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ORIGINAL ARTICLE

LncRNA-ATB promotes apoptosis of non-small cell lung cancer cells through MiR-200a/β-Catenin

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Summary

Purpose: *Long non-coding RNAs (lncRNAs) play important roles in cancer, but the effects of lncRNA-ATB on the proliferation and apoptosis of non-small cell lung cancer (NSCLC) cells remain unclear. Therefore, the present study was conducted to explore the role of this lnc-RNA in NSCLC.*

Methods: *The NSCLC NCI-H838 cell line cultured in vitro were used as the objects of study. First, the expressions of lncRNA-ATB, miR-200a and β-catenin in cells were detected. Then, the expression of lncRNA-ATB was knocked down using siRNA, and the effects of low expression of lncRNA-ATB on miR-200a/β-catenin pathway and apoptosis were studied.*

Results: *Compared with normal lung epithelial cells BEAS-2B, NCI-H838 cells had a significantly increased level of lncRNA-ATB ($p<0.01$), a significantly decreased level of miR-200a ($p<0.01$) and also a significantly increased level of β-catenin ($p<0.01$). After knockdown of lncRNA-ATB using si-ATB, the expression level of miR-200a was significantly increased, while that of β-catenin was significantly decreased. Besides, si-ATB remarkably increased the expression of Bcl-2 ($p<0.01$), and reduced the expressions of cleaved-caspase3 and Cytochrome C ($p<0.01$) and the apoptosis. In addition, the miR-200a mimic lowered obviously the expression of β-catenin ($p<0.05$) and reduced apoptosis.*

Conclusions: *This study suggests that lncRNA-ATB promotes the apoptosis of NSCLC cells through inhibiting the expression of miR-200a and reversely promoting the expression of β-catenin.*

Key words: *lncRNA-ATB, miR-200a, β-catenin, non-small cell lung cancer, proliferation and apoptosis*

Introduction

Lung cancer, one of the cancers with the highest mortality rate in the world, is divided into non-small cell lung cancer (NSCLC) and small cell lung cancer [1,2]. Although great progress has been made in the related research on lung cancer, its overall survival rate is still unsatisfactory [3]. The high mortality rate of lung cancer may be associated with early metastasis [4].

There are increasingly more studies showing that lncRNAs play crucial roles in extensive biological functions and a variety of diseases [5,6], and it is believed that lncRNAs are involved in the occurrence and development of human cancer [7,8]. Moreover, lncRNAs in the central nervous system participate widely in neuronal differentiation, and regulation of synaptic plasticity and behaviors [9,10]. Recently, changes in lncRNAs have been often reported in liver fibrosis [11,12]. LncRNA-ATB activated has been identified to be involved in many pathological conditions. For example, lncRNA-ATB

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promotes the progression of colon cancer, which is also associated with poor prognosis [13,14]. It has also been found that lncRNA-ATB acts as an oncogene in colorectal cancer and can be used as an index for poor prognosis [15]. Moreover, low expression of lncRNA-ATB is associated with clinical progression and unfavorable prognosis in pancreatic cancer [16]. Studies have demonstrated that lncRNA-ATB/miR-200s can promote the invasion of hepatocellular carcinoma (HCC) through regulating zinc finger E-box binding Homeobox 1 (ZEB1) and ZEB2, and facilitate the invasion-metastasis cascade of breast cancer [17].

According to studies, lncRNA-ATB and β-catenin share the common binding site of miR-200a, while miR-200a can directly regulate the protein expression of β-catenin [18,19]. β-catenin, an important regulator of cell proliferation and differentiation, is involved in the development of various cancers and fibrotic diseases [19]. However, there are no related studies on the effects of lncRNA-ATB/miR-200a/β-catenin on the proliferation and apoptosis of NSCLC cells.

In the present study, therefore, NSCLC cells were used as objects of study, and the expressions of lncRNA-ATB, miR-200a and β-catenin were detected first. Then the expression of lncRNA-ATB was down-regulated using siRNA, and the effects of low expression of lncRNA-ATB on the miR-200a/β-catenin pathway and apoptosis were explored.

Methods

Materials

Main laboratory reagents

M-MLV reverse transcriptase was purchased from TaKaRa (Tokyo, Japan), short-hairpin RNA (si-ATB) targeting lncRNA-ATB and its negative control (si-NC), miR-200 mimic and mimic control from GenePharma (Shanghai, China), lipofectamine 2000 reagent from Life Technologies (Gaithersburg, MD, USA), and Annexin V-fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) kit from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA).

Cell source and culture method

NCI-H838 and BEAS-2B cell lines were purchased from Shanghai E-Research Biotechnology Co., Ltd. (Shanghai, China), and they were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing double antibody + 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in an incubator with 5% CO_2 at 37°C.

RNA extraction and fluorescence quantitative polymerase chain reaction (PCR)

RNA was extracted from NCI-H838 or BEAS-2B cells using TRIzol (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using M-MLV reverse transcriptase. Then lncRNA-ATB was detected *via* fluorescence quantitative PCR, and miR-200a was detected *via* one-step real-

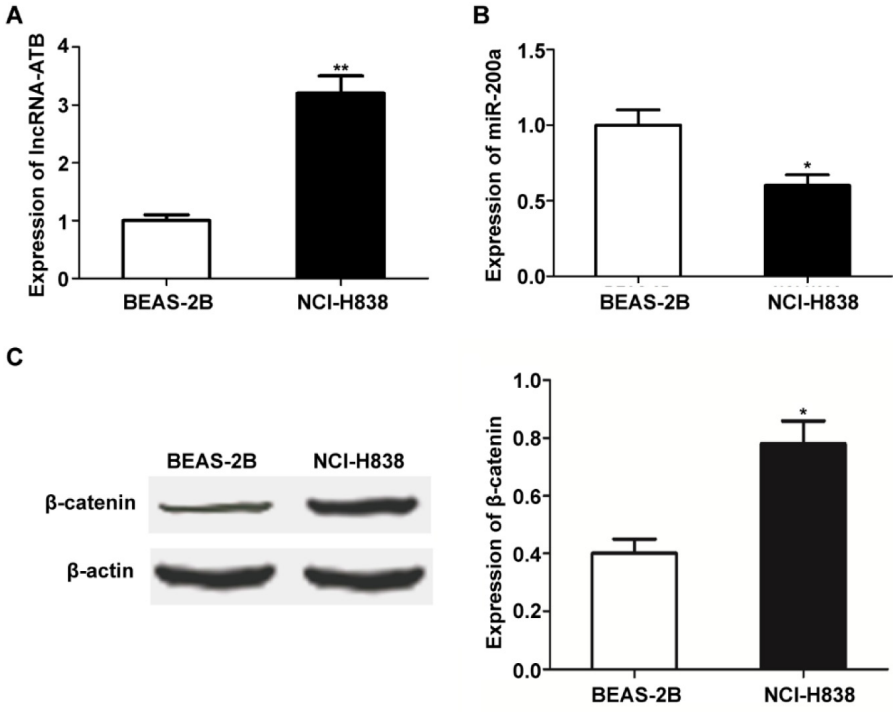

Figure 1. Expressions of lncRNA-ATB, miR-200a and β-catenin in NSCLC cells. **(A):** Expression of lncRNA-ATB in NCI-H838 cells detected *via* RT-PCR, **p<0.01. **(B):** Expression of miR-200a in NCI-H838 cells detected *via* RT-PCR, *p<0.05. **(C):** Expression of β-catenin in NCI-H838 cells detected *via* Western blotting, *p<0.05.

time PCR. The primer sequences are as follows: lncRNA-ATB (sense: 5'-TCTGGCTGAGGCTGGTTGAC-3' and antisense: 5'-ATCTCTGGGTGCTGGTGAAGG-3'), β-actin (sense: 5'-GGCATGGACTGTGGTCATGAG-3' and antisense: 5'-TGCACCACCAACTGCTTAGC-3'), and miR-200a (sense: 5'-ACGTACGCTAGCGAAGCAGATTGA-3' and antisense: 5'-ACGTACCTCGAGATGCGATGGGCAAA-3'). Each test was performed in triplicate and the relative expression of RNA was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

The total protein was extracted from NCI-H838 or BEAS-2B cells using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). After separation *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein was transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), incubated with the primary antibodies (rabbit anti-β-catenin, Bcl-2, cleaved-caspase3, CytC and β-actin) at 4°C overnight, and incubated again with the horse raddish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 2 h, followed by image development using electrochemiluminescence (ECL) solution (Thermo Fisher Scientific, Waltham, MA, USA).

Transient transfection

NCI-H838 cells (1×10^6) were inoculated into a 6-well plate, cultured in antibiotic-free DMEM for 24 h and transfected with 100 nM of siRNA-ATB, 100 nM of si-NC, 50 nM of miR-200a mimic and 100 nM of mimic control using Lipofectamine 2000 Reagent according to the manufacturer's instructions. The primer sequences of si-ATB are as follows: sense: 5'-GGUCUUUAUCUUGGAUGUUdTdT-3', antisense: 3'-dTdTCCAGAAAUAGAACCUACAA-5'.

Flow cytometry

The apoptosis was detected using the Annexin V-FITC/PI kit. NCI-H838 cells were inoculated into a 96-well plate. After treatment with the reagent, the cells were digested with trypsin and resuspended in 1 × binding buffer, followed by staining with Annexin V-FITC and PI at room temperature in the dark for 20 min. Then, apoptotic cells were identified using a FACScan flow cytometer. Finally, the data obtained were analyzed using CellQuest software.

Statistics

Three replicates were set in each test, and the data were expressed as mean ± standard deviation (SD) in three independent repeated experiments. The difference between two groups was analyzed using one-way analysis of variance (ANOVA) using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). $P<0.05$ suggested the difference was statistically significant.

Results

Expressions of lncRNA-ATB, miR-200a and β-catenin in NSCLC cells

Human NSCLC NCI-H838 cell line and normal lung epithelial cell BEAS-2B line were used as objects of study to examine the expressions of lncRNA-ATB, miR-200a and β-catenin. As shown in

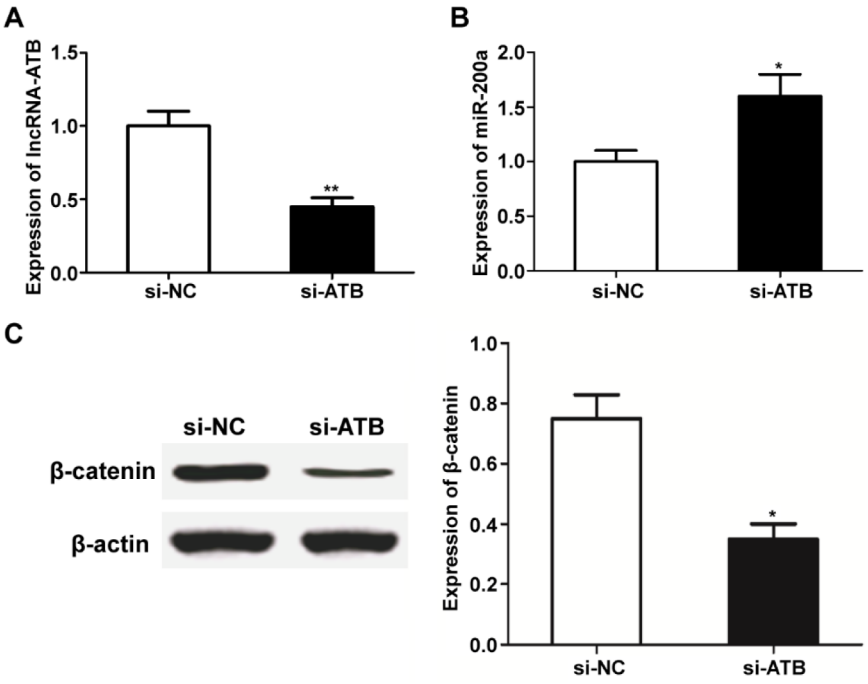

Figure 2. Effect of knockdown using si-ATB on miR-200a/β-catenin signaling pathway. **(A):** Knockdown of lncRNA-ATB using si-ATB detected *via* RT-PCR, **p<0.01. **(B)**: Expression of miR-200a in cells after knockdown of lncRNA-ATB using si-ATB detected *via* RT-PCR, *p<0.05. **(C):** Protein expression of β-catenin in cells after knockdown of lncRNA-ATB using si-ATB detected *via* Western blotting, *p<0.05.

Figure 1, compared with BEAS-2B cells, NCI-H838 cells had a significantly increased level of lncRNA-ATB ($p<0.01$; Figure 1A), a significantly decreased level of miR-200a ($p<0.01$; Figure 1B) and also a significantly increased level of β-catenin ($p<0.01$; Figure 1C).

Effect of knockdown using si-ATB on miR-200a/β-catenin signaling pathway

To study the relation among lncRNA-ATB, miR-200a and β-catenin in NSCLC, the expression of lncRNA-ATB was knocked down using si-ATB, and then the expressions of miR-200a and β-catenin were observed. The results revealed that si-ATB significantly inhibited the expression of lncRNA-ATB compared with si-NC ($p<0.01$; Figure 2A). At the same time, the expression of miR-200a was significantly increased ($p<0.05$; Figure 2B), while that of β-catenin was significantly decreased ($p<0.05$; Figure 2C). The above findings indicate that down-regulating the expression of lncRNA-ATB can promote the expression of miR-200a, while the increased expression of miR-200a reduces the expression of β-catenin.

Effect of knockdown using si-ATB on proliferation and apoptosis of NSCLC cells

The effects of lncRNA-ATB on proliferation and apoptosis of NSCLC cells were further explored. After the expression of lncRNA-ATB was knocked down using si-ATB, the expressions of Bcl-2, cleaved-caspase3 and CytC in NCI-H838 cells

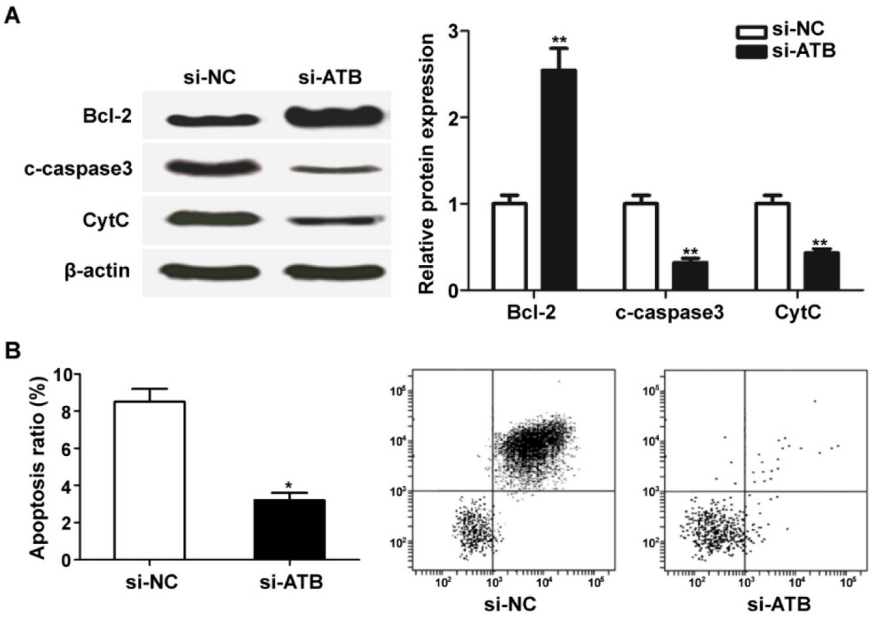

Figure 3. Effect of knockdown using si-ATB on proliferation and apoptosis of NSCLC cells. **(A):** Effects of knockdown of lncRNA-ATB using si-ATB on protein expressions of Bcl-2, cleaved-caspase3 and CytC detected *via* Western blotting, **p<0.01. **(B):** Effect of knockdown of lncRNA-ATB using si-ATB on apoptosis detected *via* flow cytometry, *p<0.05.

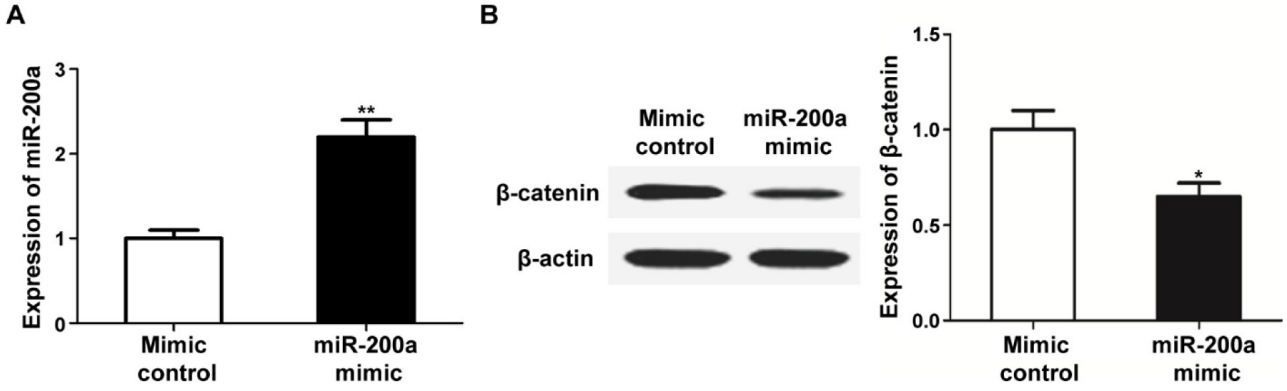

Figure 4. Effect of miR-200a overexpression on the expression of β-catenin. **(A):** Effect of miR-200a mimic on the expression of miR-200a detected *via* RT-PCR, **p<0.01. **(B):** Protein expression of β-catenin after overexpression of miR-200a using miR-200a mimic detected *via* Western blotting, *p<0.05.

were detected and the apoptosis was detected *via* flow cytometry. It was found that compared with si-NC, si-ATB remarkably increased the expression of Bcl-2 ($p<0.01$), and reduced the expressions of cleaved-caspase3 and CytC ($p<0.01$; Figure 3A) and the apoptosis (Figures 3B and 3C).

Effect of miR-200a overexpression on the expression of β-catenin

The cascade relation between miR-200a and β-catenin was further verified. After NSCLC cells were treated with miR-200a mimic, the expression of β-catenin in cells was detected. Compared with mimic control, the miR-200a mimic promoted obviously the expression of miR-200a in NCI-H838 cells ($p<0.01$; Figure 4A), and reduced the expression of β-catenin ($p<0.05$; Figure 4B). The above results confirm that miR-200a is able to negatively regulate the expression of β-catenin in NSCLC.

Effects of miR-200a overexpression on the proliferation and apoptosis of NSCLC cells

The effects of miR-200a overexpression on the proliferation and apoptosis of NSCLC cells were also studied. After overexpression of miR-200a, the expressions of Bcl-2, cleaved-caspase3 and CytC in NCI-H838 cells and the apoptosis were detected and the results showed that compared with mimic control, miR-200a mimic significantly promoted the expression of Bcl-2 ($p<0.01$) and decreased the expressions of cleaved-caspase3 and CytC ($p<0.05$; Figure 5A) and the apoptosis ratio ($p<0.05$; Figure 5B).

Discussion

In recent years, it has been proved that lncRNAs are closely related to tumor invasion and metastasis and play important roles in the diagnosis of cancer, so they have attracted increasingly more attention of researchers [5]. Chen et al [20] showed that the low expression of lncRNA TUBA4B in NSCLC is an important index for poor prognosis. Moreover, the research results of Wei et al [21] confirmed that lncRNA ANRIL can promote the proliferation and inhibit apoptosis of NSCLC cells. Therefore, the role and function of lncRNAs in tumors are constantly being revealed.

LncRNA-ATB can mediate the role of transforming growth factor-β (TGF-β) in epithelial-mesenchymal transition (EMT) and promote the metastasis of HCC, colorectal cancer and breast cancer. Moreover, lncRNA-ATB has also been proved to be a direct target of the TGF-β/Smad pathway [22]. It

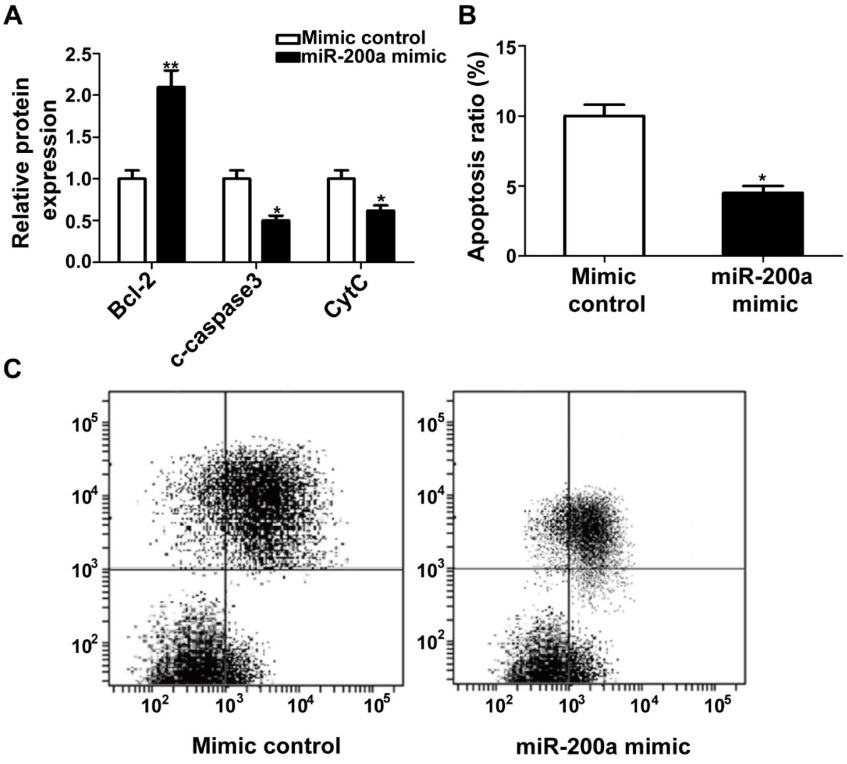

Figure 5. Effects of miR-200a overexpression on the proliferation and apoptosis of NSCLC cells. **(A):** Effects of miR-200a overexpression using miR-200a mimic on protein expressions of Bcl-2, cleaved-caspase3 and CytC, **p<0.01. **(B,C):** Effect of miR-200a overexpression using miR-200a mimic on apoptosis of NCI-H838 cells detected *via* flow cytometry, *p<0.05.

is reported that miR-200a synthesizes the extracellular matrix protein in fibrotic diseases, including lung fibrosis and liver fibrosis [18]. In the present study, it was found that the NSCLC NCI-H838 cells had a significantly increased level of lncRNA-ATB, a significantly decreased level of miR-200a and also a significantly increased level of β-catenin. After knockdown of lncRNA-ATB using si-ATB, the expression level of miR-200a was significantly increased, while that of β-catenin was significantly decreased. Besides, si-ATB remarkably increased the expression of Bcl-2, and reduced the expressions of cleaved-caspase3 and CytC and apoptosis. In addition, the miR-200a mimic obviously lowered the expression of β-catenin and obviously reduced apoptosis. The above findings demonstrate that lncRNA-ATB promotes the apoptosis of NSCLC cells through suppressing the expression of miR-200a and negatively increasing the expression of β-catenin, and it acts as a tumor suppressor in the progression of NSCLC. The results imply that lncRNA-ATB may be associated with inhibition in NSCLC. In addition, it is also reported that lncRNA-ATB/miR-200s can promote the invasion of HCC through regulating ZEB1 and ZEB2, and facilitate the invasion-metastasis cascade of breast cancer [17].

According to previous studies, miR-200a has low activity in activated hematopoietic stem cells (HSCs), and the overexpression of miR-200a can down-regulate the expression of Keap1, thereby reactivating the Nrf2-dependent antioxidant pathway [23]. Another mechanism of miR-200a in inhibiting activation of HSCs and improving liver fibrosis is provided in this study. However, there are some controversial reports. For example, Yang et al [11] argued that the miR-200 family may facilitate the progression of liver fibrosis. It is also observed that the expression of miR-200a is significantly increased in HCV patients with liver fibrosis [11]. β-catenin is the core element of the classical Wnt signaling pathway as well as an important regulator of cell proliferation and differentiation [24]. Previous studies showed that β-catenin is greatly involved in the pathogenesis of cancer and fibrotic diseases [25].

Based on this knowledge, it is believed that inhibiting β-catenin may exert an anti-apoptotic effect. In the present study it has been clearly proved that the knockdown of lncRNA-ATB can increase the expression of endogenous miR-200a and decrease the expression of β-catenin, thereby reducing the apoptosis of NSCLC cells.

Conclusions

In conclusion, the regulatory mechanism of lncRNA-ATB in the apoptosis of NSCLC cells was confirmed in this study. LncRNA-ATB can suppress the expression of miR-200a and up-regulate the expression of β-catenin, so that the expression of Bcl-2 declines and the expressions of cleaved-caspase3 and CytC are increased, thus promoting apoptosis.

Conflict of interests

The authors declare no conflict of interests.

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