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# The effect and mechanism of miR-210 in down-regulating the autophagy of lung cancer cells

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ABSTRACT

This project aims to investigate the roles of miR-210 in autophagy of lung cancer cells and the related mechanism. The expressions of miR-210 and ATG7 in 30 cancer tissues and the adjacent tissues in patients with lung cancer were compared using RT-qPCR methods, Western Blot assay was carried out to test the expression of ATG7 in protein. Moreover, the dual luciferase reporter gene assay system was used to confirm ATG7 is a target gene of miR-210. Furthermore, lung cancer cell line A549 was transfected with either miR-210 mimics or inhibitors and RT-qPCR methods was used to detect the expression of miR-210 and ATG7. Next, MTT assay was used to examine the effect of miR-210 on the growth of the lung cancer cells, and finally, the expression of autophagy related genes, ATG7, LC3-II/LC3-I and Beclin-1 were detected by Western Blot and ICC assay. We observed that miR-210 was significantly increased and ATG7 was markedly decreased in cancer tissue of patients with lung cancer compared with normal tissue. Moreover, results of dual luciferase reporter assay indicated that ATG7 is a direct target of miR-210. Next, transfection of miR-210 mimics in lung cancer cells induced significant increase in cell proliferation, and transfection of miR-210 inhibitors lead to inhibited cell proliferation. Furthermore, over-expression of miR-210 induced marked decrease in the expression of ATG7, LC3-II/LC3-I and Beclin-1, while transfection of miR-210 inhibitors induced significant increase in the expression of ATG7, LC3-II/LC3-I and beclin-1. Our results suggested that miR-210 plays a great role in autophagy of lung cancer cell by targeting ATG7.

## 1. Introduction

Lung cancer is one of the most common malignancies, and it has become the leading cause of cancer deaths worldwide. In recent years, the incidence of lung cancer is rising rapidly, and each year there are about 1 million new cases diagnosed with lung cancer, causing large burden to the public healthcare system. Lung cancer can be divided into two subtypes, non-small cell lung cancer and small cell lung cancer (NSCLC). NSCLC can be further divided into adenocarcinoma, squamous cell carcinoma, large cell carcinoma, etc [1], and it accounts for about 80% of all lung cancer cases. At present, the treatments of lung cancer are mainly the traditional methods, including surgery, radiotherapy and chemotherapy. In spite of the advances in the diagnosis and treatment in recent years, due to various reasons (for example individual differences, drug resistance), the prognosis of NSCLC remains poor [2]. Therefore, to further explore the pathogenesis of NSCLC and identify novel therapeutic targets has profound implications for the development of new anti-lung cancer therapies.

In the recent years, increasing evidences have shown that epigenetic factors such as microRNA (miRNA) may be involved in process of carcinogenesis via regulating the growth and metastasis of the tumors [3]. MicroRNAs are endogenous small RNA molecules of 20–25 nucleotides in length, they can regulate gene expression at post-transcriptional level [4] via repressing mRNA translation and triggering mRNA degradation [5]. Aberrant expressions of miRNAs in different cancers have been discussed previously, and the roles of miRNAs either as oncogenic miRNAs or tumor suppressors have been reported [6]. Recent studies have shown that microRNA-210 (miRNA-210) plays an important role in many various of cancers, including NSCLC [7–10], however, the underlying mechanism is still unclear.

Autophagy, also named type-II programmed cell death, is one of the normal life activities of cells. In normal state, cells often maintain a low level of autophagy as a protective mechanism for the survival and growth; however, when the cells were subjected by certain inside or outside stimulus, abnormal autophagy would happen [11,12]. In this study, we found the miRNA-210 was up-regulated in NSCLC, and miR-

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**Table 1**
Clinical information of the patients.

| | Number | miR-210 High | miR-210 Low | P value |
|---|---|---|---|---|
| Age (years) | | | | 0.7321 |
| ≥50 | 14 | 7 | 7 | |
| < 50 | 16 | 7 | 9 | |
| Tumor size (cm) | | | | 0.0111 |
| < 3 | 12 | 3 | 9 | |
| ≥3 | 18 | 13 | 5 | |
| Degree of differentiation | | | | 0.0099 |
| Low | 15 | 3 | 12 | |
| High | 15 | 10 | 5 | |
| Lymph node metastasis | | | | 0.1533 |
| No | 17 | 6 | 11 | |
| Yes | 13 | 8 | 5 | |

210 can regulate the proliferation and autophagy of NSCLC cells via targeting autophagy-related protein 7 (ATG7).

## 2. Materials and methods

### 2.1. Patients

A number of 30 paired NSCLC tissue and the non-tumor adjacent tissues were obtained from NSCLC patients who have performed surgery at Department of Thoracic Surgery, The First Affiliated Hospital of Soochow University. The tissue samples were immediately frozen in liquid nitrogen after surgery until needed. This study has been proved by the ethical committee of The First Affiliated Hospital of Soochow University, and the informed consent was signed by each patient. The clinical information of the patients was shown in Table 1.

### 2.2. Cell culture

A549 cells (human lung cancer cell line), were cultured in RPMI-1640 medium supplied with 10% FBS (Tianhang Biotechnology, Zhejiang, China) and 1% penicillin/streptomycin (Solarbio, Beijing, China). And these cells were maintained in an incubator with 5% $CO_2$ at 37 °C.

### 2.3. MiRNA transfection

A549 cells were cultured for 24 h in 6-well plates before transfection. The miR-210 mimics and miR-210 inhibitors were transfected into cells by Lipofectamine 2000 (Invitrogen, USA). Four groups were set up, consisting of the hsa-miR-210 mimic transfected group (transfected with mimic), hsa-miR-210 inhibitor transfected group (transfected with inhibitor), negative control transfected group (transfected with lipofectamin, NC) and non-transfected control group (blank/no transfection preformed). Cells were harvested at 48 h following transfection.

### 2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol reagent (Invitrogen) was used to extract the total RNA from the tissue samples and cells based on the manufacturer's instructions. Spectrophotometrically was carried out to measure the concentration of RNA at 260 nm through poly (A) polymerase (Thermo Fisher Scientific, USA). Reverse Transcriptase was performed using a RT kit (Beijing, China). The qRT-PCR was performed by TransStart Top Green qPCR SuperMix (TransGen Biotech, China). With U6 as internal control, all of the primers were synthesized by the GenePharma (Shanghai, China). The sequences of the primers were: miR-210-3p, forward 5′-GTGCAG GGTCCGAGGT-3′, reverse 5′-TATCTGTGCGTGTGACAGCGGCT-3′; U6, forward 5′-CTCGCTTCGGCAGCACA-3′, reverse 5;-AACGCTTCACGAAT TTGCGT-3;; ATG7, forward 5′-AGTGTCACTCTGGAGCAAGC-3′, reverse 5′-AAGAAAGTTGGGCAGATGGTCT-3′; GAPDH, forward 5′- CATTTCC TGGTATGACAACGA-3′, reverse 5′- GTCTACATGGCAACTGTGAG-3′.

### 2.5. Western blot

RIPA buffer (Biomed, China) was used for cell lysis at 4 °C. The total protein samples were collected, and SDS-PAGE was performed to separate the proteins. After that, the separated proteins were transferred onto PVDF membranes and then blocked for 2 h with 5% non-fat dried milk dissolved in 0.1% PBST. The PVDF membranes were then incubated with the primary antibodies (anti-ATG7, ab133528, 1:1000, anti-LC3-II/LC3-I, ab51520, 1:1000 and anti-Beclin-1, ab207612, 1:1000, Abcam, Cambridge, USA) overnight at 4 °C. GAPDH (Sigma, USA) was used as a protein loading control. On day 2, the membranes were incubated with the secondary antibodies (ab6721, 1:5000, Abcam, Cambridge, USA), and then visualized with ECL Western Blotting Substrate (Solarbio, Beijing, China) via Tanon 5200 imaging system (Tanon, Shanghai, China). All experiments were repeated three times at least.

### 2.6. Immunocytochemistry

A549 cells were cultured and fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were then incubated with the primary antibodies (anti-LC3, Abcam, Cambridge, USA) for 30 min, washed and then incubated with the Alexa Fluor 488-conjugated secondary antibodies. The results were visualized and imaged using a fluorescent microscope.

### 2.7. Cell viability assay

MTT assay was performed to test cell viability. After cultivated according to the manufacturer's protocol. Briefly, 10 μl methylthiazolyl tetrazolium (MTT) was added into each well and incubated at 37 °C for 4 h. After that, the absorbance value was measured at 490 nm and each experiment was performed three times.

### 2.8. Bioinformatics

To predict the potential target gene of miR-210, the online bioinformatic tool Targetscan (www.targetscan.org) was used. Based on the results, miR-210 has a paring site at the 3′-UTR of ATG7 thus ATG7 has been predicted as a target of miR-210. The results were shown in Fig. 2B.

### 2.9. Luciferase reportor assay

A549 cells were seeded in 24-well plates and cultivated for 24 h. One hundred nanogram of p3x IRS-MLP -luciferase plasmid, or pGL3-ATG7-3′UTR, or the control-luciferase plasmid, plus 1 ng of pRL-TK renilla plasmid (Promega, Madison, WI), were transfected into the cells using the Lipofectamine 2000 reagent (Invitrogen Co., Carlsbad, CA) according to the manufacturer's instructions. Luciferase and renilla signals were measured 48 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI) according to a protocol provided by the manufacturer. Three independent experiments were performed.

### 2.10. Statistical analysis

All experiments were repeated in triplicate. Results were thought as significant by comparing mean values ( ± standard deviation, SD) by *t*-test or analysis of variance with Turkey's post-hoc test as appropriate with the program SPSS 11.0. $P < 0.05$ was considered as statistically significant.

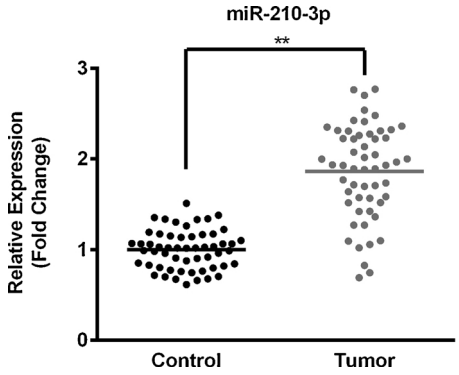


**Fig. 1.** Comparison of the expression of miR-210 in NSCLC tissue and the adjacent tissues by RT-qPCR method. **P < 0.01.

## 3. Results

### 3.1. Up-regulation of miR-210 in NSCLC

In order to explore the roles of miR-210 in NSCLC and the related mechanism, we first compared the expressions of miR-210 in 30 paired lung cancer tissues and the adjacent non-cancer using real-time qRT-PCR. Compared with the normal tissues, the expression of miR-210 was significantly higher (P < 0.01; Fig. 1). Moreover, the expression of miR-210 was positively correlated with the size and differentiation of the tumor (Table 1, p < 0.05).

### 3.2. MiR-210 directly targets ATG7 in lung cancer cells

ATG7 has been predicted as a target gene of miR-210 by online prediction tool Targetscan. Next, to further investigate the relationship between miR-210 and ATG7 in NSCLC, the expression of ATG7 in tumor tissues and the adjacent normal tissues were compared by RT-qPCR and Western-Blot assays. As shown in Fig. 2A, the expression of ATG7 was significantly down-regulate in NSCLC on both mRNA and protein levels (p < 0.01). To further validate the relationship between miR-210 and ATG7, we performed luciferase reporter assay test. It was observed that miR-210 mimics regulate the activity of luciferase in cells carried wild-

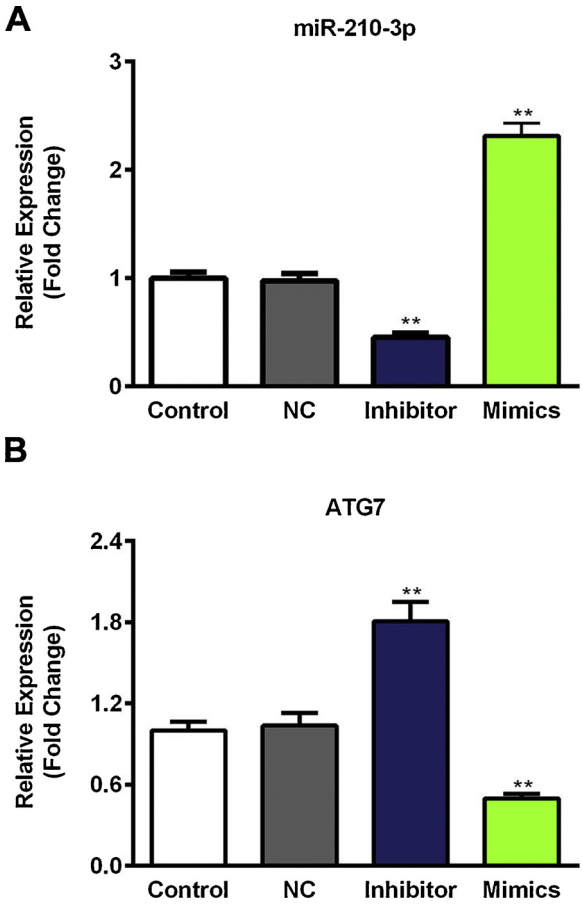


**Fig. 3.** miR-210 depresses ATG7 expression in lung cancer cells. (A) Effect of miR-210 mimics and inhibitors on the expression of miR-210 in A549 cells by RT-qPCR methods. (B) Effect of miR-210 mimics and inhibitors on the expression of ATG7 in A549 cells by RT-qPCR methods. Control, un-treated cells; NC, cells transfected with lipofectamine only; inhibitor, cells transfected with miR-210 inhibitor; mimics, cells transfected with miR-210 mimics. **p < 0.01 v.s. control.

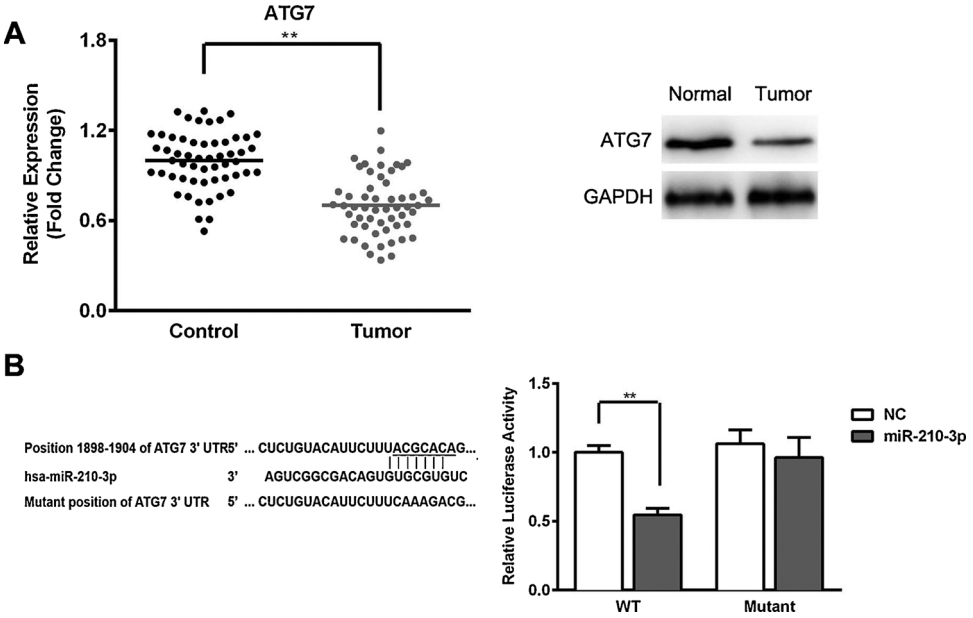


**Fig. 2.** ATG7 is a target of miR-210. (A) Relative mRNA and protein expression of ATG7 in NSCLC tissue and the adjacent tissues by RT-qPCR and western blot methods. (B) Results of luciferase reporter assay. **p < 0.01.

type ATG7 3′UTR (ATG7-3′UTR) ($p < 0.01$; Fig. 2B). After mutation of the binding site, the regulatory relationship disappeared. Taken together, the results certified that ATG7 is a direct target of miR-210.

### 3.3. MiR-210 depresses ATG7 expression in lung cancer cells

To further investigate the function of miR-210 in lung cancer, the miR-210 mimics and miR-210 inhibitor were transfected into A549 cells, and the relative levels of miR-210 and ATG7 were examined by real-time qRT-PCR. When compared with the NC and blank groups, transfection of miR-210 mimics in A549 cells resulted in the significant increase in the expression of miR-210; on the contrary, transfection of miR-210 inhibitor resulted in obvious decrease in miR-210 expression ($P < 0.01$; Fig. 3A). Moreover, miR-210 mimics decreased levels of ATG7 in A549 cell, whereas, ATG7 was elevated when cellular miR-210 levels were inhibited by miR-210 inhibitors ($P < 0.01$; Fig. 3B).

### 3.4. MiR-210 regulates the cell viability of A549 cells in vitro

Next, we further studied the effect of miR-210 on the cell viability of A549 cells. We transfected A549 cells with miR-210 mimics and miR-210 inhibitors and tested its effect on cell viability by MTT assay. When compared with the NC and blank groups, transfection of miR-210 mimics in A549 cells caused remarkably increase in cell proliferation; on the other hand, inhibited expression of miR-210 resulted in inhibited cell proliferation. ($P < 0.05$; Fig. 4)

### 3.5. MiR-210 regulates the autophagy of A549 cells in vitro

Autophagy is a conserved process amongst eukaryotes for degradating and recycling, by which the unwanted and damaged cellular components including proteins and organells in response to diverse stress are eliminated [13]. Autophagy plays a key role in cancer [14–16]. ATG7 is closely associated with the formation of phagophore [17–19], and it can regulate the expression of autophagy related genes Beclin-1 and LC3-II/LC3-I to cause the increase of autophagy [20,21]. To further validate the roles of miR-210 in autophagy of A549 cells, the expression of ATG7, LC3-II/LC3-I and Beclin-1 were examined by Western Blot assay (Fig. 5). The result show that miR-210 inhibitor enhanced the expression of ATG7, LC3-II/LC3-I and beclin-1 ($P < 0.01$); however, over-expression of miR-210 caused observably decrease in the expression of these proteins. ($P < 0.01$; Fig. 6).

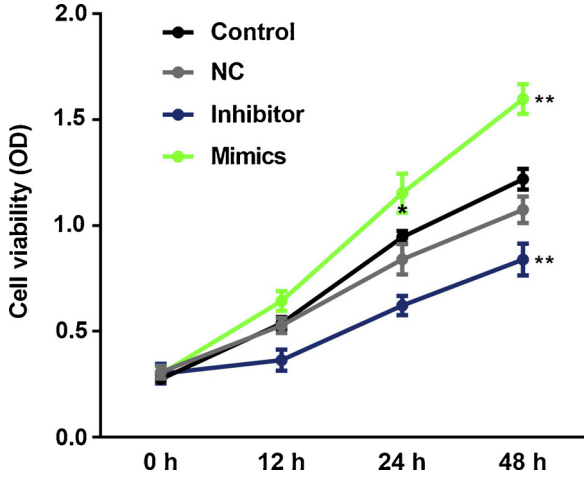


**Fig. 4.** miR-210 regulates the cell viability of A549 cells in vitro. The viability of the cells was examined by MTT assay. Control, un-treated cells; NC, cells transfected with lipofectamine only; inhibitor, cells transfected with miR-210 inhibitor; mimics, cells transfected with miR-210 mimics. **p < 0.01 v.s. control.

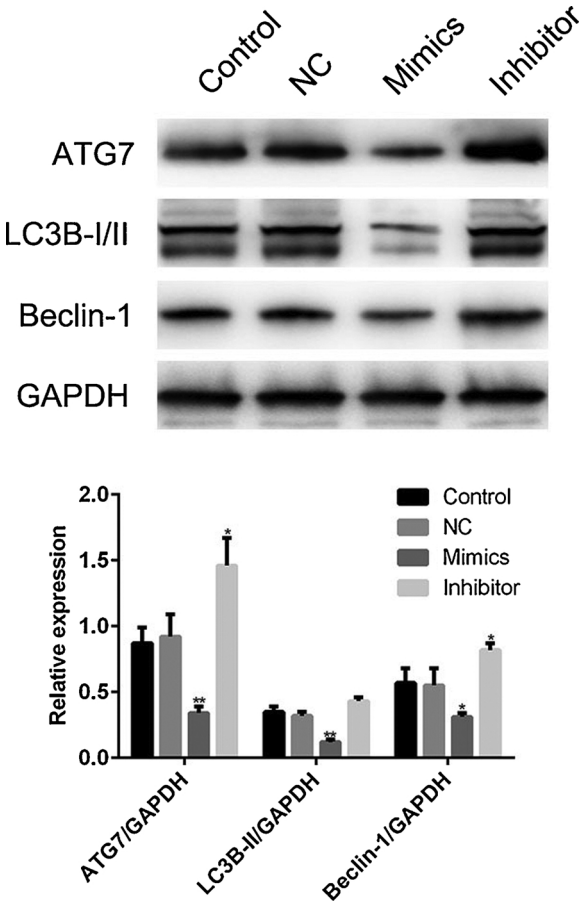


**Fig. 5.** miR-210 regulates the autophagy of A549 cells in vitro. The expressions of autophagy related proteins were examined by western blot methods. Control, un-treated cells; NC, cells transfected with lipofectamine only; inhibitor, cells transfected with miR-210 inhibitor; mimics, cells transfected with miR-210 mimics. *p < 0.05 v.s. control, **p < 0.01 v.s. control.

Moreover, the expression of LC3 was examined by ICC methods. Same as the WB results, it was observed that miR-210 mimics lead to decreased expression of LC3, while miR-210 inhibitors have shown the opposite effects (Fig. 6).

## 4. Discussion

Collectively, the present study demonstrated the aberrant expression of miR-210 in NSCLC and the potential roles of miR-210 in modulating cancer growth by regulating autophagy. Our study may provide a novel therapeutic target for the treatment of lung cancer.

Autophagy is a highly conserved catabolic process that degrades misfloded or damaged proteins or organelles to provide energy for cells. The activity of autophagy is generally low in normal cells. However, in different stages and types of tumor, autophagy plays different roles. On the one hand, autophagy plays a crucial role in preventing the accumulation of damaged proteins and organelles which are toxic and carcinogenic. As reported, autophagy related protein Beclin1 is a key protein promoter of autophagy, was the first to be confirmed, and autophagy activation is directly related to the tumor suppressor gene Beclin1 deletion caused by tumor [22]. LC3 is the only protein that has been found to be involved in the regulation of signal transduction, and is a good marker of autophagy [23].

MicroRNAs can bind to the target gene 3′-UTR and induce mRNA degradation or transcriptional suppression, then inhibiting the synthesis of the target protein and regulating cell proliferation, apoptosis and autophagy. Particularly, miRNAs expression profiles are able to change

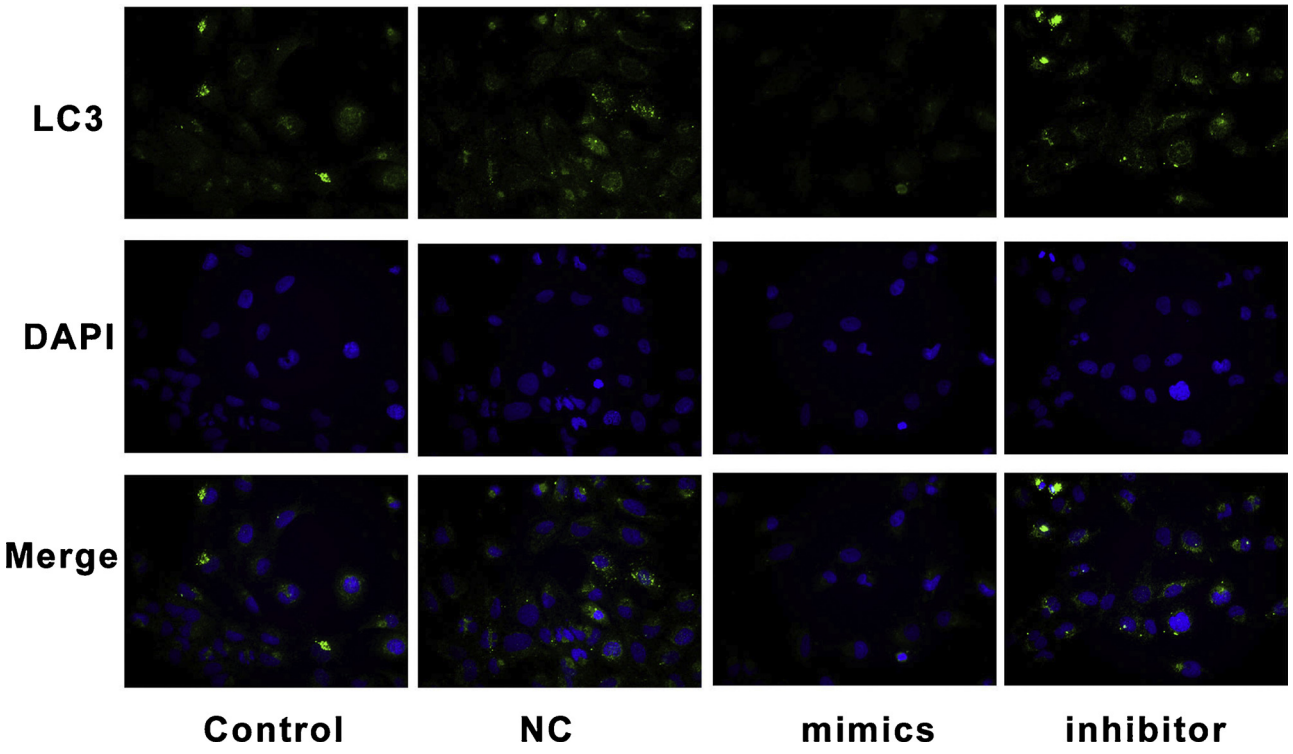


**Fig. 6.** Effect of miR-210 on the expression of LC3 by ICC methods. Magnification, 200×.

the level of autophagy and then influences cancer growth and development [24–27]. In recent years, several studies have proved that miR-210 regulates cancer gene in breast, prostate cancer, colon cancer, gliblastoma and lung cancer as well [28–31]. Functions and target genes of miR-210 are largely unknown especially in autophagy process. In this study, we demonstrated that miR-210 expression was noticeably upregulated in lung cancer cells and tissues as compared with that in normal cells and tissues, moreover, up-regulation of miR-210 induced significant decrease in the expression of autophagy-related protein, LC3 and Beclin-1, while down-regulation of miR-210 has shown the opposite effects. These results indicated that miR-210 can regulate the autophagy of A549 cells in vitro

miRNAs were known to exert their function via inhibiting the expression of their target genes. To further explore the underlying mechanism of miR-210 induced inhibition of autophagy in A549 cells, we performed bioinformatic analysis, and ATG7 was predicted as a target of miR-210. ATG7 was known as an autophagy related gene [32], and previous study indicated that knockdown of ATG7 by siRNA decreased the autophagic activity of A549 cells [34]. However, it remains unclear if miR-210 can regulate the autophagy of A549 cells via targeting ATG7. In the present study, we found that ATG7 was down-regulated in NSCLC tissue; moreover, transfection of miR-210 mimics inhibited the expression of ATG7 in A549 cells, while transfection of miR-210 inhibitor has shown the opposite effects; finally, results of dual-luciferase reporter assay confirm that ATG7 is a direct target of miR-210. Taken together, these results suggested that miR-210 can directly target ATG7 to reduce the expression of autophagy related proteins LC3-II/LC3-I and Beclin-1 in lung cancer.

To sum up, we reported for the first time that miR-210 can regulate the proliferation and autophagy of A549 cells via targeting ATG7. Our findings presented the mechanism of miR-210 regulate the autophagy process and may provide a new perspective for the clinical therapy.

## Conflict of interest

None.

## Acknowledgements

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