

Original Article

MicroRNA-652 inhibits proliferation and induces apoptosis of non-small cell lung cancer A549 cells

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Abstract: Increasing evidence suggests the down-regulation of microRNA-652 (miR-652) in various diseases, addressing its involvement in tumorigenesis, but its role in non-small cell lung cancer (NSCLC) is largely unexplored. This study therefore aims to uncover the function of miR-652 in regulating lung cancer cells. Human NSCLC A549 cells were transfected with the mimic or inhibitor of miR-652 to change miR-652 levels. MTT, 5-Bromo-2-deoxyuridine (BrdU), flow cytometry assays were performed to assess viability, proliferation and apoptosis of the transfected cells. Expression of p27, p21 and apoptotic factors including B-cell lymphoma 2 (BCL2), BCL2 associated X protein (BAX) and caspase 3 were examined by Western blot. Result showed that, miR-652 mimic inhibited, while miR-652 inhibitor increased A549 cell viability on the second and third days post transfection ($P < 0.05$). BrdU staining suggested that cell proliferative ability was suppressed by miR-652 mimic ($P < 0.001$) and induced by the inhibitor ($P < 0.05$). Expression of p21 appeared to be unaffected, whereas p27 was increased by miR-652, which was consistent with cell proliferative changes. Percent of apoptotic cells was increased by miR-652 mimic and reduced by the inhibitor ($P < 0.01$). Western blot further showed that miR-652 mimic decreased BCL2/BAX but elevated the proportion of cleaved caspase 3, and miR-652 inhibitor induced the opposite changes ($P < 0.001$). In conclusion, these results addressed the anti-proliferative and pro-apoptotic functions of miR-652 in A549 cells, providing a promising strategy for the molecular therapy of NSCLC.

Keywords: Non-small cell lung cancer, microRNA-652, cell proliferation, cell apoptosis, bax

Introduction

Lung cancer is a malignancy of high morbidity and mortality with a very poor 5-year survival rate. Among lung cancer patients, over 85% were diagnosed with non-small cell lung cancer (NSCLC) [1], which is the most common type of lung cancer. Compared to small cell lung cancer, NSCLC spreads more slowly. It includes 3 major types: adenocarcinoma, squamous cell carcinoma and large cell carcinoma. The high morbidity of lung cancer is mainly attributed to smoking and pollution in air and drinking water [2]. At present, treatment for lung cancer relies on surgery, chemotherapy and sometimes radiation therapy. Molecular-targeted therapy is a burgeoning field for attenuating NSCLC and has made great achievements in these years, such as Gefitinib and Erlotinib that target epidermal growth factor receptor [3, 4].

The uncontrolled proliferation and hindered apoptosis of lung cancer cells accelerate cancer growth and metastasis. A growing body of

research has corroborated the pivotal functions of some factors in modulating cancer cell proliferation and apoptosis, among which microRNAs (miRNAs) are considered to play key roles as regulators of NSCLC cells. For example, miR-30b/c and miR-203 inhibit proliferation [5, 6], whilst miR-34a and miR-15a induce apoptosis of NSCLC cells [7, 8]. These miRNAs recognize and target important oncogenes or apoptotic factors that facilitate cancer cell proliferation and apoptosis, such as RAB18 and factors in the B-cell lymphoma 2 (BCL2) family. More in-depth studies are under way to reveal the sophisticated regulatory network in NSCLC pathogenesis and progression.

MiR-652 has been reported in several diseases acting as a diagnostic marker or a cellular regulator. Circulating miR-652 is a potent marker for acute coronary syndromes and breast cancer [9]. miR-652 was down-regulated in nasopharyngeal carcinoma, suggesting its potential in suppressing this disease [10]. It was also down-regulated in the serum of liver cirrhosis patients,

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which reflects its putative roles in the pathogenesis of liver cirrhosis [11]. Inhibition of miR-652 was capable of attenuating cardiac hypertrophy and improving cardiac functions of transverse aortic constriction mice [12]. Moreover, it is an inhibitor of acidic microenvironment-induced epithelial mesenchymal transition, retarding the growth and liver metastasis of pancreatic cancer [13]. Despite these associations between miR-652 and diseases, functions and potential mechanisms of miR-652 in NSCLC remain enigmatic.

This study was performed to uncover the effects of miR-652 on NSCLC cells and its functional mechanisms. Human NSCLC A549 cells were cultured and transfected with miR-652 mimic or inhibitor to alter miR-652 level, and then cell viability, proliferation and apoptosis were assessed. Cell cycle factors p21 and p27, as well as important apoptotic factors including BCL2, BCL2 associated X protein (BAX) and caspase 3 were detected by Western blot. By this means, this study manifested the anti-proliferative and pro-apoptotic roles of miR-652 in A549 cells, and proposed miR-652 to be a potential strategy for the molecular therapy of NSCLC.

Materials and methods

Cell culture

Human NSCLC A549 cells (ATCC, Manassas, VA, USA) were cultured in Roswell Park Memorial Institute-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco), 0.1 g/L sodium pyruvate (Gibco), 2.0 g/L NaHCO₃ and 100 U/mL penicillin-streptomycin (Corning, New York, NY, USA). Cells were incubated in humid air containing 5% CO₂ at 37°C and passaged at a confluency of 90%.

Cell transfection

Cell transfection was conducted to alter the level of miR-652 in A549 cells. The cells were seeded in a 24-well plate 1 d before transfection, with 2×10^5 cells in each well. When the confluency reached 90%, miR-652 mimic (50 nM), miR-652 inhibitor (100 nM) or the negative control (50 nM), all synthesized by Sangon Biotech (Shanghai, China), was transfected to the cells using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Cells were incubat-

ed at 37°C for 48 h, and then the following detection was performed.

MTT assay

MTT assay was performed at 0, 1, 2 and 3 d post transfection to analyze A549 cell viability. Cells were adjusted to a density of 5×10^4 /mL and seeded in a 96-well plate (100 µL in each well). Then 10 µL of MTT Formazan (5 mg/mL, Sigma-Aldrich, Saint Louis, MO, USA) was added to each well and the cells were incubated for 4 h at 37°C. The supernatant was discarded and 150 µL of dimethyl sulfoxide (Sigma-Aldrich) was added. The plates were slowly shaken for 10 min to dissolve the crystals. The absorbance at 490 nm was measured by a microplate reader iMark (Bio-Rad, Hercules, CA, USA).

5-Bromo-2-deoxyuridine (BrdU) assay

BrdU assay was conducted to assess A549 cell proliferation at 48 h post transfection using BrdU Cell Proliferation Assay Kit (BioVision, Milpitas, CA, USA) based on the manufacturer's instruction with minor changes. Briefly, the cells were seeded in a 96-well plate (5×10^3 cells/well) and incubated for 24 h. BrdU Solution was added and the plate was incubated for another 4 h, after which the medium was discarded, and 100 µL of Denaturing Solution was added, and the plate was slowly shaken for 30 min. The BrdU Detection Antibody was added for incubation with gentle shaking for 1 h, and the cells were washed in Wash Buffer for 2 times. Then Goat Anti-Mouse IgG H&L (Alexa Fluor® 647, ab150115, Abcam, Cambridge, UK) was added and the plate was incubated in the dark for 1 h at room temperature. Cells were then dual-stained with 4',6-diamidino-2-phenylindole (DAPI, 0.5 µg/mL, Sigma-Aldrich) for 2 min and immediately observed using a fluorescent microscope (Olympus, Tokyo, Japan).

Cell apoptosis assay

Fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining was performed to detect apoptotic A549 cells at 48 h post transfection using Annexin V-FITC/PI Apoptosis Detection Kit (Yeasen, Shanghai, China). For each sample, 1×10^5 cells were collected and washed twice in cold phosphate-buffered saline (PBS). After resuspension, the cells were stained by 5 µL of Annexin V-FITC and 5 µL of PI Staining Solution for 10 min in the dark. Then 400 µL of

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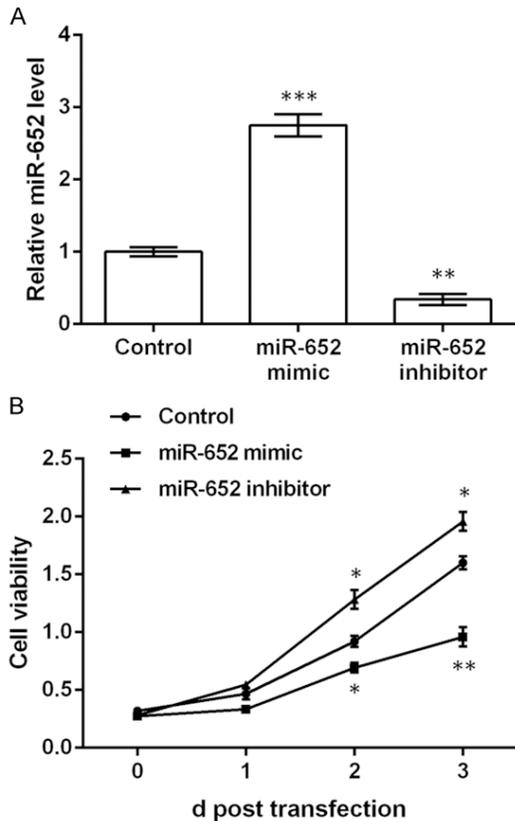


Figure 1. MiR-652 inhibits A549 cell viability. Human non-small cell lung cancer A549 cells were transfected with miR-652 mimic, inhibitor or negative control to alter miR-652 expression level. Cell viability was detected by MTT method on 0, 1, 2 and 3 d post transfection. A. qRT-PCR shows that miR-652 mimic and inhibitor alter the level of miR-652 significantly. B. Significant suppression by miR-652 mimic and promotion by miR-652 inhibitor in cell viability are detected on 2 and 3 d post transfection. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the Control group.

Binding Buffer was added and the cells were analyzed by a flow cytometer BD FACSCalibur (San Jose, CA, USA). The FITC-positive and PI-negative cells were counted as apoptotic cells.

Western blot

Total protein lysates of A549 cells were prepared by Radio-Immunoprecipitation Assay (RIPA) Lysis Buffer (ShineGene, Shanghai, China) according to the manufacturer's instruction at 48 h post transfection. The protein was quantified by Bio-Rad Protein Assay Kit (Bio-Rad) and 20 μg of protein for each sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, after which the protein bands were transferred to a polyvinyl-

dene fluoride membrane (Roche, Basel, Switzerland). The membrane was blocked in 5% milk in PBS for 2 h at room temperature and then incubated in the primary antibodies (1:1000) for p27 (ab193379), p21 (ab80633), BCL2 (ab16779), BAX (ab77566), pro-caspase 3 (ab2171) and cleaved-caspase 3 (ab204944, Abcam) at 4°C overnight. Anti-GAPDH (ab8245) was used as an internal control. The membrane was washed in PBS for 5 times and then incubated in the secondary antibodies Goat Anti-Mouse IgG H&L conjugated with horseradish peroxidase (1:2000, ab6789) for 1 h at room temperature. After washed in PBS for 5 times, signals on the membrane was developed using EasyBlot ECL Kit (Sangon Biotech) and analyzed by ImageJ 1.49 (National Institutes of Health, Bethesda, MD, USA).

qRT-PCR

At 48 h post transfection, miRNAs were extracted using RNAiso for Small RNA (Takara, Dalian, China) according to the manufacturer's instruction. Reverse transcription was catalyzed by PrimeScript Reverse Transcriptase (Takara) and guided by the primer for hsa-miR-652-5p (5'-CTCAA CTGGT GTCGT GGAGT CGGCA ATTCA GTTGA GTGAA TGGC-3'). qRT-PCR was performed on QuantStudio 6 Flex Real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) to quantify the level of miR-652 using the specific primers (forward: 5'-ACACT CCAGC TGGGC AACCC TAGGA GAGGG TGC-3' and reverse: 5'-TGGTG TCGTG GAGTC G-3'). Data were calculated by $2^{-\Delta\Delta C_t}$ method normalized by U6 (forward: 5'-CTCGC TTCGG CAGCA CATAT ACT-3' and reverse: 5'-ACGCT TCACG AATTT GCGTG TC-3').

Statistical analysis

All the experiments were performed in triplicate. Values were presented as mean \pm standard error of the mean. Data were analyzed using one-way analysis of variance and Student's *t* test by SPSS 20 (IBM, New York, NY, USA). Statistical significance was identified if $P < 0.05$.

Results

MiR-652 inhibits proliferation and promotes apoptosis of A549 cells

At 48 h post transfection, miR-652 level was detected by qRT-PCR, and results showed that

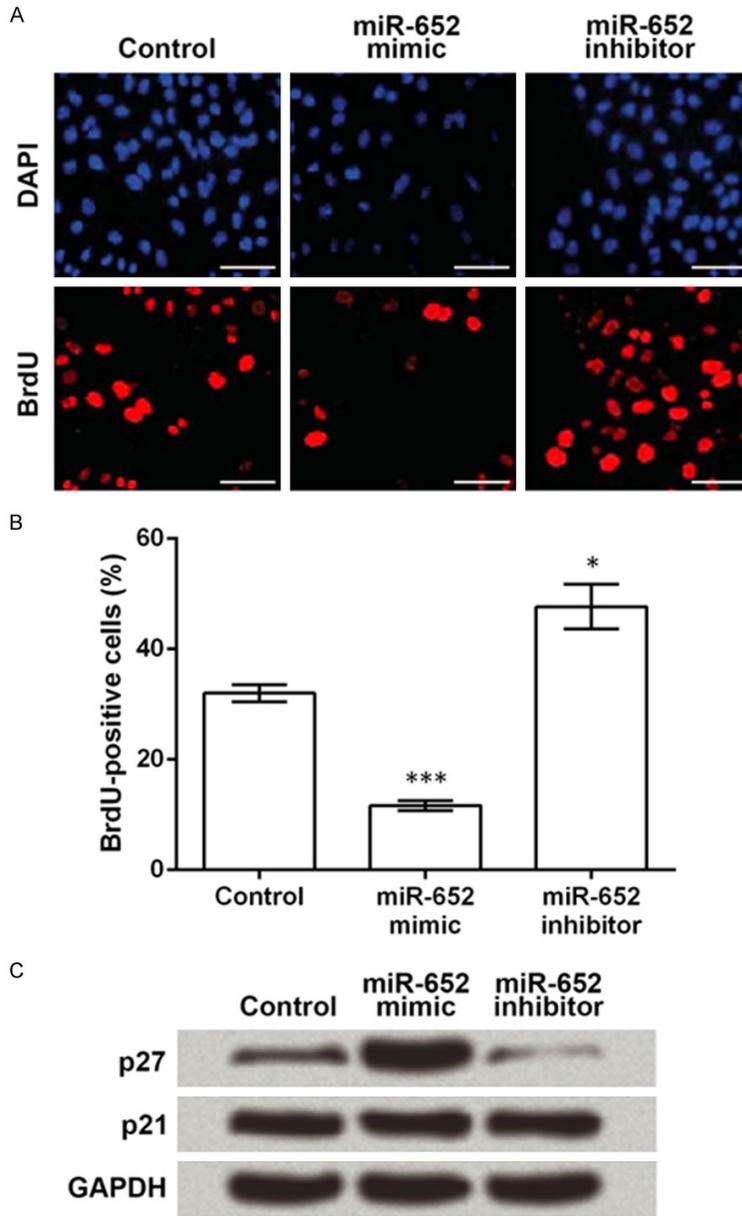


Figure 2. MiR-652 inhibits A549 cell proliferation. Human non-small cell lung cancer A549 cells were transfected with miR-652 mimic, inhibitor or negative control to alter miR-652 expression level. Cell proliferation was detected by 5-Bromo-2-deoxyuridine (BrdU) staining at 48 h post transfection. A. BrdU staining indicates the decreased proliferative cells by miR-652 mimic. Cells were dual-stained with 4',6-diamidino-2-phenylindole (DAPI) to indicate the nucleus. Bar = 50 μ m. B. Percent of BrdU-positive cells. * $P < 0.05$ and *** $P < 0.001$ compared to the Control group. C. Western blot shows that the protein expression of p27 is promoted by miR-652 mimic, while p21 is almost unchanged. GAPDH is used as an internal control.

miR-652 mimic significantly elevated miR-652 level ($P < 0.001$) and that the inhibitor obviously suppressed miR-652 ($P < 0.01$, **Figure 1A**).

Hence these transfected cells were valid for further analysis. Cell viability was detected at different time points post transfection. No significant change could be observed on the first day post transfection ($P > 0.05$), whereas cell viability was obviously suppressed by miR-652 mimic and promoted by miR-652 inhibitor when detected on the second and third days post transfection ($P < 0.05$ or $P < 0.01$, **Figure 1B**), suggesting that miR-652 might inhibit A549 cell viability.

Similarly, A549 cell proliferative ability assessed by BrdU method indicated consistent changes. MiR-652 mimic reduced while its inhibitor increased proliferative cells (**Figure 2A**), with significant difference in the percent of BrdU-positive cells compared to the Control group ($P < 0.001$ or $P < 0.05$, **Figure 2B**), implying the anti-proliferative function of miR-652 in A549 cells. Here two cell cycle regulators p27 and p21, which have been studied intensively in cell cycle, were detected, and results showed that p21 protein was hardly changed by transfection, while p27 protein was induced by miR-652 mimic and suppressed by the inhibitor (**Figure 2C**), which was in accordance with cell proliferation changes.

Cell apoptosis was further investigated to reveal the function of miR-652. Flow cytometry detection indicated that miR-652 mimic obviously increased the percent of FITC-positive and PI-negative cells, and its inhibitor decreased the cells in this quadrant (**Figure 3A**) with statistical significance compared to the

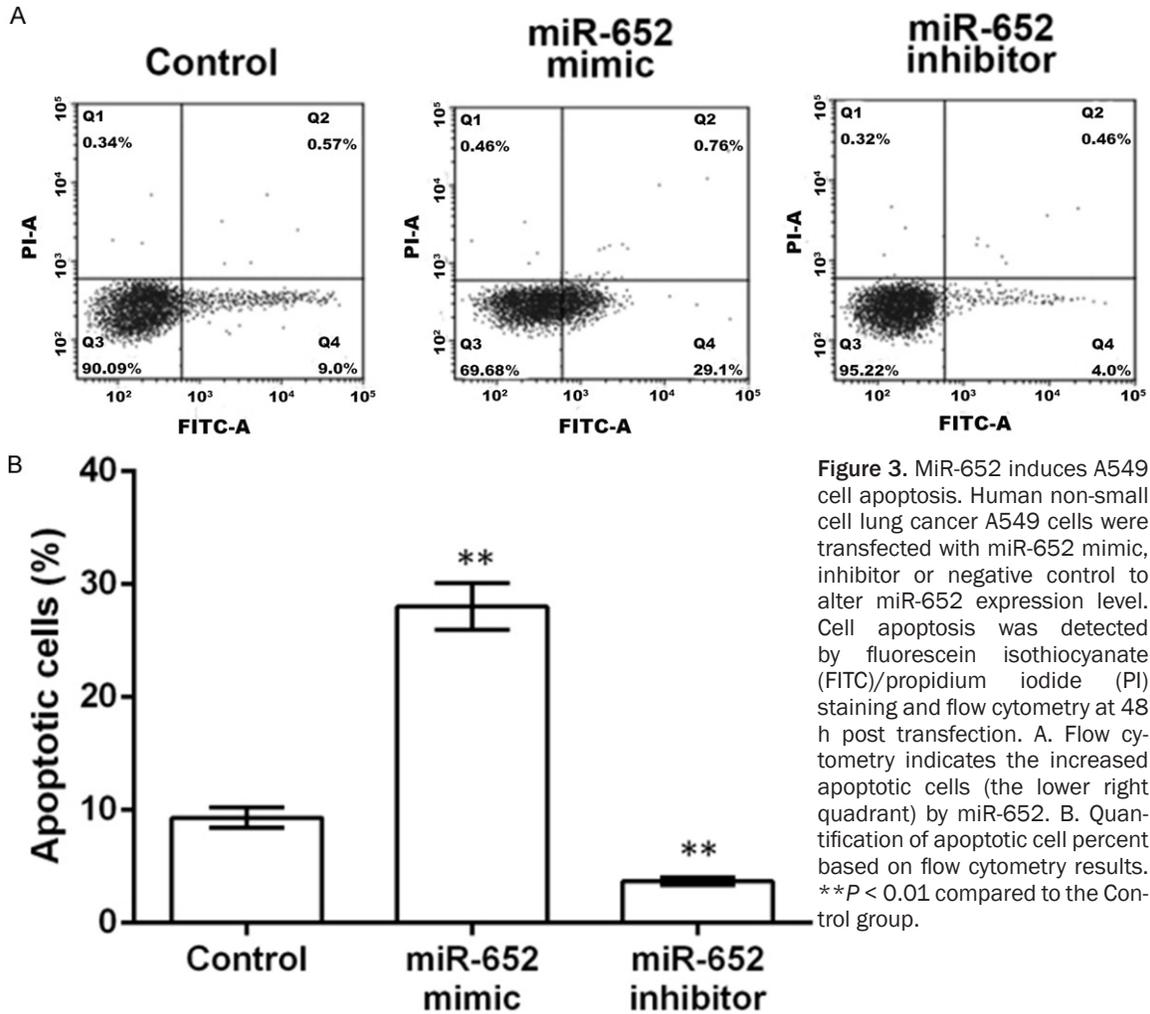


Figure 3. MiR-652 induces A549 cell apoptosis. Human non-small cell lung cancer A549 cells were transfected with miR-652 mimic, inhibitor or negative control to alter miR-652 expression level. Cell apoptosis was detected by fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining and flow cytometry at 48 h post transfection. A. Flow cytometry indicates the increased apoptotic cells (the lower right quadrant) by miR-652. B. Quantification of apoptotic cell percent based on flow cytometry results. ** $P < 0.01$ compared to the Control group.

control group ($P < 0.01$, **Figure 3B**). Taken together, miR-652 was able to suppress proliferation and accelerate apoptosis of A549 cells.

MiR-652 regulates apoptotic factors in A549 cells

In order to verify the apoptotic changes by miR-652 from the molecular level and analyze the potential functional mechanism of miR-652 in A549 cells, we detected the expression of several important apoptotic factors including the anti-apoptotic BCL2 and the pro-apoptotic BAX and caspase 3. Western blot showed that miR-652 decayed BCL2 protein and promoted BAX, pro-caspase 3 and cleaved caspase 3, and meanwhile its inhibitor induced the opposite changes in these factors (**Figure 4A**). In the quantitated results of Western blot, significant differences could be found in the BCL2/BAX

and cleaved/pro-caspase 3 ratios ($P < 0.001$, **Figure 4B**), which further supported the apoptotic changes induced by miR-652 and implied the involvement of these factors in the functional mechanism of miR-652.

Discussion

Compared to most of the other miRNAs, our knowledge of miR-652 is relatively poor, especially in lung cancer. This study provided pioneer work on the role of miR-652 in NSCLC A549 cells. miR-652 mimic was capable of suppressing A549 cell viability and proliferation, and promoting apoptosis, while its inhibitor induced opposite changes in A549 cells. Furthermore, miR-652 induced changes in the expression of p27 and apoptotic factors including BCL2, BAX and caspase 3, which gave a hint on its regulatory mechanisms in A549 cells.

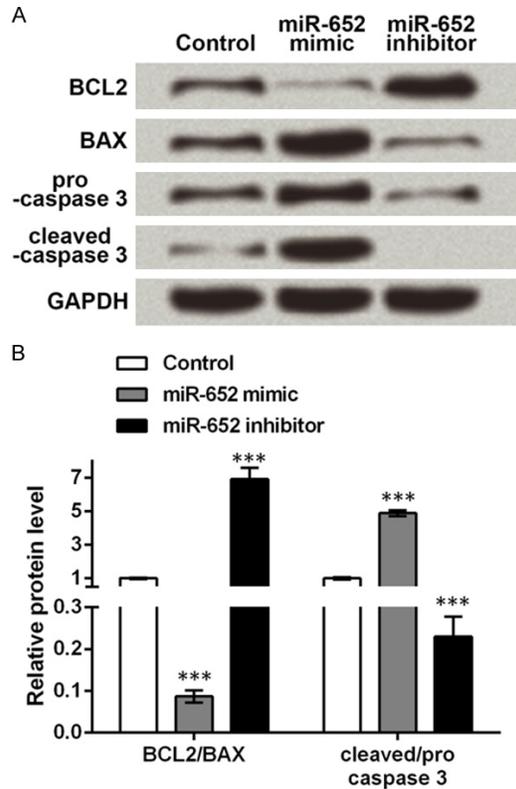


Figure 4. MiR-652 regulates apoptotic factors in A549 cells. Human non-small cell lung cancer A549 cells were transfected with miR-652 mimic, inhibitor or negative control to alter miR-652 expression level. Protein expression of apoptotic factors was detected by Western blot at 48 h post transfection. A. Western blot shows that miR-652 mimic reduces B-cell CLL/lymphoma 2 (BCL2) but increases BCL associated X protein (BAX), pro-caspase 3 and cleaved-caspase 3. GAPDH is used as an internal control. B. Quantitative of the ratio BCL2/BAX and cleaved/pro caspase 3 based on Western blot results. *** $P < 0.001$ compared to the Control group.

MiR-652 has been reported in the regulation of some diseases. In combination with miR-301a, miR-454, miR-223 and miR-139, miR-652 was a strong predictor of metastasis in prostate cancer [14]. Besides, transduction of miR-652 to bone marrow cells was able to suppress the recovery of myeloid and platelet [15]. Our *prima facie* results indicated that the effective promotion of miR-652 suppressed A549 cell viability on the second and third days post transfection, and meanwhile inhibited cell proliferation as revealed by BrdU staining. The repression of miR-652 level by its inhibitor induced A549 cell proliferation. Furthermore, up-regulation of miR-652 increased and its inhibitor

decreased the percent of apoptotic A549 cells, which was reflected from FITC and PI dual-staining results, suggesting that miR-652 can induce A549 cell apoptosis. Taken together, these findings suggest the anti-proliferative and pro-apoptotic role of miR-652 in A549 cells, which is consistent with the implications of existed studies on miR-652, implying its suppressive effects on NSCLC.

The expression of two cell cycle regulators, p21 and p27, was detected by Western blot in light of their significant involvement of cell proliferation and survival. p21, which is encoded by the gene cyclin-dependent kinase inhibitor 1A (*CDKN1A*), can be induced by p53 and block cell cycle progression at G1 phase [16]. In NSCLC, p21 was reported to be targeted by miR-208a and long noncoding RNA ANRIL, resulting in accelerated cell proliferation [17, 18]. Similarly p27, encoded by *CDKN1B*, has been found up-regulated in A549 cells, accompanied by the promoted miR-340 level and suppressed proliferation and promote apoptosis [19, 20]. In light of these evidences, this study also detected the elevated p27 expression by miR-652 mimic and its decrease by miR-652 inhibitor, further suggesting that A549 proliferation was suppressed by miR-652. However, the p21 protein level was barely changed, which was possible because the roles of p21 and p27 are not identical [21]. We suspect that the regulation of miR-652 on p21 or p27 was via different pathways, which needs to be tested in further research.

Apoptotic factors BCL2, BAX and caspase 3 were detected by Western blot to confirm changes in A549 cell apoptosis and investigate the potential mechanism of miR-652. BCL2 and BAX are factors of the BCL2 family, occupying important positions in cellular apoptosis [22, 23]. The two factors have been widely used as apoptotic markers: a lower BCL2/BAX ratio is usually associated with promoted apoptosis, while elevation in the ratio indicates suppressed apoptotic progression [24, 25]. As to caspase 3, it is a crucial executor of cell apoptosis, which is cleaved into active forms upon apoptosis [26-28]. Western blot of this study showed that miR-652 elevation in A549 cells significantly decreased BCL2/BAX and increased cleaved/pro caspase 3, consistent with the A549 apoptotic changes induced by

miR-652. These findings also implicate that miR-652 may regulate key factors in apoptotic pathways and lead to the expression alteration in BCL2, BAX and caspase 3. The elaborate regulatory relationship is to be investigated in the following studies.

A recent study propounded that miR-652-3p had higher levels in NSCLC and exhibited different functions of promoting proliferation and metastasis [29] compared to roles of miR-652-5p revealed in this study. The contradiction may rest in the sequence and target disparities between miR-652-5p and miR-652-3p, which generates regulatory effects on different factors and pathways. Thus the two miRNAs need to be carefully distinguished in future research.

To sum up, this study provides evidences for the anti-proliferative and pro-apoptotic roles of miR-652 in A549 cells, implying that miR-652 is a promising candidate for the molecular therapy in treating NSCLC. miR-652 regulates the expression of p27 and apoptotic factors BCL2, BAX and caspase 3, which highlights the importance of more detailed research on its functional mechanisms.

Disclosure of conflict of interest

None.

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