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LncRNA NEAT1/miR-1224/KLF3 contributes to cell proliferation, apoptosis and invasion in lung cancer

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Abstract. – OBJECTIVE: The aim of this study was to detect the relationship between long-chain non-coding RNA (lncRNA) NEAT1 and microRNA-1224 (miR-1224) in lung cancer and to explore its underlying mechanism.

MATERIALS AND METHODS: The expression levels of lncRNA NEAT1 and miR-1224 in lung cancer tissues and cells were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The interaction between lncRNA NEAT1 with miR-1224, miR-1224, and KLF3 was detected by Dual-Luciferase Reporter Gene Assay. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and flow cytometry were used to detect the changes in the proliferative and apoptosis abilities of lung cancer cells after silencing lncRNA NEAT1 or up-regulating miR-1224, respectively.

RESULTS: Compared with adjacent normal tissues, lncRNA NEAT1 was significantly up-regulated, while miR-1224 was significantly down-regulated in lung cancer tissues. LncRNA NEAT1 could specifically bind to the 3'UTR of miR-1224 and regulate its expression. The inhibition of lncRNA NEAT1 remarkably reduced the proliferation and enhanced the apoptosis of lung cancer cells. However, the upregulation of the expression of miR-1224 level could significantly inhibit proliferation and promote the apoptosis rate of lung cancer cells. Furthermore, miR-1224 could downregulate KLF3 expression by directly binding to its 3'UTR.

CONCLUSIONS: LncRNA NEAT1 can sponge the expression of miR-1224, thereby affecting the proliferation and apoptosis of lung cancer.

Key Words:
Lung cancer, LncRNA NEAT1, MiR-1224, KLF3, Proliferation.

Introduction

Worldwide, lung cancer is the most common malignancy with a mortality rate of 92%. Meanwhile, its morbidity and mortality remain the highest among males[1]. In recent years, the research on targeted therapy of lung cancer has made great progress. However, its 5-year survival rate is still lower than 16%[2]. This is mainly due to the reason that most patients have already been in advanced stage when diagnosed. Moreover, the prognosis of patients with lymph node metastasis is extremely poor[3]. Therefore, a search for suitable early diagnosis targets and prognostic markers is of great significance to improve the survival rate of patients with lung cancer.

Long-chain non-coding RNA (lncRNA) is a type of non-coding RNA with over than 200 nt in length. Current studies have found that lncRNA plays an important role in transcriptional silencing, transcriptional activation, intranuclear transport, and chromosomal modification[4]. In recent years, research on the roles of lncRNAs in tumors has attracted extensive attention. For example, long noncoding RNA LINC00675 enhances the phosphorylation of vimentin on Ser83 and suppresses

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gastric cancer progression[5]. The overexpression of lncRNA AFAP1-AS1 predicts poor prognosis and promotes the proliferation and invasion of gallbladder cancer cells[6]. As a novel lncRNA, LL22NC03-N64E9.1 promotes the proliferation of lung cancer cells. Meanwhile, it is also a potential prognostic molecular biomarker for lung cancer[7]. The main function of lncRNA is not to encode proteins. However, they can regulate the biological processes of tumors by regulating the expression of the target genes or competitively targeting miRNAs[8,9]. Ha and Kim[10] have demonstrated that non-coding regulatory RNAs play important roles in the development and progression of tumors. MicroRNAs have also been confirmed as a promising biomarker for lung cancer[11]. For instance, microRNA-661 promotes non-small cell lung cancer progression by directly targeting RUNX3[12]. MicroRNA-376c suppresses the growth and invasion of non-small-cell lung cancer cells by targeting LRH-1-mediated Wnt signaling pathway[13].

In this study, we first detected the expressions of lncRNA NEAT1 and miR-1224 in lung cancer cells. Furthermore, the interaction between lncRNA NEAT1 and miR-1224 in lung cancer was explored.

Materials and Methods

Cell Culture and Passage

Lung cancer cell line (A549) was used for *in vitro* experiments. The cells in the logarithmic phase were first inoculated into 6-well plates. When the degree of cell fusion reached 80% to 90%, the corresponding siRNA was transfected into the cells according to the instructions of Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA). Briefly, lncRNA NEAT1 siRNA and Lipofectamine™ 2000 were diluted with serum-free medium, mixed, and incubated at room temperature for 5 min. Subsequently, the transfection reagent was added to the culture plate. After 6-8 h of culture, the transfected culture medium was discarded. Fresh serum-containing medium without anti-antibiotics was then added. Oligo miR-1224 was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China) and transfected into the cells by Lipofectamine™ 2000.

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

Total RNA was extracted from the transfected cells according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA samples were determined by spectroscopy. Subsequently, the extracted RNA was reverse transcribed using the TaKaRa (Otsu, Shiga, Japan) reverse transcription kit. PCR detection was performed in accordance with the real time PCR kit. U6 was used as an internal control. The relative expressions of lncRNA NEAT1 and miR-1224 in lung cancer cells were calculated. The experiment was repeated three times. The primer sequences used in this study were as follows: lncRNA NEAT1, F: 5'-CAGGAGCAGGCCGCTGCGTAATC-3', R: 5'-CCGTAGGCATCTGTGCCACGCA-3'; microRNA-1224, F: 5'-GCCTGTGACCATCATCCACTG-3', R: 5'-GGATTAAGTGACGAGGCTCAG-3'; MKRN3, F: 5'-AAGCAGCGGCATTTGGACAA-3', R: 5'-CCGTGCGAATAGCGACAGTTCT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Dual-Luciferase Reporter Gene Assay

To verify the correlation between lncRNA NEAT1 and miR-1224, the Dual-Luciferase Reporter Gene Assay was performed. Firstly, we constructed a luciferase reporter with the miR-1224 3′-untranslated region (3′-UTR), in which the binding site of corresponded to lncRNA NEAT1 binding site (lncRNA NEAT1 wild). Meanwhile, the control group was lncRNA NEAT1-mutant. After adding specific luciferase substrate, luciferase reacted with the substrate to generate fluorescence. The activity of luciferase was determined by detecting the intensity of fluorescence.

Using Lipofectamine™ 2000, the cells were transfected with miRNA-1224 mimics and plasmids containing pMIR-KLF3-3′UTR wild or pMIR-KLF3-3′UTR mutant, respectively. The activities were detected by a Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA).

MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide Assay

The proliferative ability of the cells was detected by MTT assay (Sigma-Aldrich, St. Louis, MO, USA). The transfected cells were seeded into 96-well plates at a density of 1×10^3 cells/well. Subsequently, MTT reagent (20 μL) was added in each well with dimethyl sulfoxide (DMSO,

150 μL; Sigma-Aldrich, St. Louis, MO, USA). The absorbance at the wavelength of 490 nm was detected. The experiment was repeated for three times.

Cell Apoptosis Detection

The transfected cells in each group were first collected. Single cell suspension (100 μL) was prepared by binding buffer containing fluorescein isothiocyanate (FITC) Annexin V (5 μL) and propidium iodide (PI). Flow Cytometry (BD Biosciences, Franklin Lakes, NJ, USA) was used to measure the apoptosis rate. The experiment was repeated three times.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Prism software was applied for image plotting. The *t*-test was used to compare the difference between the two groups. $p<0.05$ was considered statistically significant.

Results

Expression and Influence of LncRNA NEAT1 in Lung Cancer

StarBase showed that lncRNA NEAT1 significantly increased in lung cancer tissues (Figure 1A). In this study, we first detected the expression of lncRNA NEAT1 in lung cancer tissues and adjacent normal tissues by qRT-PCR. The results showed that the expression level of lncRNA NEAT1 in lung cancer tissues was significantly higher than that of adjacent tissues (Figure 1B), suggesting the abnormal expression of lncRNA NEAT1 in lung cancer. Fur-

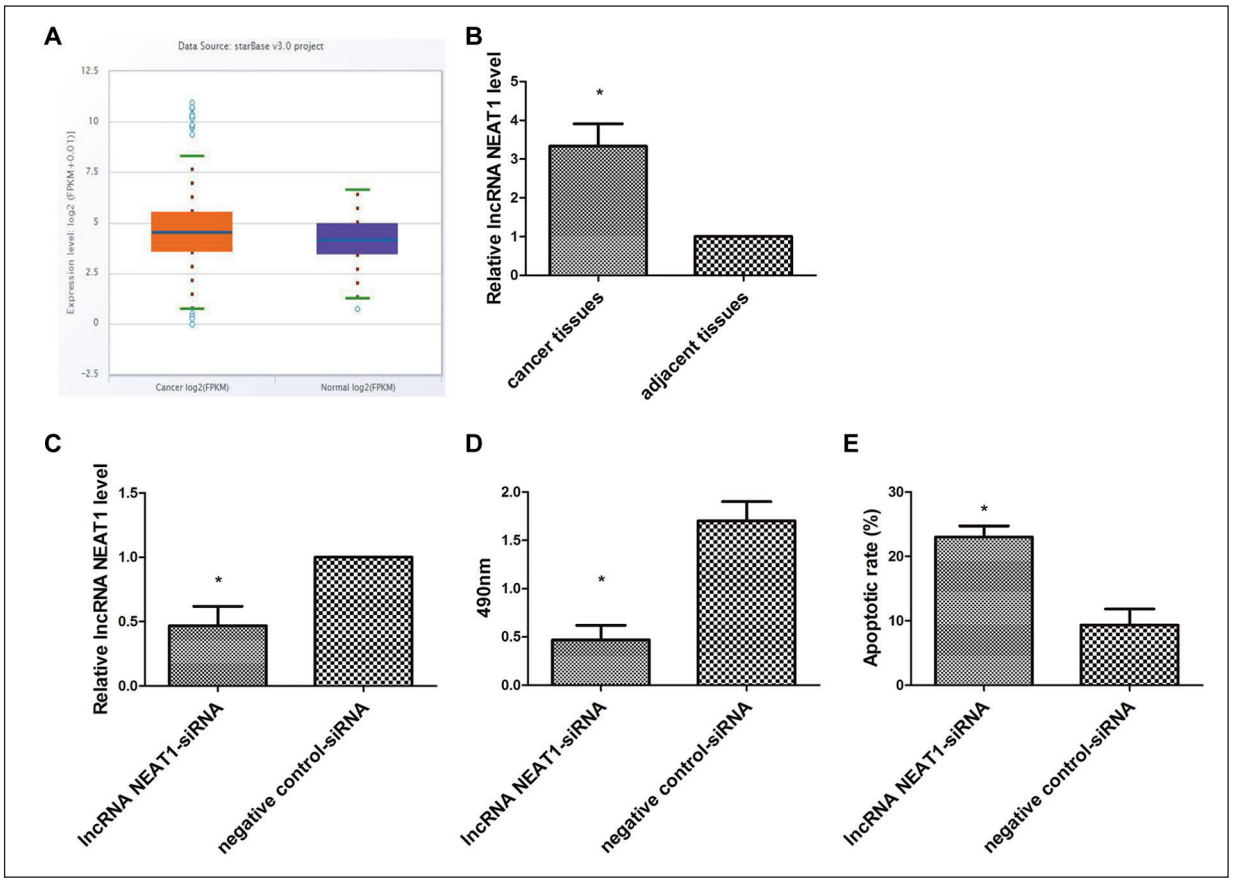

Figure 1. Expression and influence of lncRNA NEAT1 in lung cancer. **A,** StarBase showed that lncRNA NEAT1 significantly increased in lung cancer tissues. **B,** The expression of lncRNA NEAT1 in lung cancer tissues and adjacent tissues was detected by qRT-PCR. **C,** LncRNA NEAT1 siRNA was effectively transfected into cells. **D,** MTT assay was used to detect cell proliferation. **E,** Flow cytometry method was applied to detect cell apoptosis.

thermore, to detect the effect of lncRNA NEAT1 knockdown on the proliferation and apoptosis of lung cancer cells, lncRNA NEAT1 siRNA was transfected into cells. MTT and flow cytometry assays were then performed. The results showed that the expression level of lncRNA NEAT1 siRNA in lncRNA NEAT1 siRNA group was significantly lower than that of the control group (Figure 1C). MTT assay demonstrated that the silence of the expression of lncRNA NEAT1 significantly inhibited the proliferation of lung cancer cells (Figure 1D). Flow cytometry assay also indicated that the knockdown of lncRNA NEAT1 remarkably promoted cell apoptosis (Figure 1E). All the results showed that lncRNA NEAT1 was highly expressed in lung cancer and could influence cell proliferation and apoptosis.

Relationship Between LncRNA NEAT1 and MiR-1224

To clarify the expression of miRNAs associated with lncRNA NEAT1 in lung cancer cells, the bioinformatics prediction tools were first used to search for miRNAs. The results indicated that lncRNA NEAT1 might have a direct effect on miR-1224 due to a similar binding sequence (Figure A). To verify whether lncRNA NEAT1 could bind to miR-1224 3'UTR, the Dual-Luciferase Reporter Gene Assay showed that the luciferase activity of lncRNA NEAT1-wild cells with miR-1224 mimics was significantly reduced. However, no evident changes were observed in the luciferase activity of lncRNA NEAT1-mutant cells with miR-1224 mimics (Figure 2B). Furthermore, the qRT-PCR assay showed that miR-1224 was highly expressed in the cells transfected with lncRNA NEAT1-siRNA (Figure 2C). The results showed that lncRNA NEAT1-siRNA could specifically bind to the 3'UTR of miR-1224 and downregulate its expression level.

Expression and Effect of MiR-1224 in Lung Cancer

StarBase showed that miR-1224 remarkably decreased in lung cancer tissues (Figure 3A). In this study, we detected the expression of miR-1224 in lung cancer tissues and adjacent tissues by qRT-PCR. As shown in Figure 3B, the ex-

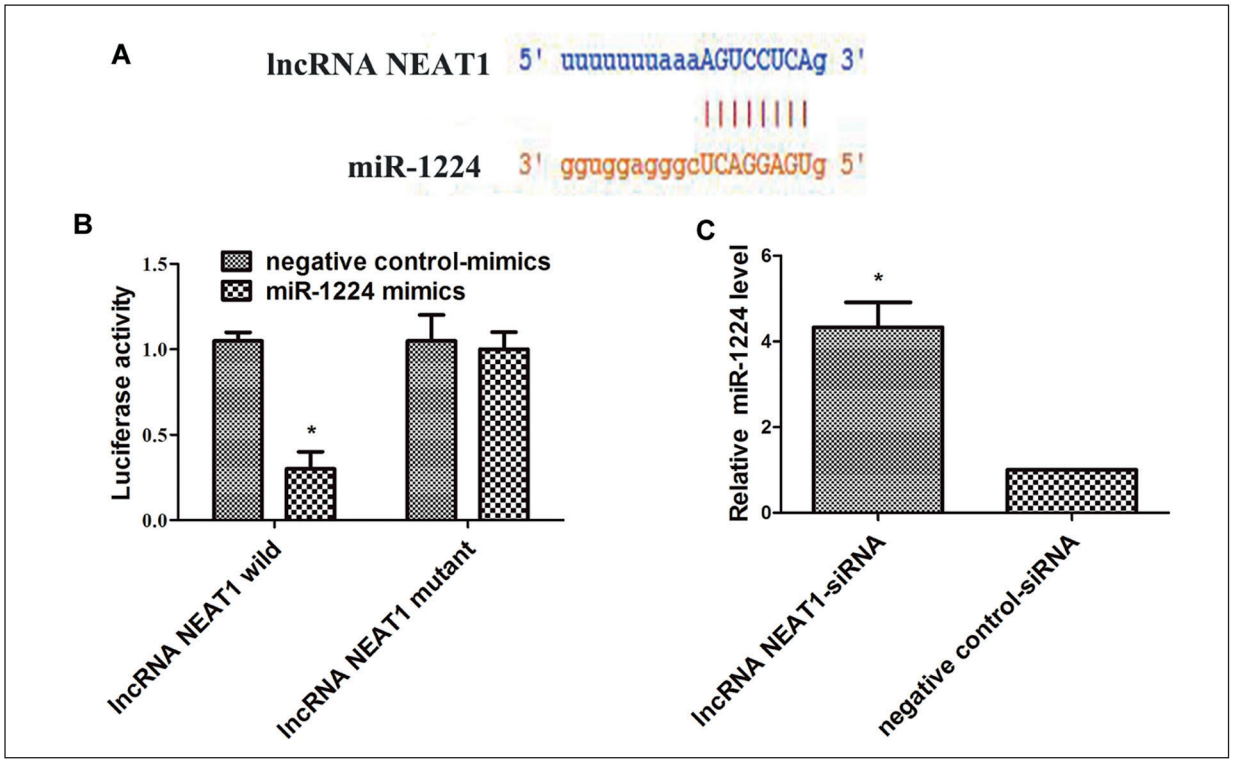

Figure 2. Relationship between lncRNA NEAT1 and miR-1224. **A,** Bioinformatics prediction tools showed that lncRNA NEAT1 might have a direct effect on miR-1224 due to a similar binding sequence. **B,** Dual-luciferase reporter gene assay was used to verify whether lncRNA NEAT1 could bind to miR-1224 3'UTR. **C,** QRT-PCR assay was applied to detect the expression of miR-1224.

pression of miR-1224 in lung cancer tissues was significantly lower than that of adjacent normal tissues ($p<0.05$). This suggested that miR-1224 might play a role in lung cancer. Subsequently, miR-1224 mimics were transfected into the cells to upregulate miR-1224 expression *in vitro* (Figure 3C). The effect of miR-1224 on cell proliferation and apoptosis was then evaluated. MTT assay showed that miR-1224 significantly up-regulated and suppressed the proliferation of lung cancer cells (Figure 3D). As shown in Figure 3E, the flow cytometry assay demonstrated that the number of apoptotic cells in miR-1224 mimics group was significantly higher than that of the control group. In conclusion, these experiments indicated that the proliferative ability of A549 cells was inhibited, and the apoptosis rate of the cells was enhanced after miR-1224 up-regulation.

Regulation of MiR-1224 on KLF3

The online software predicted that miR-1224 might be bound to the 3'UTR of KLF3 (Figure 4A). The Luciferase reporter gene assay was then used to verify this prediction. The result showed that the luciferase activity of the cells transfected with miR-1224 mimics and pMIR-KLF3-3′UTR wild was significantly inhibited. However, no evident changes were observed in the luciferase activity in the cells transfected with miR-1224 mimics and pMIR-KLF3-3′UTR mutant (Figure 4B). To detect the regulation of abnormal miR-1224 expression on KLF3, we performed the qRT-PCR assay. The results pointed out that the mRNA expression level of KLF3 was remarkably down-regulated in miR-1224 over-expression group when compared with the control group (Figure 4C). These data demonstrated that miR-1224 could down-regulate KLF3 by directly binding to its 3'UTR.

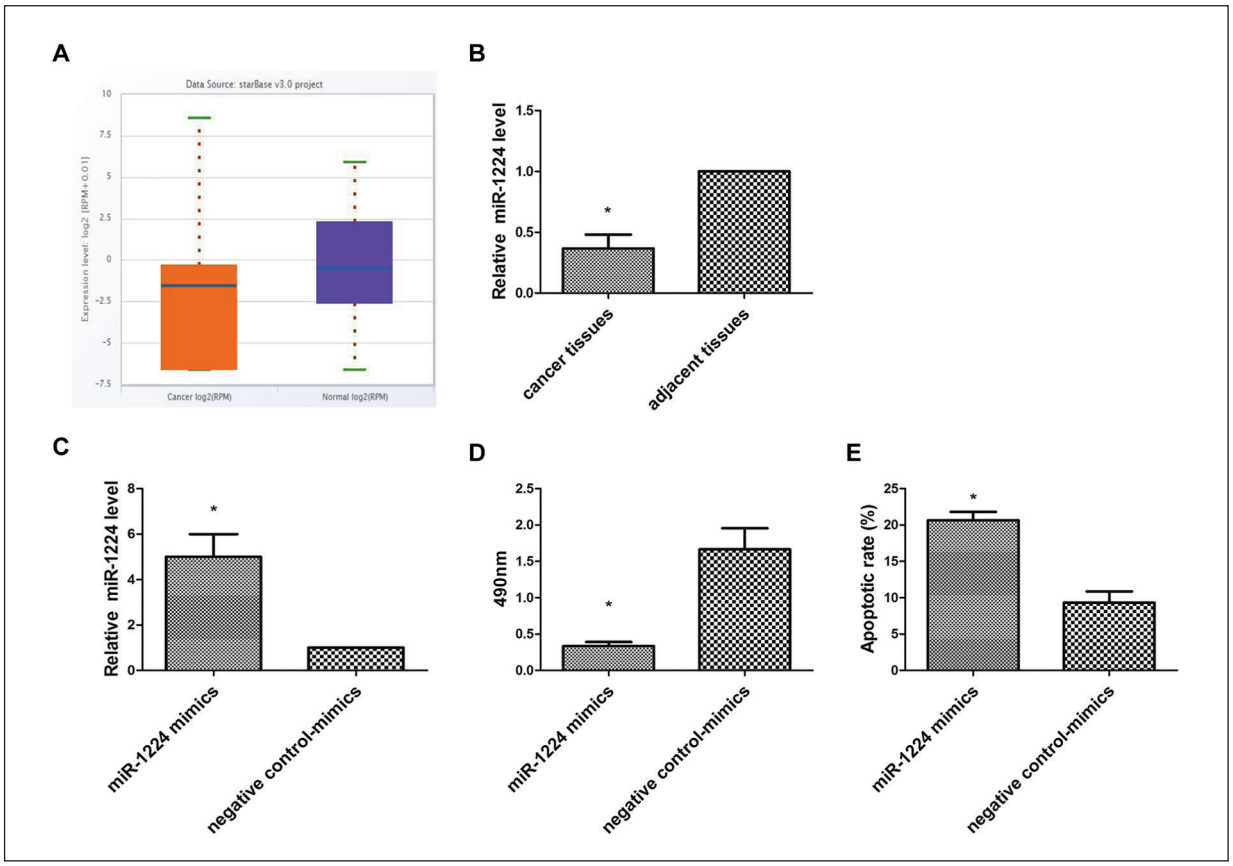

Figure 3. Expression and effect of miR-1224 in lung cancer. **A,** StarBase showed that miR-1224 significantly decreased in lung cancer tissues. **B,** The expression of miR-1224 in lung cancer tissues and adjacent tissues was detected by qRT-PCR. **C,** MiR-1224 mimics was effectively transfected into cells. **D,** MTT assay was used to detect cell proliferation. **E,** Flow cytometry method was applied to detect cell apoptosis.

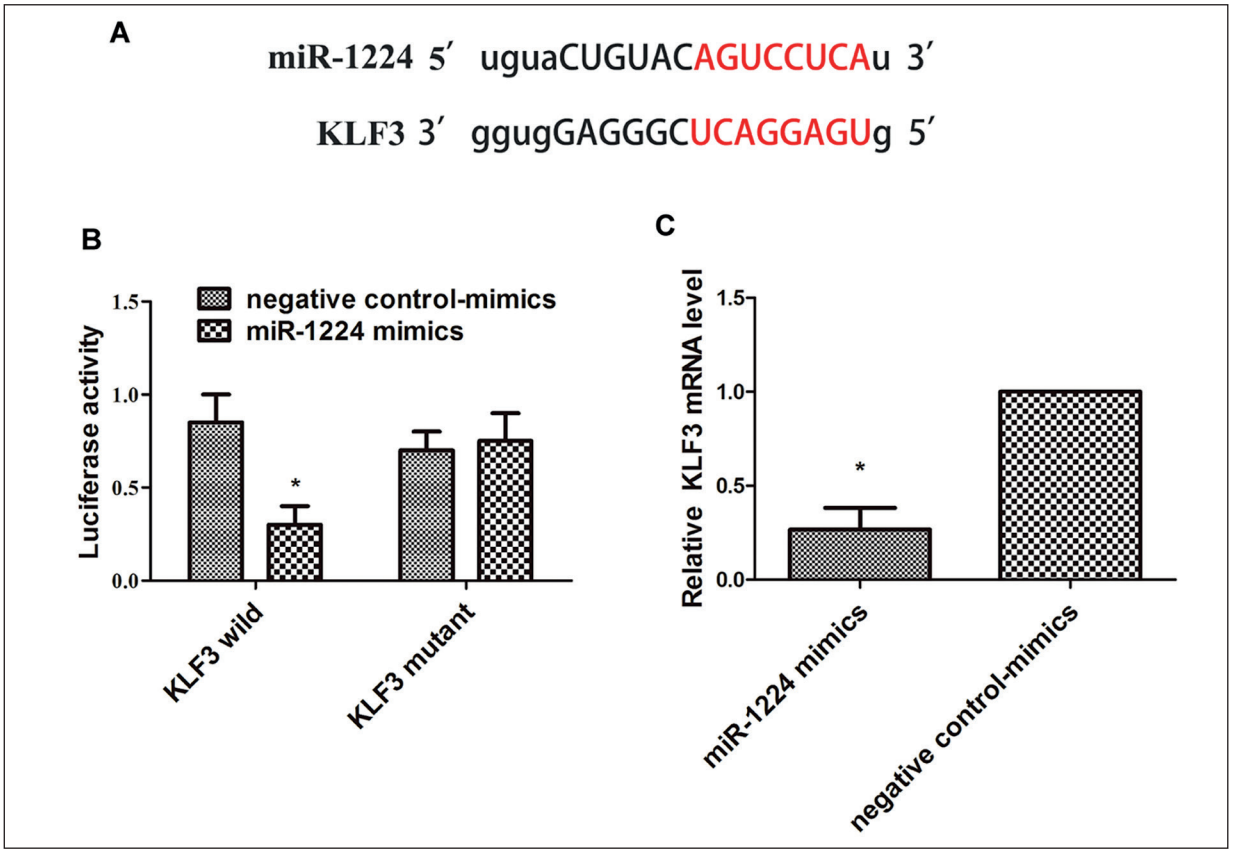

Figure 4. Regulation of miR-1224 on KLF3. **A,** Online software predicted that miR-1224 might be bound to KLF3 3'UTR. **B,** Luciferase reporter gene assay was used to verify this prediction. **C,** QRT-PCR was performed to detect the regulation of abnormal miR-1224 expression on KLF3.

Discussion

With the continuous improvement of molecular biology theory and research technology, the treatment of lung cancer has developed into a comprehensive treatment, including surgery, radiotherapy and chemotherapy, gene therapy, and other treatments[14]. However, the effect is far from satisfactory. LncRNA or microRNA as a target for early diagnosis or drug therapy has become a new model for lung cancer treatment. In this study, we demonstrated that lncRNA NEAT1/miR-1224/KLF3 contributed to cell proliferation, apoptosis, and invasion in lung cancer.

Firstly, we found that the expression level of lncRNA NEAT1 in lung cancer tissues was significantly higher than that of the adjacent normal tissues. Meanwhile, silencing lncRNA NEAT1 significantly inhibited the proliferation and promoted the apoptosis of lung cancer cells.

Previous studies have shown that approximately 98% of DNA is transcribed into non-coding RNA, including short-chain non-coding RNA (ncRNA) and long-chain non-coding RNA (lncRNA). MicroRNAs (miRNAs) are a type of short-chain non-coding RNAs with about 22 nucleotides in length. They have been found in most plants, animals, and some viruses[15]. Rozovski et al[16] have indicated that miRNAs play important roles in regulating RNA silencing and post-transcriptional gene expression. For example, miR-1260b, mediated by YY1, activates the KIT signaling by targeting SOCS6 to regulate cell proliferation and apoptosis in NSCLC[17]. Most miRNAs are located in cells. However, some miRNAs, considered as circulating miRNAs or extracellular miRNAs, have also been found in the extracellular environment[18]. Moreover, our results illustrated that the expression of miR-1224 in lung cancer tissues was significantly lower than that of the adjacent normal tissues. This suggested that miR-1224 might play a role

in lung cancer. High expression of miR-1224 significantly inhibited the proliferation of lung cancer cells. Meanwhile, the apoptosis rate of the cells was remarkably enhanced after miR-1224 up-regulation.

Although there are significant differences in function and structure between lncRNA and endogenous small RNAs (such as microRNAs), there are still some links between them[19]. Long noncoding RNA SNHG15 promotes the proliferation, migration, and invasion of human breast cancer by sponging miR-211-3p[20]. Long noncoding RNA neuroblastoma-associated transcript 1 gene inhibits malignant cellular phenotypes of bladder cancer through miR-21/SOCS6 axis[21]. The bioinformatics prediction tool showed that lncRNA NEAT1 might have a direct effect on miR-1224 due to a similar binding sequence. The Dual-Luciferase reporter gene and qRT-PCR assays showed that lncRNA NEAT1 could specifically bind to the 3'UTR of miR-1224 and downregulate its expression level.

Furthermore, the online software predicted that miR-1224 might be bound to KLF3 3'UTR. Krüppel-like factor 3 (KLF3/BKLF) is a member of the KLF family[22]. The loss of KLF3 has been found to be correlated with aggressive phenotypes and poor survival outcomes of colorectal cancer and may be a potential new predictor and therapeutic target[23]. Our luciferase reporter gene assay and qRT-PCR disclosed that miR-1224 could downregulate KLF3 by directly binding to its 3'UTR. However, the potential regulatory mechanism between them needs further investigation.

Conclusions

We provided clues to reveal the mechanism of lncRNA NEAT1 and miR-1224 in tumorigenesis and progression for the first time. LncRNA NEAT1 could sponge the expression of miR-1224 and affect the proliferation and apoptosis of lung cancer. However, there were still some limitations to this study. The relationship between lncRNA NEAT1 or miR-1224 with prognosis was still unclear and needed further investigation *in vivo*.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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