diagnosis and treatment of ESCC.

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Disclosure

The authors declare that they have no financial or non-financial conflicts of interest for this work.

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