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High expression of lncRNA MEG3 participates in non-small cell lung cancer by regulating microRNA-7-5p

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Abstract. – OBJECTIVE: This study was made to investigate whether long noncoding RNA (lncRNA) MEG3 could participate in the occurrence and development of non-small cell lung cancer (NSCLC) by regulating the expression of BRCA1 through competitive binding to microRNA-7-5p.

PATIENTS AND METHODS: We used quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) to explore the expression of lncRNA MEG3 and BRCA1 in NSCLC tissues and adjacent normal tissues, as well as NSCLC cell lines. The dual luciferase reporter gene assay was used to detect the binding of microRNA-7-5p to lncRNA MEG3 and BRCA1. Meanwhile, the expression of BRCA1, B-cell lymphoma-2 (Bcl-2) and BCL2-associated X (Bax) was detected by Western blot after the cells were overexpressed or knocked down of lncRNA MEG3. All these experiments were designed to investigate whether lncRNA MEG3 participated in the pathogenesis of NSCLC through inhibiting the expression of BRCA1 and Bcl-2 and promoting Bax expression.

RESULTS: The expressions of lncRNA MEG3 and BRCA1 in NSCLC tissues and A549 and HCC823 cell lines were significantly lower than those in the normal group. Overexpression of lncRNA MEG3 and BRCA1 in A549 and HCC823 cell lines resulted in increased apoptosis of lung cancer cells. Dual luciferase reporter assay demonstrated that lncRNA MEG3 can regulate the expression of BRCA1 through competitively binding to microRNA-7-5p to form the lncRNA MEG3/microRNA-7-5p/BRCA1 regulatory network. Besides, lncRNA MEG3 could inhibit the apoptosis inhibitory protein Bcl-2 and promote the expression of apoptosis-promoting factor Bax.

CONCLUSIONS: LncRNA MEG3 was significantly downregulated in NSCLC, and it could regulate the BRCA1 expression by competitive binding to microRNA-7-5p.

Key Words:
lncRNA MEG3, miRNA, NSCLC, ceRNA.

Introduction

Lung cancer is the most common pulmonary primary malignancy. Since most lung cancers originate in the bronchial epithelium, it is also called bronchial lung cancer. It can be divided into two main types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC)[1], of which about 80-85% of lung cancers are NSCLC. The most common NSCLCs are adenocarcinoma and squamous cell carcinoma[2]. High incidence, rapid growth, high mortality, and poor prognosis are prominent features of the epidemiology of NSCLC[3]. As NSCLC seriously affects the health of people, the diagnosis and treatment of NSCLC have become a crucial issue in the respiratory research. Recent studies have found that the reduction of tumor cell apoptosis is an important pathogenic factor in the development of NSCLC[4]. B-cell lymphoma-2 (Bcl-2) is the most important apoptosis-inhibitory gene, and BCL2-associated X (Bax) is the most important apoptosis-promoting gene. The ratio of Bcl-2/Bax affects the occurrence of apoptosis[5].

Non-coding RNAs (ncRNA) including microRNA (miRNA) and long noncoding RNA (lncRNA) and account for about 98% of gene transcription products. MicroRNAs have been shown to act as crucial roles in the pathogenesis of a variety of inflammatory diseases, neoplastic diseases, cardiovascular and cerebrovascular diseases[6,7]. Long-chain non-coding RNA is a type of non-coding RNA molecules and is widely present in the nucleus and cytoplasm. Due to the lack of the open reading frame, lncRNAs do not participate in protein-coding or are rarely involved in protein-coding[8]. However, lncRNAs can act as a crucial role in various biological

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processes such as cell proliferation, cell cycle, differentiation, and apoptosis through various molecular mechanisms[9]. For instance, MALAT1 inhibits apoptosis in tumors such as pancreatic and prostate cancers[10]. Decreased HOTAIR expression leads to cell cycle arrest in G0/G1 and *in situ* tumor growth suppression[11]. LncRNA-PVT1 oncogene can promote cell proliferation, cell cycle progression, and stem cell performance[12]. Long non-coding RNAs have been illustrated to participate in gene expression regulation at epigenetic, transcriptional, post-transcriptional and translational levels. In recent years, many studies have found that lncRNAs can exert its biological function as competing endogenous RNA (ceRNA). CeRNAs, also known as miRNA "molecular sponges," have revealed a novel regulatory mechanism for long non-coding RNAs which "absorb" miRNAs and affect miRNA downstream functions.

LncRNA MEG3 is a lncRNA with tumor suppressor function discovered in recent years. Studies[13-15] have shown that lncRNA MEG3 is lowly expressed in a variety of tumor tissues, suggesting its role of tumor suppressor gene. Overexpression of lncRNA MEG3 could inhibit tumor growth, invasion, and metastasis. Meanwhile, overexpression of lncRNA MEG3 could induce apoptosis, increase the sensitivity of cells to chemotherapy drugs. Xia et al[16] have confirmed that high expression of lncRNA MEG3 can lead to apoptosis of NSCLC cells; however, its specific regulatory role in NSCLC remains unknown.

Patients and Methods

Specimen Collection and Processing

72 cases of NSCLC tissues and adjacent tissues were obtained from the specimens after radical NSCLC. The specialized doctors used special clamps to take fresh tissue from the lesions, which were immediately rinsed with diethyl pyrocarbonate (DEPC) water and stored into liquid nitrogen tanks. Subsequently, patient-related clinical data were collected in the Department of Pathology and Medical Records. All of the above-mentioned tissue samples were solicited for patient consent and authorized by the Ethics Committee of Shan County Central Hospital.

Cell Culture and Transfection

BEAS-2B, A549, and HCC823 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) (HyClone, South Logan, UT, USA) containing 10% inactivated newborn calf serum (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were placed in a 37°C, 5% CO_2 incubator with a relative humidity of 90%.

Recombinant lentiviral vectors were constructed by Gene Pharma (Shanghai, China). A549 and HCC823 cells were digested into single cells with 1 mL of trypsin analog TrypLE Express. Cells were then cultured in a 24-well plate (5×10^5 cells per well) and added with a certain volume of virus fluid according to the multiplicity of infection (MOI) value of 40. Transfection enhancer Polybrene was added in a ratio of 1:200. After 24 hours, the fresh culture medium was added to replace the medium containing lentivirus. For cell transfection, cells were transfected with microRNA-7-5p mimics or inhibitors according to the Lipofectamine 2000 instructions (Invitrogen, Carlsbad, CA, USA).

Apoptosis Detection

Cells were digested, washed and adjusted to a cell density of 10^6/mL. Then, cell suspensions were stained with 10 μL of Annexin-V in the dark for 15 min. After adding 10 μL propidium iodide solution in each tube, cells apoptosis was immediately determined by a FACS flow cytometer (Arlesheim, Switzerland).

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

Total RNA of tissues or cells was extracted by the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary Deoxyribose Nucleic Acid (cDNA) was synthesized by reverse transcription according to TaKaRa PrimeScript RT Master Mix instructions (Otsu, Shiga, Japan). The microRNA quantitative PCR procedure was performed according to the miScript SYBR Green PCR Kit instructions (TaKaRa, Otsu, Shiga, Japan). PCR amplification conditions were: pre-denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and 30 s. The primer sequences were listed below: lncRNA MEG3 (F: 5'-GGCTGCAGACGTTAATGAGG-3', R: 5'-GAAATGTCGGCTCCATCACC-3'), GAPDH (F: 5'-AGCCACATCGCTCAGACAC-3', R: 5'-GCCCAATACGACCAAATCC-3'), BRCA1 (F: 5'-GAAACCGTGCCAAAAGACTTC-3', R: 5'-CCAAGGTTAGAGAGTTGGACAC-3').

Dual Luciferase Reporter Assay

The 3'UTR sequences of lncRNA MEG3 and BRCA1 were downloaded from the NCBI website to construct lncRNA MEG3 and BRCA1 wild-type sequences lncRNA MEG3 WT 3'UTR, BRCA1 WT 3'UTR and mutant sequences lncRNA MEG3 MUT 3'UTR, BRCA1 MUT 3'UTR. The cells were then cultured and 50 pmol/L microRNA-7-5p mimics or negative controls were co-transfected with the constructed 80 ng lncRNA MEG3 and BRCA1 wild-type or mutant plasmids. After 48 hours of transfection, the fluorescence intensity was detected by dual luciferase reporter gene assay.

Western Blot

Total proteins were extracted by radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China). Proteins were separated in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) while the immunoblots were incubated overnight at 4°C with the primary antibodies. The immunoblots were incubated with horseradish peroxidase (HRP)-labeled antibodies for 2 h at room temperature after washed 3 times in Tris-Buffered Saline-Tween (TBST). Protein bands were determined by imaging analysis system with enhanced chemiluminescence (ECL) imaging (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was used for statistical analysis. The chi-square test was used for classification data, and the *t*-test was used for measurement data. Data were expressed as mean ± standard deviation. The difference was statistically significant at $p < 0.05$.

Results

LncRNA MEG3 is Lowly Expressed in NSCLC Tissues and Cell Lines

We detected the expression of lncRNA MEG3 in NSCLC tissues and normal tissues by Real-Time quantitative PCR. Results demonstrated that lncRNA MEG3 was lowly expressed in NSCLC tissues (Figure 1A). Subsequently, we found that lncRNA MEG3 was also lowly expressed in A549 and HCC823 cells (Figure 1B). The prognosis of patients with high expression of lncRNA MEG3 was worse than that of patients with low expression of lncRNA MEG3 (Figure 1C, 1D). Next, we used LV-sh lncRNA MEG3 and LV-lncRNA MEG3 to achieve lncRNA MEG3 knockdown or overexpression in A549 cells (Figure 1E). Flow cytometry revealed that the number of apoptotic cells in the LV-sh lncRNA MEG3 group was decreased compared to the LV-Vector group, while the apoptotic cells in the LV-lncRNA MEG3 group were increased (Figure 1F). These findings demonstrated that lncRNA MEG3 was lowly expressed in NSCLC tissues and cells and may function through regulating cell apoptosis.

LncRNA MEG3 Regulates MicroRNA-7-5p Expression

By bioinformatics prediction of miRNAs that can bind to lncRNA MEG3, we found that microRNA-7-5p had the highest binding fraction. By luciferase reporter gene assay, we also found that after transfection of microRNA-7-5p, the fluorescence intensity of the lncRNA MEG3-WT 3'UTR group was decreased, while no difference was observed in that of the lncRNA MEG3-MUT 3'UTR (Figure 2A), suggesting that lncRNA MEG3 could bind to microRNA-7-5p. Then, we predicted the microRNA-7-5p target gene and performed a functional analysis. Reporter gene results demonstrated that after transfection of microRNA-7-5p cells, the fluorescence intensity in the BRCA1-WT 3'UTR group was decreased, while no significant difference was found in BRCA1-MUT 3'UTR luciferase (Figure 2B), indicating that BRCA1 could bind to microRNA-7-5p. Furthermore, we detected the expression of microRNA-7-5p after infection of LV-sh lncRNA MEG3 and LV-lncRNA MEG3 on A549 cells. We found that microRNA-7-5p expression was markedly increased in the LV-Vector group compared with LV-sh lncRNA MEG3 group while the expression of microRNA-7-5p in LV-lncRNA MEG3 group was decreased (Figure 2C), indicating that lncRNA MEG3 could negatively regulate the expression of microRNA-7-5p. Meanwhile, we found that BRCA1 expression was significantly decreased after transfection of microRNA-7-5p mimics and that microRNA-7-5p inhibitor increased the expression of BRAC1 in A549 cells (Figure 2D), suggesting that microRNA-7-5p could directly target BRCA1.

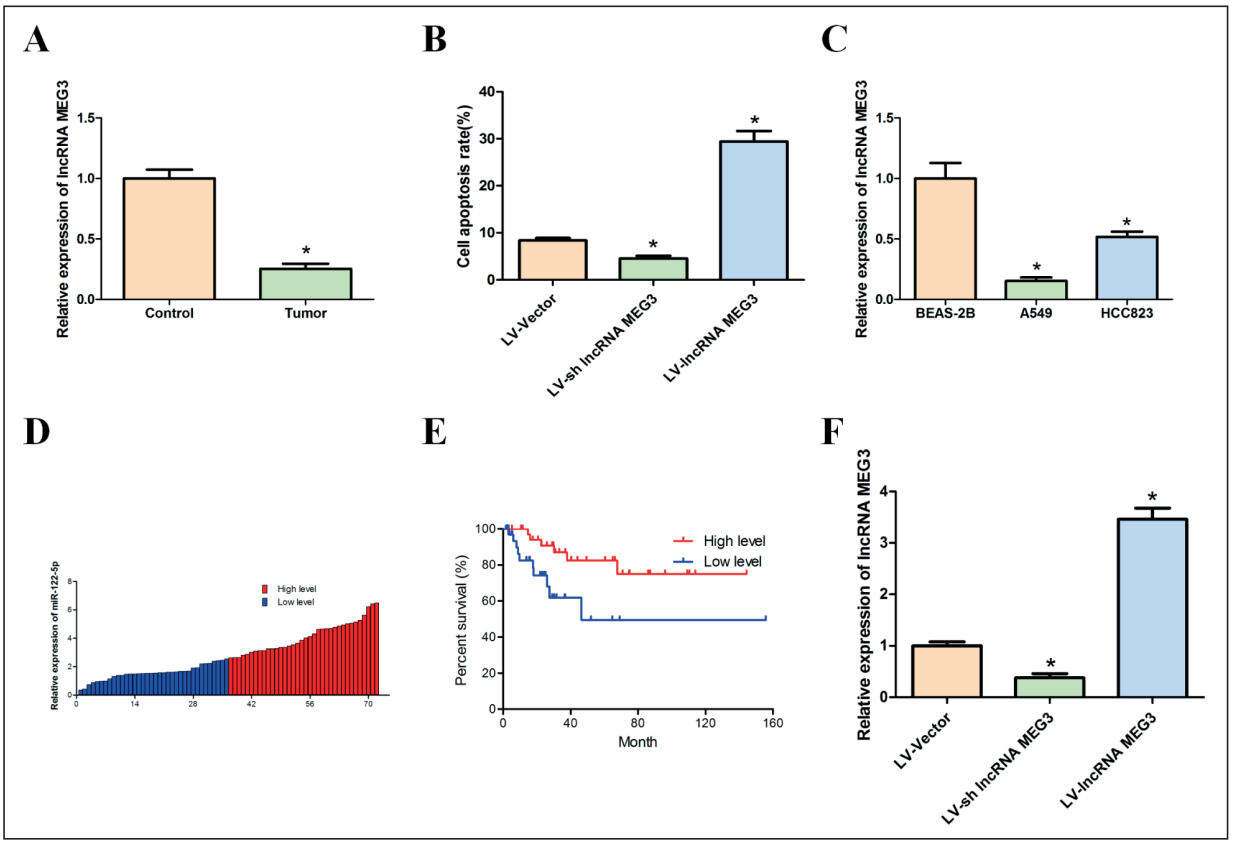

Figure 1. LncRNA MEG3 is lowly expressed in non-small cell lung cancer tissues and cell lines. ***A,*** The expression of lncRNA MEG3 in non-small cell lung cancer was detected by qRT-PCR. ***B,*** The expression of lncRNA MEG3 in A549 and HCC823 cells was detected by qRT-PCR. ***C,*** Prognosis of patients with non-small cell lung cancer with low or high expression of lncRNA MEG3. ***D,*** Prognosis of patients with non-small cell lung cancer with low or high expression of miR-122-5p. ***E,*** lncRNA MEG3 expression was detected after transfection of LV-shlncRNA MEG3 and LV-lncRNA MEG3 on A549 cells. ***F,*** cells apoptosis was detected by flow cytometry after LV-shlncRNA MEG3 and LV-lncRNA MEG3 transfection.

BRCA1 Promotes Apoptosis of Lung Cancer Cells

In A549 cells, the mRNA expression of BRCA1 was detected in LV-Vector, LV-sh lncRNA MEG3, and LV-lncRNA MEG3 group; we found that compared with the LV-Vector group, the expression of BRCA1 was decreased in LV-sh lncRNA MEG3 group while increased in LV-lncRNA MEG3 group (Figure 3A). The same result was verified at the protein level (Figure 3B). These results indicated that lncRNA MEG3 can positively regulate the expression of BRCA1. Next, we detected the expression of BRCA1 in NSCLC tissues and adjacent normal tissues by Real-Time quantitative PCR. Results illustrated that BRCA1 was lowly expressed in NSCLC tissues (Figure 3C). We also found that BRCA1 was lowly expressed in A549 and HCC823 cells as well (Figure 3D). In addition, we used LV-sh BRCA1 and LV-BRCA1 to achieve BRAC1 knockdown and overexpression (Figure 3E). Then, we examined the effect of BRCA1 on apoptosis of lung cancer cells by flow cytometry. Results demonstrated that compared with the LV-shBRCA1 group, the number of apoptotic cells in the LV-Vector group decreased, while the apoptotic cells in the LV-BRCA1 group increased (Figure 3F). These results suggested that BRCA1 promoted apoptosis of lung cancer cells.

LncRNA MEG3 Regulated Bcl-2 and Bax Expression

To investigate the effect of lncRNA MEG3 on apoptosis, we detected the expression of the pro-apoptotic protein Bax and apoptosis-inhibiting protein Bcl-2 in A549 cells. Results showed that LV-shlncRNA MEG3 significantly inhibited Bax but promoted Bcl-2 expression, whereas LV-lncRNA MEG3 promoted the Bax but inhibit-

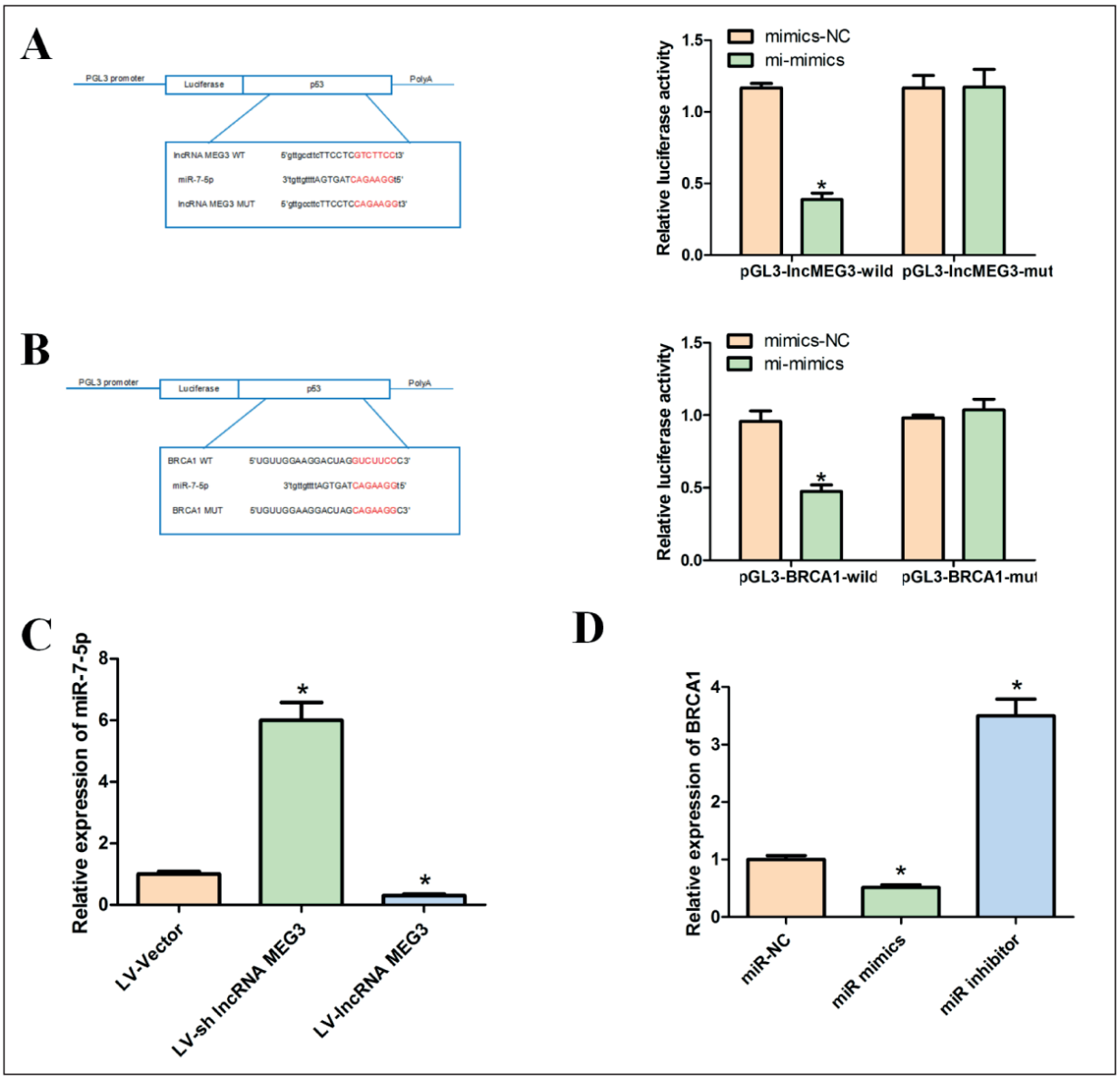

Figure 2. LncRNA MEG3 regulates microRNA-7-5p expression. ***A,*** Dual luciferase assay was performed to detect the binding of lncRNA MEG3-WT and lncRNA MEG3-MUT reporter plasmids microRNA-7-5p. ***B,*** Dual luciferase assay was performed to detect the binding of BRCA1-WT and BRCA1-MUT reporter plasmids with microRNA-7-5p. ***C,*** Detection of microRNA-7-5p expression in A549 cells transfected with LV-shlncRNA MEG3 and LV-lncRNA MEG3. ***D,*** Detection of BRCA1 expression after transfection of microRNA-7-5p mimics and microRNA-7-5p inhibitor in A549 cells.

ed Bcl-2 expression (Figure 4A, 4B). These above results suggested that lncRNA MEG3 regulates the expression of Bcl-2 and Bax.

Discussion

Several reports show that lncRNA acts as a crucial role in maintaining normal cell growth and function, and changes in lncRNA expression are also closely related to cancer. It has been confirmed that lncRNA inhibits oncogene function[17]. Qin et al[18] found that lncRNA TSLC1-AS1 is a novel tumor suppressor in gliomas. Besides, MT1DP inhibits tumors by regulating cell proliferation and migration in liver cancer[19]. Since lncRNA is involved in a variety of important physiological processes, their functional disorders may have important effects on the homeostasis of cells.

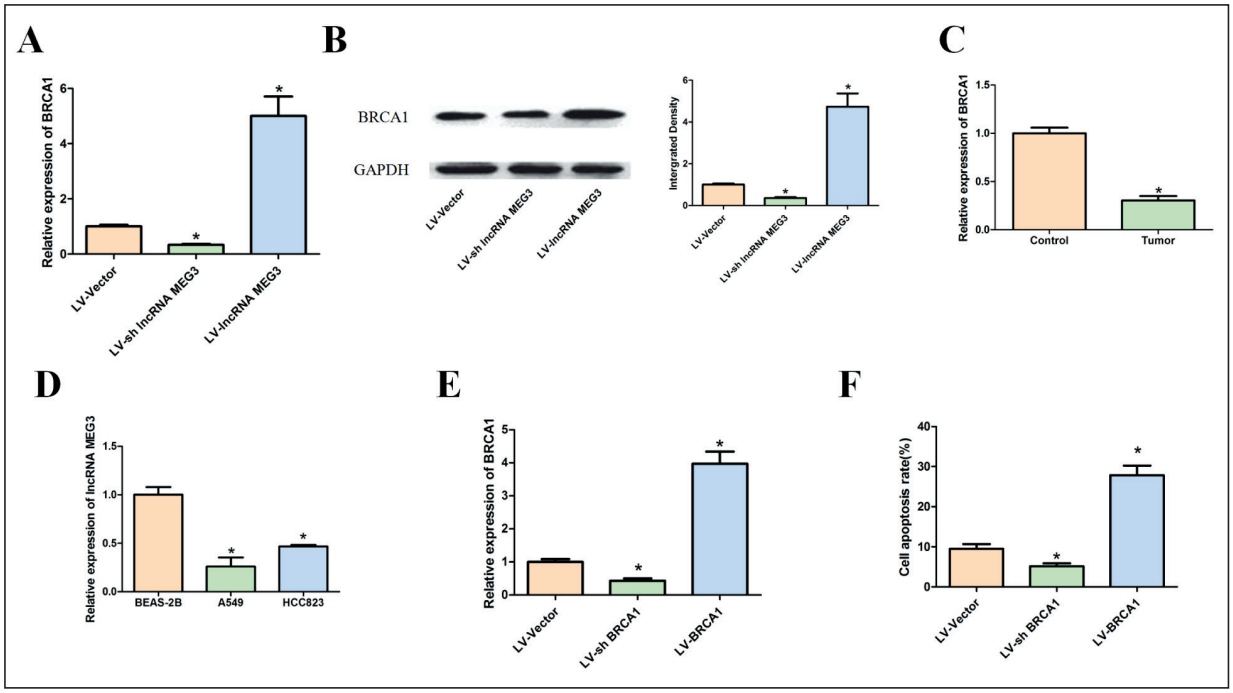

Figure 3. BRCA1 promotes apoptosis of lung cancer cells. ***A,*** RNA expression of BRCA1 was detected in A549 cells transfected with LV-shlncRNA MEG3 and LV-lncRNA MEG3. ***B,*** Protein expression of BRCA1 was detected in A549 cells transfected with LV-shlncRNA MEG3 and LV-lncRNA MEG3. *C,* BRCA1 was lowly expressed in non-small cell lung cancer. ***D,*** BRCA1 was lowly expressed in A549 and HCC823 cells. ***E,*** LV-shBRCA1 and LV-BRCA1 were transfected into A549 cells to detect the expression of lncRNA MEG3.

LncRNA MEG3 is located on human chromosome 14q32.2. A study found that lncRNA MEG3 expression was reduced in a variety of malignant tumors and that it functioned as a tumor suppressor gene. A large number of studies have illustrated that overexpressing lncRNA MEG3 can lead to cell growth arrest of tumor cells by inducing apoptosis and regulating cell cycle[20]. Overexpression of lncRNA MEG3 can also increase the sensitivity of tumor cells to chemotherapy drugs[21].

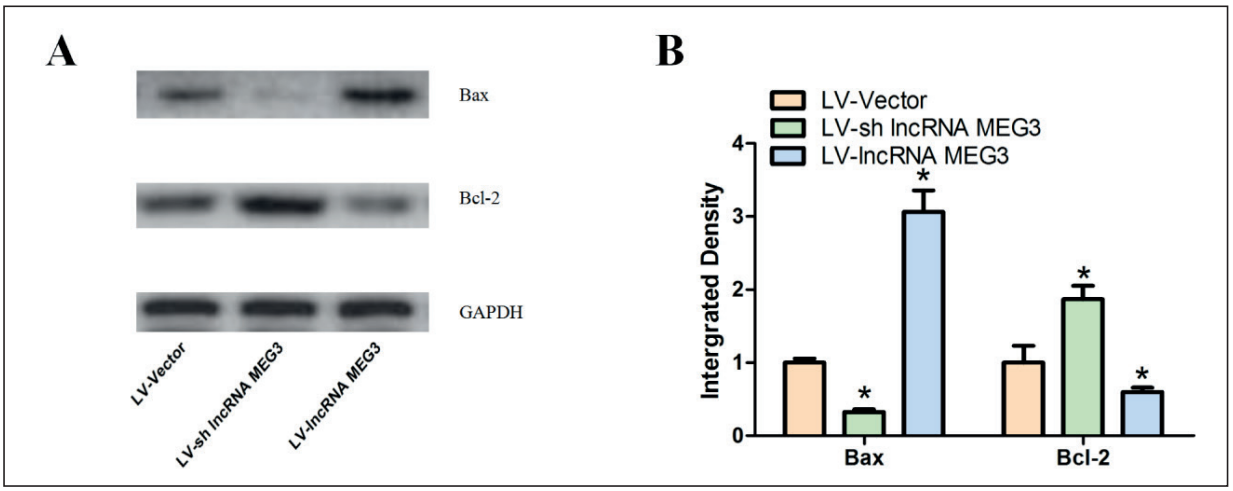

Figure 4. LncRNA MEG3 regulates the expression of Bcl-2 and Bax. The expression of Bax and Bcl-2 were detected by Western blot after transfection of LV-shlncRNA MEG3 and LV-lncRNA MEG3 on A549 cells.

MicroRNAs have been regarded as a new class of molecules that regulate the expression of genes in stem cells, and many miRNAs have been shown to be important intermediates in the regulation of related signaling pathways. Many studies have found that in addition to being directly involved in regulating target gene expression, lncRNAs can also interact with microRNAs as a competing endogenous RNA (ceRNA) and participate in the development of malignancies, resulting in cell proliferation, apoptosis, angiogenesis, invasion, and metastasis. For example, in prostate cancer, lncRNA PTENP1 has been shown to absorb miR-19 and miR-20a so as to release inhibition of the well-known tumor suppressor gene PTEN, thereby inhibiting the PI3K signaling pathway and inhibiting cell growth[22]. Besides, lncRNA HOTAIR targets miR-331 in the form of ceRNAs in gastric cancer, thereby regulating the expression of the well-known oncogene HER2[23]. In this work, we demonstrated that the expression of lncRNA MEG3 was strikingly decreased in NSCLC tissues and lung cancer cell lines and that highly expressed lncRNA MEG3 can promote apoptosis. Dual luciferase reporter assay demonstrated that lncRNA MEG3 can target microRNA-7-5p and regulate its expression, thereby mitigating the degradation of BRCA1 by microRNA-7-5p.

Apoptosis inhibitory protein Bcl-2 and apoptosis-promoting factor Bax are important members of the apoptotic molecules family, which have been widely confirmed in regulating apoptosis of brain cells. Of the many apoptosis-regulating genes, the roles of Bcl-2 and Bax have been widely confirmed and are considered to be one of the last common pathways of apoptosis[24,25]. In 1990, researchers discovered a gene directly associated with hereditary breast cancer. It was named Breast Cancer No. 1 gene, which has been referred to as BRCA1. BRCA1 is an excellent gene that inhibits the development of malignant tumors (called "suppressor gene") and acts as a significant role in the repair of damage and normal cell growth[26]. In this study, we found that the expression of BRCA1 was strikingly downregulated in NSCLC tissues and lung cancer cell lines. Overexpression of BRCA1 in A549 cells can promote cell apoptosis. Through overexpressing and knocking down of lncRNA MEG3, we found that lncRNA MEG3 promoted apoptosis of A549 cells by inhibiting Bcl-2 and promoting Bax, thereby participating in the occurrence and development NSCLC.

Conclusions

We showed that lncRNA MEG3 expression was significantly decreased in NSCLC, and it was involved in the occurrence of NSCLC through lncRNA MEG3/microRNA-7-5p/BRCA1 pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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