

Targeting the HMGA2 oncogene by miR-498 inhibits non-small cell lung cancer biological behaviors

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Abstract. – OBJECTIVE: Previous study reported that miR-498 served as a tumor suppressor in non-small cell lung cancer (NSCLC), but the underlying mechanism remains largely unknown. The aim of this study is to investigate the role of miR-498 and its target gene HMGA2 in NSCLC progression.

PATIENTS AND METHODS: The expression of miR-498 was assessed in clinical NSCLC specimens and cell lines using RT-PCR. Overexpression of miR-498 and transfection of pLenti-HMGA2 were performed in A549 cells. Cell proliferation, apoptosis, migration, and invasion were determined using cell counting kit-8 (CCK-8) assay, clone formation assay, flow cytometry, and transwell assay, respectively. Luciferase reporter assays were performed to analyze the regulation of putative target of miR-498. Western blot was used to detect the levels of HMGA2 in A549 cells.

RESULTS: MiR-498 was found to be down-regulated in NSCLC tissues and cell lines. After miR-498 mimics transfection, cell proliferation, migration, and invasion were significantly suppressed in the NSCLC cells. Mechanistically, bioinformatic analysis predicted that miR-498 may target the 3'-UTR of HMGA2 and suppressed its translation, and was further confirmed by luciferase assay. Furthermore, restoration of HMGA2 expression completely rescued the inhibitory effect of miR-498 in NSCLC cells.

CONCLUSIONS: This paper revealed that miR-498 may serve as a tumor suppressor in NSCLC through targeting HMGA2, suggesting that miR-498 could represent a novel target for effective therapies.

Key Words:

miR-498, NSCLC, HMGA2, Proliferation, Metastasis.

Introduction

Lung cancer has ranked as the first cause of cancer-related death worldwide, accounting for

1.59 million estimated deaths per year¹. Non-small cell lung cancer (NSCLC) represents approximately 85% of all cases of lung cancer². Although advances in combination treatment strategies involving surgery, radiotherapy, and chemotherapy, NSCLC still remains as the most aggressive malignant tumor with a poor 5-year overall survival rate^{3,4}. The leading cause of death from lung cancer is metastasis⁵. Thus, it is critical to understand the molecular mechanisms involved in NSCLC metastasis.

MicroRNAs (miRNAs) are a class of small non-coding RNAs, 17-25 nucleotides long, that associate with 3'-UTR of specific target mRNAs⁶. It has been confirmed that miRNAs were involved in many crucial cellular processes, including apoptosis, differentiation, invasion, and proliferation^{7,8}. Recently, growing evidence showed that the aberrant expression of miRNAs had been linked to tumor initiation, progression, and prognosis^{9,10}. Indeed, depending on their targets, miRNAs act as tumor suppressors or oncogenes. In human NSCLC, the clinical significance and biological function of several miRNAs have been identified, such as miR-204¹¹, miR-377¹², and miR-448¹³. MiR-498, similarly, has been found to function as a potential tumor suppressor in NSCLC¹⁴, while its underlying molecular mechanism in NSCLC remains unclear.

The high mobility group AT-hook (HMGA) proteins, a family of DNA architectural factors, can modulate transcription by binding to DNA¹⁵. It has been confirmed that HMGA2 served as a key regulator of cellular processes, such as cell cycle, proliferation, and apoptosis^{16,17}. More and more evidence showed that HMGA2 served as oncogenes and to be overexpressed in almost all human tumors, including breast cancer, thyroid carcinomas, and NSCLC¹⁸⁻²⁰. On the other hand,

many studies^{21,22} reported that several miRNAs can bind to the 3'UTR of HMGA2 mRNA. However, the reports to date have not described a relationship between HMGA2 and miR-498. In this study, we aimed at investigating the exact roles of miR-498 and its underlying molecular mechanisms in NSCLC.

Patients and Methods

Patients and Samples

Primary NSCLC samples and matched normal lung tissues were collected from 44 patients. These patients were diagnosed with NSCLC and accepted surgery operation at Jining No. 1 People's Hospital. These tissues were collected following surgical resection, and stored at -80°C immediately for further RNA. Informed consent was obtained from all individual participants included in the study. All samples were approved for use in this research by the Ethics Committee of Jining No. 1 People's Hospital

Cell Culture and Transfection

A total of four NSCLC cell lines (A549, SK-MES-1, H1299, and 95D) and human embryonic lung fibroblast cells (HELFL) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. All cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C .

MiR-498 mimic and corresponding negative control miRNA (miR-NC), plasmids carrying human HMGA2 were purchased from Shanghai GenePharma (Pudong, Shanghai, China). About 1×10^5 A549 cells were seeded in 6-well plates and transfected with miR-498 mimics and plasmids using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Quantitative Real-time PCR

Total RNA was extracted from the tissues and cells using a miRcute miRNA isolation kit (Jiangning, Nanjing, China) according to the manufacturer's instructions. For the detection of miR-498, cDNA was synthesized from 1 mg of total RNA by means of a reverse reaction kit. Quantitative Real-time PCR was performed using 2 \times SYBR Green PCR Mix (Aidlab, Haidian, Beijing, China) following the procedure of the manufacturer. GAPDH was used

as the internal control. The primer sequences used were: miR-498, 5'-TTTCAAGCCAGGGGGC-GTTTTTC-3' (forward) and

5' - G C T T C A A G C T C T G G A G G T - G C T T T T C - 3' (reverse); GAPDH,

5' - A T G T C G T G G A G T C T A C T G G C - 3' (forward) and

5'-TGACCTTGCCCACAGCCTTG-3' (reverse). Fold change of miR-498 was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.

Cell Proliferation Assay

Cell counting kit-8 (CCK-8) assay was utilized to evaluate the proliferation of NSCLC cells. Cells were cultured in a 96-well plate at a concentration of 1×10^4 cells/mL. Subsequently, 10 μL of CCK-8 reagent was added to each well, followed by incubation for another 1.5 hours. Then, absorbance was measured at 450 nm and each experiment was performed three times.

Colony Formation

Cells were placed in a 6-well plate at a density of 1×10^3 cells/well and cultured for 15 days. The colonies were stained with 1% crystal violet for 10 mins after fixation with 10% formaldehyde for 5 mins. Colonies containing at least 50 cells were scored. Each experiment was performed in triplicates.

Flow Cytometry Analysis

Flow cytometry was used to detect the cell apoptosis. The procedure was shown in the previous study²³.

Migration and Invasion Assays

For migration assay, 5×10^4 transfected cells were placed in the upper chamber of each insert. For invasion assay, 5×10^4 transfected cells were placed in the upper chamber of each insert coated with 150 mg Matrigel. For both assays, 500 ml Dulbecco's Modified Eagle Medium (DMEM) culture medium containing 10% FBS was added into the lower chamber. Cells were plated in the upper chamber of quadruplicate wells at a density of $1 \times 10^5 \text{ mL}^{-1}$ and incubated at 37°C for 24 h. After overnight incubation, top cells were removed, and bottom (migrated) cells were fixed and stained with crystal violet to visualize nuclei. The cell number was determined using IPP software (Media Cybernetics, Bethesda, MD, USA).

Luciferase Activity Assay

For the dual luciferase assay, cells were seeded in 24-well plates at a density of 1×10^5 cells

per well. The cells were then co-transfected with 0.2 mg/ml of vector with the wild-type or mutant 3'UTR of HMGA2 gene. Luciferase activity levels were measured using the Dual-Luciferase Reporter Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol.

Western Blot

Western blotting was performed as described previously²⁴. Briefly, after 48-h transfection, cell debris was removed by centrifugation, and total protein was extracted using radioimmunoprecipitation assay (RIPA) cell lysis buffer. Then, the protein was separated with 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which was then transferred to polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the membrane with a primary monoclonal antibody to HMGA2 was incubated for 2 h at room temperature. The next day, the horseradish peroxidase (HRP) conjugated secondary antibodies with a proper dilution were incubated for 1 h at room temperature. The fold changes of protein levels were analyzed by the Image J software (Rawak Software, Stuttgart, BW, Germany). GAPDH was used as an internal control, and each experiment was repeated at least thrice.

Statistical Analysis

Statistical analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). The *t*-test

was used to analyze the statistical significance between different groups. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

MiR-498 is Downregulated in Both NSCLC Tissues and Cell Lines

MiR-498 expression levels in 44 pairs of NSCLC tissues and pair-matched adjacent normal tissues were examined by qRT-PCR. As shown in Figure 1A, we found that miR-498 expression was significantly down-regulated in NSCLC tissues compared to their non-tumor lung tissues ($p < 0.01$). In addition to NSCLC tissues, we further detected the expression levels of miR-498 in four NSCLC cells and normal HELF cells. As shown in Figure 1B, a significantly lower expression of miR-498 was found in NSCLC cell lines compared to normal HELF cells (all $p < 0.01$). Those results revealed that miR-498 may be involved in the progression of NSCLC.

Effects of miR-498 Upregulation on the Biological Behaviors of A549 Cells

Then, we further explored the biological role of miR-498 in A549 cells. A549 cells were transfected with NC or miR-498 mimics. As shown in Figure 2A, there was a significant increase in the miR-498 level in the A549 cells post-transfected with miR-21 mimics. Then, the results of CCK-8 showed that the proliferation rate of A549 cells infected with miR-498 was significantly decreased.

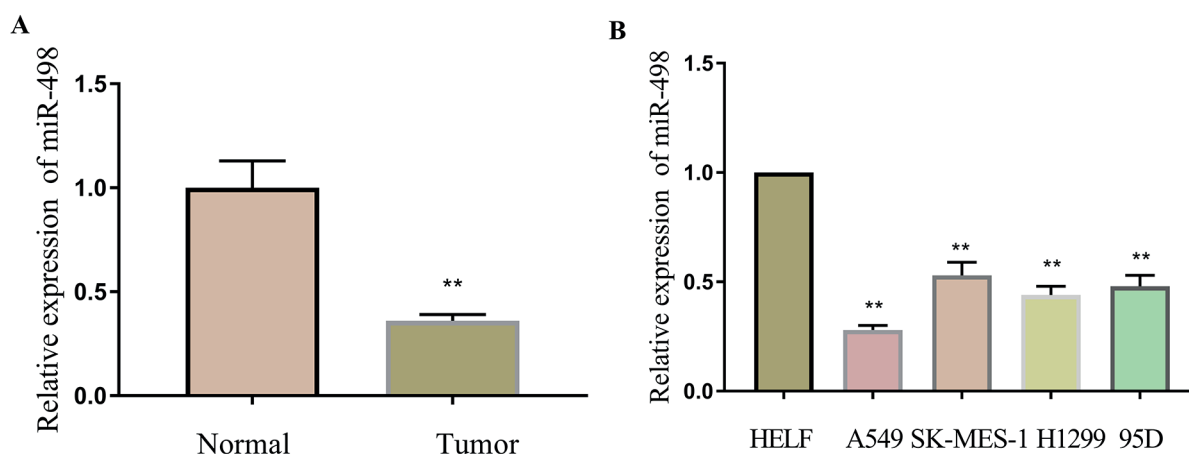


Figure 1. Expression miR-498 is significantly down-regulated in primary human NSCLC and cell lines. *A*, Relative expression of miR-498 in NSCLC tissues in comparison with adjacent non-tumor tissues. *B*, qRT-PCR analysis of miR-498 expression levels in four NSCLC cell lines compared with the normal HELF cells. miR-498 expression was examined by qRT-PCR and normalized to GAPDH expression. * $p < 0.05$; ** $p < 0.01$.

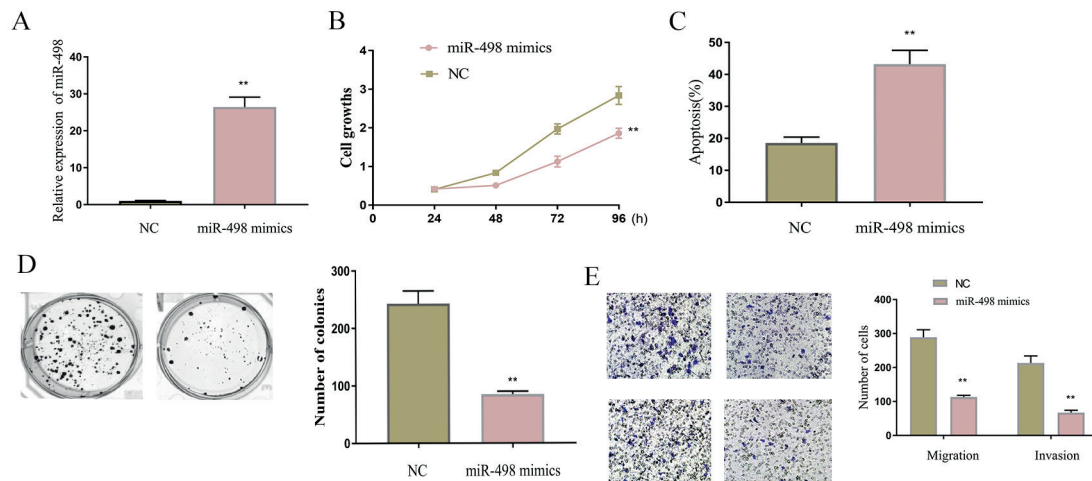


Figure 2. Effects of miR-498 expression on growth, colony formation, apoptosis and metastasis in NSCLC. **A**, The miR-498 expression level in A549 cells after transfected with miR-498 mimics or miR-NC were detected by qRT-PCR. **B**, CCK-8 assay was performed to detect the effect of miR-498 on the cell viability in A549 cells following transfection. **C**, Up-regulation of miR-498 promoted apoptosis in A549 cells. **D**, Colony formation assay was performed to detect the effect of miR-498 on cell growth in A549 cells. **E**, Transwell assays were performed to detect the effect of miR-498 on cell migration and invasion of A549 cells. * $p < 0.05$; ** $p < 0.01$.

sed compared with NC (Figure 2B). Next, Flow cytometry analysis showed that miR-498 upregulation promoted cell apoptosis compared to the si-NC group (Figure 2C). Colony formation assays were also performed. Upregulation of miR-498 resulted in a significant increase in foci number in GC cells (Figure 2D). Finally, by migration and invasion assay, we observed that the migration and invasion decreased in cells transfected with miR-498 compared with control cells (Figure 2E).

Those results indicated that miR-498 may serve as a tumor suppressor in NSCLC.

miR-498 Directly Targeted HMGA2 Expression

To identify the potential target genes of miR-498, TargetScan, and miRanda were used in combination. Our analysis revealed that HMGA2 was a potential target of miR-498 (Figure 3A). Then, dual luciferase reporter assay was used to iden-

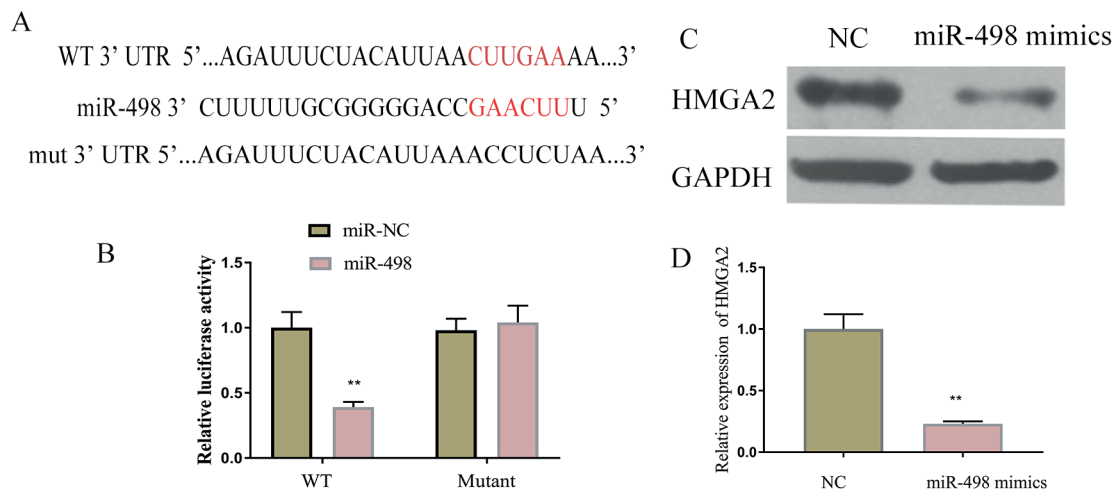


Figure 3. HMGA2 is a direct target of miR-498 in NSCLC cells. **A**, Diagram of seed sequence of miR-498 matched the 3'UTR of the HMGA2 gene. **B**, The wild-type or mutated HMGA2 3'-UTR was transfected into gastric cancer cells with or without synthetic miR-498 mimic. Luciferase activity was determined 48 h after transfection. **(C-D)** A549 cells transfected with miR-498 mimics and the expression level of HMGA2 was detected by Western blot. * $p < 0.05$; ** $p < 0.01$.

tify whether the 3'UTR of HMGA2 was a binding target of HMGA2. Our results showed that miR-498 mimics significantly suppressed the luciferase activity of reporter genes which contains wild-type of 3'-UTR of HMGA2 (Figure 3B). Subsequently, Western blot was used to detect the expression of HMGA2. As shown in Figure 3C and 3D, the expression of HMGA2 protein was decreased in A549 cells transfected with miR-498 mimics compared with the control. These findings suggested that miR-498 played an important role in NSCLC via regulation of HMGA2.

HMGA2 Overexpression Attenuated miR-498 Tumor Suppressor Role

Since HMGA2 is the target gene of miR-498, we wonder whether miR-498 regulates proliferation, migration, and invasion of NSCLC cells by targeting HMGA2. MiR-498-overexpressing A549 cells were further transfected with HMGA2 plasmid to restore its expression. As shown in Figure 4A, the protein expression of HMGA2 was significantly higher in the miR-498+ HMGA2 group. Then, we found that miR-498-related decrease in cell proliferation, apoptosis, migration, and invasion were reversed by restoration of HMGA2 expression by CKK-8 (Figure 4B), apop-

tosis assays (Figure 4C), migration (Figure 4D), and invasion (Figure 4E). Our findings indicated that inhibition of HMGA2 expression induced by miR-498 is responsible for proliferation and metastasis ability in NSCLC cells.

Discussion

Growing studies^{25,26} have concluded that miRNAs are closely correlated with tumor development and progression. Thus, they could function as important molecular biomarkers for NSCLC diagnosis, prognosis, and therapy. For example, Zhu et al²⁷ found that miR-520e exerted tumor-suppressive role in NSCLC by targeting Zbtb7a-mediated Wnt signaling pathway. Xiang et al²⁸ reported that the expression of miR-1271 was inversely correlated with clinical stage and its upregulation inhibited cell proliferation, invasion, and EMT in NSCLC by directly suppressing FOXQ1 expression. In the current study, we observed that miR-498 expression was significantly down-regulated in human NSCLC tissues and cell lines. In addition, *in vitro* assay indicated that overexpression of miR-498 enhanced cellular proliferation, migration, and invasion of NSCLC.

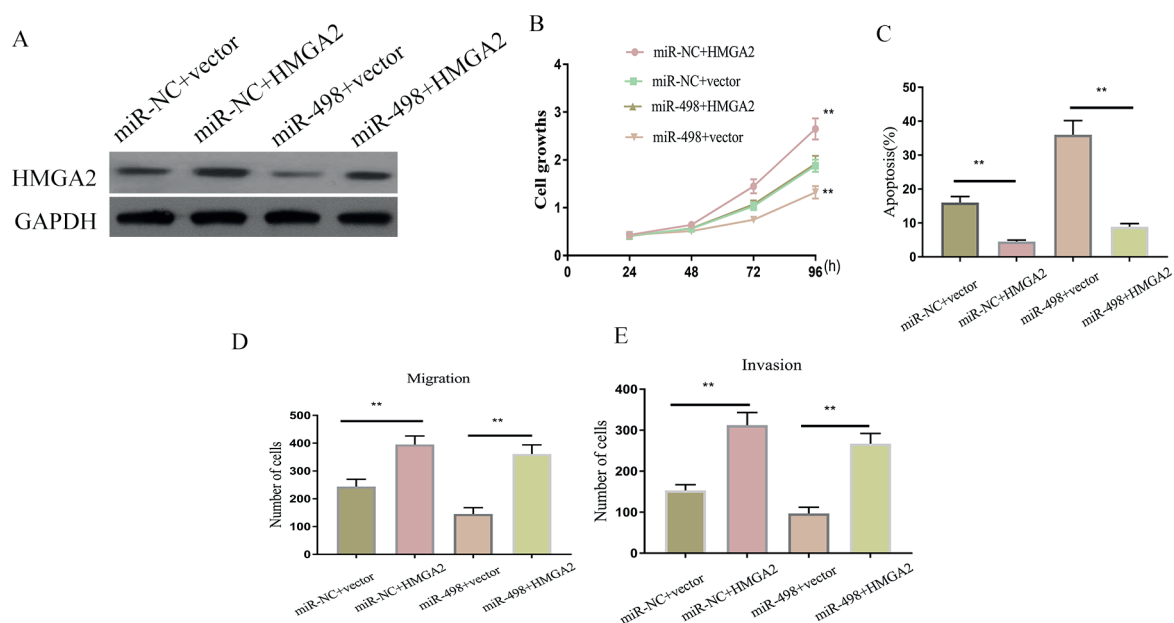


Figure 4. Restoration of HMGA2 expression completely rescued the inhibitory effect of miR-498 in CRC cells. **A**, Endogenous HMGA2 expression levels were determined by Western blot in A549 cells transfected with miR-498 or miR-NC mimics in the presence of HMGA2 or vector control. **B**, Cell proliferation was measured using CCK-8 assays in A549 cells. **C**, The cell apoptosis of A549 cells was performed by flow cytometry. **D-E**, Transwell invasion assay was used to detect the ability of migration and invasion in A549 cells. * $p < 0.05$; ** $p < 0.01$.

Our results revealed that miR-498 may serve as an anti-oncogene in NSCLC.

MiR-498, located in 19q13.42, have been reported to be involved in progression of several tumors. For instance, Liu et al²⁹ found that ectopic expression of miR-498 suppressed cell proliferation of ovarian cancer cells by targeting FOXO3. Cong et al³⁰ found that down-regulation of miR-498 was associated with poor prognosis in ovarian cancer patients. Islam et al³¹ reported that miR-498 served as a tumor suppressor in esophageal squamous cell carcinoma by modulating the FOXO1/KLF6 signaling pathway. Gopalan et al³² showed that the levels of miR-498 were significantly down-regulated in colorectal cancer and its overexpression could suppress cell proliferation. Notably, in lung cancer, miR-498 was found to be down-regulated. Furthermore, *in vitro* assay showed that ectopic expression of miR-498 inhibited NSCLC cell proliferation¹⁴. Those results were in line with our results. However, previous studies have not reported potential molecular mechanism of miR-498 in NSCLC.

It is known that each miRNA modulates the expression of hundreds of gene transcripts³³. According to bioinformatic analysis, we found that HMGA2 may be a target of miR-498. Previous studies^{34,35} showed that HMGA2 was overexpressed in NSCLC tissues and its ectopic expression promoted the ectopic cell tumor formation, cell proliferation, and metastasis, suggesting that HMGA2 may function as an oncogene in NSCLC. Thus, we wondered whether miR-498 exerted its tumor-suppressive role by targeting HMGA2. To answer this question, we performed luciferase assays and the results showed that miR-498 can repress the expression of HMGA2 by targeting its 3'-UTR. Moreover, we perform *in vitro* assay to confirm our results. Our data showed that re-expression of HMGA2 could significantly reverse the miR-498-imposed inhibition on proliferation, apoptosis, and metastasis.

Conclusions

We demonstrated that ectopic miR-498 expression inhibited NSCLC cell proliferation, migration, and invasion through directly targeting of HMGA2. Our data provided new insight into the mechanism responsible for the progression of human NSCLC. So, miR-498 might serve as a potential target for NSCLC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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