

MicroRNA-30a-3p inhibits the progression of lung cancer via the PI3K/AKT by targeting DNA methyltransferase 3a

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Background: MicroRNAs (miRNAs) are small non-coding RNAs, involved in pathological and physiological processes via regulating target genes expression. Abnormally expressed miR-30a-3p has been verified in several tumors, such as liver cancer, esophageal cancer and lung cancer. It was reported that DNA methylation plays a critical role in the tumorigenesis of lung cancer through regulated tumor suppressor genes silencing. Nevertheless, the potential mechanism of miR-30a-3p in restoring abnormal DNA methylation patterns is still unclear in lung cancer. Therefore, because the miR-30a-3p is complementary to the 3'-untranslated regions (3'-UTR) of DNA methyltransferase 3A (DNMT3A), we investigated whether miRNA-30a-3p could target DNMT3a to regulate the progression of lung cancer cell.

Methods: qRT-PCR was used to evaluate miR-30a-3p and DNMT3a mRNA expression levels in A549 lung cancer cells and normal cell line BEAS-2B. MiR-30a-3p expression plasmid was transferred into A549 cells. The target of miR-30a-3p was detected by luciferase reporter assay. Western blot was used to measure related protein expression levels. MTT assay was used to measure the proliferation of cells in each group. The cycle and apoptosis of cells were detected by flow cytometry.

Results: We found down-regulation of miR-30a-3p mRNA expression and up-regulation of DNMT3a mRNA expression in A549 cells. Overexpression of miR-30a-3p downregulates DNMT3a or blocked DNMT3a by interference vector, significantly inhibited the proliferation and G1/S transition in A549 cells via regulating p38 MAPK pathway, and induced the apoptosis in A549 cells via regulating Bcl-2/Bax protein levels. Furthermore, we observed the opposite phenomenon in A549 cells transfected with both miR-30a-3p and DNMT3a vector.

Conclusion: Our data show that miR-30a-3p suppressed the progression of lung cancer via regulating p38 MAPK pathway by targeting DNMT3A in A549 cells, indicating that miR-30a-3p might be a novel potential therapeutic strategy in the treatment of lung cancer.

Keywords: DNA methylation, MiR-30a-3p, lung cancer, DNA methyltransferases, tumor suppressor genes

Introduction

DNA methylation as a conserved epigenetic silencing mechanism in mammalian cells is involved in numerous biological processes.¹ Abnormal DNA methylation regulates the expression of tumor suppressor genes (TSGs) or oncogenes, of which translating proteins participate in genomic instability, malignant cell growth, metastasis and differentiation.^{2,3} It has been well known that aberrant DNA

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hypermethylation in TSGs leads to inhibits of transcription and induce in tumorigenesis. DNA methylation is carried out by DNA methyltransferases (DNMT). Among all members of DNMT family, abnormal DNMT3a expression has been reported in many types of tumors.^{4,5} A number of reports have confirmed the overexpression of DNMT3a mRNA or protein levels in human tumors, including hepatocellular carcinomas,⁶ prostate cancer,⁷ non-small cell lung carcinoma⁸ and breast cancer.⁹ However, the potential mechanism of DNMT3a in lung cancer needs further study.

MicroRNAs (miRNAs) are a class of 12–25-nucleotide endogenous non-coding RNAs that cause mRNA degradation or inhibiting translation by interacting with the 3'-untranslated region (3'-UTR) of the target genes.¹⁰ MiRNA play important regulatory role in various fundamental biological processes, such as development, differentiation and apoptosis.^{11,12} Furthermore, miRNAs can act as either oncogenes or tumor suppressors, and due to its potential on regulation of numerous genes, miRNAs represent powerful discovery tools to pioneer new ways impacting cancer.¹³ MiR-30a as a tumor suppressor can regulate proliferation, apoptosis, invasion and migration of various tumor cells.^{14,15} MiR-30a can attenuate the progression of breast cancer by inhibiting the expression of the downstream target gene Notch1.¹⁶ In *H. pylori* gastric cancer models, miR-30a inhibited tumor growth via targeting COX-2 and BCL-9.¹⁷ MiR-30a-3p as a member of miR-30a family has been reported to be down-regulated in several tumors.¹⁸ Qi et al reported that down-regulation of miR-30a-3p/5p promotes esophageal squamous cell carcinoma (ESCC) cell proliferation by activating the Wnt signaling pathway.¹⁹ However, little is known about the role and underlying molecular mechanism of action of miR-30a-3p in lung cancer.

In this study, we investigate the effect and the potential mechanism of miR-30a-3p in the progression of lung cancer. The results indicated that miR30a-3p suppresses the development of A549 cells by targeting DNMT3a via the p38 MAPK pathway. These findings suggest miR-30a-3p have value as therapeutic strategies for lung cancer and warrants continued investigation in this regard.

Materials and methods

Cell culture

A549 (BNCC337696) and BEAS-2B (BNCC100240) cells were obtained from BeNa Culture Collection. Cells were

cultured in RPMI-1640 medium (ThermoFisher, Waltham, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, ThermoFisher, Waltham, USA) under the condition with 95% air and 5% CO₂ at 37°C in a humidified chamber.

Plasmid constructions and transfection

The pc-DNATM6.2-GW/EmGFP-miR vector (Invitrogen, Waltham, USA) was used to generate vectors of re-expression of miR-30a-3p and DNMT3a. The gene coding for miR-30a-3p was chemically synthesized and cloned in to pc-DNATM6.2-GW/EmGFP-miR vector between the EcoRI and HindIII sites using sense primer 5'-TGCC CTGG CTTAGTTATCACAGTGCTGATGCTGTCTACT CTAAAGCTAGAGTACTGTGAT AACTCAAGGATGG CA-3' and anti-sense primer 5'-TGCCATCCTTGAGTTA GTT ATCACACTACTGTACCTTTAGATTAGTCAGCA TCAGCACAGTGATAACTGAGCCAGGGCA-3'. Target Plus Smart pool siRNAs (Dharmacon, Waltham, USA) containing a mixture of oligonucleotides with potential for DNMT3a abrogation was used to silence DNMT3a expression. The luciferase-UTR reporter constructions which were generated by introducing the wild-type (wt)/mutant (mut) EGFR 3'-UTR carrying a putative miR-30a-3p binding site were used to identify the DNMT3a by miR-30a-3p targeting.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted by TRIzol reagent (Takara, Dalian, China) according to the manufacturer's instructions, and RNase-free DNase was used to remove DNA contamination. RNA was quantified by an ultraviolet spectrophotometer and 1% agarose electrophoresis. 1 µg RNA from each sample was reverse transcribed to obtain cDNA by using PrimeScriptTM RT reagent kit (Takara). The expression of relative genes was analyzed using 2×SYBR Premix ExTaqTM (Takara) and the specific primers were miR-30a-3p forward: 5'-CCCTGCTCTGGCTGGTCAAA CG GA-3', miR-30a-3p reverse: 5'-TTGCCAGCCCT GCTGTAGCTGGTTGAAG-3' and DNMT3a forward: 5'-CGGCAGAATAGCCAAGTTCA-3', miR-30a-3p reverse: 5'-GGGAAGCCAAACACCCTTTC-3'. The threshold cycle (Ct) was determined using ΔΔCt method, which makes Ct values normalized to the endogenous control gene (*GAPDH*). For each group, the quantification was triplicated.

Dual luciferase assay

A549 cells were co-transfected with 500 ng DNMT3a-3'-UTR (wild type and mutant type) and 50 nM miR-30a-3p mimics or 100 nM inhibitor by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. After 48 hrs, cells were lysed to Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA), and luciferase activity was detected by GloMax20/20 Luminometer (Promega). Luciferase activity was normalized by Renilla/Firefly luciferase signal in lymphocytes.

Western blot

For total protein extraction, cells were washed with phosphate-buffered saline (PBS) and lysed with RIPA buffer (Beyotime, Shanghai, China) supplemented with protease inhibitor. Cell lysate was centrifuged at $12,000\times g$ for 15 mins and the supernatants were collected. The concentration of proteins was measured by BCA kit. 30 μg protein in each group were separated by 10% SDS-polyacrylamide gels. After electrophoresis at 200 mA for 2 hrs, proteins were transferred onto PVDF membrane (Millipore, USA). The membranes were blocked with 5% skim milk for 2 hrs at room temperature. Subsequently, the membrane was incubated with primary antibodies against DNMT3a (ab188470, 1:2000 dilution), p-38 (ab27986, 1:1000 dilution), p-p38 (ab178867, 1:1000 dilution), p21CIP1 (ab212247, 1:1000 dilution), p27KIP1 (ab32034, 1:5000 dilution), cyclin-dependent kinase 2 (CDK2) (ab32147, 1:2000), cyclin D (ab134175, 1:10,000), Bcl-2 (ab32124, 1:1000) and Bax (ab32503, 1:2000 dilution) overnight at 4°C. Anti-GAPDH (ab128915, 1:10,000) antibody was selected as internal reference. Then, the membranes were washed with Tris-buffered saline and incubated in biotinylated goat IgG secondary antibody (ab7090) for 2 hrs at room temperature. All antibodies were purchased from Abcam (USA). Immunoreactivity was visualized by colorimetric reaction using ECL substrate buffer (Millipore, Massachusetts, USA). Membranes were scanned with Gel Doz EZ imager (Bio-rad, USA).

MTT assay

The proliferation of A549 cells was measured by MTT colorimetric method. Cells were seeded in 96-well cell culture plates with 1×10^4 cells per well. After treatment for 24 hrs, 48 hrs and 72 hrs, cells were incubated with

20 μL MTT for 2–4 hrs. Then, the purple precipitate was visible, the medium was removed and 150 μL DMSO was added into each well. After shaking at low speed for 10 mins, the absorbance was recorded at 570 nm. For each detect, the total procedure was repeated 3 times.

Cell cycle analysis

After treatment for 48 hrs, cells were collected by trypsinization, and 1×10^6 cells were used for analysis. The cells were washed twice with PBS and fixed in ice-cold ethanol overnight at 4°C. Then, the cells were washed with PBS and incubated in 1 mL staining solution (10 U/mL RNaseA and 20 $\mu g/mL$ propidium iodide) for 30 mins at room temperature. Cell cycle distributions were assayed by fluorescence-activated cell sorting using a flow cytometer (FACSort; BD Biosciences, Franklin Lakes, USA).

Cell apoptosis assay

The apoptosis of A549 cells was detected by AnnexinV-FITC/PI (propidium iodide) flow cytometry kit (BD) according to the manufacturer's instructions. Cells were washed twice with ice-cold PBS and resuspend 200 μl of binding buffer at a concentration of 1×10^6 cells/mL. Cells were incubated in 10 μL PI and 10 μL Annexin V-FITC for 30 mins at 4°C in the dark. Finally, 300 μl binding buffer was added and analyzed by flow cytometry (Beckman Coulter, Cytomics FC 500, CA) within 1 hr.

Statistical analysis

All numerical data were expressed as mean \pm S.D. The statistical differences were analyzed by one-way analysis of variance (ANOVA) using SPSS 19.0 software package. Differences were considered as statistically significant at $p<0.05$ and very significant at $p<0.01$.

Results

The expression of miR-30a-3p decreased, while DNMT3a increased in the A549 cells

We detected the miR-30a-3p and DNMT3a expressions in A549 cells and normal BEAS-2B cells. As shown in [Figure 1A](#), the level of miR-30a-3p in A549 cells was lower than that in BEAS-2B cells. The DNMT3a mRNA ([Figure 1B](#)) and protein ([Figure 1C](#) and [D](#)) levels in A549

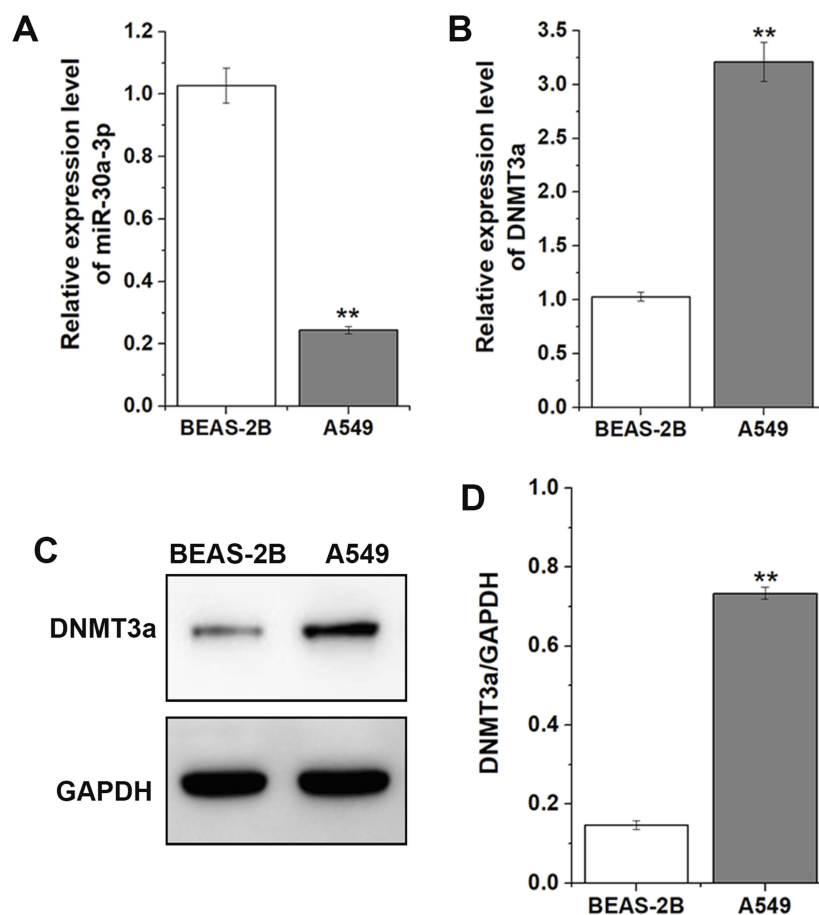


Figure 1 The differential expression of miR-30a-3p and DNMT3a in lung cancer A549 cells and normal BEAS-2B cells. **(A)** The mRNA levels of miR-30a-3p in A549 cells and normal BEAS-2B cells. **(B)** The mRNA levels of DNMT3a in A549 cells and normal BEAS-2B cells. **(C, D)** Western blot analysis of DNMT3a protein expression in A549 cells and normal BEAS-2B cells. Data are reported as mean \pm SD (n=3). **P<0.1 versus BEAS-2B cell group.

cells increased significantly compared with that in BEAS-2B cells. These data suggest that miR-30a-3p and DNMT3a might involve the progression of A549.

miR-30a-3p targets DNMT3a

We constructed the miR-30a-3p overexpression vector and transfected with A549 cells. The results showed that the expression of miR-30a-3p increased significantly in A549 cells after transfection (Figure 2A). The protein expression levels of DNMT3a in A549 cells transfected with miR30a-3p overexpression vector decreased significantly (Figure 2B and C). Moreover, we co-transfected A549 cells with miR-control, miR-30a-3p mimics, miR-30a-3p inhibitor and pmirGLO-DNMT3a-3'-UTR-wt or pmirGLO-DNMT3a-3'-UTR-mut. We found that miR-30a-3p inhibited the firefly luciferase activity of pmirGLO-DNMT3a-3'-UTR-wt, whereas miR-control did not (Figure 2D).

miR-30a-3p inhibits the growth of A549 cells by suppressing the p-38 MAPK signaling pathway

To study the effect of miR-30a-3p on A549 cell proliferation, we performed MTT and cell cycle assays. We found that upregulation of miR-30a-3p significantly inhibited the proliferation of A549 cells at 48 hrs and 72 hrs (Figure 3A). Up-regulation of miR-30a-3p resulted in a significant repression from the G1 phase to S phase and G2 phase in A549 cells (Figure 3B). Furthermore, we analyzed the expression of cell cycle regulators related with the p-38 MAPK pathway. The results demonstrated that the p-38 MAPK pathway was activated by transfection with miR-30a-3p (Figure 3C), which induced the expression of p21CIP1, p27KIP1 and inhibited the expression of CDK2, cyclin D in A594 cells (Figure 3D). Those results suggest that miR-30a-3p arrested the cell cycle at the G1 phase and suppressed cell proliferation in vitro.

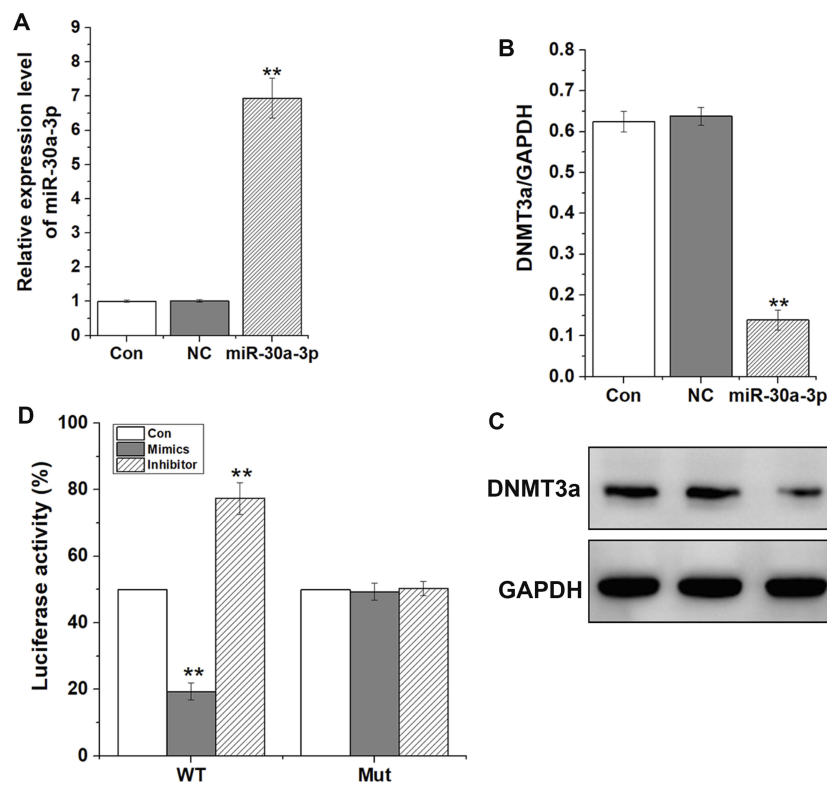


Figure 2 miR-30a-3p targets DNMT3a. **(A)** The mRNA levels of miR-30a-3p in A549 cells measured by RT-qPCR 48 hrs after transfection with miR-30a-3p. **(B, C)** Overexpression of miR-30a-3p inhibited the protein expression levels of DNMT3a in A549 cells. Data are reported as mean \pm SD (n=3). ** P <0.1 versus NC group. **(D)** Luciferase assay miR-30a-3p binding sites in the WT-3'UTR and Mut-3'UTR of DNMT3A were cloned into pmirGLO Dual-Luciferase miRNA Target Expression vector. The normalized firefly luciferase activity was obtained by firefly luciferase activity/Renilla luciferase activity. Data are reported as mean \pm SD (n=3). ** P <0.1 versus Con group. **Abbreviations:** Con, control group; NC, cells transfected with miR-control vector group; miR-30a-3p, cells transfected miR-30a-3p vector group.

miR-30a-3p induces apoptosis in A549 cells

The effect of miR-30a-3p on A549 cell apoptosis was detected by our in vitro experiments, the results indicating up-regulation of miR-30a-3p-induced cell apoptosis. Compared with cells in control group, miR-30q-3p overexpression-vector-transfected cells exhibited higher apoptosis rates (Figure 4A). In addition, we observed that miR-30a-3p regulated the expression of the proteins of apoptosis-related genes. As a result, up-regulation of miR-30a-3p promoted apoptosis by inactivating Bcl-2 and accelerating Bax (Figure 4B).

DNMT3a silencing suppresses A549 cell development and up-regulating DNMT3a suppresses miR-30a-3p-induced A549 cell cycle arrest and apoptosis

As reported previously, upregulation of miR-30a-3p affects cell proliferation, cycle and apoptosis in A549

cells. We identified that DNMT3A was a direct target of miR-30a-3p. Therefore, we silenced or activated the expression of DNMT3A via RNA interference or overexpression vector to confirm that DNMT3A is involved in the antitumor effects of miR-30a-3p. RNA interference or overexpression vector significantly silenced or activated the protein expression of DNMT3a (Figure 5A and B). Moreover, silencing DNMT3A significantly suppressed cell growth, arrest of the G1 phase and inhibited apoptosis (Figure 5C–G). These results follow the same trend as those obtained with miR-30a-3p-transfected A549 cells, but this effect was reduced by up-regulation of DNMT3a.

In addition, we examined the expression genes associated with cell cycle and apoptosis. As shown in Figure 5H and I, the protein levels of the cell cycle regulators CDK2 and cyclin D were reduced by siDNMT3a, whereas those expressions were increased by up-regulation of DNMT3a. Moreover, siDNMT3a promoted cell apoptosis by inactivating the anti-apoptotic protein Bcl-2 and activating the pro-apoptotic protein

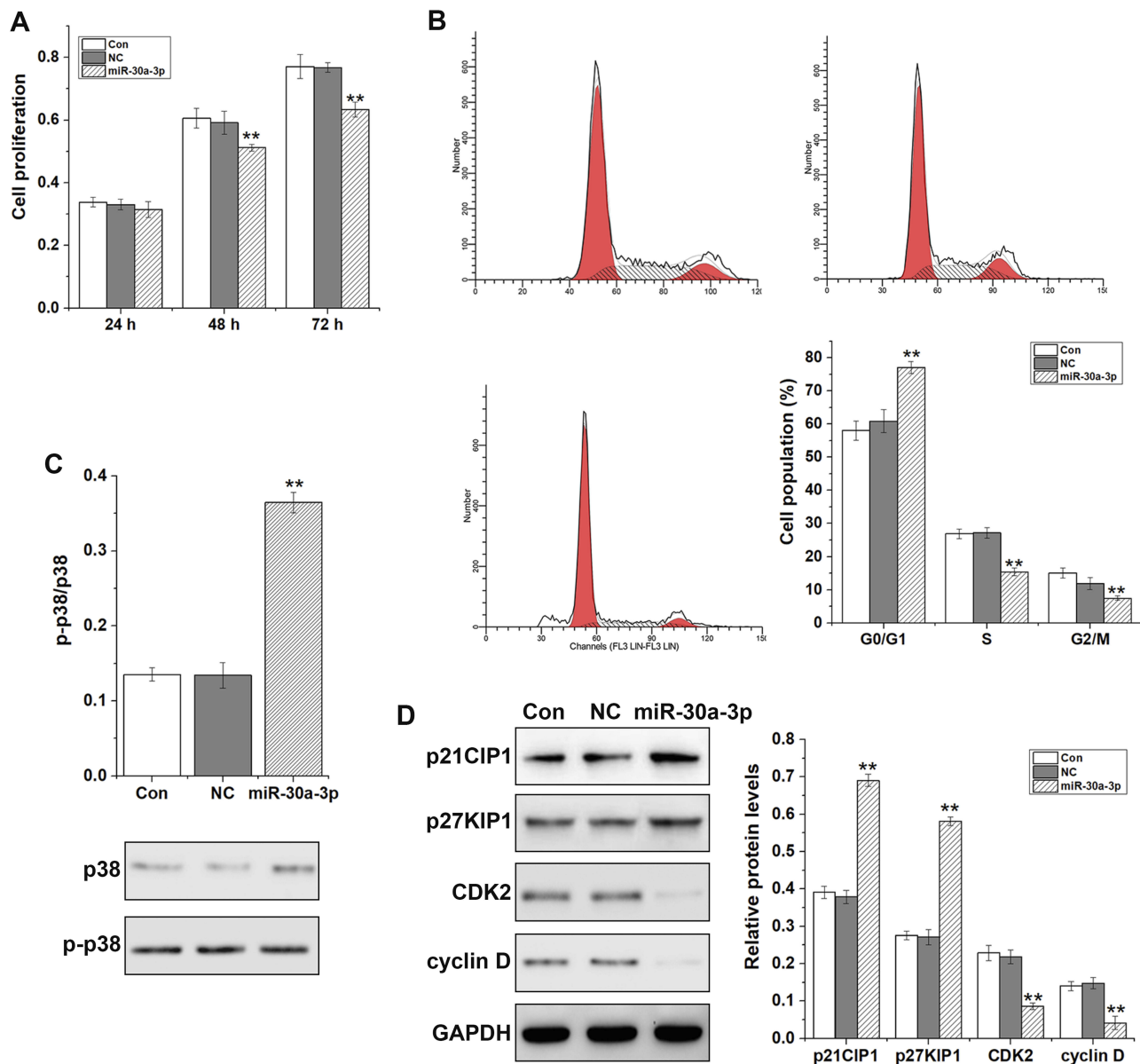


Figure 3 miR-30a-3p inhibited A549 cell proliferation and induced G1-phase arrest in vitro. **(A)** The effects of miR-30a-3p on A549 cell proliferation were evaluated by MTT assay at 24, 48 and 72 hrs. **(B)** The cell cycle was determined in A549 cells 48 hrs after transfection. **(C, D)** Analysis of the expression of cell cycle regulatory protein in A549 cells following transfection with miR-30a-3p overexpression vector. Data are reported as mean \pm SD (n=3). **P<0.1 versus NC group.

Abbreviations: Con, control group; NC, cells transfected with miR-control vector group; miR-30a-3p, cells transfected miR-30a-3p vector group.

Bax. Compared with miR-30a-3p transfected A549 cells, the cell cycle regulators CDK2 and cyclin D and anti-apoptotic protein Bcl-2 increased significantly in A549 cells transfected with miR-30a-3p and DNMT3a, and the pro-apoptotic protein Bax decreased significantly.

Discussion

Downregulation of miR-30 family has been frequently reported in various tumors, including lung, liver, prostate and thyroid, in a large-scale miRNA expression arrays.^{20–22}

MiR-30a-3p is a member of the miR-30 family, and has been demonstrated to be downregulated in several tumors, such as colorectal cancer, breast cancer and ovarian cancer.^{23–25} Consistent with the above results, we found that miR-30a-3p is downregulated in lung cancer A549 cells, and miR-30a-3p exert anti-lung cancer tumor effect by targeted DNMT3a to induced cell apoptosis and inhibited cell proliferation and cycle. Apart from the expression pattern, it has been observed that miR-30a-3p exhibited multiple roles in the regulation of tumor progression. MiR-30a-3p is

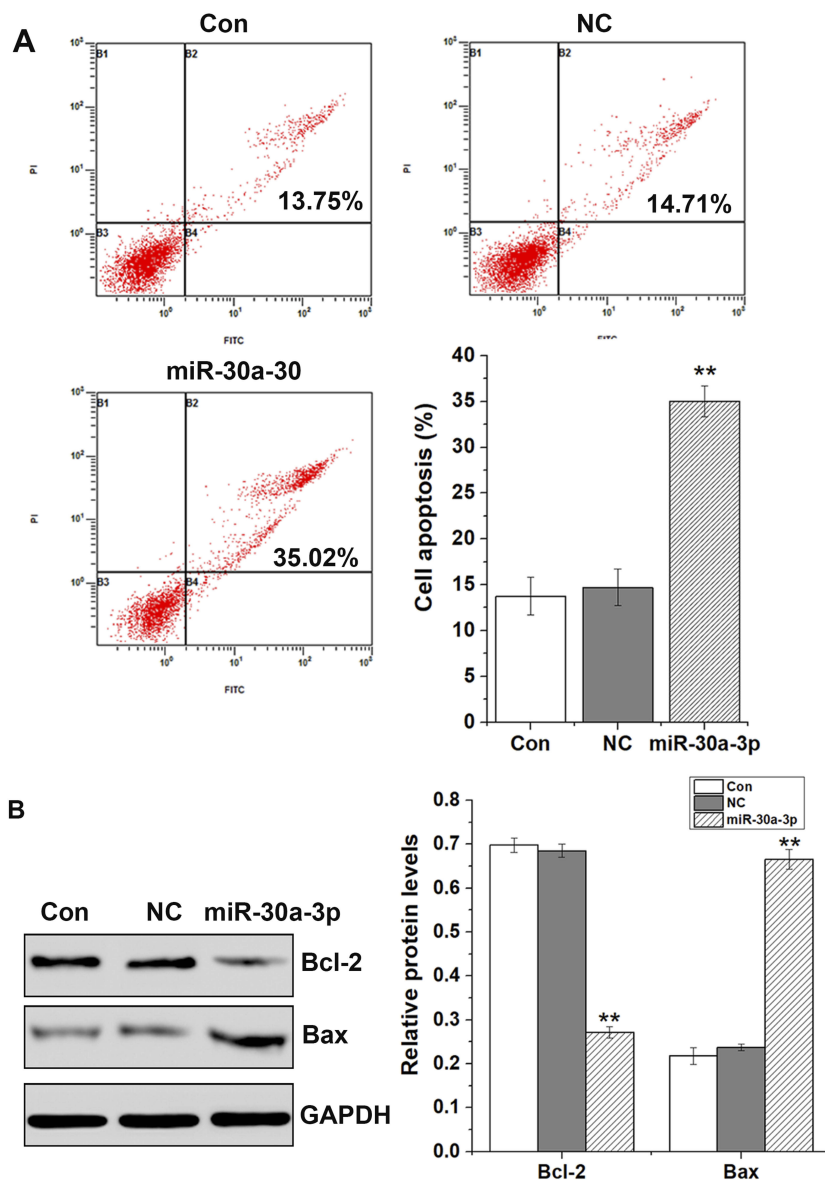


Figure 4 miR-30a-3p induces apoptosis in A549 cells. **(A)** Cell apoptosis was detected by Annexin VI/propidium iodide combined labeling flow cytometry in A549 cells at 48 hrs after transfection with miR-30a-3p. Apoptotic evaluation was carried out by the percentage of the apoptotic cell number of the total cell number. **(B)** Expression of cell apoptosis-associated proteins in A549 cells 48 hrs after transfection of miR-30a-3p by Western blot analysis. Data are reported as mean \pm SD (n=3). ** $P < 0.1$ versus NC group. **Abbreviations:** Con, control group; NC, cells transfected with miR-control vector group; miR-30a-3p, cells transfected miR-30a-3p vector group.

downregulated in hepatocellular carcinoma and acts as a tumor suppressor via regulation of cell proliferation and apoptosis.¹⁴ In breast cancer, miR-30a could suppress breast cancer cell migration and invasion and overexpression of miR-30a is associated with improved survival of breast cancer patients.^{26,27} In line with most observations, our results demonstrated that overexpression of miR-30a-3p in A549 cells dramatically reduced the proliferation and induced the apoptosis of A549 cells.

It has been reported that DNMTs-mediated hypermethylation of promoters is one of the primary reason for

inactivation of TSGs. Hypermethylation is responsible for the inactivation of TSGs involved in tumorigenesis.²⁸ DNMT3a, which act as other DNMT family members, participated in tumorigenesis, metastasis and differentiation.²⁹ In the present study, we found that miR-30a-3p was downregulated in lung cancer A549 cells, and overexpression of miR-30a-3p inhibited the DNMT3a expressions in A549 cells. As previously mentioned, p38 MAPKs were found to up-regulated in the DNMT3a depletion cells, which suppresses tumor formation by negatively regulating cell proliferation and cycle progression, or by inducing apoptosis.³⁰ Our

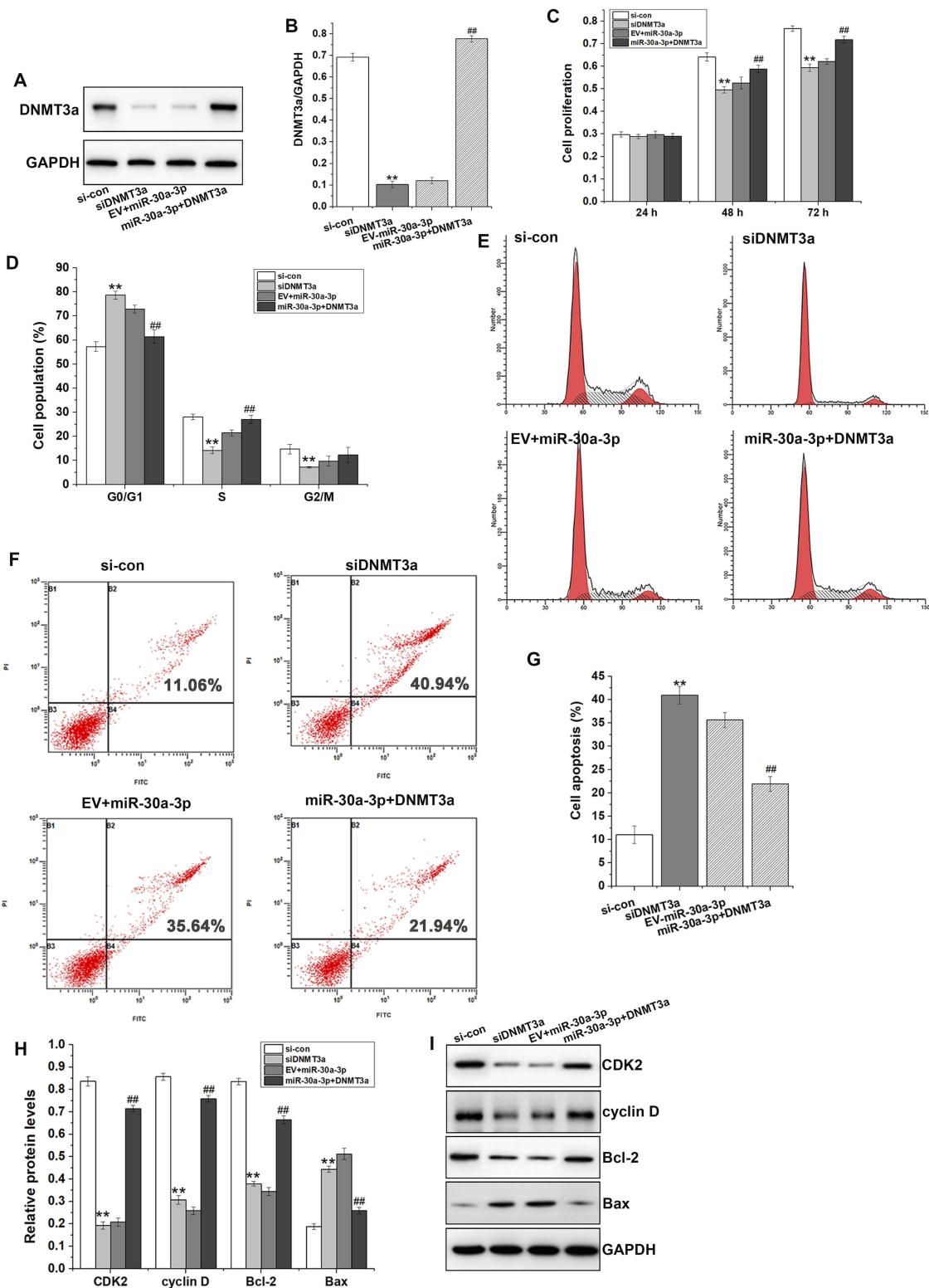


Figure 5 Silencing DNMT3a suppresses lung cancer cell proliferation and induces G1-phase arrest and cell apoptosis in A549 cells, and upregulating DNMT3a induces lung cancer cell proliferation and cycle progression and inhibits cell apoptosis in overexpression miR-30a-3p A549 cells. **(A, B)** Analysis of protein expression of DNMT3a in A549 cells at 48 hrs after transfection. **(C)** The effect of DNMT3a knockdown or overexpression on the proliferation of A549 cells were evaluated by MTT assay at 24, 48 and 72 hrs. **(D, E)** The cell cycle was determined in A549 cells at 48 hrs after DNMT3a knockdown or overexpression. **(F, G)** Cell apoptosis was detected in A549 cells at 48 hrs after DNMT3a knockdown or overexpression. **(H, I)** Analysis of protein expression associated with cell cycle and apoptosis in A549 cells at 48 hrs after transfection. Data are reported as mean \pm SD (n=3). **P<0.1 versus si-con group. ###P<0.01 versus EV + miR-30a-3p group.

Abbreviations: Si-con, cells transfected with DNMT3a empty vector group; siDNMT3a, cells transfected with DNMT3a interference vector; EV+miR-30a-3p, cells transfected with miR-30a-3p overexpression vector and DNMT3a empty vector group; miR-30a-3p+DNMT3a, cells transfected miR-30a-3p and DNMT3a overexpression vector group.

results showed that p-38 MAPK pathway was activated in A549 cells overexpression miR-30a-3p or siDNMT3a, resulting in inhibition of cell proliferation and cell cycle arrest. Evidence indicates that activation of p38-involved cell cycle regulates expression of p21CIP1, p27KIP1, cyclin D and CDKs.^{31,32} p21CIP1 and p27KIP1 are typical tumor suppressors that regulate G0 to S phase transitions by regulating the activity of CDKs.³³ Upregulation of p27KIP1 and p21CIP1 are often induced by cell–cell contact-dependent fashion to inhibit cyclin-D and CDKs and hence promotes the inhibition of cell proliferation.³⁴ In the present study, overexpression of miR-30a-3p or siDNMT3A inhibited DNMT3A expression, which leads to p38 activation. Next, we verified the effects of p38 on the downstream target genes p21CIP1, p27KIP1, cyclin D and CDK2, which are key transcriptional factors in the G0/S phase. From these results, we noted an increased expression of p21CIP1, p27KIP1 and a reduced expression of cyclin D and CDK in A549 cells transfected with miR-30a-3p.

Moreover, to explore the effect of DNMT3a and miR-30a-3p in the apoptosis of lung cancer A549 cells, we evaluated Bcl-2 and Bax protein expression levels, and suggested that miR-30a-3p inhibited the expression of DNMT3a which plays a critical role in the regulation of cell apoptosis. Bcl-2 is a proto-oncogene does not trigger cell proliferation but elevates the survival ability of cells under deleterious conditions.³⁵ Bax as pro-apoptotic member can initiate cell death pathways, although it has similar sequence homology with Bcl-2.³⁶ The ratio of Bcl-2 and Bax determines the response to a death signal via modulating membrane permeability transition pore opening. Our results indicate that miR-30a-3p can inhibit A549 cell apoptosis by upregulation of Bcl-2 and downregulation of Bax.

In addition, in order to further validate our experiments, both miR-30a-3p and DNMT3a were transfected with A549 cells; the results suggested that overexpression of DNMT3a induced the growth of lung cancer cells and suppressed miR-30a-3p expression inhibited cell proliferation and included cell cycle arrest and apoptosis. The results support our findings that miR-30a-3p targets DNMT3a and suppresses lung cancer cell growth in the opposite direction.

In conclusion, we explored the roles of miR-30a-3p and its target gene DNMT3a in the proliferation, cell cycle and apoptosis of A549 cells. Those findings indicate that miR-30a-3p might act as a novel tumor suppressor via blocking the proliferation of lung cancer cells through the p38

MAPK pathway through targeting DNMT3a. Our findings highlight the functional association of miR-30a-3p, provide new insight into the regulatory network of the cell proliferation, cell cycle and apoptosis and open possibilities for future therapeutic interventions.

Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Disclosure

The authors report no conflicts of interest in this work.

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