Long non-coding RNA FAM201A promotes lung squamous cell carcinoma progression through interaction with miR-101

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Abstract. – OBJECTIVE: This study aimed to investigate the mechanism of LncRNA FAM201A mediating lung squamous cell carcinoma progression through interaction with miR-101.

PATIENTS AND METHODS: NCI-H520 cells and SK-MES-1 cells were transfected with miRNA-101-mimics and miRNA-101-inhibitor, the quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect FAM201A and miR-101 expression. CCK-8, Wound healing assay and transwell assay were utilized to detect the influence of FAM201A on the malignancy of NCI-H520NCI-H520 and SK-MES-1SK-MES-1 cells. Cell apoptosis was determined by flow cytometry. The underlying pathways of FAM201A were measured using Western blot. Xenograft tumor experiments were conducted to detect tumor growth and metastasis *in vivo*.NCI-H520SK-MES-1 Kaplan-Meier method calculated patient survival.

RESULTS: (1) Silencing of FAM201A inhibited the proliferation, migration and invasion of NCI-H520 and SK-MES-1cells and stimulated cell apoptosis significantly. Furthermore, FAM201A elimination hindered tumor growth and metastasis *in vivo*. (2) Compared with the si-control group, the protein expression of Ki67, Vimentin, Cleaved-caspase-3 and N-cadherin were decreased in the si-FAM201A group. (3) After transfection of miR-101-mimics, the expression level of Vimentin protein was significantly increased, while the expression level of Vimentin protein was significantly decreased after miR-101-inhibitor transfection. (4) MiR-101 mimics could alleviate FAM201A silencing-induced inhibitive effects on cell proliferation, migration, invasion and promotive effects on cell apoptosis.

CONCLUSIONS: FAM201A could target miR-101 and upregulate Vimentin to inhibit lung cancer progression. FAM201A was expected to be a potential biomarker and therapeutic target for lung cancer.

Key Words:
Lung squamous cell carcinoma, Long-chain non-coding RNA, FAM201A, MiR-101.

Introduction

Lung cancer, which accounts for about 10.3% of malignant tumors in adults, is the most common cancer in the world[1]. The mortality rate of lung cancer is also higher. Squamous carcinoma of the lung is the most common pathological type of lung cancer, accounting for about 40-55% of lung tumors. Of note, the incidence and mortality of lung cancer in the world has been increasing gradually in recent years[2]. At least one-third of lung cancer patients were diagnosed with distant metastases at the first time, 20-40% were prone to metastasis after surgical resection, and less than 20% had a satisfactory 5-year survival rate after surgery[3]. Therefore, the current treatment of lung cancer remains a serious challenge. It is of great importance to disclose the molecular mechanism implicated in lung cancer to develop novel targeted therapeutic strategies against lung cancer.

The eukaryotic genome encodes a large number of long non-coding RNAs (lncRNAs), which are defined as endogenous cell RNAs with a length of more than 200 nucleotides. However, in the absence of a long open reading framework, lncRNA was initially considered as the "dark matter" of the genome[4]. In recent years, they have become an indispensable functional component of the mammalian transcriptome. LncRNAs play an important role in regulating gene expression at different levels, such as chromatin modification, transcription and post-tran-

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scriptional processing[5]. A large number of lncRNAs displayed specificity in embryonic stem cell differentiation, pathogenesis, or tumor formation. In recent years, the function of lncRNAs in tumors has become increasingly specific with the successful application of new methods such as genome-wide gene expression screening, genome-wide association studies, regional targeted association analysis and routine linkage screening, design of lncRNA arrays, RIP-RNA sequencing, and transgenic expression and gene knockout[6,7]. A growing number of data show that many identified lncRNAs play a key role in the carcinogenesis, invasion and metastasis of various tissues. LncRNA FAM201A was initially isolated from NIH3T3 cells by subtractive hybridization. Liu et al[8] have found that many of the important biological activities of FAM201A must be mediated by introns encoding microRNAs (miRNAs). In some prostate and breast cancer cell lines, FAM201A expression can be independent of other stimuli-induced growth arrest and withering. Downregulation of FAM201A through RNA interference protects leukemia and primary human cells against the anti-proliferative effects of rapamycin but is less studied in lung cancer.

Patients and Methods

Clinical Samples and Cell Culture

The patients with lung cancer treated in our hospital from January 1, 2019 to January 1, 2020 were studied. Inclusion criteria: (1) First-time. (2) Surgical treatment. (3) Lung scales were diagnosed by pathological examination. (4) No chemotherapy and radiotherapy were performed before operation. (5) All participants in this study signed a written informed consent. Exclusion criteria: (1) Age ≥80. (2) A distinct far-off turn has occurred. Eventually ,76 patients were included in the study, of which 49 were male and 27 were female, with an average age of 56. This study was approved by the Medical Ethics Committee of China-Japan Union Hospital of Jilin University.

Cell culture: normal human lung cancer cell line SK-MES-1NCI-H520 cells, NCI-H520SK-MES-1 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured with Roswell Park Memorial Institute-1640 medium (RPIM-1640; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, Utah, USA),100 U/ml penicillin and 0.1 mg/ml streptomycin in a 37°C, 5% CO_2 incubator.

Modeling and Grouping of Rats

40 SPF-grade SD rats (8 to 10 weeks, 230±60 g) were purchased from the Field Surgery Institute of the Third Affiliated Hospital of the Third Military Medical University, animal qualification number: 0001517, laboratory animal use license number: SYXK (Chungking) 2017-000. The 40 rats were randomly divided into NCI-H520-NC group, NCI-H520-miRNA-101-mimics group, SK-MES-1-NC group, SK-MES-1-miRNA-101-inhibitor group using different treatment methods, respectively. NCI-H520-NC group: injection of 1×10^5 NCI-H520 tumor cells. NCI-H520-miRNA-101-mimics group: injection of 1×10^5 NCI-H520NCI-H520-miRNA-101-mimics cells. SK-MES-1SK-MES-1-NC group: 1×10^5 SK-MES-1 cells were injected. SK-MES-1-miRNA-101-inhibitor group: injection of 1×10^5 SK-MES-1-miRNA-101-inhibitor fineness. All rat tumors were inoculated under the armpit.

Quantitative Real-Time PCR

Total RNA was extracted from the cell, patient serum, and rat tissue specimens, Reverse transcription reaction and qRT-PCR reaction was performed following instructions of the reverse transcript kit and the amplification kit. miRNA-101 primer sequences: forward: 5'-CTGTATTC AAAA GGCCACTGAA-3', reverse: 5'-GTCTATCTCTTGATCGCCA-3'. FAM201 primer sequence: forward: 5'- CTCATCCAGGTTTCTTCGTC-3', reverse: 5'-GCCAGTT CAACGTAGGATCC-3'. All primer sequences were synthesized by Shanghai Bioengineering Technology CO., LTD. The qRT-PCR reaction conditions were as follows: pre-denaturation at 95°C for 10 min, then denaturation at 95°C for 30 s, annealing at 50°C for 30 s, 40 cycles, and the final extension at 70°C for 10 min ended.

Cell Transfection

Small interfering RNA (SiRNA) targeting FAM201a (Si-FAM201a), miR-101 mimics, and inhibitors were transfected into SK-MES-1 cells and NCI-H520 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 24 hours, the transfected cells were collected for further analysis. The siRNA sequences are as follows: forward: 5'-GATCGCGTTCTGTGTGGAACTTACT-3', reverse: 5'-GGATCAAGAGTCCAGTAAGTTCCACA-3'.

CCK-8 Detection and Flow Cytometry

The transfected SK-MES-1 cells, NCI-H520 cells were inoculated in 96-well plates with 5000 cells/well. 10 μl cholecystokinin was added after transfection for 0 h, 24 h, 48 h, 72 h. After incubation for 2 h, the optical density value (OD value) was measured at 490 nm using a microplate instrument and recorded in the cell growth curve. There were 6 multiple holes in each group. Transfected SK-MES-1 cells, NCI-H520 cells were collected and suspended in a binding buffer of 500 μl. With 5 μl of fluorescein isothiocyanate tagged Annexin V (FITC-Annexin V) and 5 μl of propidium iodide (PI) double-labeled apoptotic cells stained for 30 min. Then, the cells were washed for 3 times and the apoptotic rate was analyzed by BD FACS Calibur flow cytometer (BD Biosciences, Franklin, NJ, USA). The apoptosis rate was expressed as the percentage of apoptotic cells in Annexin V+/PI− and Annexin V+/PI+. Each sample was repeated three times.

Transwell Assay

Cell invasion ability was measured using transwell chamber coated with Matrigel. The transfected SK-MES-1 cells, NCI-H520 cells (400 cells) were inoculated into 200 μl medium without FBS. The upper cavity was implanted into 500 μl medium containing 10% (Franklin Lakes, NJ, USA) and the inferior cavity was inoculated with 500 mmol/L culture containing 10% FBS. For cell migration analysis, the compartment was not coated with matrix, and other steps were associated with invasion analysis phase. All transwell experiments were incubated at 37°C for 24 min, the cells migrating and invading on the lower surface were stained with crystal violet to photograph five predefined fields of vision under a microscope (×200). Each sample was repeated 3 times.

Hematoxylin and Eosin (H&E) Staining

Paraffin-embedded lung tissues from rats were sliced, baked and frozen. Hematoxylin stained for 30 to 60 s, washed for 5 min, eosin stained for 30 s, washed for 5 min; ethanol gradient dehydrated, dried, xylene transparent, neutral gum sealed, dried, observed under optical microscope.

Western Blotting Test

The cells and rat lung tissues of each group were collected, the total protein was extracted, and the protein concentration was determined by bicinchoninic acid assay (BCA) method. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking by 5% nonfat milk, the membranes were incubated with primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibody (Sangon, Shanghai, China). All antibodies were diluted at 1:1000. Following three washes with Tris-Buffered Saline and Tween-20 (TBST), detection was made with the enhanced chemiluminescence system (BioRad, Richmond, CA, USA). The protein expression of Ki67, Vimentin Cleaved-caspase-3 and N-cadherin was detected with β-actin as internal reference.

Results

Effect of si-FAM201A Transfection on Lung Cancer Cell Activity

SK-MES-1 cells and NCI-H520 cells were transfected with si-FAM201A. CCK-8 and flow cytometry results showed that the proliferation ability of SK-MES-1 cells and NCI-H520 cells decreased significantly after transfection of si-FAM201A (Figure 1A). The apoptosis rate of SK-MES-1 cells and NCI-H520 cells increased significantly by FAM201A silencing (Figure 1B). Transwell result revealed that depletion of FAM201A displayed negative effects on cell migration and invasionSK-MES-1 (Figure 1C).

Effect of si-FAM201A Transfection on Protein Expression

Western blot was further conducted to investigate the importance of FAM201A in protein expression of ki67, cleaved-caspase-3, N-cadherin and Vimentin in SK-MES-1 cells and NCI-H520 cells. Compared with si-control group, the relative protein expression of ki67 and Vimentin in the transfected group was significantly decreased ($p<0.05$), and the relative protein expression of cleaved-caspase-3 and N-cadherin was significantly increased ($p<0.05$) (Figure 2).

Effect of Transfection of MiR-101 on Lung Cancer

RT-PCR found that the mRNA expression of miR-101 in serum of lung cancer patients was significantly higher than that of healthy volunteers. Moreover, there was a negative correlation between miRNA-101 and FAM201A expression

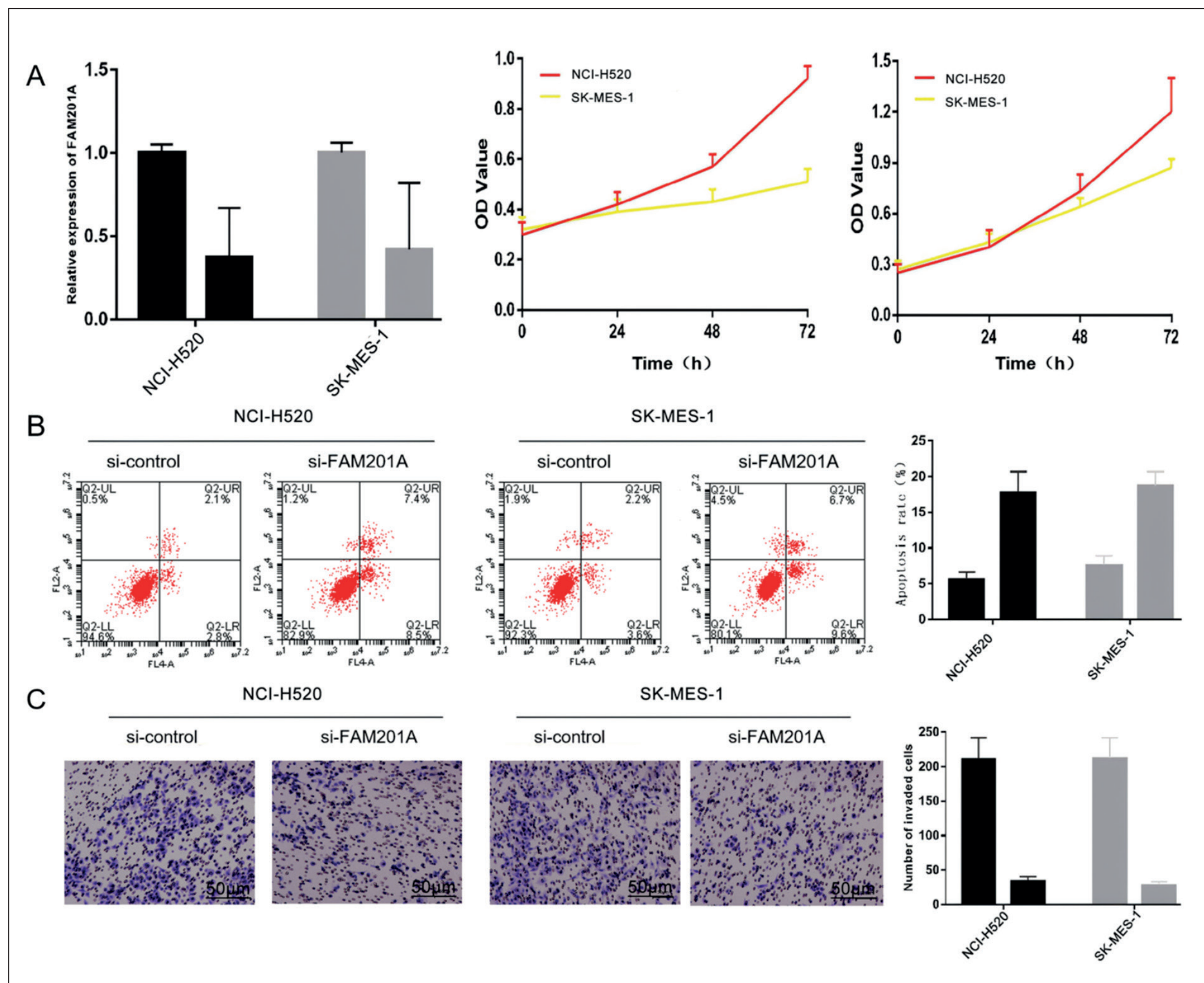

Figure 1. Effect of si-FAM201A transfection on lung cancer cell activity. si-FAM201A or si-control was transfected into SK-MES-1 cells and NCI-H520 cells. **A**, qRT-PCR analysis of FAM201A expression. CCK-8 assay was used to detect cell viability. **B**, Apoptotic rates were analyzed by using flow cytometry. **C**, Transwell assay was conducted to evaluate cell migration and invasion (magnification 200×).

level (Figure 3A). CCK-8 assay indicated inhibition of miR-101 weakened cell viability while miRNA-101 mimics strengthened cell viability (Figure 3B). Wound-healing assay and transwell assay showed that inhibition of miR-101 inhibited cell migration activity while miRNA-101 mimics stimulated cell migration activity, ($p<0.05$) (Figure 3C-D).

Effect of MiR-101 on Tumorigenesis In Vivo

To confirm the effect of miR-101 on tumor growth of lung cancer *in vivo*, the xenograft tumor model was constructed. NCI-H520 and SK-MES-1 cells transfected with NC, miRNA-101 mimics or miR-101 inhibitor were inoculated into rats. The results showed that tumor volume in miRNA-101 inhibitor group was significantly lower than that in NC group, while tumor volume of miRNA-101 mimics group was greatly higher than that of NC group ($p<0.05$) (Figure 4A). Immunohistochemical detection found that the miR-101-inhibitor group had fewer pulmonary metastatic lesions in rats (Figure 4B).

Effect of MiR-101 Transfection on Vimentin Protein Expression

Vimentin is the downstream target gene of miR-101. Western-blot detection found that the expression level of Vimentin protein was significantly increased after transfection of miR-101-mimics ($p<0.05$), while the protein expression of Vimentin was significantly decreased after transfection of miR-101-inhibitor

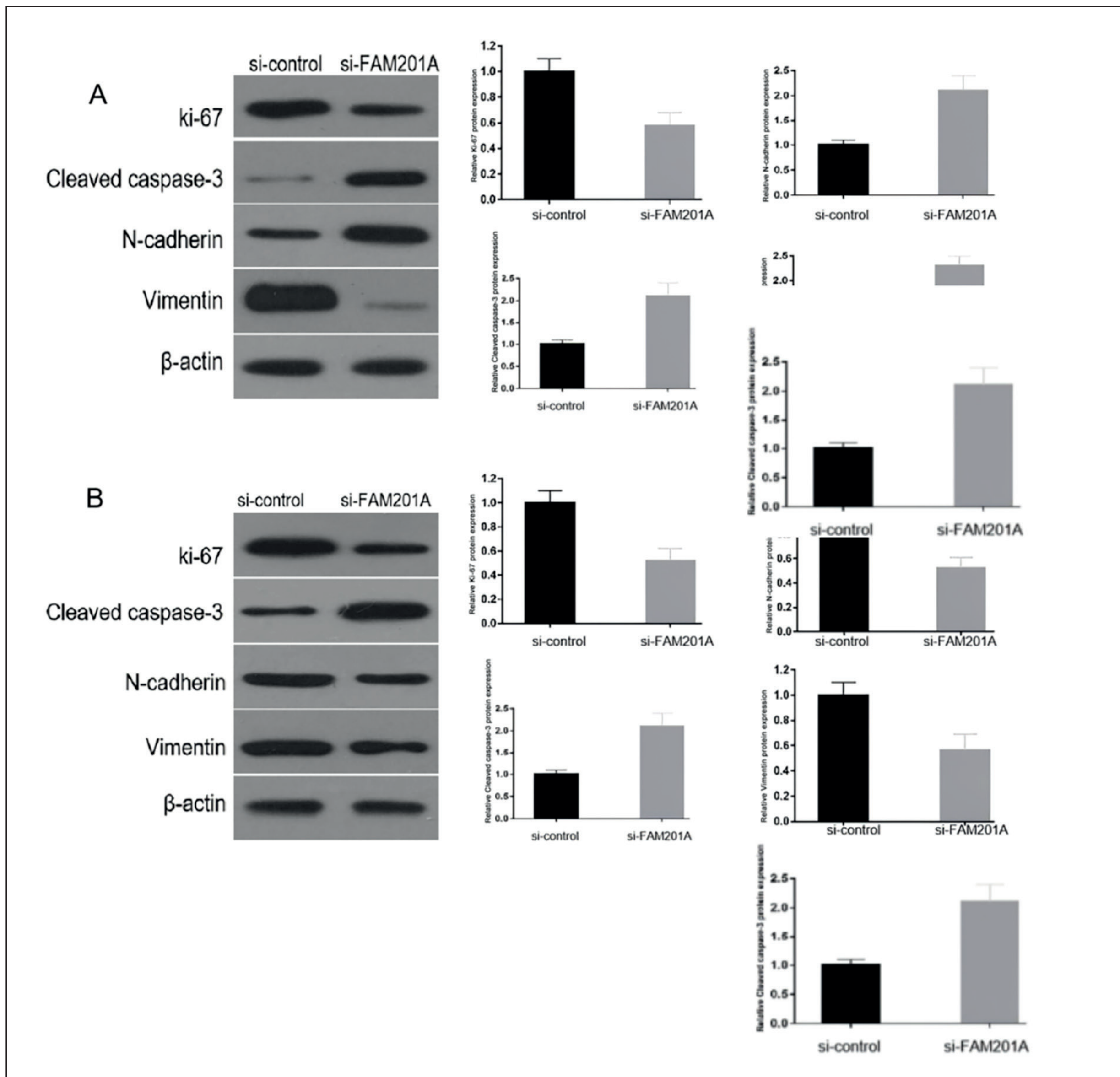

Figure 2. Effect of transfection of si-FAM201A on protein expression. si-FAM201A or si-control was transfected into SK-MES-1 cells and NCI-H520 cells. Protein expression of ki67, cleaved-caspase-3, N-cadherin and Vimentin in SK-MES-1 cells (**A**) and NCI-H520 cells (**B**) was analyzed by Western blot respectively.

($p<0.05$) (Figure 5A-B). Immunofluorescence detection also found the same trend, and the miR-101-mimics transfection group had more fluorescein-carrying Vimentin protein expression (Figure 5C).

Effect of MiR-101 and FAM201A on Lung Cancer Cell Activity

The survival curve showed that the overall survival rate and tumor-free survival rate of patients with high expression of serum miR-101 were significantly lower than those in the low expression group ($p<0.05$) (Figure 6A). To disclose the molecular mechanism of Lung cancer progression, cells were transfected with miR-control, miR-101, miR-101+Vector and miR-101+FAM201A (Figure 6B). CCK-8 and Flow cytometry showed that after co-transfection of miR-101-mimics and FAM201A, the tumor-promoting effect of miRNA101 was partially attenuated (Figure 6C), the proportion of apoptotic cells decreased significantly (Figure 6D), and the ability of cell migration and invasion decreased significantly (Figure 6E-F). Western-blot detection found that com-

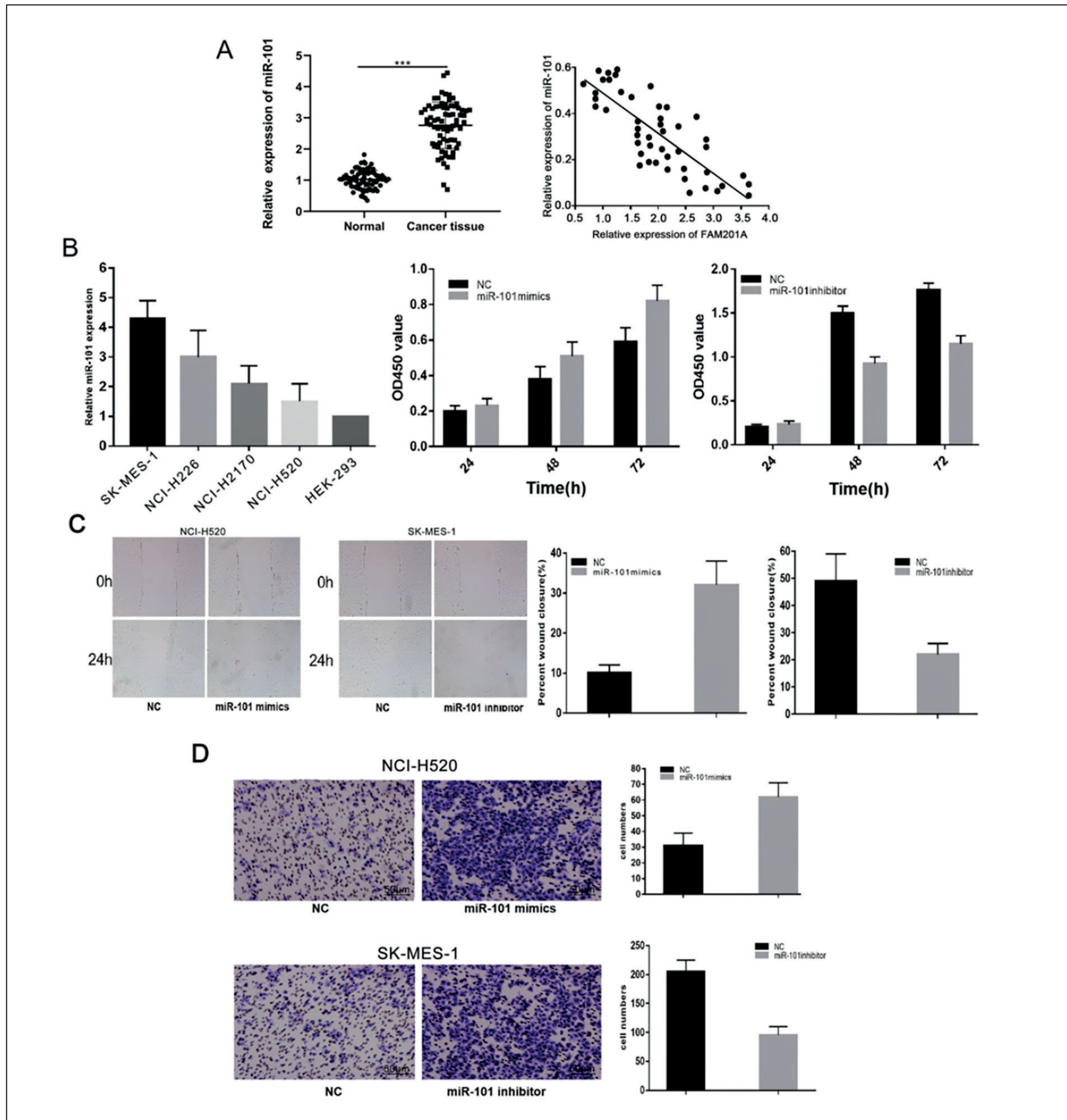

Figure 3. Effect of transfection of miR-101 on lung cancer cells. **A**, RT-PCR analysis of miRNA-101 expression in lung cancer and paired adjacent normal lung tissues; pearson's correlation scatter plots of miR-101 and FAM201A in lung cancer. **B**, The expression of miRNA-101 in lung cancer cells was assessed by RT-PCR. Cell viability was measured by CCK-8 assay. **C-D**, Wound-healing assay and transwell assay were conducted to evaluate cell migration activity (magnification 200×).

pared with the single transfection group, the relative protein expression of Ki67, vimentin in the co-transfected group was significantly reduced ($p<0.05$), and the relative protein expression of Cleaved-caspase-3 and N-cadherin was significantly increased ($p<0.05$) (Figure 6G).

Discussion

Long-chain non-coding RNAs are RNA transcripts of more than 200 nucleotides and do not encode protein. Increasing studies have shown that the molecular mechanism of carcinogenesis is

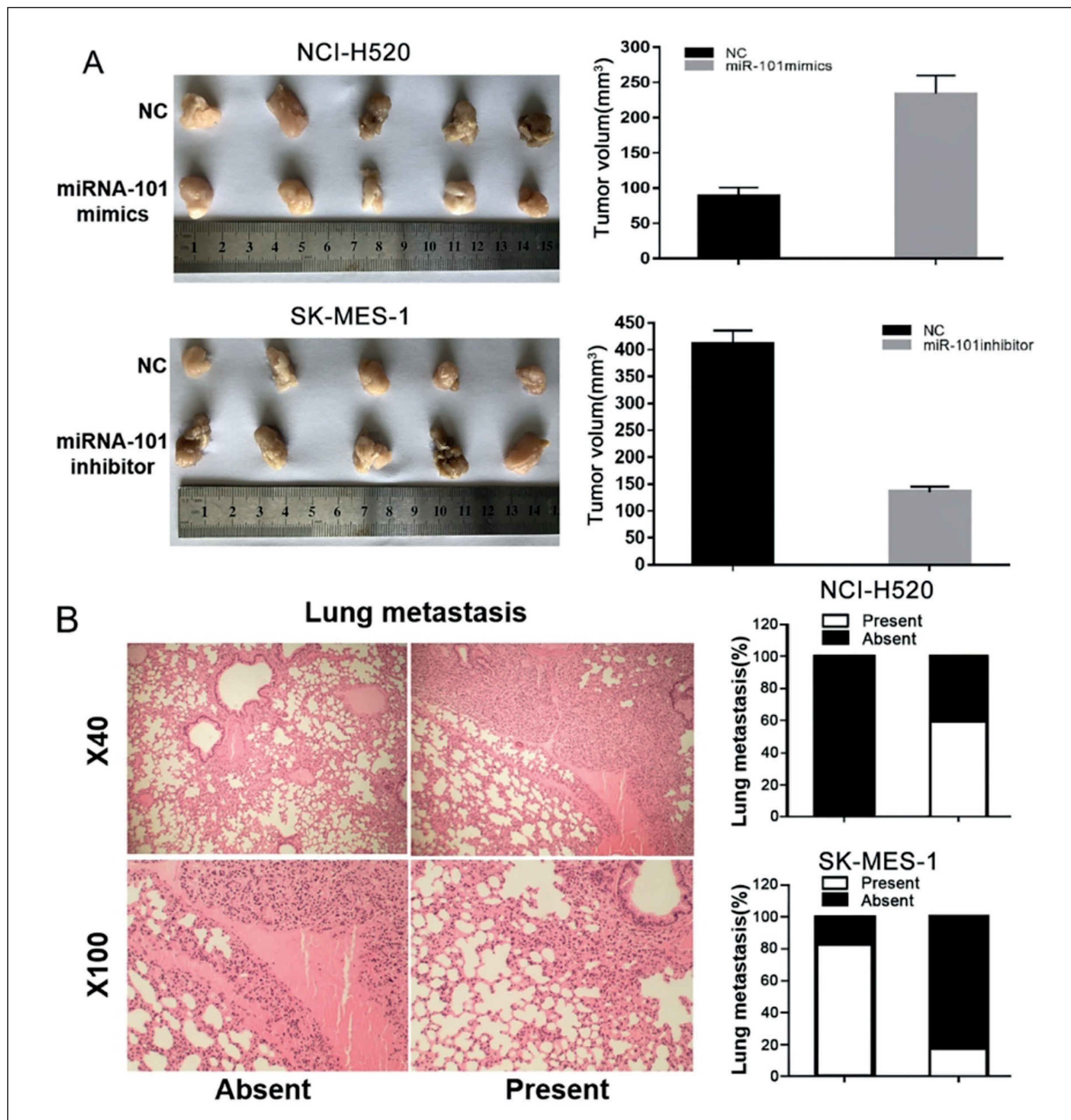

Figure 4. Effect of transfection of miR-101 on growth of lung cancer. Cells transfected with miR-101 mimics or miRNA-101 inhibitor were subcutaneously injected in nude mice. **A**, Tumor volume was evaluated. **B**, Lung metastases were validated by H&E staining (magnification 100×; 40×).

not only related to protein-coding genes, but also to noncoding regulatory RNA[9]. Some lncRNAs have been found to play a key role in the development of cancer. It has been shown[10] that a large number of lncRNAs are downregulated in various tumors, and can regulate tumor metastasis, suggesting that abnormal expression of lncRNAs mediated tumorigenesis and development. However, at present, only a few lncRNAs have been studied in detail in function, and many important issues remain to be solved. Previous studies have shown[11] that FAM201A genetic abnormalities are associated with many types of tumors, including melanoma, breast and prostate cancer. In our study, whether the abnormal expression of FAM201A is associated with the occurrence of lung

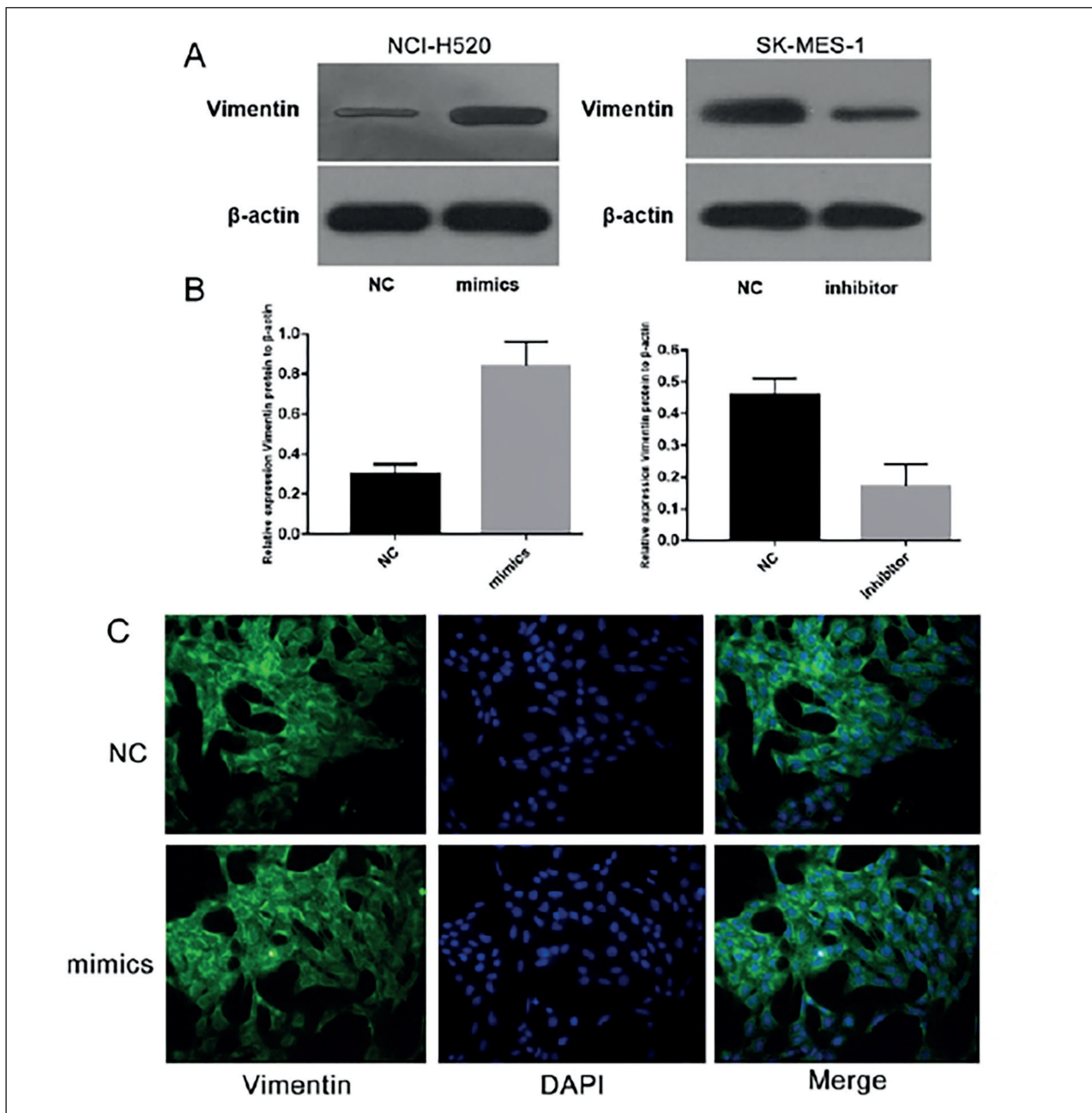

Figure 5. Effect of miR-101 transfection on Vimentin protein expression. MiR-101-mimics or miR-101-inhibitor were transfected into NCI-H520 and SK-MES-1 cells. **A-B**, The protein expression of Vimentin in transfected cells was examined by Western blot. **C**, Immunofluorescence detection of Vimentin expression (magnification 100×).

cancer has been explored. MicroRNAs (miRNAs) are a 22-nt family of noncoding endogenous single-stranded RNAs involved in regulating the gene table. Mature miRNAs and Argonaute (AGO) proteins form RNA-induced silencing complexes (RISC) that mediate post-transcriptional gene silencing by inducing mRNA degradation or translational inhibition[12,13]. It has been found that some miRNAs play an important role in promoting lung cancer growth, but the relationship of miRNAs and lncRNAs in the process of tumor biology needs further investigated.

In this study, we first explored the role of LncRNA FAM201A in lung cancer *in vitro*, the results showed that the proliferation ability of SK-MES-1 cells and NCI-H520 cells decreased significantly after transfection of si-FAM201A, and the apoptosis rate of SK-MES-1 cells and

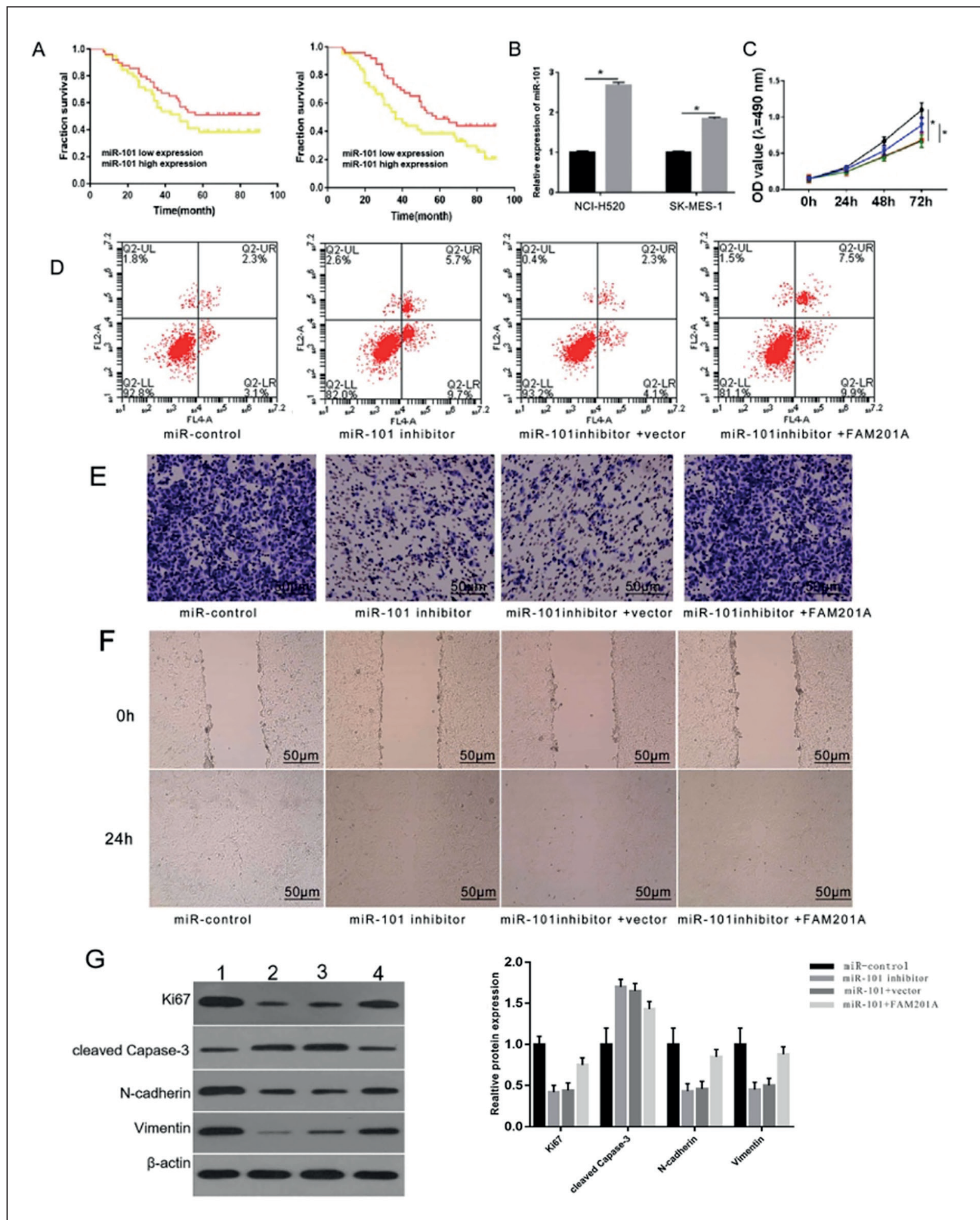

Figure 6. Effect of interaction between miR-101 inhibitor and FAM201A on lung cancer cell activity. Cells were transfected with miR-control, miR-101-inhibitor, miR-101-inhibitor+Vector and miR-101-inhibitor+FAM201A. **A**, The overall survival analysis of patients with Lung cancer. **B**, The expression of miR-101 in lung cancer cells was assessed by RT-PCR. **C**, Cell viability was measured by CCK-8 assay. **D**, Apoptotic rates were analyzed by using flow cytometry. **E-F**, Transwell assay and Wound-healing assay were used to evaluate cell migration and invasion (magnification 200×). **G**, Protein expression of ki67, cleaved-caspase-3, N-cadherin and Vimentin was determined by Western blot.

NCI-H520 cells increased significantly after 24 h after transfection. Transwell assay also found that SK-MES-1 migration and invasion ability of SK-MES-1 cells and NCI-H520 cells significantly decreased. These results indicated that the deregulation of FAM201A may play a certain role in the occurrence and development of lung cancer. Western-blot results show that compared with si-control group, the protein expression of Ki67 and Vimentin in si-FAM201A group was significantly decreased, while the protein expression of cleaved-caspase-3 and N-cadherin was significantly increased. Ki67/Cleaved-caspase-3 signaling pathway has previously been suggested to play a central role in determining cell survival[14]. It has now been found that the Ki67/Cleaved-caspase-3 pathway has been altered in many types of cancer. After Ki67 binds to its receptor, it causes the Dsh/DVL complex to be activated, leading to the inactivation of the glycogen synthase kinase (GSK)3b complex that prevents β-catenin degradation[15]. This results in stabilized b-catenin translocated to the nucleus where it binds to members of the T cell factor/lymphoid enhancer-binding factor (TCF/LEF) family of transcriptional factors, and is able to modulate the expression of a wide range of target genes to regulate cell function[16,17]. Accordingly, we hypothesized that FAM201A has tumor oncogene in lung cancer. That is, *in vivo* induction of FAM201A down-expression in lung cancer tissues will be an attractive strategy for the treatment of lung cancer. Therefore, finding an effective way or designing a vector to inhibit FAM201A expression in lung cancer, may provide an attractive therapeutic pathway. For example, the expression of LncRNA FAM201A is increased in a wide range of human cancers. Intra-tumoral injection of BC-819 (DTA-H19) plasmid carrying the subunit gene of diphtheria toxin A under the regulation of H19 promoter induces high levels of specific expression of diphtheria toxin in tumors, thus reducing tumor volume. Some studies[18,19] have found encouraging results in a variety of cancers including colon, bladder, pancreatic and ovarian cancer. This success undoubtedly provides us with a confident and good way to treat lung cancer by regulating the expression of FAM201A *in vivo*.

After the cell experiments were verified, we conducted *in vivo* studies. The results showed that compared with the NC group, the tumor growth of rats injected intravenously with miR-101-inhibitor was significantly inhibited, the diameter was significantly smaller, and the tumor growth of rats injected with miR-101-mimics was significantly promoted, and the tumor diameter was significantly more. Immunohistochemical detection found that the miR-101-inhibitor group had fewer intrapulmonary metastatic lesions and more diameter in rats. It suggests that miRNA101 may act as a tumor-inhibiting action in lung cancer that is completely contrary to FAM201A. Therefore, we further co-transfected NCI-H520 cells with miR-101-inhibitor and FAM201A. The results showed that the tumor-inhibiting effect of miRNA-101-inhibitor was partially offset, the proportion of apoptotic cells decreased significantly, and the ability of cell migration and invasion was also significantly reduced. This suggests that the functional activity of tumor cells is significantly inhibited. Western-blot detection found that compared with the single-transfected group, the relative expression of Ki67 protein, Vimentin protein and Cleaved-caspase-3 protein, N-cadherin protein in the co-transfected group was significantly reduced which is consistent with the conclusion of the cell experiment. Therefore, FAM201A may regulate miRNA101 function through the Ki67/Cleaved-caspase-3 signaling pathway, exerting an antitumor effect on lung cancer.

Taken together, this study found that FAM201A knockdown inhibited cell proliferation, migration, and targeted to miR-101 and upregulated Vimentin, promising a new therapeutic target of lung cancer.

Conclusions

We discovered the molecular mechanism of FAM201A in lung cancer progression. The results demonstrated that FAM201A acted as an oncogene to facilitate proliferation, migration, invasion and block apoptosis of lung cancer cells by regulating miRNA101 and enhancing Ki67, Cleaved-caspase-3, Vimentin protein expression. Our study provided a potential biomarker for the diagnosis and prospective targeted therapies of lung cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding Statements

(1) Jilin Province Science and Technology Development Plan Project (Project No. 20180101162JC). (2) “Thirteenth Five-Year” Science and Technology Project of Jilin Provincial Department of Education (Project No. JJKH20170849KJ).

Ethical Statement

The study was approved by the Ethics Committee of the China-Japan Union Hospital of Jilin Universi (NO.ERC-20213), and all patients participating in this study provided written informed consent in accordance with the "Helsinki Declaration".

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