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MiR-142 inhibits lung cancer cell proliferation and promotes apoptosis by targeting XIAP

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Abstract. – OBJECTIVE: The X-linked inhibitor of apoptosis protein (XIAP) is associated with the development of various tumors. The abnormal miR-142 expression is associated with the onset of lung cancer. Bioinformatics analysis revealed a targeted relationship between miR-142 and XIAP. This report investigated whether miR-142 plays a role in regulating XIAP expression and affecting the biological processes of lung cancer cells.

PATIENTS AND METHODS: The tumor tissues of lung cancer patients were collected, and the adjacent tissues were used as controls. The dual luciferase reporter gene assay validated the targeted regulation between miR-142 and XIAP. Using BEAS-2B cells as control, qRT-PCR was used to detect the expression of miR-142 and XIAP in lung cancer cells A549 and H1650. Lung cancer H1650 cells were cultured and divided into miR-NC group and miR-142 mimic group followed by an analysis of cell proliferation by EdU staining.

RESULTS: Compared with those in adjacent tissues, miR-142 expression was significantly decreased and XIAP expression was increased in lung cancer tissues. The Dual-Luciferase Reporter Assay confirmed a targeted regulation relationship between miR-142 and XIAP. Compared with BEAS-2B cells, miR-142 expression in lung cancer A549 and H1650 cells was significantly decreased, and XIAP expression was significantly increased. Transfection of miR-142 mimic significantly inhibited the expression of XIAP in H1650 cells, promoted apoptosis and inhibited cell proliferation.

CONCLUSIONS: Decreased miR-142 expression and increased XIAP expression is associated with the onset of lung cancer. MiR-142 can inhibit lung cancer cell proliferation and induce apoptosis through inhibition of XIAP expression.

Key Words:
Lung cancer, MiR-142, XIAP, Proliferation, Apoptosis.

Introduction

Lung cancer (LC) is one of the most common human malignancies. In male populations, the incidence and mortality of lung cancer ranks first in systemic malignancies. In female populations, the incidence of lung cancer and mortality ranks second in the body of malignant tumors[1-3], posing a serious threat to human life, but also bringing a huge burden on the society[4].

X-linked Inhibitor of Apoptosis Protein (XIAP) can simultaneously inhibit the expression of Caspases-9 in the key proteases Caspases-3, Caspases-7, and mitochondria-dependent apoptotic pathways, thus exerting anti-apoptotic effect[5-7].

MicroRNA (microRNA) is an endogenous non-coding small RNA molecule in eukaryotes and regulates the expression of the target genes through complementary binding to the 3'-untranslated region (3'-UTR) of the target gene mRNA, leading to degradation of mRNA or inhibition of mRNA translation, thereby participating in the regulation of the biological processes, such as cell survival, proliferation, apoptosis, migration. Abnormal microRNA expression and dysfunction were found in lung cancer, ovarian cancer, and bladder cancer[8-11]. Previous works[12-14] found that the expression of miR-142 in lung cancer patients was significantly decreased, suggesting that miR-142 may play a role as a tumor suppressor gene in

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the pathogenesis of lung cancer. The bioinformatics analysis showed that there is a targeted complementary binding site between miR-142 and XIAP mRNA, suggesting a possible regulatory effect between them. This analysis analyzed and detected the expression characteristics of miR-142 and XIAP in tumor tissues of lung cancer patients and explored whether miR-142 plays a role in regulating XIAP expression and affecting proliferation and apoptosis of lung cancer cells.

Materials and Methods

Main Reagents and Materials

The human normal lung epithelial cells BEAS-2B were purchased from Shanghai Meixuan Biotechnology Co., Ltd. (Shanghai, China); lung cancer cells A549 and H1650 cells were purchased from Beijing Beina (Beijing, China); HEK293T cells were purchased from Shanghai Jihe (Shanghai, China); DMEM medium, fetal bovine serum (FBS), and Opti-MEM were purchased from Gibco (Grand Island, NY, USA); RNA extraction reagent RNAiso Plus was purchased from Dalian TaKaRa (Dalian, China); qRT-PCR SuperMix was purchased from Beijing TransGen Biotechnology (Beijing, China); Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA); miR-NC and miR-142 mimic were purchased from Guangzhou Ruibo Bio (Guangzhou, China). The EdU cell proliferation flow assay kit was purchased from Sigma-Aldrich (St. Louis, MO, USA); the rabbit anti-human XIAP polyclonal was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the rabbit anti-human β-catenin and HRP polyclonal conjugated secondary antibody were purchased from Shanghai Shenggong (Shanghai, China); pMIR plasmid was purchased from Changsha Youbao Bio (Changsha, China); Luciferase activity assay kit Dual-Glo Luciferase Assay System was purchased from Promega (Madison, WI, USA); Annexin V/PI apoptosis assay kit was purchased from Jiangsu Biyuntian Bio (Nantong, China); UV-1100 UV spectrophotometer was purchased from Shanghai Meifida Instrument Co., Ltd (Shanghai, China).

Clinical Data

50 patients with non-small cell lung cancer who were treated in our hospital from March 2018 to October 2018 were enrolled. Non-small cell lung cancer tissue specimens confirmed by histopathological examination were collected and the adjacent tissues were collected as a control. This study was approved by the Ethnic Committee of our Hospital and the informed consents were obtained from all patients.

Cell Culture

BEAS-2B, A549, and H1650 cells were cultured in DMEM medium containing 10% FBS and 1% streptomycin in a cell culture incubator containing 5% CO_2 at 37°C. The 1:4~1:5 ratio was sub-cultured, and the experiment was performed when the cells were in the logarithmic growth phase.

Dual-Luciferase Reporter Gene Test

Using the HEK293T cell genome as a template, the full-length 3'-UTR fragment or the mutant-containing fragment of XIAP gene was amplified and cloned into pMIR vector, transformed into DH5α competent cells, and the correct plasmid was sequenced and named as pMIR-XIAP-WT, pMIR-XIAP-MUT. pMIR-XIAP-WT (or pMIR-XIAP-MUT) and miR-142 mimic (or miR-NC) were co-transfected into HEK293T cells with Lipofectamine 2000. After 48 h of culture, the relative Luciferase activity was detected to follow the instructions of the Dual-Glo Luciferase Assay System Kit.

Cell Transfection and Grouping

A549 and H1650 cells were cultured *in vitro* and divided into two transfection groups: miR-NC transfection group and miR-142 mimic transfection group. The general procedure for the transfection was as follows: 10 μL of Lipofectamine 2000, 50 nmoL miR-NC, and 50 nmoL miR-142 mimic were diluted with 100 μL of Opti-MEM, respectively, and incubated for 5 min at room temperature, respectively. We gently mixed, incubated for 20 min at room temperature, added the transfectants to DMEM cell culture medium containing 10% FBS and 1% streptomycin, continued to culture for 72 hours, and collected the cells.

Flow Cytometry Detection of Cell Proliferation

The two transfected cells were harvested by trypsinization, and the cells were resuspended in Roswell Park Memorial Institute-1640 (RPMI-1640) complete medium containing 10% FBS. After incubating with 10 μM of EdU at 37°C for 2 h, the cells were inoculated into the culture plate

for 48 h, for trypsin digestion. We then collected the cells, added PBS to wash once, added paraformaldehyde to fix the cells, washed once in PBS, added 100 μL permeabilization solution and 500 μL reaction test solution, incubated at room temperature for 30 min in the dark, and finally, added 3 mL. The solution was washed once with centrifugation, the cells were resuspended by adding 500 μL of wash buffer, and cell proliferation was measured by Beckman Coulter FC500MCL flow cytometry (Brea, CA, USA).

Flow Cytometry Detection of Apoptosis

The two transfected cells were collected by trypsinization and resuspended in 100 μL Annexin V Binding Buffer, according to the instructions, followed by the addition of 5 μL of FITC Annexin V and 10 μL of PI and subsequent incubation for 15 min at room temperature. After that, 400 μL of Annexin V Binding Buffer was added and cell apoptosis was measured by flow cytometry.

qRT-PCR detection of Gene Expression

The RNA was extracted with RNAiso Plus reagent, and the relative expression of the gene was detected by One-Step qRT-PCR using thr TransScript Green One-Step qRT-PCR SuperMix in the 20 μL reaction system including: 1 μg of Template RNA, 0.2 μM of pre-primer, 0.2 μM of post-primer, 10 μL of 2×TransStart Tip Green qPCR SuperMix, 0.4 μL of One-Step RT Enzyme Mix, 0.4 μL of Passive Reference Dye II, and RNase-free water. The qRT-PCR reaction conditions were: 45°C, for 5 min; 94°C, for 30 s; (94°C, for 5 s; 60°C, for 30 s) × 40 cycles, and the gene expression was detected on a Bio-Rad CFX96 Real Time-PCR instrument (Hercules, CA, USA).

Western Blot

The total protein in the cells was extracted by the RIPA lysis method. After the concentration was determined, 40 μg was separated by electrophoresis on SDS-PAGE (12% separation gel, 5% concentrated gel), transferred to polyvinylidene difluoride (PVDF) membrane (250 mA, 100 min), blocked with 5% skim milk powder at room temperature for 60 min, and incubated with the primary antibody at 4°C overnight (XAP, β-actin dilution ratio: 1:2000, 1:5000) followed by washing three times with PBST, and incubation with HRP-conjugated secondary antibody (1:5000 dilution) at room temperature for 60 min. After 3 times PBST washing, a chemiluminescence solution was added to the protein blotting membrane and incubated for 2-3 minutes under the dark followed by exposure and development.

Statistical Analysis

Statistical analysis was performed using the Statistical Product and Service Solution (SPSS Inc, Chicago, IL, USA) 18.0 software. The measurement data were expressed as mean ± standard deviation (SD). The Student's *t*-test was used to compare the measurement data between the two groups. The comparison between the measurement data of multiple groups was done by One-way ANOVA with Bonferroni as post-hoc analysis. The Mann-Whitney U test was used to compare the expression of miR-142 and XIAP mRNA in lung cancer and adjacent tissues. $p < 0.05$ was considered statistically significant.

Results

Abnormal Expression of MiR-142 and XIAP in Lung Cancer Tissues

The results of qRT-PCR showed that the expression of miR-142 in lung cancer patients was significantly lower than that in the adjacent tissues (Figure 1A), while the expression of XIAP mRNA was significantly increased (Figure 1B).

Abnormal Expression of MiR-142 and XIAP in Lung Cancer Cells

The results of qRT-PCR showed that, compared with normal lung epithelial BEAS-2B cells, the expression of miR-142 in lung cancer A549 and H1650 cells was significantly decreased (Figure 2A), while the expression of XIAP mRNA was significantly increased (Figure 2A). The Western blot analysis showed that the expression of XIAP protein in lung cancer A549 and H1650 cells was significantly higher than that of BEAS-2B cells (Figure 2B).

Targeted Regulation Relationship Between MiR-142 and XIAP

The bioinformatics analysis revealed a targeted complementary binding site between miR-142 and the 3'-UTR of XIAP mRNA (Figure 3A). The Dual-Luciferase Gene Reporter Assay showed that the transfection of miR-142 mimic significantly reduced the relative Luciferase activity of pMIR-XIAP-WT transfected HEK293T cells. However, miR-NC or miR-142 mimic did not have a significant effect on the relative Lu-

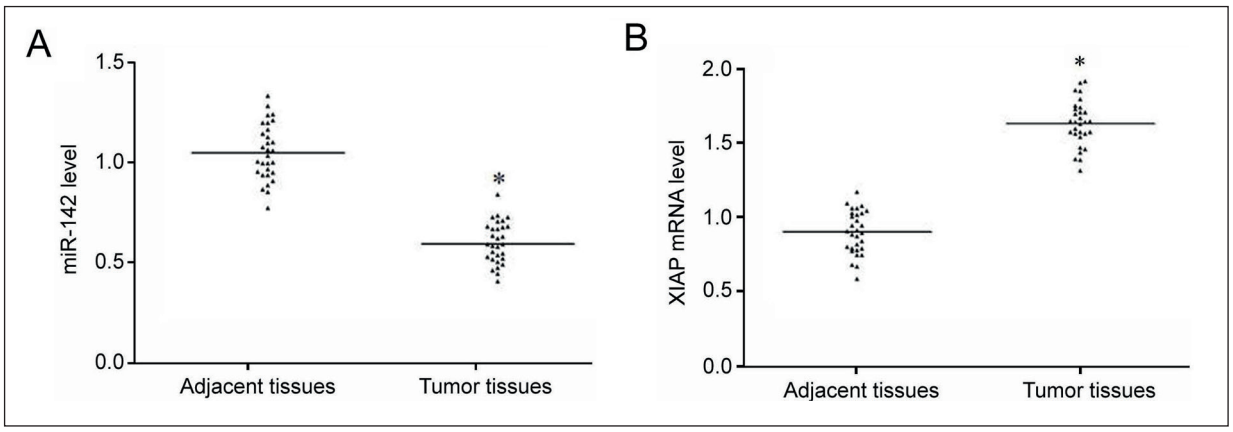

Figure 1. Abnormal expression of miR-142 and XIAP in lung cancer tissues. ***A,*** qRT-PCR detection of miR-142 expression in lung cancer tissues. ***B,*** qRT-PCR detection of XIAP mRNA expression in lung cancer tissues. *Represents $p < 0.05$ compared to the two groups.

ciferase activity in HEK293T cells transfected with pMIR-XIAP-MUT (Figure 3B), indicating a targeted regulatory relationship between miR-142 and XIAP mRNA.

Increased MiR-142 Level Inhibits XIAP Expression and Proliferation of Lung Cancer Cells and Promotes Apoptosis

The qRT-PCR assay showed that miR-142 mimic transfection significantly upregulated the expression of miR-142 compared to the miR-NC group (Figure 4A), resulting in a significant decrease in XIAP mRNA expression in H1650 cells (Figure 4B). The Western blot analysis showed that the expression of XIAP protein in H1650 cells was significantly decreased in miR-142 mimic transfection group compared with miR-NC group (Figure 4C). The spectrophotometry analysis showed that caspase-3 activity was significantly increased in H1650 cells in the miR-142 mimic group compared with the miR-NC group (Figure 4D). The flow cytometry analysis showed that the transfection of miR-142 mimic significantly increased apoptosis in H1650 cells (Figure 4E) and reduced cell proliferation (Figure 4F).

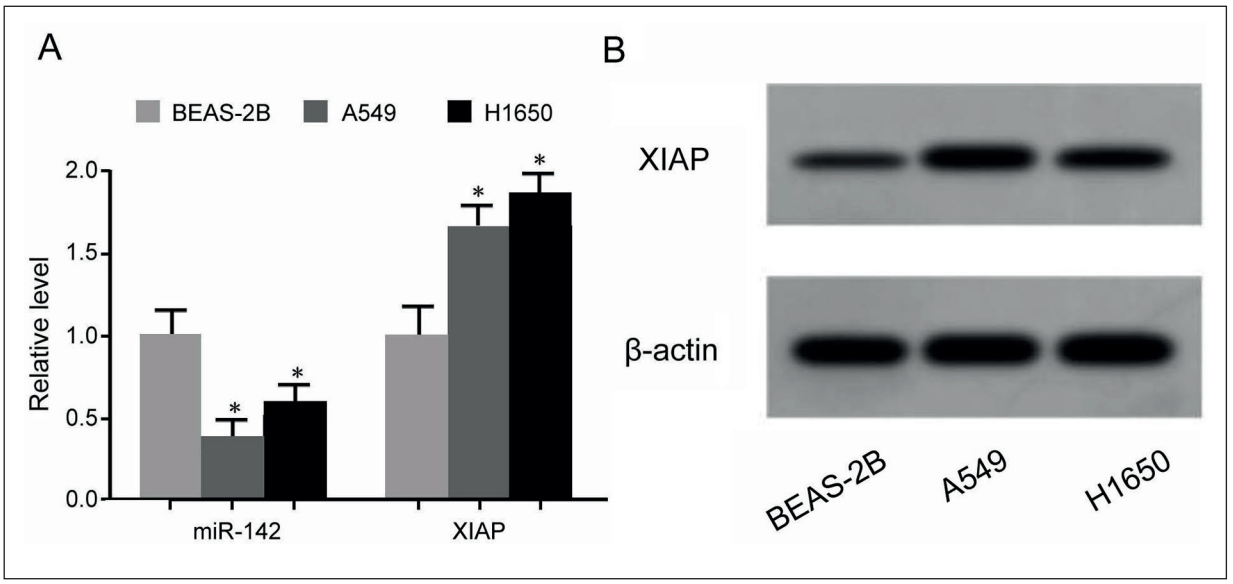

Figure 2. Abnormal expression of miR-142 and XIAP in lung cancer cells. ***A,*** qRT-PCR was used to detect the expression of miR-142 and XIAP mRNA in lung cancer cells. ***B,*** Western blot analysis of XIAP protein expression in lung cancer cells. *Represents $p < 0.05$ compared to BEAS-2B cells.

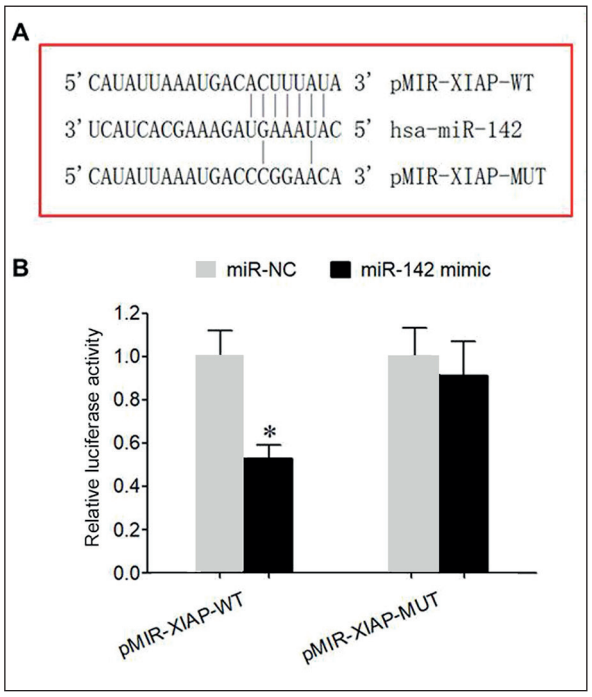

Figure 3. Targeted regulatory relationship between miR-142 and XIAP mRNA. ***A,*** The binding site between miR-142 and the 3'-UTR of XIAP mRNA, ***B,*** Dual-Luciferase Gene Reporter Assay. *$p < 0.05$ compared to the miR-NC group.

Discussion

Lung cancer is one of the most common malignant tumors in the clinic, and it is also the malignant tumor with the fastest growth in morbidity and mortality. In addition, with the development of industry, the increase in the number of pollutants in the environment and the increase in the number of lung cancer, the incidence, and mortality of lung cancer are getting higher and higher[15]. Although surgical treatment, radiotherapy and chemotherapy, immunotherapy and other comprehensive treatment methods have improved the survival of lung cancer patients, the survival and prognosis of patients are still poor, and their 5-year survival rate is only 15-20%. In case of metastasis, the 5-year survival rate is reduced to less than 5%[16,17]. Therefore, exploring the signal molecules of the abnormal changes in the pathogenesis of lung cancer is of great significance to improve the early diagnosis, therapeutic effect, and the prognosis.

Inhibitor of apoptosis proteins (IAPs) are a family of proteins that are structurally homologous. XIAP is the most potent inhibitor of apoptosis in the IAPs family. At the same time, it inhibits the apoptosis antagonistic factor in the initial stage of apoptosis and the stage of apoptosis effect[18,19]. XIAP has a characteristic BIR (baculovirus inhibitor of apoptosis repeat) domain, which exerts an anti-apoptotic effect through three IAP repeats in the N-terminus of the BIR domain and a zinc finger domain in the C-terminus. XIAP can directly bind to and inhibit the apoptosis initiation molecule Caspase-9 and the apoptosis-executing molecule Caspase-3/7, thereby blocking the transmission of the downstream signaling pathways and protecting the cells from apoptosis caused by various factors[5-7]. The overexpression of XIAP is closely related to the occurrence, progression, metastasis, and prognosis of several tumors such as ovarian cancer[5], gastric cancer[20], and esophageal cancer[21].

Studies have shown that an abnormal expression of miR-142 is associated with the occurrence, progression, and metastasis of various tumors such as gastric cancer, prostate cancer and breast cancer, and it plays a role in tumor suppression in various tumors. This investigation found that the expression of miR-142 in lung cancer patients was significantly decreased, suggesting that miR-142 may play a role as a tumor suppressor gene in the pathogenesis of lung cancer[12-14]. This study analyzed and detected the expression of miR-142 and XIAP in tumor tissues of lung cancer patients and explored whether miR-142 plays a role in regulating XIAP expression and affecting proliferation and apoptosis of lung cancer cells.

In this investigation, the Dual-Luciferase Gene Reporter Assay showed that the transfection of miR-142 mimic significantly reduced the relative Luciferase activity of pMIR-XIAP-WT transfected HEK293T cells, without affecting the relative Luciferase activity in HEK293T cells transfected with pMIR-XIAP-MUT, indicating that there is a targeted regulation relationship between miR-142 and XIAP mRNA. The results of this work showed that the expression of miR-142 was significantly decreased in lung cancer tissues compared with adjacent tissues, and the expression of XIAP was significantly increased. In addition, the expression of miR-142 in lung cancer A549 and H1650 cells was significantly lower than that in normal lung epithelial BEAS-2B cells, and the expression of XIAP mRNA and protein was significantly higher than the latter, indicating that a decreased expression of miR-142 may play a role in increasing XIAP expression and promoting lung cancer. In the study of the relationship between miR-142 and lung cancer, Liu et al[22] showed that the expression of miR-142 in lung

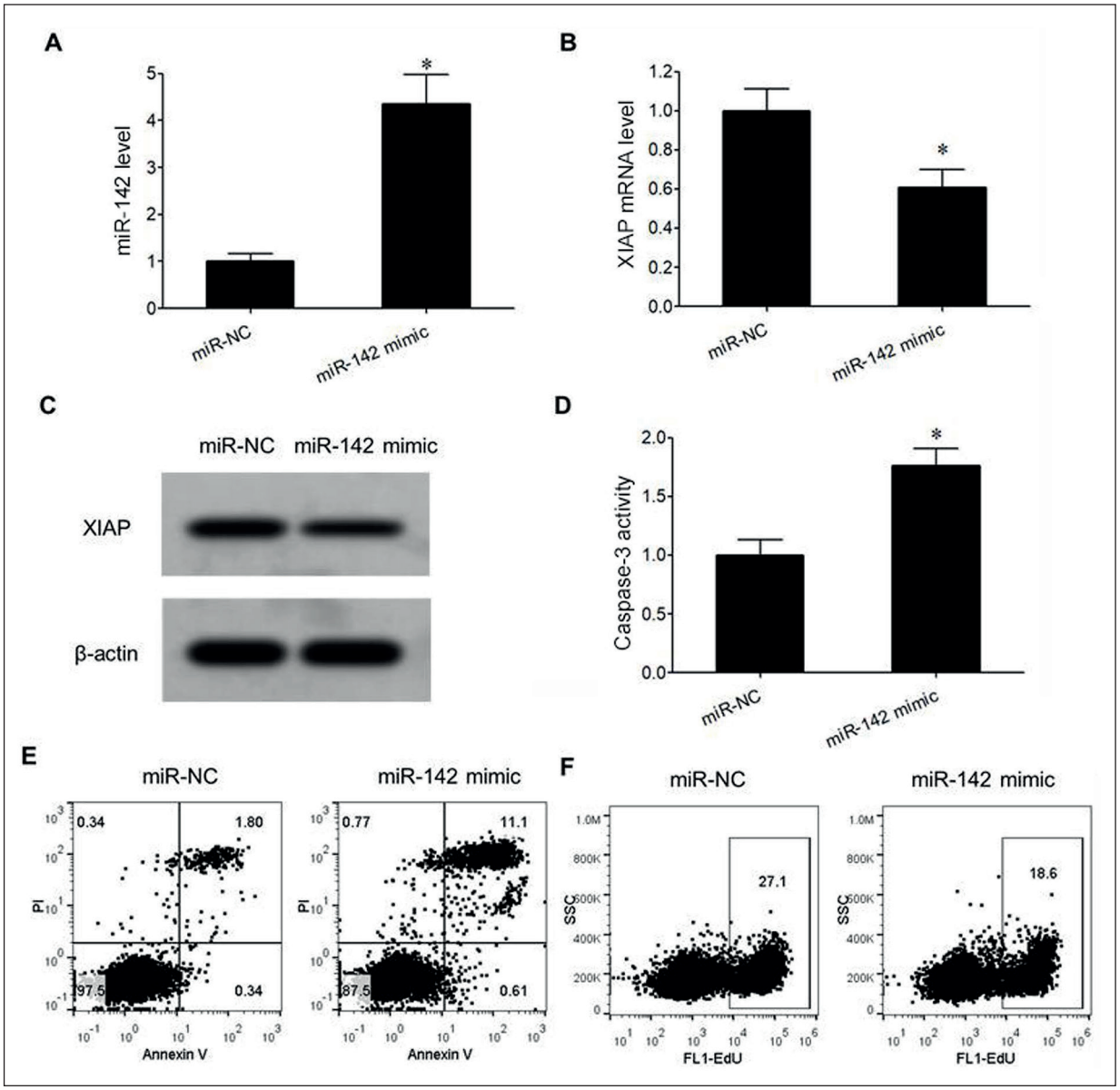

Figure 4. Increased miR-142 inhibits XIAP expression and proliferation of lung cancer cells and promotes apoptosis. ***A,*** qRT-PCR detects the expression of miR-203. ***B,*** qRT-PCR detection of XIAP mRNA expression. ***C,*** Western blot analysis of XIAP protein expression. ***D,*** Spectrophotometric detection of caspase-3 activity. ***E,*** Flow detection of apoptosis. ***F,*** Flow detection of cell proliferation. *Represents $p < 0.05$ compared to the miR-NC group.

cancer patients was significantly lower than that in normal lung tissues. Wang et al[13] showed that compared with normal lung tissue, the expression of miR-142 in lung cancer patients was significantly reduced, and the expression of miR-142 in lung cancer cells was also significantly reduced. In the current report, the expression of miR-142 in lung cancer tissues and cells was decreased, and the decrease in miR-142 was associated with lung cancer, which was consistent with the work by Liu et al[22] and Wang et al[13].

This investigation further explored the role of miR-142 and XIAP in the biological processes of lung cancer cell proliferation and apoptosis. The results showed that the overexpression of miR-142 inhibited cell proliferation and promoted apoptosis by significantly inhibiting the expression of XIAP in lung cancer cells, indicating that miR-142 exerts an inhibitory effect on lung cancer by targeting XIAP. In the study on the relationship between miR-142 and the biological effects of lung cancer cells, Chen et al[12] showed

that the overexpression of miR-142 in lung cancer cells can attenuate HMGB1-mediated expression by targeting the inhibition of HMGB1 expression. The cells are autophagy, thereby enhancing the sensitivity of lung cancer cells to chemotherapeutic drugs. Liu et al[22] showed that there is a targeted regulation between miR-142 and cyclin E in lung cancer ED-1 and ED-2 cells, and the overexpression of miR-142 in lung cancer cells inhibited the expression of cyclin E and attenuated the proliferation of ED-1 and ED-2 cells in lung cancer. Sempere et al[23] observed that miR-142 plays a role as a tumor suppressor gene in lung cancer cells and that the increasing the expression of miR-142 can significantly inhibit the proliferation of lung cancer cells. Wang et al[13] found that there is a targeted regulation between miR-142 and PIK3CA in lung cancer cells. The increase in the expression of miR-142 can inhibit the proliferation of lung cancer cells and the growth and tumorigenicity of lung cancer cells in animals by inhibiting the expression of PIK3CA. The downregulation of miR-142 expression can promote the proliferation of lung cancer cells. This study combines the targeted regulatory relationship between miR-142 and XIAP, revealing that miR-142 plays a role in targeting XIAP to regulate the biological effects of proliferation and apoptosis of lung cancer cells. No reports have been analyzed in the study. However, the presence of miR-142 targeting XIAP affecting the biological effects of lung cancer cells *in vivo* remains unclear and needs further studies to be confirmed.

Conclusions

The decreased expression of miR-142 plays a role in the upregulation of the expression of XIAP and the promotion of the pathogenesis of lung cancer. MiR-142 can inhibit the expression of XIAP and lung cancer cell proliferation and induce apoptosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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