

miR-641 Functions as a Tumor Suppressor by Targeting MDM2 in Human Lung Cancer

Qinglong Kong, Nan Shu, Jun Li, and Ning Xu

Department of Thoracic Surgery, Dalian Municipal Central Hospital Affiliated of Dalian Medical University, Dalian, P.R. China

Lung cancer is the leading cause of deaths due to cancer. Studies suggest an important role of microRNAs (miRNAs) in a variety of cancers, including lung cancer. In the present study, we evaluated the role of miR-641 in human lung cancer A549 cells. Quantitative RT-PCR and Western blot were used to measure mRNA and protein expression, respectively. Cell viability and cell apoptosis were respectively measured by MTT assay and flow cytometry. In addition, luciferase activity assay was used to identify the target of miR-641. The expression of miR-641 was downregulated in lung cancer tissues and lung cancer cell lines ($p < 0.05$ or $p < 0.01$). Overexpression of miR-641 significantly inhibited proliferation and induced apoptosis of lung cancer cells ($p < 0.05$, $p < 0.01$, or $p < 0.001$). MDM2 was identified as a direct target of miR-641. Overexpression of miR-641 decreased the expression of MDM2 and increased the expression of p53 in lung cancer cells.

Key words: miR-641; Cell proliferation; Murine double minute 2 (MDM2); Lung cancer; p53; Apoptosis

INTRODUCTION

Lung cancer is the leading cause of cancer deaths. It is histologically classified into small cell lung cancer (SCLC), which is a neuroendocrine tumor, and non-small cell lung cancer (NSCLC), which arises from epithelial cells of the airways, and represents 80% of cases, including major subtypes such as lung adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma (LCC)^{1,2}. By the time of diagnosis, most of the patients have developed an aggressive form of the disease, and it is often difficult to diagnose at late stages because it has metastasized to other organs. Therefore, it is necessary to explore novel biomarkers for early detection and diagnosis.

MicroRNAs (miRNAs) are a class of small (~22 nt), noncoding, endogenous single RNA molecules that play important roles in gene expression through binding to the 3'-UTR of the target gene mRNA, leading to mRNA cleavage or translational repression³. Numerous studies have shown that miRNAs participate in various biological processes, such as cell differentiation, cell growth, apoptosis, and development^{4,5}. Aberrations in the expressions of miRNAs are associated with different diseases including cancer. About 50% of miRNAs are located in the cancer-related gene region or fragile region^{6,7}. miRNA-99b acts

as a tumor suppressor in NSCLC by directly targeting fibroblast growth factor receptor 3⁸. miRNA-7 inhibits the growth of human NSCLC A549 cells through targeting Bcl-2⁹. In a meta-analysis for miRNA expression profiling between lung cancer tissues and normal tissues, many miRNAs were found to be deregulated in lung cancer¹⁰. Previous studies have demonstrated the role of miR-641 in normal chondrocytes and in cervical tissues^{11,12}; however, the potential role of miR-641 in lung cancer cells is still unclear.

Murine double minute 2 (MDM2) is an oncogene that was originally discovered in a locus amplified on double minute chromosomes in transformed mouse fibroblasts¹³. MDM2 contains several conserved functional domains, which provide the structural basis for MDM2's oncogenic properties. MDM2 expression is deregulated in a variety of human cancers. MDM2 exerts oncogenic activity predominantly by inhibiting the p53 tumor suppressor, which is essential for mediating apoptosis¹⁴.

In this study, we assessed the downregulation of miR-641 in lung cancer tissues. We also suggested that miR-641 plays antiproliferation and antimetastasis roles in lung cancer cells. We further identified MDM2 as a novel target of miR-641, which would help to explore the regulation mechanism of miR-641 in lung cancer.

MATERIALS AND METHODS

Patients and Specimens

Seventy-five NSCLC tissues and matched neighboring normal tissues were obtained from the Dalian Municipal Central Hospital Affiliated to Dalian Medical University (Dalian, P.R. China) between January 2015 and April 2017. Patients were diagnosed with NSCLC according to histopathological evaluation. The enrolled patients had not received preoperative radiotherapy or chemotherapy. There were a total of 40 male patients and 35 females, with an average age of 65.34 years. The collected tissue samples were immediately stored at liquid nitrogen until use. Informed consent was obtained from all patients. Our study was approved by the ethics committee of the Dalian Municipal Central Hospital Affiliated to Dalian Medical University, P.R. China.

Cell Culture

Human normal embryonic lung WI-38 cells and lung cancer cell lines H157, H4006, A549, H1299, and H1650 were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). The cell lines were grown in RPMI medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and penicillin/streptavidin (Sigma-Aldrich). All cells were grown at 37°C in a humidified incubator under 5% CO₂.

Cell Proliferation

The MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide] assay was used to measure cell proliferation. On each day of the MTT assay, 100 µl of cells was taken from each of the culture conditions and placed in a 96-well plate, in triplicate. Fifty micrograms of MTT (Sigma-Aldrich) was added to each well, and this mix was incubated for 4 h at 37°C. At the end of incubation, 100 µl of 0.04 N HCl in 2-propanol was mixed thoroughly into each well. Plates were read on a Molecular Devices microplate reader (Sunnyvale, CA, USA) at a wavelength of 570 nm, with a background reading at 650 nm subtracted. Triplicate readings for each sample were averaged.

MicroRNA Transfection

Synthetic miR-641 mimic and scrambled negative control RNA (control mimic and control inhibitor) were purchased from GenePharma (Shanghai, P.R. China). Cells were seeded in six-well plates and were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) on the following day when the cells were approximately 70% confluent. In each well, equal amounts (100 pmol) of miR-641 mimic or the scrambled negative control

RNAs were used. The efficiency of downregulation or overexpression of miR-641 was evaluated by real-time polymerase chain reaction (RT-PCR).

Apoptosis Assay

Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining was used to measure cell apoptosis. Cells (3×10^6) were stained using the FITC Annexin-V/Dead Cell Apoptosis Kit (V13242; Invitrogen) according to the manufacturer's instructions. Stained cells were diluted in annexin V binding buffer (Invitrogen). Suspended cells were used to perform flow cytometry. Annexin V–FITC/PI-stained cells were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). In total, 10,000 cells were analyzed per measurement. Data were analyzed using FlowJo 10.0 software (Treestar Inc., Ashland, OR, USA).

Western Blot Analysis

The cells were washed two times with PBS and then lysed with 1× SDS loading buffer (50 mM Tris-Cl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, and 0.1% bromophenol blue) as the whole-cell sample. The protein samples were subjected to SDS-PAGE gel electrophoresis. Immunoblotting was carried out with primary antibodies. Anti-MDM2 (sc-812; 1:1,000), p53 (sc-47698; 1:1,000), and anti-β-actin (sc-58673; 1:1,000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-cleaved PARP (#9532; 1:1,000) and anti-caspase 3 (9662; 1:1,000) were both obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). The proteins were detected by enhanced chemiluminescence (ECL Plus; Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Quantitative Real-Time PCR RT-PCR Analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Grand Island, NY, USA). RNA (500 ng) was polyadenylated and reverse transcribed to cDNA using an N Code miRNA First-Strand cDNA Synthesis Kit (Invitrogen). cDNA was used as the template for RT-PCR. FastStart Universal SYBR Green Master (Roche Applied Science, Indianapolis, IN, USA) with the universal reverse primer provided in the kit. RT-PCR was performed on Applied Biosystems real-time detection system (Applied Biosystems, Foster City, CA, USA), and the thermocycling parameters were 95°C for 3 min and 40 cycles of 95°C for 15 s followed by 60°C for 30 s. Each sample was run in triplicate and was normalized to U6 snRNA levels [U6 primers 5'-CTTCGGCAGCACATATACT-3' (forward) and 5'-AAAATATGGAACGCTTCACG-3' (reverse)]. Melting curve analysis was performed to confirm the specificity of the PCR products. The replicates were

then averaged, and fold induction was determined by a $\Delta\Delta C_T$ -based fold change calculation.

Luciferase Activity Assay

The 3'-UTR segment of the MDM2 gene, containing the miR-641 binding site, was amplified through PCR and inserted into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) to form the reporter vector MDM2 wild type (MDM2-wt). To mutate the putative binding site of miR-641 in the MDM2, the sequence of putative binding site was replaced, which was named MDM2 mutated type (MDM2-mt). Thereafter, A549 cells were cotransfected with the MDM2 3'-UTR and miR-641 mimic or miR-NC using Lipofectamine 2000 (Invitrogen). The luciferase activity was analyzed at 48 h posttransfection using the Dual-Luciferase Reporter Assay System (Promega). For each transfection, the luciferase activity was averaged from three replicates.

Statistical Analysis

All experiments were repeated at least three times. Statistical analyses were performed using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). The data were presented as the mean \pm standard deviation (SD), and the data were analyzed using independent two-tailed *t*-test one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

miR-641 Expression Is Downregulated in Human Lung Cancer Tissues and Cell Lines

RNA was isolated from lung cancer, neighboring normal lung tissues, and cancer cell lines. The expression levels of miR-641 were measured by RT-PCR. As shown in Figure 1A, miR-641 expression was statistically

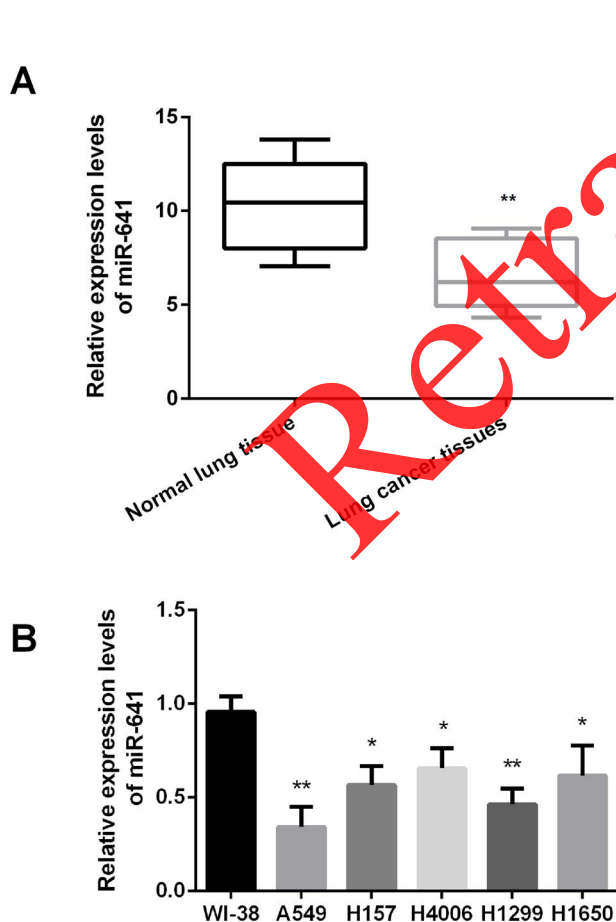


Figure 1. MicroRNA-641 (miR-641) expression is downregulated in human lung cancer tissues and cell lines. (A) miR-641 expression is significantly downregulated in human lung cancer tissues, compared with neighboring normal lung tissues. (B) miR-641 expression is statistically downregulated in human lung cancer cell lines. * $p < 0.05$; ** $p < 0.01$.

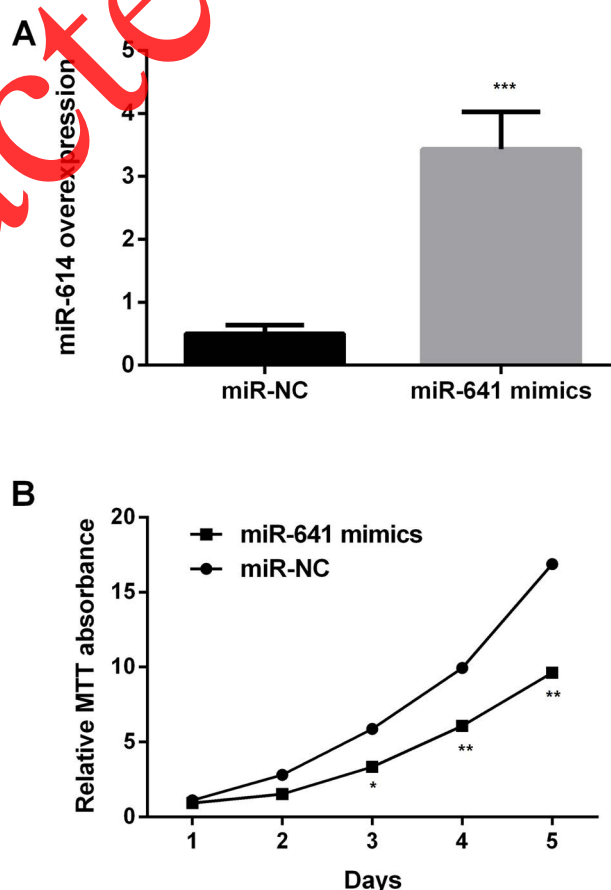


Figure 2. Overexpression of miR-641 inhibits the proliferation of lung cancer cells. (A) miR-641 was statistically increased in the mimic group compared to miR-NC. (B) Overexpression of miR-641 mimics inhibits the proliferation of human lung cancer A549 cells. Relative MTT absorbance. NC, negative control; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

downregulated in human lung cancer tissues compared to normal lung tissues ($p < 0.01$). In addition, the results showed that the expression levels of miR-641 were significantly decreased in lung cancer cell lines compared to the normal embryonic lung WI-38 cells ($p < 0.05$ or $p < 0.01$). The results indicated that miR-641 might act as a tumor suppressor in lung cancer.

Overexpression of miR-641 Inhibits the Proliferation of Lung Cancer Cells

Control miR-NC and miR-641 mimics were transfected into A549 cells, and the expression levels of miR-641 were measured by RT-PCR (Fig. 2A). The results showed that miR-641 was statistically increased in the mimic group compared to miR-NC ($p < 0.001$). The results implied that the transfection efficiency was high. The cell proliferation was then determined using MTT

assay (Fig. 2B). The results showed that overexpression of miR-641 significantly inhibited the proliferation of A549 cells compared to miR-NC ($p < 0.05$ or $p < 0.01$).

Overexpression of miR-641 Induces Apoptosis in Lung Cancer Cells

After transfection of miR-641 mimic into A549 cells, flow cytometric analysis was done to determine the percentage of apoptotic cell population (Fig. 3A). The apoptotic cell percentage was increased in the miR-641 mimics group compared to the miR-NC group ($p < 0.001$). The levels of cleaved PARP and caspase 3 were determined by Western blot and PCR to measure apoptosis. Western blotting results showed increased expression of cleaved PARP and caspase 3 in the miR-641 mimics group compared to the miR-NC group (Fig. 3B). Similarly, overexpression of miR-641 also significantly elevated

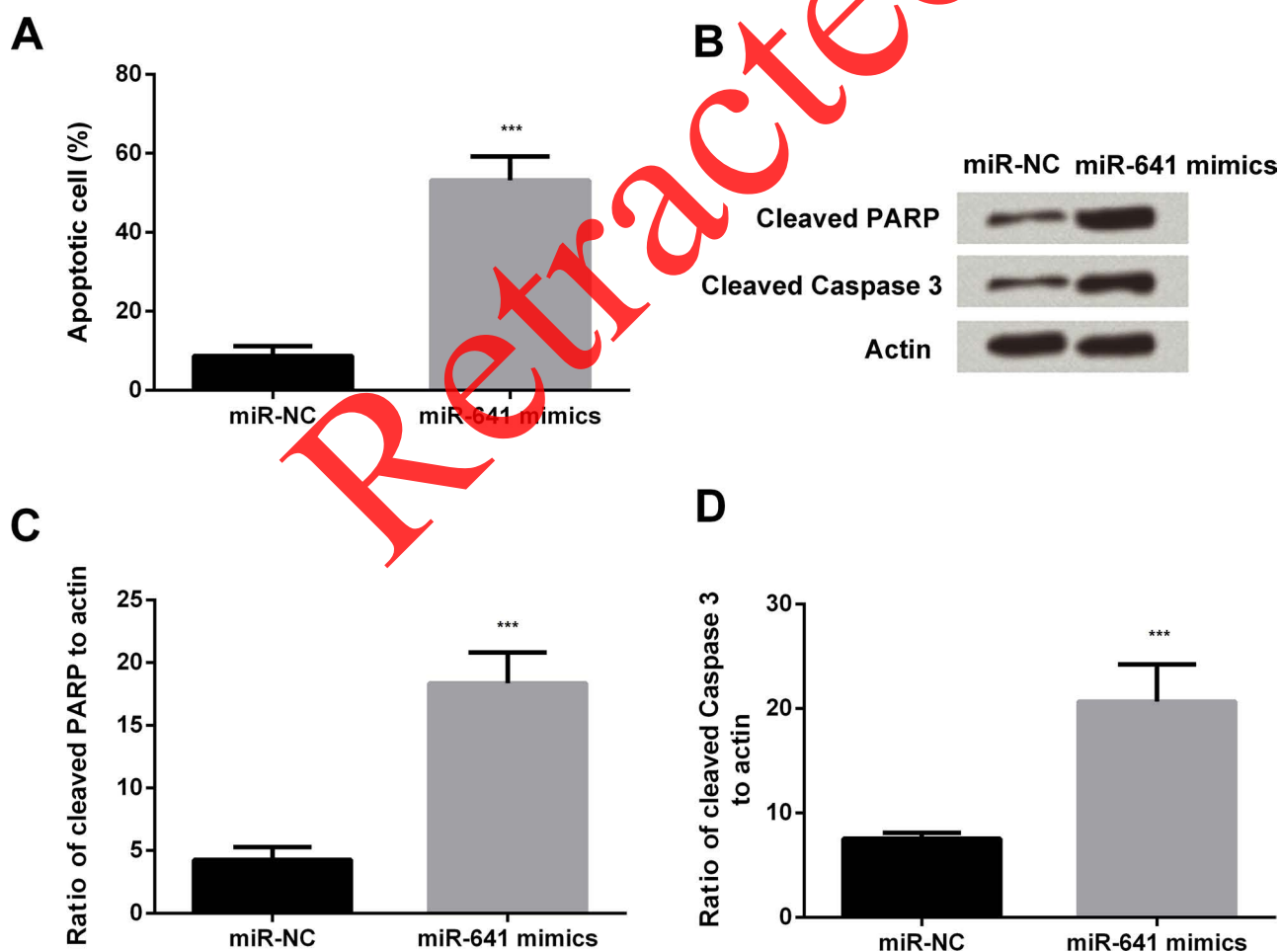


Figure 3. Overexpression of miR-641 induces apoptosis in lung cancer cells. (A) Overexpression of miR-641 induces apoptotic cell death of human lung cancer A549 cells. (B) Overexpression of miR-641 induces apoptotic cell death of human lung cancer A549 cells. Western blotting analysis. (C) Overexpression of miR-641 induces apoptotic cell death of human lung cancer A549 cells. (D) Overexpression of miR-641 induces apoptotic cell death of human lung cancer A549 cells. PARP, poly ADP-ribose polymerase. *** $p < 0.001$.

the mRNA levels of PARP and caspase 3 (both $p < 0.001$) (Fig. 3C and D).

MDM2 Is a Direct Target of miR-641 in Lung Cancer Cells

Bioinformatics analysis (Fig. 4A) showed that miR-641 forms base pairs with the 3'-UTR of MDM2. In further experiments, miR-641 mimic or miR-NC and the 3'-UTR of MDM2 were cotransfected into A549 cells, and luciferase activities were then performed. We found that cotransfection with the miR-641 mimic and MDM2-wt significantly decreased the relative luciferase activity compared to the miR-NC group ($p<0.05$) (Fig. 4B). These results suggested that miR-641 targets MDM2.

Overexpression of miR-641 Decreases MDM2 Expression and Increases p53 Expression in Lung Cancer Cells

Western blot analysis and PCR were conducted to examine the expression levels of MDM2 and p53 in the cells. The results showed decreased expression of MDM2 and increased expression of p53 in the miR-641 mimic group compared to miR-NC (Fig. 5A). Similar results were observed in Figure 5B and C; the expression of MDM2 was significantly decreased by the miR-641 mimic ($p < 0.001$), and p53 was statistically increased by

the overexpression of miR-641 ($p < 0.001$). The results implied that miR-641 negatively regulated the expression of MDM2 and positively regulated the expression of p53.

DISCUSSION

Our data demonstrate that miR-641 may function as a tumor suppressor by negatively regulating the expression of MDM2, which is involved in the degradation of p53 in human lung cancer. We showed that miR-641 expression is downregulated in human lung cancer tissues and cancer cell lines compared with normal lung tissues and cells (Fig. 1), and overexpression of miR-641 inhibits proliferation of human lung cancer A549 cells (Fig. 2), induces apoptosis (Fig. 3), downregulates the expression of MDM2 (Fig. 4), and increases the expression of p53 in the lung cancer cells (Fig. 5).

Expression of aberrant miRNAs occurs more frequently in human cancer, which play important roles in carcinogenesis and tumor progression^{15,16}. Kong et al. demonstrated that miRNA-140-3p inhibited proliferation, migration, and invasion of lung cancer cells by targeting ATP6AP2¹. Hence, it is necessary to evaluate the effects of the aberrant expression of miRNAs in cancers. The tumor suppressor role of miR-641 has been suggested in a previous

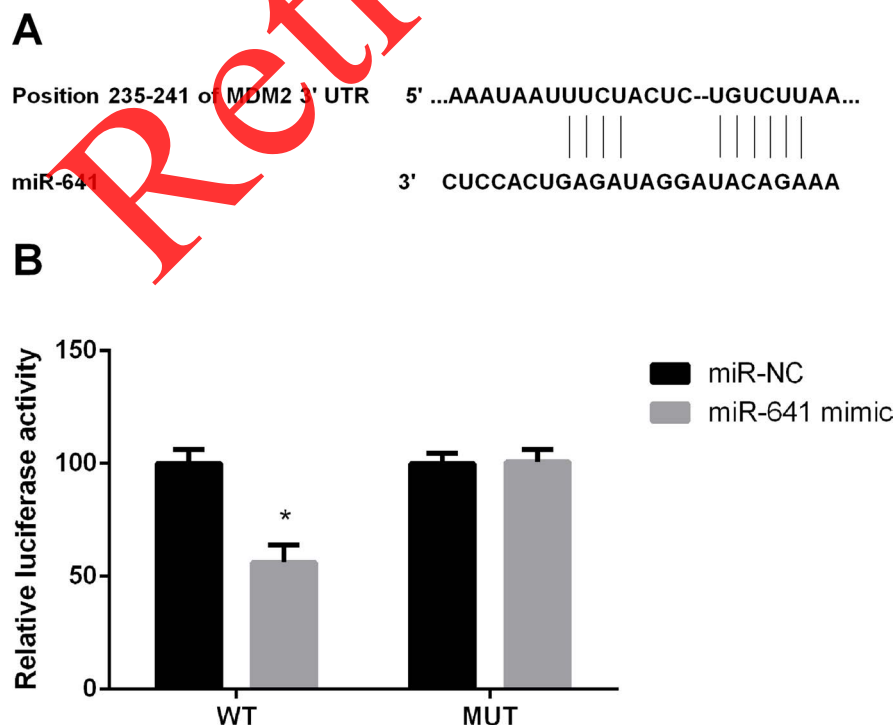


Figure 4. MDM2 is a direct target of miR-641 in lung cancer cells. (A) MDM2 is a direct target of miR-641 in human lung cancer A549 cells. (B) MDM2 is a direct target of miR-641 in human lung cancer A549 cells. MDM2, murine double minute 2; MUT, mutant type; WT, wide type. * $p < 0.05$.

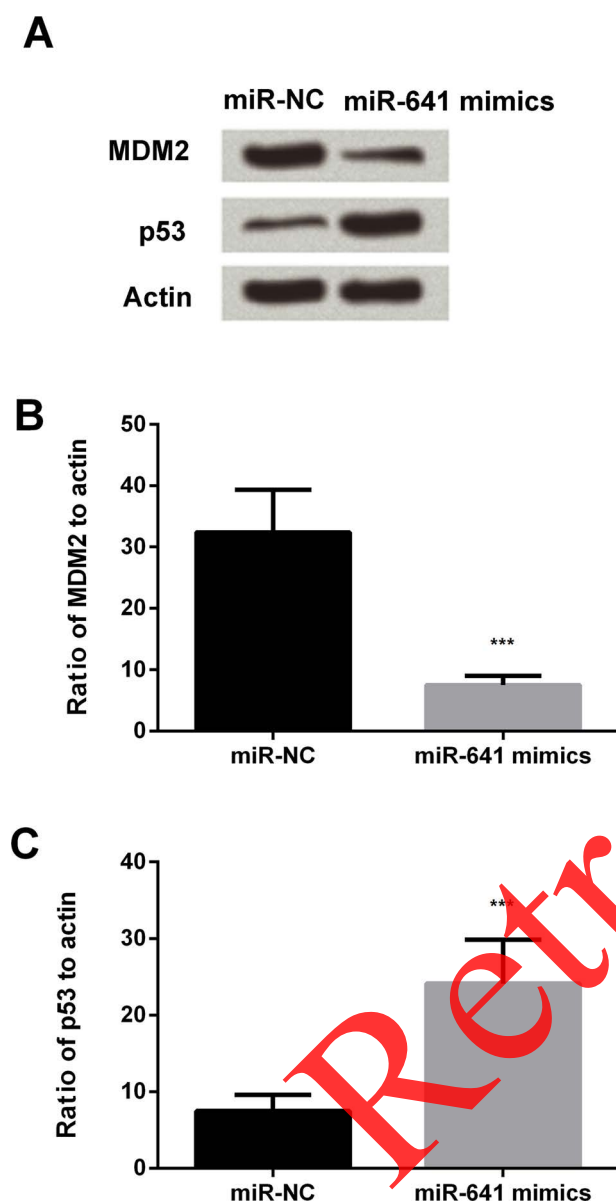


Figure 5. Overexpression of miR-641 decreases MDM2 expression and increases p53 expression in lung cancer cells. (A) Overexpression of miR-641 reduces the expression levels of MDM2 and increases p53 expression in human lung cancer A549 cells. Western blotting analysis. (B) Overexpression of miR-641 reduces the expression levels of MDM2 in human lung cancer A549 cells. (C) Overexpression of miR-641 increases p53 expression in human lung cancer A549 cells. *** $p < 0.001$.

study: Yao et al. showed that overexpression of miR-641 inhibited cell proliferation in cervical cancer cells¹². According to the study by Richards et al., suppression of miR-641 sensitizes tumor cells to cisplatin-induced apoptosis¹⁷. However, the role of miR-641 in lung cancer has not yet been studied. To our knowledge, this is the first study to evaluate the effects of miR-641 in lung cancer.

Our study results demonstrated that overexpression of miR-641 decreases MDM2 expression levels and increases p53 expression in human lung cancer cells. Also, expression of MDM2 varies in different types of cancer. MDM2 is classified as an oncogene. Previous studies have demonstrated overexpression of MDM2 in a variety of human tumors, including sarcoma, leukemia, breast carcinoma, melanoma, and glioblastoma¹⁸. MDM2 acts as a negative regulator of p53; high expression levels of MDM2 decrease p53 protein levels and function. This in turn leads to increased risk of cancer and augmented tumor formation and progression. In human tumors, overexpression of MDM2 and p53 mutations are often mutually exclusive¹⁹. This indicates that inactivation of p53 by MDM2 contributes to the effect of MDM2 on tumor development. A previous study in mice and cultured cells showed that MDM2 has p53-independent oncogenic functions, which control proliferation, apoptosis, and tumor invasion and metastasis¹⁸.

MDM2 is a key player in human cancer and is considered to be an important cancer therapeutic target. Higashiyama et al. reported that MDM2 gene amplification and expression are considered as favorable prognostic markers in NSCLC²⁰. Increased expression of MDM2 in human tumors is mainly caused by gene amplification. Increased transcription and/or enhanced translation of MDM2 also contribute to MDM2 overexpression in human tumors¹⁹. MDM2 is highly regulated at both the mRNA and protein levels in cells. MDM2 is a transcriptional target of p53. p53 binds to p53 consensus DNA-binding element in the first intron of the MDM2 gene to transcriptionally induce the expression of MDM2 and forms an autoregulatory negative feedback loop with MDM2^{21–23}. MDM2 expression levels are also transcriptionally regulated by various oncogenic and tumor-suppressive pathways. In addition, many stress signals, including DNA damage, oncogenic activation, ribosomal biogenesis, and chronic stress, and miRNAs regulate MDM2 protein levels, activity, and cellular localization²⁴.

In conclusion, the present study showed that miR-641 is downregulated in lung cancer cells, and overexpression of miR-641 inhibits cell growth and induces apoptosis in lung cancer cells by decreasing the expression of MDM2 and increasing the expression of p53. These results suggest miR-641 as a novel therapeutic target for lung cancer.

REFERENCES

- Kong XM, Zhang GH, Huo YK, Zhao XH, Cao DW, Guo SF, Li AM, Zhang XR. MicroRNA-140-3p inhibits proliferation, migration and invasion of lung cancer cells by targeting ATP6AP2. *Int J Clin Exp Pathol*. 2015; 8(10):12845–52.
- Shi X, Zhan L, Xiao C, Lei Z, Yang H, Wang L, Zhao J, Zhang HT. miR-1238 inhibits cell proliferation by targeting

- LHX2 in non-small cell lung cancer. *Oncotarget* 2015; 6(22):19043–54.
3. Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 2004;116(2):281–97.
4. Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM. Bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene *hid* in *Drosophila*. *Cell* 2003;113(1):25–36.
5. Ambros V. The functions of animal microRNAs. *Nature* 2004;431(7006):350–55.
6. Zhang LY, Liu M, Li X, Tang H. miR-490-3p modulates cell growth and epithelial to mesenchymal transition of hepatocellular carcinoma cells by targeting endoplasmic reticulum-Golgi intermediate compartment protein 3 (ERGIC3). *J Biol Chem*. 2013;288(6):4035–47.
7. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, Croce CM. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA* 2004;101(9):2999–3004.
8. Kang J, Lee SY, Lee SY, Kim YJ, Park JY, Kwon SJ, Na MJ, Lee EJ, Jeon HS, Son JW. MicroRNA-99b acts as a tumor suppressor in non-small cell lung cancer by directly targeting fibroblast growth factor receptor 3. *Exp Ther Med*. 2012;3(1):149–53.
9. Xiong S, Zheng Y, Jiang P, Liu R, Liu X, Chu Y. MicroRNA-7 inhibits the growth of human non-small cell lung cancer A549 cells through targeting BCL-2. *Int J Biol Sci*. 2011;7(6):805–14.
10. Guan P, Yin Z, Li X, Wu W, Zhou B. Meta-analysis of human lung cancer microRNA expression profiling studies comparing cancer tissues with normal tissues. *J Exp Clin Cancer Res*. 2012;31:54.
11. Diaz-Prado S, Cicione C, Muinos-Lopez E, Hermida-Gomez T, Oreiro N, Fernandez-Lopez C, Blanco FJ. Characterization of microRNA expression profiles in normal and osteoarthritic human chondrocytes. *BMC Musculoskelet Disord*. 2012;13:144.
12. Yao T, Rao Q, Liu L, Zheng C, Xie Q, Liang J, Lin Z. Exploration of tumor suppressive microRNAs silenced by DNA hypermethylation in cervical cancer. *Virol J*. 2013; 10:175.
13. Cahilly-Snyder L, Yang-Feng T, Francke U, George DL. Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. *Somat Cell Mol Genet*. 1987;13(3):235–44.
14. Wade M, Li YC, Wahl GM. MDM2, MDMX and p53 in oncogenesis and cancer therapy. *Nat Rev Cancer* 2013; 13(2):83–96.
15. Ryan BM, Robles AI, Harris CC. Genetic variation in microRNA networks: The implications for cancer research. *Nat Rev Cancer* 2010;10(6):389–402.
16. Croce CM. Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet*. 2009;10(10):704–14.
17. Richards EJ, Coppola M, Guo J, Kong W, Cheng JQ. Abstract B28: MicroRNA-641 activates MAPK by targeting NF1 and cooperates with its host gene AKT2 in human cancer. *Cancer Res*. 2012;72(2 Suppl):B28.
18. Jones SN, Hancock AR, Vogel H, Donehower LA, Bradley A. Overexpression of Mdm2 in mice reveals a p53-independent role for Mdm2 in tumorigenesis. *Proc Natl Acad Sci USA* 1998;95(26):15608–12.
19. Momand J, Jung D, Wilczynski S, Niland J. The MDM2 gene amplification database. *Nucleic Acids Res*. 1998; 26(15):3453–9.
20. Higashiyama M, Doi O, Kodama K, Yokouchi H, Kasugai T, Ishiguro S, Nakami K, Nakayama T, Nishisho I. MDM2 gene amplification and expression in non-small-cell lung cancer. Immunohistochemical expression of its protein is a favourable prognostic marker in patients without p53 protein accumulation. *Br J Cancer* 1997;75(9):1302–8.
21. Perry ME, Piette J, Zawadzki JA, Harvey D, Levine AJ. The mdm-2 gene is induced in response to UV light in a p53-dependent manner. *Proc Natl Acad Sci USA* 1993; 90(24):11623–7.
22. Wu X, Bayle JH, Olson D, Levine AJ. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev*. 1993;7(7a): 1126–32.
23. Barak Y, Juven T, Haffner R, Oren M. mdm2 expression is induced by wild type p53 activity. *EMBO J*. 1993;12(2): 461–8.
24. Zhao Y, Yu H, Hu W. The regulation of MDM2 oncogene and its impact on human cancers. *Acta Biochim Biophys Sin*. (Shanghai) 2014;46(3):180–9.