


# miR-96-5p regulates autophagy through targeting ATG9A in lung cancer

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## ABSTRACT

**Objective:** Lung cancer is the leading cause of cancer-related deaths among all types of cancer worldwide. Autophagy is a cellular process involving lysosomal degradation of damaged organelles and long-lived proteins. It provides nutrients and energy to cancer cells by breaking down damaged proteins and organelles and contributes to tumor-cell survival by enhancing stress tolerance and supplying nutrients to meet the metabolic demands of tumors. Therefore, the combination of cytotoxic chemotherapy and autophagy inhibition strategies has been proposed. This study aimed to investigate how miR-96-5p regulates autophagy in A549 and HTB-54 lung cancer cell lines.

**Methods:** MiR-96-5p expression levels in lung cancer cell lines and normal bronchial epithelium were measured by qPCR. The functional role of miR-96-5p on autophagy and its modulatory effects were investigated in vitro by overexpression studies.

**Results:** miR-96-5p was found to be overexpressed in A549 and HTB-54 lung cancer cell lines compared to the normal Beas2B cell line. Overexpression of miR-96-5p resulted in the attenuation of starvation-induced autophagy. It was shown that miR-96-5p suppressed autophagy by targeting ATG9A. Both mRNA and protein cellular levels of ATG9A were decreased in cells upon miR-96-5p overexpression.

**Conclusion:** This study demonstrated that miR-96-5p might be a candidate for autophagy inhibition in lung cancer. The effects of autophagy inhibition by miR-96-5p and cytotoxic chemotherapy should be further examined.

**Keywords:** Autophagy, microRNA, miR-96-5p, lung cancer

## INTRODUCTION

Lung cancer is the most common type of cancer worldwide, contributing to 11,6% of all new cancer cases according to the Globocan 2018 (1). Surgery, radiotherapy, and chemotherapy are the main therapeutic strategies. However, despite treatment, the survival rate is approximately 5 years, which is still at a very low level (2). Treatments can reduce tumor growth but usually relapse occurs because genetic heterogeneity and tumor plasticity contribute to drug resistance and metastasis, which are responsible for mortality (3).

The term autophagy was first used by Christian de Duve in 1963, shortly after lysosome discovery. It is derived from ancient Greek and literally means “self-eating” (4). Autophagy is an evolutionarily conserved mechanism from yeast to mammals and it is a stress response regulating cellular metabolism and homeostasis by eliminating toxic components (5). Three different types of autophagy were defined mechanistically and morphologically: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy (herein referred to as autophagy) involves lysosomal degradation of intracellular damaged proteins and organelles by sequestering them into autophagosomal

double membrane vesicles first, fusing with lysosomes and then the re-introduction of the building blocks to the cell. Microautophagy uptakes cargo directly into the lysosome to be degraded by invagination of the lysosomal membrane. Chaperone-mediated autophagy relies on the transportation of unfolded proteins to the lysosomal membrane (6).

When cells are exposed to stress conditions such as growth factor deficiency, oxygen deficiency, and starvation, autophagy is stimulated for the destruction of organelles and proteins, and the resulting building blocks are involved in cell homeostasis by providing nutrients and energy to cells (7). Recent studies have shown that autophagy contributes to physiological processes such as cell differentiation, regulation of cell metabolism, and destruction of intracellular pathogens. Furthermore, it has been shown that defects in the autophagy mechanism are associated with pathophysiological conditions such as cancer, neurodegenerative, and infectious diseases (8).

Autophagy has both oncogenic and tumor-suppressor roles in cancer. Its role in tumorigenesis is context dependent and is affected by cellular conditions, type, and stage of the tumor. It

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functions as a tumor suppressor by eliminating damaged proteins and organelles at early stages and prevents cancer formation by assisting in reducing genomic instability. However, autophagy supports cancer cells against cellular stresses such as oxygen and nutrient deficiency, endoplasmic reticulum and mitochondrial stress, and chemotherapy and radiotherapy at later stages. It contributes to chemotherapy resistance by supplying energy to the cancer cells through recycling of damaged proteins and organelles (9). The currently used chemotherapeutic agents for treatment of lung cancer promote cell death. However, relapse and drug resistance are common. Therefore, autophagy inhibition strategies have been proposed together with cytotoxic chemotherapy in order to increase the effectiveness of treatment. However, the only autophagy inhibitor approved by the United States Food and Drug Administration (FDA) is hydroxychloroquine, a chloroquine derivative (10). Because hydroxychloroquine is a chemical compound and causes various adverse effects, researchers have focused on finding miRNAs targeting autophagic genes.

MicroRNAs (miRNAs) are single-stranded noncoding RNAs of 17–25 nucleotides in length. They exert their functions by degrading their target mRNAs or suppressing their translation into protein through base-pairing with targets. Therefore, miRNAs can be defined as post-transcriptional negative regulators of genes. miRNAs have been shown to be associated with several diseases, including cancer (11). As noted in all cancers, there are miRNAs correlated with lung cancer that promote cancer or suppress invasiveness (12). Further, several miRNAs have already been identified, patented as biomarkers, phase I and phase II studies have been conducted on them and can be found in Chakraborty's review for further reading (13).

This study aimed at revealing whether miR-96-5p has a role in suppressing autophagy pathway in non-small-cell lung carcinoma model A549 and HTB-54 cell lines.

In order to produce mechanistic data explaining the mechanism by which gene autophagy is regulated, ATG9A, one of the autophagy genes, was selected as a target gene of miR-96-5p by using bioinformatic data tools. The miR-96-5p mimic transfection was performed on lung cancer cell lines and its effect on autophagy was investigated. It has been shown to suppress autophagy by regulating ATG9A.

## METHODS

### Cell lines and Cell Culture

For cancer cell lines, A549 (CCL-185) and HTB-54 (Calu-1) were used and Beas2B (CRL-9609) was used as a normal bronchial epithelium. They were purchased from ATCC (American Type Cul-

ture Collection, VA, USA). Beas2B was cultured in Roswell Park Memorial Institute; A549 and HTB-54 were cultured in Dulbecco's modified eagle's medium, both supplemented with 10% fetal bovine serum and 50 U/ml penicillin/streptomycin and cultured in a 5% CO<sub>2</sub>-humidified incubator at 37°C. Research procedures were in accordance with the ethical standards of the Declaration of Helsinki of 2018.

### Selection of ATG9A as a Target of miR-96-5p

The miRNAs interact with their target mRNAs through base-pairing. miRBase, TargetScan, and miRDB bioinformatic databases were used to select miR-96-5p target based on base-pairing strength and the presence of conserved 7-mer and 8-mer sites that match the seed region of the miR-96-5p. ATG9A was computationally selected as a strong predicted target which plays a role in autophagosome formation.

### Transfection

Cells were transfected with miR96-5p mimic (Cat no. MSY0000095 Qiagen, Germany) using HiPerFect Transfection Reagent (Qiagen, Germany) following the manufacturer's instructions. All-Stars Negative Control siRNA (Cat:1027281 Qiagen, Germany) was used as negative control for fluorescence microscopy analysis of autophagy pMRX-IP-GFP-LC3-RFP-LC3-ΔG plasmid cotransfected with miR-96-5p mimic using Lipofectamine 3000 (Thermo Fisher Sci. US).

### RNA Isolation and RT-PCR Analysis

Total RNA was isolated using miScript miRNeasy Mini Kit (Qiagen, Germany), cDNA was synthesized using miScript II RT Kit (Qiagen, Germany) following the manufacturer's instructions. The microRNA and mRNA gene expression analysis were performed using miScript SYBR Green PCR kit (Qiagen, Germany). For the analysis of relative gene expression data and calculation of fold changes, 2<sup>-ΔΔCt</sup> method was used. To normalize data in terms of RNA added to the reverse transcription reactions, internal control genes were used. Whereas RNU-6 (Cat: 218300 Qiagen, Germany) was used as an internal control for miR-96-5p (MS00003360, Qiagen, Germany) expression analyses, GAPGH (Cat:QT00079247 Qiagen, Germany) was used for ATG9A (QT01025262 Qiagen, Germany). In order to normalize data and to calculate ΔCt, difference between Ct values of the gene of interest and the housekeeping gene was calculated. Then, to calculate ΔΔCt; difference of ΔCt values between the test and control samples was calculated. The fold change was then equal to 2<sup>-ΔΔCt</sup>.

### Western Blot

Cells were washed with cold 1X PBS, and then total protein was extracted using RIPA buffer containing protease inhibitors cocktail. Total protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Sci. US). An SDS-PAGE gel was loaded with 20 μg total protein and transferred using wet transfer electroblotting (Bio-Rad, America) under a constant current overnight at +4 °C. After membranes were incubated in 5% (w/v) skimmed milk, incubated with primary antibodies overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Chemiluminescent imaging system was used to obtain protein bands. (Vilmer Lourmat, Germany).

#### Main Points:

- miR-96-5p is a modulator of autophagy.
- miR-96-5p is repressor of ATG9A.
- miR-96-5p might be used to sensitize cancer cells to chemotherapeutic agents.

**GFP-LC3 Analysis**

Cells were seeded on cover slides. After 48 hours of cotransfection of mimic and the plasmid, cells were fixed with 3.7% formaldehyde for 20 min. Images were obtained with Leica DMIL Led DFC450 microscope (Leica, Germany). After normalization with a threshold, the GFP dots were counted and compared between groups.

**Statistical Analysis**

Statistical analyses and comparisons between groups were performed by Student’s t-test and Mann-Whitney U test using SPSS Statistics V22.0 (IBM SPSS Corp.; Armonk, NY, USA) programme. A  $p < 0.05$  was considered statistically significant.

**RESULTS**

**miR-96-5p was Overexpressed in Lung Cancer Cell Lines**

Expression levels of miR-96-5p were determined by RT-PCR. Normal lung cell line and lung cancer cell lines were compared and values were normalized with RNA-U6. The miR-96-5p was found 2.5 fold increased in A549 and 2.3 fold increased in HTB-54 cell line compared to Beas2B normal cell line (Figure 1) ( $p = 0.0023$  and  $p = 0.0053$ , respectively).

**miR-96-5p Blocked Starvation-Induced Autophagy**

Protein levels of LC3, marker of the autophagy, were detected by western blot, and the cells were cotransfected with GFP-LC3-containing plasmid and the mimic in order to show whether miR-96-5p regulates autophagy. The cells were incubated with HBSS for 4 hours before fixation to induce autophagy. Change in autophagosome numbers were evaluated. It was shown that overexpression of miR-96-5p repressed starvation-induced GFP-LC3 dot accumulation (Figure 2) which represents autophagic vesicles. These results showed that miR-96-5p is an autophagy regulating microRNA. This was also confirmed by western-blot analysis. When autophagy is induced, Arginine amino acid at the C-terminus of LC3 is cleaved by an enzyme called Atg4 (cysteine

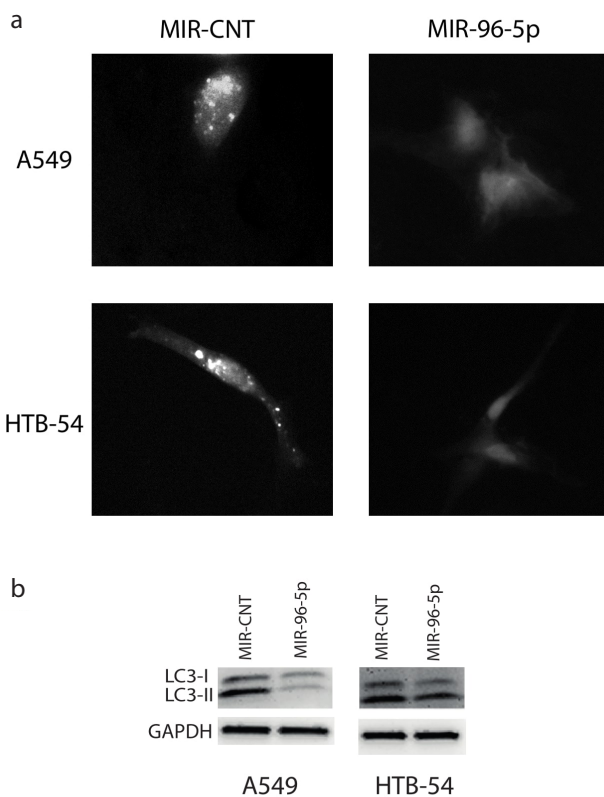
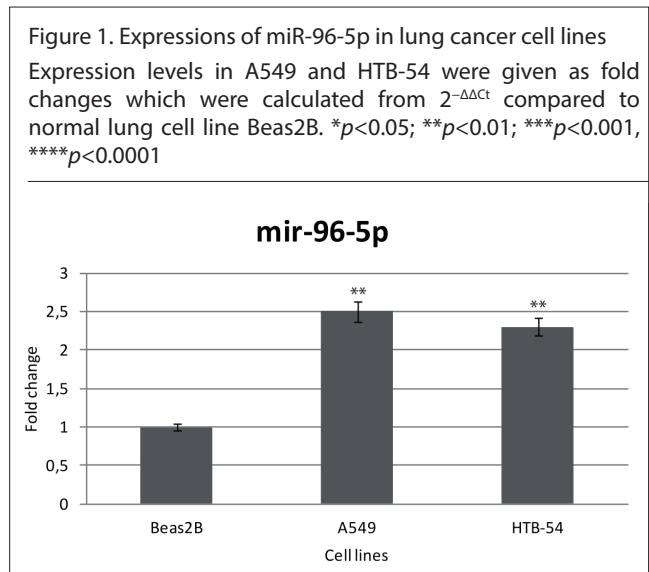
protease) and LC3-I type is formed. Binding LC3-I to phosphatidylethanolamine (PE) leads to the formation of LC3-II. As shown in Figure 3, lipid conjugation of LC3-I was attenuated after miR-96-5p transfection.

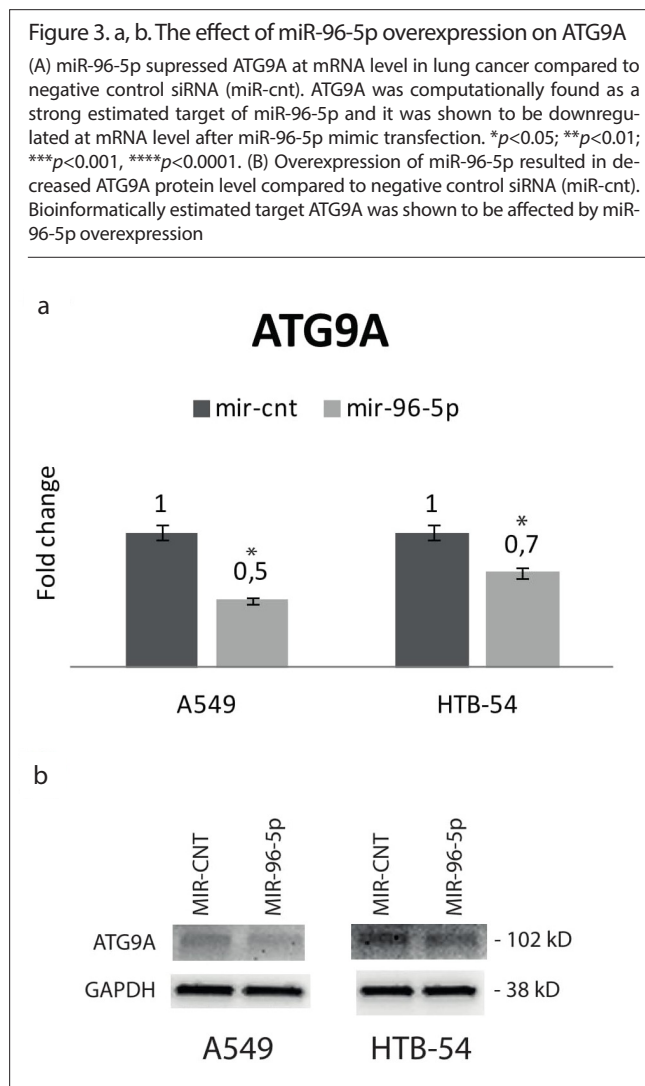
**miR-96-5p Repressed Autophagy-Related Target ATG9A**

Bioinformatics tools were used to identify a predicted target of miR-96-5p, and autophagy-related gene—ATG9A—the only transmembrane autophagy protein required for autophagosome formation, was selected as a target to demonstrate the mechanism by which autophagy is regulated. Cells were transfected with miR-96-5p and the effects on ATG9A was evaluated. MiR-96-5p has been shown to suppress autophagy by targeting ATG9A. Both ATG9A mRNA (Figure 4) and protein levels (Figure 5) were decreased after transfection in both cell lines.

Figure 2. a, b. The effect of miR-96-5p overexpression on starvation induced autophagy

(A) Immunofluorescence imaging of autophagosomes. For immunofluorescence analysis, cells were transfected with miR-96-5p mimic or negative control siRNA (miR-cnt) simultaneously with the plasmid containing GFP-LC3. Cells were starved for 4 hours prior to fixation to induce autophagy. Decreased numbers of GFP-LC3 puncta is evidence of decreased autophagosomes. (B) miR-96-5p suppressed autophagy in lung cancer compared to negative control siRNA (miR-cnt). Since LC3-II is degraded by autophagy mechanism, it is used as a marker of autophagy and band density of LC3-I, and LC3-II was evaluated. When autophagy is stimulated, arginine amino acid at the C-terminus of LC3 is cleaved by an enzyme ATG4 (cysteine protease) and LC3-I type is formed. LC3-I is bound to phosphatidylethanolamine (PE) to form LC3-II. The increase in LC3-II band density indicates that autophagy is stimulated if there is no problem with lysosomal fusion and degradation, whereas the decrease of LC3 is interpreted vice versa





**DISCUSSION**

MicroRNAs are small noncoding RNAs that negatively regulate gene expression at the transcriptional or post-transcriptional level. They have been found to regulate many cellular processes, including autophagy, and have been associated with many diseases, including cancer (14, 15). The role of miRNA-mediated autophagy in cancer treatment is controversial. Whether autophagy needs to be suppressed or induced should be decided based on tumor type, stage, and tumor microenvironment.

Many cancers have been associated with miR-96-5p in previous studies (16-19). miR-96-5p was found to be overexpressed in breast, prostate, bladder, and colorectal cancers compared with that observed in normal tissue and is defined as an oncogenic miRNA (16-19). In addition, overexpression of miR-96-5p has been shown to increase cell proliferation in prostate, lung, and breast cancer and also induce cell migration in lung and breast cancer (17, 20, 21). Conversely, miR-96-5p suppresses cell migration in osteosarcoma and renal cancer and has been reported as a tumor suppressor (22, 23). The expression level of miR-96-5p was found higher in non-small cell lung cancer and non-small cell epidermoid cancer cell lines compared to the normal lung

cell line in this study. Furthermore, miR-96-5p has been revealed to inhibit autophagy by suppressing Atg9A when it is overexpressed by mimic transfection. ATG9A suppression was shown at both mRNA and protein levels. It is important to show protein expression levels when studying miRNAs because at the molecular level, miRNAs mainly exert their function by affecting target mRNA stability and/or by down regulating their translation.

In humans, ATG9 has two different isoforms; ATG9A and ATG9B. ATG9A is found in all tissues, while ATG9B is concentrated in the placenta and pituitary, and is stimulated by hypoxic conditions (24). ATG9 is the only transmembrane autophagy protein required for autophagosome formation. It plays an important role in organization of nucleation site for formation of precursor autophagosomal structures and in phagophore elongation (25). Previous studies have shown that there are numerous membrane sources that contribute to autophagosome formation, such as endoplasmic reticulum, golgi apparatus, and plasma membrane (26). ATG9 is thought to play an important role in autophagosome formation by directing membrane from these donor organelles (27). Therefore it is important to understand ATG9-related regulatory mechanisms to better understand autophagic machinery. There have been studies in which autophagy was associated with both cell survival and cell death. Therefore, the contribution of autophagy to cancer cell death is controversial and context-dependent. Many anti-cancer treatments including chemotherapy, radiotherapy, and targeted therapies have been shown to induce autophagy (28, 29). In response to these anti-cancer treatments, autophagy is activated to protect cells from stress-related damages and it appears as a mechanism that contributes drug resistancy in cancer cells as in the study of Chen *et al* (30). Therefore, targeting of autophagy with combination of chemotherapy has been proposed as a new strategy and clinical studies based on this approach are being conducted (10). Since hydroxychloroquine is the only FDA approved chemical compound that inhibits autophagy and causes various adverse effects, researchers have been focused on finding miRNAs targeting autophagic genes. As miRNAs are already present physiologically in the human body, they are thought to lead fewer adverse effects. Our study showed that miR-96-5p has a regulatory role on autophagy by downregulating ATG9A. Structural analysis of microRNA-target interaction is needed in order to show whether ATG9A is a direct target of miR-96-5p. A mutation should be introduced in miRNA target recognition site by site directed mutagenesis and then direct targeting could be finally confirmed. We also bioinformatically checked for regulatory roles of miR-96-5p on other autophagy genes and we found that ATG7 might be a predicted target gene of miR-96-5p. Ubiquitin-like modifier-activating enzyme ATG7 is essential for autophagy and required for cytoplasm-to-vacuole targeting (Cvt). Interaction between ATG7 and miR-96-5p should be further investigated to obtain deeper mechanistic view of autophagy regulation by miR-96-5p.

**CONCLUSION**

After suppression of autophagy, cancer cells became more sensitive to chemotherapy and radiotherapy. Previous studies showed that a combination of chemotherapy and autophagy inhibition by miRNAs can be used as an effective approach for treatment.

Although miR-96-5p has been shown to induce proliferation and cell migration in lung cancer, this is a dose-dependent effect and its effect on autophagy and cell survival with chemotherapeutic agents should be investigated.

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