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Mir-451 inhibits proliferation and migration of non-small cell lung cancer cells *via* targeting LKB1/AMPK

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Abstract. – OBJECTIVE: To discuss the influence of micro ribonucleic acid (miR)-451 on cell proliferation and apoptosis of non-small cell lung cancer (NSCLC) by regulating liver kinase B1 (LKB1) signals and activating adenosine monophosphate-activated protein kinase (AMPK).

MATERIALS AND METHODS: Lung cancer A549 cells were divided into control group, miR-451 mimic group, and miR-451 inhibitor group. The cell proliferation and migration ability, as well as the expression of LKB1 and AMPK in the three groups, were determined *via* Reverse Transcription-Polymerase Chain Reaction (RT-PCR), transwell assay, and cell counting kit-8 (CCK8) assay, respectively.

RESULTS: Compared with that in control group, the number of migrating cells evidently declined in miR-451 mimic group, while that in miR-451 inhibitor group was significantly increased, and the differences were statistically significant ($p < 0.05$). At 7 d of cell culture, the cell proliferation ability in miR-451 mimic group was higher than that in control group, while it was higher in control group than that in miR-451 mimic group, and there were significant differences among the three groups ($p < 0.05$). The expression of LKB1 and AMPK was significantly decreased in miR-451 mimic group compared with that in miR-451 inhibitor group and control group, and the differences had statistical significance ($p < 0.05$). The differences in activities of LKB1 and AMPK between miR-451 inhibitor group and control group were not significant ($p > 0.05$).

CONCLUSIONS: LKB1/AMPK can be involved in the cell metabolism of NSCLC and miR-451 is negatively correlated with LKB1/AMPK. Therefore, miR-451 may inhibit cell proliferation and migration of NSCLC *via* regulating LKB1/AMPK.

Key Words:
MiR-451, Non-small cell lung cancer, LKB1/AMPK, Proliferation, Apoptosis.

Introduction

With the air quality continuously getting worse, patients with lung cancer have become younger worldwide. Every year, there are millions of deaths due to lung cancer, which seriously threatens the health of human beings[1]. In China, the morbidity rate of primary lung cancer is higher among men compared to that among women[2,3]. Patients in the advanced stage of cancer have no chance to be cured by surgery. Although there are various treatment measures, for the time being, the overall survival of patients with advanced lung cancer is only 4-6 months, while the 5-year survival rate is about 4.2%[4,5]. Some studies[6] have shown that micro ribonucleic acid (miRNA) tumor suppressor genes can be involved in the occurrence and development of tumors, while some researchers have found that multiple miRNAs including miR-451 are dysregulated in non-small cell lung cancer (NSCLC) tissues and serum[7]. Moreover, some research[8] has proved that an increased expression of miR-451 can hinder the adenosine monophosphate-activated protein kinase (AMPK) signal pathway, thus inhibiting proliferation of lung cancer cells. AMPK can promote the inflammatory factors and the activation of AMPK can promote both the proliferation and migration of cancer cells[9-11]. Jakobsen et al[12] found that liver kinase B1 (LKB1) plays an important role in the occurrence and development of lung cell carcinoma. LKB1, as the upstream kinase of AMPK, will activate the expression of AMPK *via* phosphorylation after mutation, thereby promoting the proliferation and survival of tumor cells[13]. Therefore, the correlations of miR-451, LKB1/AMPK, and NSCLC cells were further explained in the present work. With NSCLC

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cells lines as the research objects, the influence of miR-451 on cell proliferation and apoptosis of NSCLC by regulating LKB1/AMPK was investigated.

Materials and Methods

Materials, Reagents, and Instruments

NSCLC cell lines A549, H1299, epithelial cell line 11-ZQG (American Type Culture Collection; ATCC, Manassas, VA, USA), transwell chamber (Corning, Corning, NY, USA), Roswell Park Memorial Institute-1640 (RPMI-1640) culture medium and fetal bovine serum (FBS) were purchased form HyClone, South Logan, UT, USA; miR-451 mimic and miR-451 inhibitor from Ambion, Austin, TX, USA; RNA-iMAX transfection reagent from Thermo Fisher Scientific, Waltham, MA, USA; Dual-Luciferase assay kit from BD, Franklin Lakes, NJ, USA; miRNA (BD, Franklin Lakes, NJ, USA), pcDNA extraction kit (BD, Franklin Lakes, NJ, USA), LKB1 inhibitor gene (Abcam, Cambridge, MA, USA), fluorescent quantitative polymerase chain reaction (PCR; Santa Cruz Biotechnology, Santa Cruz, CA, USA), cell counting kit-8 (CCK8; Thermo Fisher Scientific, Waltham, MA, USA), miRNA quantitative reverse transcription-polymerase chain reaction (qRT-PCR) kit (Pierce, Rockford, IL, USA).

Cell Culture and Grouping

NSCLC cell lines A549, H1299, and the epithelial cell line 11-ZQG were added with 100U/mL penicillin and 0.1 μg/mL streptomycin, as well as a complete medium containing 10% FBS. The cell lines were cultured at a constant temperature, and the medium was replaced every 24 h. The cells in the growth phase were collected for the experiments when 90% of A549, H1299, and epithelial cell line 11-ZQG were fused. Three groups Control group, miR-451 mimic group, and miR-451 inhibitor group were set up.

MiR-451 Transfection

The compound was prepared by mixing 30 pmol of miR-mimic and miR-inhibitor with RNA-iMAX transfection reagents, respectively, according to the instructions of the RNA-iMAX kit and placed at room temperature for 5 min. The organic compound was added into the cells for transfection for 48 h. Each group was set up in duplicate. The normal medium was replaced for subsequent analysis. When the concentration of NSCLC A549 cell lines reached 40-50%, miR-451 mimics and miR-45 NC were transfected into A549 cell lines. After 6 h, the culture solution was replaced with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% serum, and the cells were continuously cultured for 48 h. After that, the working solution was prepared according to the instructions of RNA-iMA, and A549 cells were collected after the experiment.

Detection of LKB1 and AMPK Expression Via RT-PCR

The cells in the growth phase were collected, digested with trypsin, and washed with phosphate- buffered saline (PBS). RNAs were extracted with 2mL of miR-451 carrier reagent. A549 cells were collected into an Eppendorf (EP) tube, and RNAs were extracted using 2 mL of RNA reagent and reversely transcribed into complementary deoxyribose nucleic acid (cDNA). Then, PRC was performed as follows: 97°C for 6 min, 98°C for 28 s, 75°C for 30 s, and 80°C for 4 min, for a total of 55 cycles. The relative expression of miR-451 in the cells of different groups was quantitatively determined. The experiment was repeated for 3 times. The phosphorylation level of AMPK activated by LKB1 was detected, and 1.0 g of RNA product was taken for the reverse transcription. 2 U of DNAs were collected for qRT-PCR, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. The primer sequences of LKB1/AMPK are shown in Table I.

Table I. Primer sequence.

Gene		Primer sequence
LKB1	Forward	5'-GGCTGAGGGlTrAGTGAGCA-3'
	Reverse	5'-AGGGAGTTGGTGAAAGACATC-3'
AMPK	Forward	5'-CGGGCAGAATCATGAGCAAGT-3'
	Reverse	5'-AGGGTCTGCATrGGATGGCAT-3'
GAPDH	Forward	5'-CACCATIGGCAATGAGGGGTFC-3'
	Reverse	5'-AGGTCTTTGCGGATGTCCACGT-3'

Detection of LKB1/AMPK Expression in A549 Cells Via Transwell Chamber

At 48 h after LKB1/AMPK-activated NSCLC A549 cells were transfected with miR-451, 100 U of cell suspension was added into the upper chamber, and 300 mL of culture medium containing 10% FBS was added into the lower chamber (3 duplicates in each group). After 10 h of culture in the cell incubator, the chamber was taken out, and 6 fields were randomly selected under the inverted optical microscope to count the number of cells that penetrated the membrane. The relative number of cells that penetrated the membrane indicated the migration ability of cancer cells. Meanwhile, the database of miRNA online genetic prediction tool was adopted.

Detection of Cell Proliferation Via Cell Counting Kit-8 (CCK-8) assay

Lung cancer A549 cells (4×10^3) were inoculated into the 96-well culture plate and cultured for 24 h. Cells were starved using the serum-free medium for 24 h. MiR-451 inhibitor working solution was prepared according to the operational instructions of RNA-iMAX. The cells were transfected in accordance with the steps in "1.3" and incubated with CCK-8 reagent continuously for 4 h. The experiment was repeated for 3 times, with the proliferation level of A549 cells as the ordinate and time as the abscissa.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was adopted for statistical analysis. Control group, miR-451 mimic group, and miR-451 inhibitor group were compared using the independent-samples *t*-test. One-way ANOVA test was employed for pairwise comparison, followed by Post-Hoc Test (Least Significant Difference). The enumeration data were presented as $\bar{x} \pm s$. $p < 0.05$ suggested that the difference was statistically significant.

Results

Expressions of MiR-451 in Different Lung Cancer Cell Lines (A549 and H1299)

The expression level of miR-451 was evidently lower in the lung cancer cells lines (A549 and H1299) than that in normal epithelial cells of the pulmonary *bronchus* (11-ZQG) ($p < 0.05$). Since the expression level of miR-451 was the lowest in A549 cells (Figure 1), the subsequent experiments were carried out using A549 cells.

MiR-451 Inhibited Migration Ability of A549 Cells

Compared with that in control group, the number of migrating cells significantly declined in miR-451 mimic group, while that in miR-451 inhibitor group was evidently increased, and the differences were statistically significant ($p < 0.05$) (Figure 2A and 2B).

MiR-451 Attenuated Proliferation Ability of A549 Cells

The proliferation ability of A549 cells was determined using the CCK8 assay. The curves were plotted with the OD value as the ordinate and time as the abscissa, from which it could be observed that there were no differences in the growth of cells among control group, miR-451 mimic group, and miR-451 inhibitor group ($p > 0.05$). At 4-5 d, the proliferation of cells in the three groups was rapid and then tended to be stable. The proliferation of cells was significantly slower in miR-451 mimic group than that in control group and miR-451 inhibitor group at 6 d. Also, the differences were statistically significant ($p < 0.05$). However, control group and miR-451 inhibitor group had no difference in the cell proliferation ability ($p > 0.05$). At 7 d, the cell proliferation ability was higher in miR-451 inhibitor group than that in control group, while that in control group was higher than that in miR-451 mimic group. Also, there were significant differ-

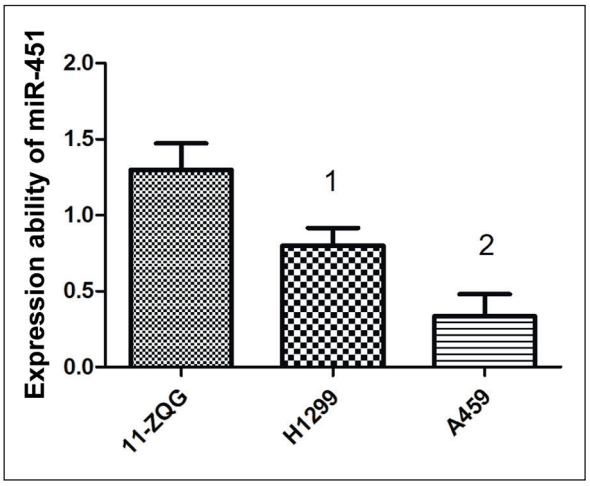

Figure 1. Expressions of miR-451 in the lung cancer cell lines. (Note: $^1p < 0.05$ *vs.* 11-ZQG group, $^2p < 0.05$ *vs.* H1299 group).

ences among the three groups ($p < 0.05$), indicating that miR-451 could effectively inhibit the proliferation ability of A549 cells (Table II and Figure 3A). The proliferation ability of A549 cells in the three groups was further detected *via* transwell assay. Results showed that the number of proliferating cells was smaller in miR-451 mimic group than that in control group and miR-451 inhibitor group ($p < 0.05$) (Figure 3B and 3C).

MiR-451 Decreased the Expressions of LKB1/AMPK in A549 Cells

Compared with that in miR-451 inhibitor group and control group, the expression of LKB1 and AMPK in miR-451 mimic group was decreased significantly, and the differences were statistically significant ($p < 0.05$). MiR-451 inhibitor group and control group had no significant difference in the activity of LKB1 and AMPK ($p > 0.05$) (Figure 4A and 4B).

Discussion

In recent years, with the influence of serious environmental air pollution, the morbidity rate of NSCLC has been rising year by year, showing a younger trend[14]. Most patients with NSCLC have been at an advanced stage at diagnosis. Therefore, early diagnosis is of great significance for prolonging the survival time of patients. How to develop reasonable individualized treatment scheme for patients with NSCLC has become a research focus. MiRNAs have been widely applied in the treatment of tumors[15]. According to related reports, miRNAs are up-regulated in NSCLC cells and play an important regulatory role in promoting metastasis, proliferation, and differentiation of lung cancer cells *via* targeted regulation[16]. Results of the present study showed that the cell proliferation was significantly slower in miR-451 mimic group than that in control group and miR-451 inhibitor group, and the differences were statistically significant ($p < 0.05$). Compared with that in control group, the number of migrating cells markedly declined in miR-451 mimic group, while that in miR-451 inhibitor group was evidently increased, and the differences were statistically significant ($p < 0.05$). Guan et al[17] found that miR-451 played an inhibitory role in the metastasis of NSCLC. According to the research by Kikkawa et al[18], miR-451 has a little effect on the treatment of brain glioma by regulating PI3K/AKT pathway through the CAB39 gene. Moreover, some works[19] have suggested that miR-181a inhibits the proliferation of tumor cells by inhibiting the formation of free radicals, thereby improving the patients' survival time. Other works[20] also indicated that the increased expression of miR-451 can significantly inhibit the proliferation of lung cancer cells and block the cancer cell cycles, which are in line with the results of the present study that miR-181a could effectively inhibit the proliferation and growth of lung cancer cells and relieve the progression of lung cancer.

Compared with that in miR-451 inhibitor group and control group, the expression of LKB1 and AMPK was decreased significantly in miR-451 mimic group, and the differences were statistically significant ($p < 0.05$). MiR-451 inhibitor group and control group had no significant difference in the activity of LKB1 and AMPK ($p > 0.05$). AMPK has the effect of protecting tumor cells. LKB1 is a kind of protein kinase mainly involved in regulating cell cycles, protecting polarity, and development of tumor cells. It was found that[21] LKB1 phosphorylates and activates AMPK. The activation of miR-451 can inhibit the development of lung cancer cells by blocking

Table II. Comparison of OD value in different groups.

Group	Control group	MiR-451 mimic group	MiR-451 inhibitor group
0 d	0.64 ± 0.08	0.64 ± 0.08	0.64 ± 0.08
1 d	0.69 ± 0.03	0.59 ± 0.01	0.59 ± 0.02
2 d	1.11 ± 0.07	1.03 ± 0.01	1.22 ± 0.04
3 d	2.10 ± 0.05	1.73 ± 0.07	2.19 ± 0.06
4 d	2.90 ± 0.04	2.36 ± 0.10	3.03 ± 0.20
5 d	3.02 ± 0.09	2.33 ± 0.08[1]	3.07 ± 0.05[2]
6 d	3.12 ± 0.08	2.32 ± 0.03[1]	3.15 ± 0.08[2]
7 d	3.06 ± 0.18	2.40 ± 0.11[1]	3.59 ± 0.08[1,2]

Note: [1]$p < 0.05$ *vs*. control group, [2]$p < 0.05$ *vs*. miR-451 mimic group.

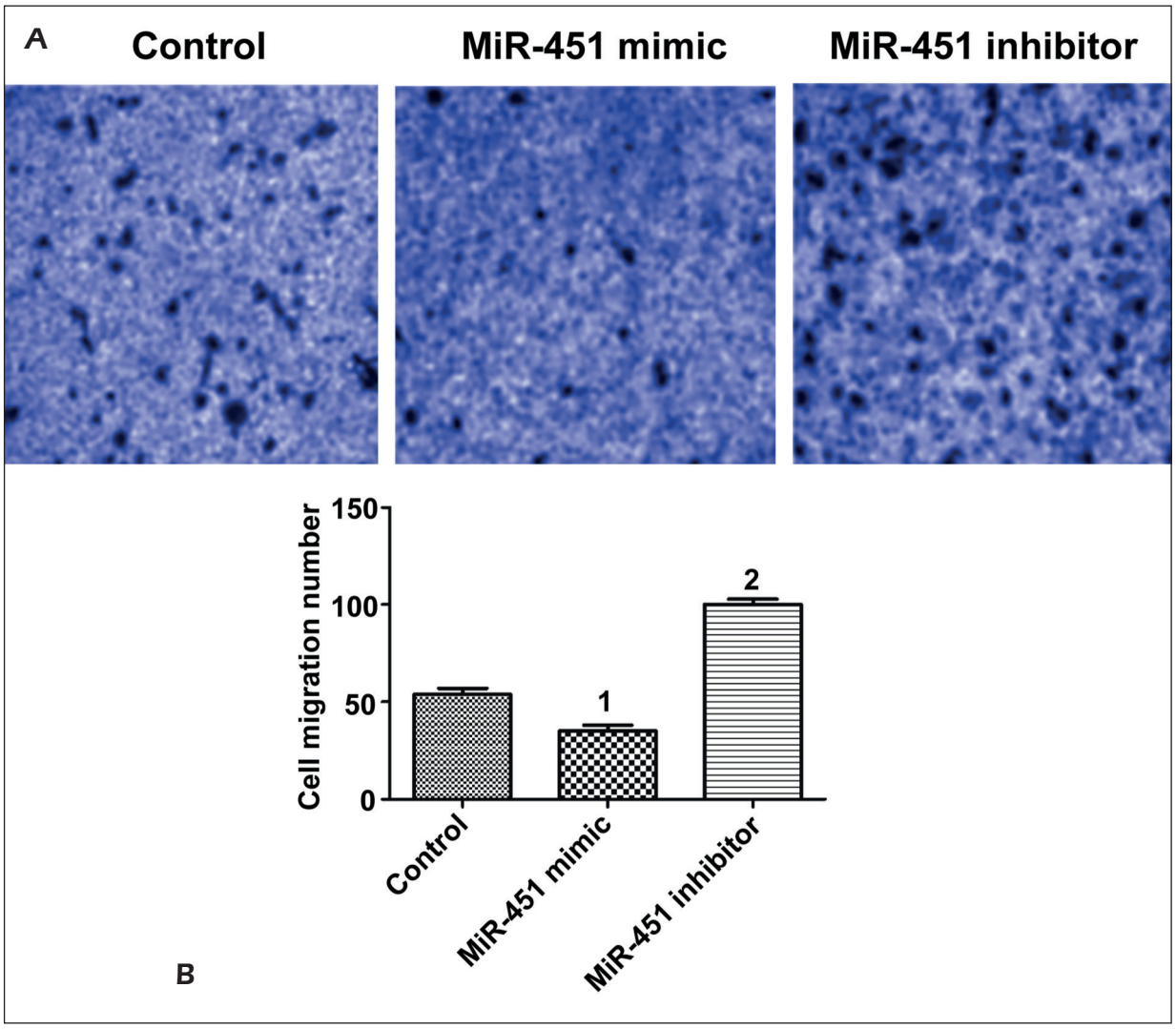

Figure 2. MiR-451 inhibited migration ability of A549 cells. ***A,*** Number of migrating A549 cells in different groups (magnification × 100); ***B,*** Comparison of cell migration ability in different groups. (Note: $^1p < 0.05$ *vs.* control group, $^2p < 0.05$ *vs.* miR-451 mimic group).

the expression of LKB1/AMPK. Yue et al[22] found that the expression of LKB1/AMPK is positively correlated with the proliferation of NSCLC cells. By regulating the expression of LKB1/AMPK in 60 patients with NSCLC, Rupaimoole et al[2] found that the expression of LKB1/AMPK can evidently up-regulate the cell activity in lung cancer group. According to Soutto et al[9], the differentiation and proliferation of cancer cells can be inhibited by blocking the activation of LKB1 signal pathway. The above views are similar to the results of the present study, proving that LKB1/AMPK has a significant effect on the proliferation of lung cancer cells. In NSCLC A549 cells, the expression level of endogenous LKB1 protein is increased, thus activating AMPK phosphorylation and promoting the proliferation of A549 cells. This confirms that miR-451 can effectively inhibit the proliferation and migration of NSCLC cells by blocking the expression of LKB1 and AMPK.

Conclusions

We showed that LKB1/AMPK can be involved in the cell metabolism of NSCLC and miR-451 is negatively correlated with LKB1/AMPK, so miR-451may inhibit cell proliferation and migration of NSCLC *via* regulating LKB1/AMPK, thus providing a new target for the treatment of lung cancer.

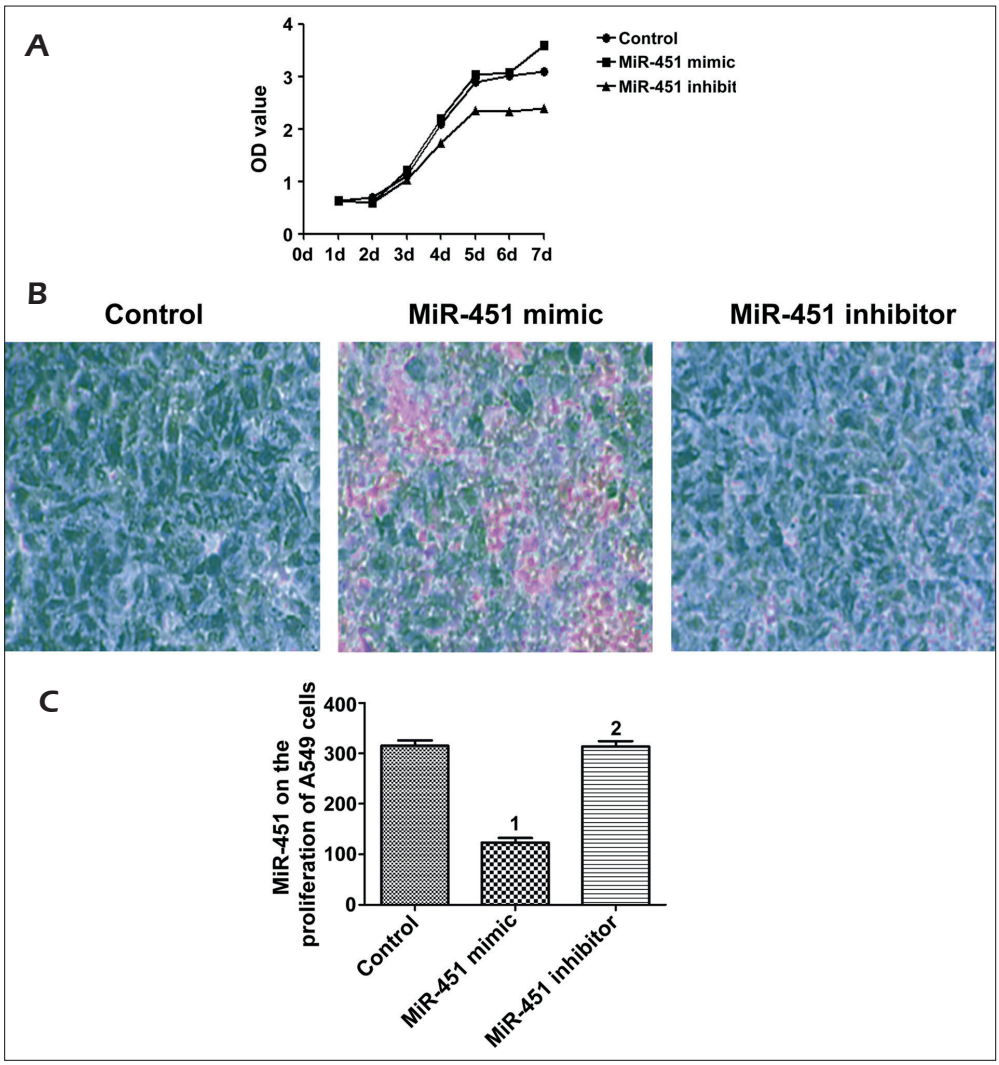

Figure 3. MiR-451 attenuated proliferation ability of A549 cells. ***A,*** Proliferation ability of A549 cells was determined using CCK8 method; ***B,*** Detection of proliferation ability of A549 cells *via* transwell chamber (magnification × 100); ***C,*** Comparison of proliferation ability of A549 cells *via* transwell chamber (Note: $^1p < 0.05$ *vs.* control group, $^2p < 0.05$ *vs.* miR-451 mimic group).

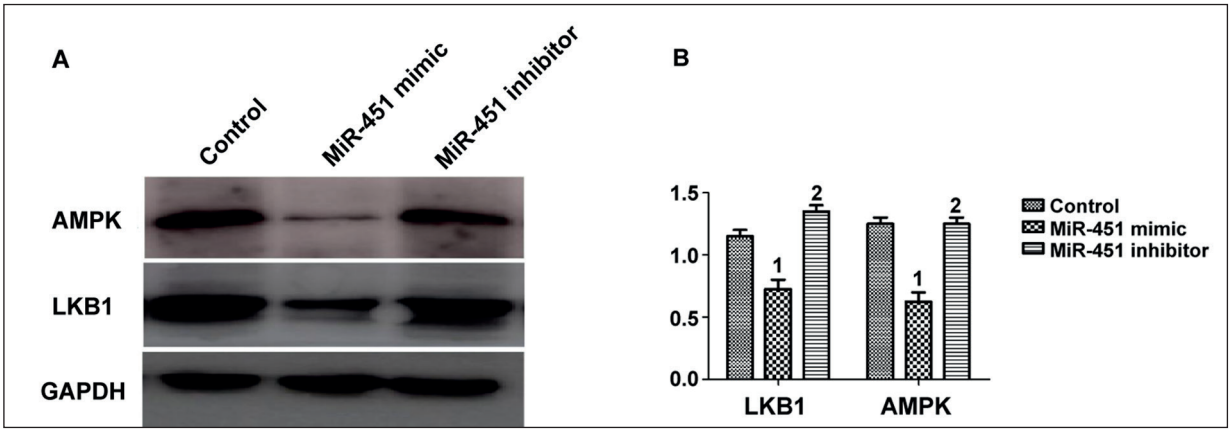

Figure 4. MiR-451 decreased the expressions of LKB1/AMPK in A549 cells. ***A,*** Expression of LKB1 and AMPK kinase activity in the three groups; ***B,*** Differences in the relative luciferase activity among the three groups. Note: $^1p < 0.05$ *vs.* control group, $^2p < 0.05$ *vs.* miR-451 mimic group.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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