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MicroRNA-218 regulates the epithelial-to-mesenchymal transition and the PI3K/Akt signaling pathway to suppress lung adenocarcinoma progression by directly targeting BMI-1

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Abstract. – OBJECTIVE: To investigate the role of miR-218 in the development of lung adenocarcinoma (LA) and its underlying mechanism.

PATIENTS AND METHODS: Fifty-two pairs of human LA samples and adjacent para-cancerous tissue samples were collected from patients in hospital between June 2015 and March 2017. Meanwhile, one normal human pulmonary epithelial cell line BEAS–2B and four human LA cell lines (H1299, PC-9, A549 and SPC-A1) were cultured. The cells' ability of proliferation, migration was detected by MTT assay and Transwell assays, respectively. The target gene was clarified by dual-luciferase reporter assay. The protein and mRNA expression levels were detected by immunohistochemistry (IHC), Western blot and quantitative real-time polymerase chain reaction (qRT-PCR), respectively. Moreover, the tumor xenograft model was made for further exploring the mechanism.

RESULTS: miR-218 expressions were notably reduced in LA tissues in comparison with controls. In addition, the declined miR-218 expressions were correlated with the poor OS and worse clinicopathological parameters of LA patients. Furthermore, miR-218 overexpression could suppress the proliferation, migration and invasion abilities of cells via regulation of PI3K/Akt signaling pathway and epithelial-mesenchymal transition (EMT) respectively. Results in the current study also revealed that miR-218 upregulation could suppress the tumor growth rate and size of LA mice. B-lymphoma Mo-MLV insertion region-1 homolog (BMI-1) was confirmed to be a direct target for miR-218 and upregulated in LA tissues, which indicated the poor prognosis of LA patients.

CONCLUSIONS: MiR-218 exerted anti-tumor functions in LA partially via the regulation of BMI-1, suggesting that BMI-1/miR-218 axis may provide a novel insight into tumorigenesis and the basis for the development of miRNA-targeted therapies against LA.

Key Words:
MicroRNA-218, Lung adenocarcinoma, Epithelial-to-mesenchymal transition, PI3K/Akt, BMI-1.

Introduction

Lung adenocarcinoma (LA), one commonest lung cancer, belongs to the histological subgroup of non-small cell lung cancer (NSCLC)[1]. Recently, despite the considerable progress in LA therapies, including surgery, chemotherapy and radiation therapies, the 5-year overall survival rates for LA patients have not been prominently improved[2]. This may be at least partially result from lacking effective diagnostic methods and the diagnosis in advanced stages when distant metastasis has already occurred or local invasion is serious[3,4]. Therefore, a thorough understanding of the mechanism underlying LA progression could provide more effective diagnostic biomarkers for LA therapies. Emerging evidence has indicated that the abnormal expressions of miRNA are closely associated with tumorigenesis *via* regulating the key anti-tumor genes or oncogenes[5,6]. It has been demonstrated that dysregulations of specific miRNA are known to

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affect the progression of various cancers. For example, Duan et al[7] showed that miR-130 promoted gastric cancer cell migration and proliferation via targeting TGFbetaR2, Xu et al[8] pointed out that miR-96 promoted prostate carcinoma cell growth by suppressing MTSS1; Ding et al[9] claimed that miR-145 repressed cell proliferation and migration in breast cancer by regulating TGF-beta1 expressions. Therefore, according to the above studies, miRNAs may have great potentials to be therapeutic targets and prognostic indicators for tumors. However, there has been little quantitative analysis of the expression and roles of miR-218 in LA. Hence, further understanding of the mechanisms involved in LA progression mediated by miR-218 might be important to improve the outcome of LA patients. As a key driver in carcinogenesis[10], the PI3K/Akt pathway has been implicated in the regulation of cellular processes, including metabolism, apoptosis survival and proliferation[11]. Akt is known to be overexpressed in multiple human cancers, which is associated with the poor outcome[12]. Additionally, over activation of the AKT pathway can facilitate cell proliferation[13]. Recently, PI3K/Akt pathway has been confirmed to be fre[illegible]t-ly activated in varieties of cancers, inclu[illegible] carcinoma[14], gastric carcinoma[15] and co[illegible]tal carcinoma[16]. However, the mechanisms by [illegible] PI3K/Akt pathway integrate miR-218 into LA [illegible] proliferation has not previously [illegible]stigated. [illegible] the progress of epithelial-mese[illegible]ransition (EMT) during tumorigenesis[illegible]helia[illegible]ls were conversed to mesenchyma[illegible][17]. Mor[illegible]r, EMT is deemed to play vital r[illegible] tum[illegible] triggering cellular mo[illegible]ity [illegible]g to the invasion of cancer cell[illegible]uring t[illegible]T event, the epithelial markers[illegible]n as E-cadhe[illegible] loss of the expressions [illegible]ease in the exp[illegible]sions of N-cadherin a[illegible]me[illegible]hich are the eminent mesenchym[illegible] markers, a[illegible]firmed[19]. B-lymphoma M[illegible]ey murine leuk[illegible]virus insertion region-[illegible]MI-1) is an importa[illegible] member of the polyc[illegible] grou[illegible]ein (PcG) family, playing oncog[illegible]oles [illegible]everal t[illegible]s of tumors[20]. BMI-1 is rela[illegible]ior pr[illegible]sion, metastasis, invasi[illegible] and [illegible]ion [illegible] senescence or apoptotic path[21,][illegible]ulation of BMI-1 has been foun[illegible] multip[illegible]mors. For example, Guo et al[23] [illegible]rted that elevated BMI-1 was found in adva[illegible]ast cancer and promoted the invasion and metastasis capacities; similarly, Li et al[24] [illegible]d BMI-1 regulated EMT in breast cancer to p[illegible] the migration and invasion abilities; Li et al[25] [illegible]d that BMI-1 overexpression contributed to the hepatocellular carcinoma invasion and metastasis by enhancing the expressions [illegible] endothelial growth factor, matrix p[illegible]oprote[illegible]ase (MMP)2 and 9 via the PTEN[illegible]K/Akt pathway. However, the precise func[illegible]of BMI-1 in LA progression remain largely un[illegible]

Patients a[illegible] Meth[illegible]ds

Tissue Samples

Fifty-two pair[illegible]f hu[illegible] sample[illegible]d adjacent para-carci[illegible]a tissue[illegible]s w[illegible] collected from the Ch[illegible]pan Union H[illegible]f Jilin University bet[illegible]2015 and Ma[illegible]2017. All patients di[illegible] rece[illegible]motherapy or radiotherapy before the tissue colle[illegible] Written informed consent[illegible]eived from an[illegible]atients. All the tissue s[illegible]es were frozen immediately in liquid nitrogen [illegible]stored at -80°C[illegible]for further use. This study was [illegible]roved by the I[illegible]s Committee of The China-Japan Union Hospi[illegible]f Jilin University.

Cel[illegible]

One normal human pulmonary epithelial cell [illegible]ine BEAS-2B and four human LA cell lines [illegible]PC-9, A549 and SPC-A1) were purchased [illegible]om [illegible]e American Type Culture Collection (Manassas, VA, USA). All the cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) which contained 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in a humidified atmosphere at 37°C with 5% CO_2.

Cell Transfection

miR-218 mimics, inhibitor as well as the corresponding controls were obtained from Gene Pharma (Shanghai, China). Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) was utilized to transfect the miRNAs into LA cells following the manufacturer's proposals.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The total RNAs from LA tissues and cultured cells were isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, PrimeScript™ RT reagent kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) was used to reversely transcribe the RNA into cDNA in line with the manufacturers' protocols. Then, complementary deoxyribose nucleic acid (cDNA) was then amplified with the SYBR Green Master Mix kit (TaKaRa, Dalian, China) on

the system of ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The gene expression was evaluated by the $2^{-\Delta\Delta CT}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were internal controls for BMI-1 and miR-218, respectively. The sequences of the primers were described in Table I.

Immunohistochemistry (IHC)

The IHC assay for BMI-1 was carried out to detect the BMI-1 expressions. Briefly, 10% formalinfixed and paraffin embedded tissue sections were deparaffinized and hydrated with xylene and graded alcohols. After antigen retrieval in a microwave oven, the activity of endogenous peroxidase was blocked with 3% hydrogen peroxide in ethanol for 10 min. Next, 5% normal goat serum in 0.01 M PBS was used to block nonspecific binding. Slides were then incubated with BMI-1 antibody (1:200, ab126783, Abcam, Cambridge, MA, USA) at 4°C in a moist chamber overnight and incubated with biotinylated goat anti-rabbit antibodies (1:500, ab7090, Abcam, Cambridge, MA, USA) at room temperature for 30 min. Next, a SP staining kit (Zhongshan Golden Brids[illegible] technology, Beijing, China) was used to [illegible] the slides with DAB as a chromogen and [illegible]n-terstained by hematoxylin following the m[illegible] facturer's proposals. The expression levels w[illegible] determined on the basis of the ratio of positi[illegible] cells: stained cells/all cells < [illegible] regarded as negative (-), while >25% [illegible]dent[illegible] as positive (+). Positive rate of e[illegible]ssion = [illegible]mber of positive cells/total numb[illegible]lls.

Cell Proliferation [illegible]says

MTT assays we[illegible]rformed to m[illegible] the proliferation ability [illegible]s with differen[illegible]atment. After transfec[illegible]with [illegible]18 mimics or inhibitor, LA cells were harvested and plated into 96-well plate. After incubated for 0 h, 24 h, 4[illegible] at 37°C, MTT (3-(4,5-dimethylthiaz[illegible])-2,5-phenyl tetrazolium bromide) (10 µl[illegible]g/mL) (Sigma-Aldrich, St. Louis, MO, US[illegible] added into each well, followed by an incubation[illegible]ther 4 h. Subsequently, 100 µL dime[illegible]sulfox[illegible]MSO) (Sigma-Aldrich, St. Louis[illegible], USA) was [illegible] solubilize the crystals. [illegible] optical[illegible]ensity ([illegible]0 was measured with a[illegible]roplate[illegible]der (Bio-Rad, Hercules, CA, USA).

Cell Migrati[illegible]nd Inv[illegible]A[illegible]s

Transwell[illegible]ys were carri[illegible] detect the LA cell in[illegible]l migration c[illegible]acities. Briefly, cell i[illegible]ion w[illegible]ssed by transwell inserts (8.0 µm pore size, C[illegible] Incorporated, Corning[illegible]USA) coated [illegible] Matrigel (BD Biosc[illegible]es, Franklin Lakes, NJ, USA) as a matrix [illegible]ier, whereas [illegible]ll migration was detected by [illegible]nswell inser[illegible]ithout Matrigel coated. The [illegible]wing steps [illegible]e the same for invasion and m[illegible]ion assa[illegible] Following 48 h of transfection, [illegible]e added in the top chambers of the inserts [illegible] serum-free medium. At the same [illegible]me, the bottom chambers were added with me-[illegible]aining 10% fetal bovine serum (FBS) [illegible]che[illegible]battractant. Being incubated for 48 h at 37°C with 5% CO_2, cells that remained on the top chambers were removed with cotton swabs and those attached to bottom chamber were fixed (10% methanol, 37°C, 15 min) and stained (0.1% crystal violet, 37°C, 10 min) for the detection under an inverted microscope (Olympus, Tokyo, Japan) from five randomly selected visual fields.

Dual-Luciferase Reporter Assay

Wild-type BMI-1 3'-UTR containing binding sites of miR-218 or the mutated BMI-1 3'-UTR

Table I. [illegible]er seq[illegible]ces for qRT-PCR.

P[illegible]	Sequence
miR-21[illegible]	5'- TTGCGGATGGTTCCGTCAAGCA-3'
[illegible]218 re[illegible]	5'- ATCCAGTGCAGGGTCCGAGG -3'
[illegible]ward	5'-CTCGCTTCGGCAGCACA-3'
U6 [illegible]se	5'- AACGCTTCACGAATTTGCGT-3'
BM[illegible]orward	5'- CCACCTGATGTGTGTGCTTTG-3'
[illegible]	5'-TTCAGTAGTGGTCTGGTCTTGT-3'
[illegible]DH for[illegible]	5'- TGTGGGCATCAATGGATTTGG -3'
[illegible]APDH reverse	5'- ACACCATGTATTCCGGGTCAAT -3'

[illegible] nuclear RNA, snRNA. BMI-1: B-lymphoma Moloney murine leukemia virus insertion region-1. GAPDH: glyce[illegible]hyde-3-phosphate dehydrogenase.

was cloned into the pGL3 plasmids (Promega, Madison, WI, USA), to get the wild-type BMI-1 -3'UTR or mutant BMI-1 -3'UTR, respectively. LA cells were cotransfected with wild-type or mutant 3'-UTR of BMI-1 along with miR-218 mimics. At 48 h after cotransfection, the luciferase activities for the wild-type or mutant BMI-1 3'-UTR was determined by luciferase reporter assays (Promega, Madison, WI, USA).

Western Blotting Analysis

The whole protein from LA cells was harvested in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (Roche, Basel, Switzerland) and subjected to bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) for protein concentration. Then, proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland), which were blocked with 5% non-fat milk in tris-buffered saline-tween (TBST) for 2 h at room temperature and incubated with specific primary anti[bod]ies at 4°C overnight. The following primary [anti]bodies were used: rabbit antibodies against [BMI]-1 (1:500, ab135713, Abcam, Cambridge, MA, U[SA]), E-cadherin (1:1000, ab15148, Abcam, Cambridge, MA, USA), Vimentin (1:1000, ab[1]27321, Abcam, Cambridge, MA, USA), PI3K (ab86714, Abcam, Cambridge, MA, U[SA]), Akt (000, sc-56878, Santa Cruz Biotechnology, S[anta] Cruz, CA, USA), p-PI3K (1:[1]000, ab182[651], Cambridge, MA, USA), p-[Akt] (, sc-81433, Santa Cruz Biotechnology, Santa [Cruz], CA, USA) and GAPDH (1:[5]000, ab70699, Abcam, Cambridge, MA, USA). Subsequently, the membranes were washed with TBS[T] 3 times, followed by being incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3000, ab2057[01], Abcam, Cambridge, MA, USA) for 2 h at room temperature. The results were determined by enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). GAPDH served as internal control.

Tumor Xenograft Model

Animal assays were approved by the Animal Committee of Jilin University (Jilin, China). Mice were randomly divided into two groups and inoculated subcutaneously on the flank with stable expressing A549 cells which was transfected either with lentiviral miR-218 (lenti-miR-218) or the negative lentiviral miR-control (lenti-control). The length and width of the diameters were measured every three days. Tumor volumes (mm^3) = length×width^2/2. Following treatments for 28 days, all of the mice were sacrificed for tumor isolation.

Statistical Analysis

All experiments were carried out at least three times. Statistical Product and Service Solutions (SPSS) software version 17.0 (SPSS Inc., Chicago, IL, USA) was applied for statistical analysis. Student's *t*-test, ANOVA and Scheffe post-hoc analysis were applied, where appropriate. Kaplan-Meier and log-rank tests were applied to estimate the survival rates and compare the survival curves respectively. $p<0.05$ was regarded as statistically significant difference.

Results

miR-218 Down-regulation in LA Was Correlated with Poor Prognosis

To elucidate the prognostic roles of miR-218 in LA, we first examined the miR-218 expressions in tissues. The qRT-PCR results showed that miR-218 expressions were notably reduced in LA tissues in comparison with normal tissues (Figure 1A). In addition, we further investigated the clinical significance of miR-218 in LA. The mean miR-218 expression was used as the cut-off to assign the LA patients into miR-218 high expressing group and low expressing group. The clinicopathological characteristics of the LA patients included in current study were shown in Table II. Data indicated that low miR218 expression was associated with malignant clinicopathologic features in LA patients. Moreover, in order to analyze the overall survival of LA patients, we performed the Kaplan-Meier analysis and found that the low miR-218 expressions were prominently associated with shorter OS in LA patients (Figure 1B).

miR-218 Repressed LA Cell Proliferation

The expressions of miR-218 in several LA cells were also detected and the results demonstrated prominent decreases of miR-218 expressions in LA cells compared to BEAS–2B (Figure 2A). To further investigate the tumor repressive roles of miR-218 in LA cells, the loss-of-function or gain-of-function assay was carried out. Briefly, the overexpression or inhibition of miR-218 was obtained by transiently transfecting miR-218 mim-

Table II. Correlation of miR-218 expression with the clinicopathological characteristics of the LA patients.

Clinicopathological features	Cases (n=52)	miR-218# expression High (n=20)	Low (n=32)	value
Age (years)				0.
> 60	30	12	18	
≤ 60	22	8	14	
Gender				2564
Male	25	9	16	
Female	27	11	16	
Tumor size (cm)				0.17
≥ 5.0	26	7	19	
< 5.0	26	13		
Lymph node metastasis				031*
Yes	29	4		
No	23	16	7	
TNM stage				0.0026*
I +II	25	15	10	
III+IV	27	5	22	
Smoker				0.2231
Yes	29	12	17	
No	23	8	15	

LA: Lung adenocarcinoma; TNM: tumor-node-metastasis. #The mean e ion level of p 18 was used as the cutoff. *Statistically significant.

ics or inhibitor into PC-9 or A549 cells, ch had the lowest and highest endogenous mi expressions respectively (Figure 2B and 2C). MTT assays revealed that miR-218 overexpre sion dramatically inhibited PC liferation whereas miR-218 inhibition d rem bly enhance the proliferation abi of A549 ls (Figure 2D).

miR-218 Inhibite A Cell ion and Migration

Next, transw was carried to determine the i sion migration abilities of LA cells w different t tions. The results showed t miR-218 upreg in PC-9 dramatica epress d the invasio and migration capa s of PC ells (Figure 3A and 3B). On the hand R-218 pression in A549 cells notab d the i on and migration abilities (Fig and

BM was a ct Target of miR-218 in Cells

mechanisms about the inhibito- fects of miR-218 in LA cells, Targetscan was ed to explore the candidate targets of miR-218 a I-1 was one predicted target for miR-218 (Fig 4A). Luciferase reporter assay was carried her determine the direct correlation be- iR-218 and BMI-1. Luciferase activity of LA cells co-transfected with BMI-1-3'UTR-WT and miR-218 mimics was significantly decreased whereas cotransfection with miR-218 mimics and BMI-1-3'UTR-MUT had no influence on the luciferase activity (Figure 4B). Furthermore, qRT-PCR was conducted to determine the regulatory effects of miR-218 on BMI-1 expressions. Results demonstrated that miR-218 overexpression in PC-9 cells markedly decreased the BMI-1 expressions (Figure 4C) while miR-218 suppression in A549 cells notably increased the BMI-1 expressions (Figure 4D). Data confirmed that BMI-1 was a direct target for miR-218 in LA cells.

miR-218 Regulated the PI3K/AKT Signaling Pathway and EMT in LA Cells

The underlying mechanisms of inhibitory effect mediated by miRA-218 in LA progression were next investigated. Firstly, IHC assays were performed to examine the BMI-1 expressions in LA tissues. Results indicated that BMI-1mainly localized at the nucleus and upregulated in LA tissues in compassion with the normal tissues (Figure 5A and 5B). Additionally, Kaplan-Meier analysis demonstrated that LA patients with high BMI-1expression levels

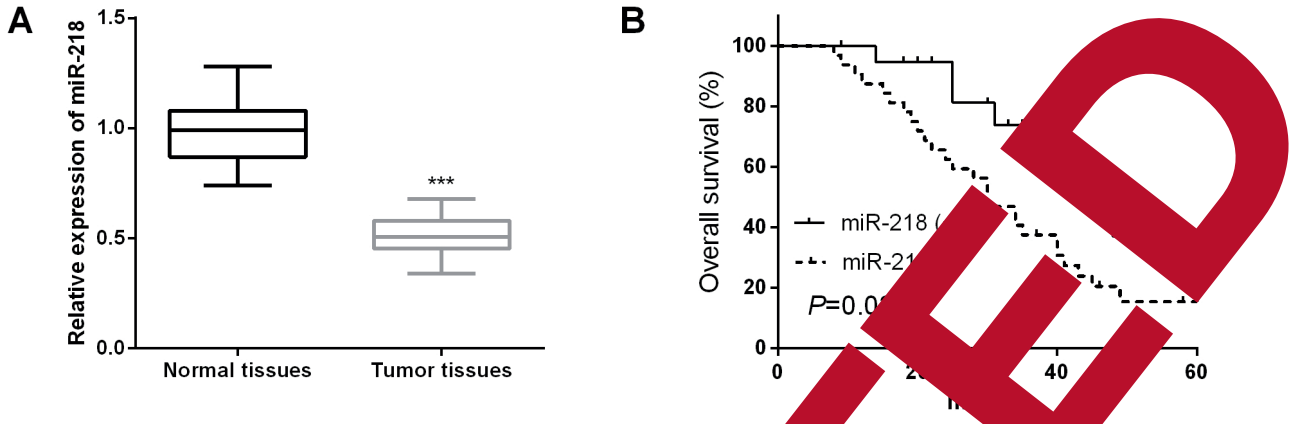


Figure 1. miR-218 was down-regulated in LA. **A,** miR-218 expression levels in LA tissues and normal tissues were detected using qRT-PCR. **B,** Kaplan-Meier analysis of LA patients with different miR-218 expression. ***p<0.001.

had shorter OS (Figure 5C). To explore whether miR-218 contributed to regulating the PI3K/Akt signaling pathway and EMT in LA cells, Western blot analysis was performed to detect the expressions of p-PI3K, PI3K, p-Akt and Akt as well as the EMT related markers in PC-9 or A549 cells treated with miR-218 mimics or inhibitor. As shown in Figure 5D, the expressions of p-PI3K and p-Akt were prominently decreased by miR-218 overexpression in PC-9 cells while there was no statistically significant effect on PI3K and Akt expressions. Meanwhile, miR-218 overexpression in PC-9 cells obviously enhanced the E-cadherin expressions whereas reduced the N-cadherin and vimentin expressions. On contrary, miR-

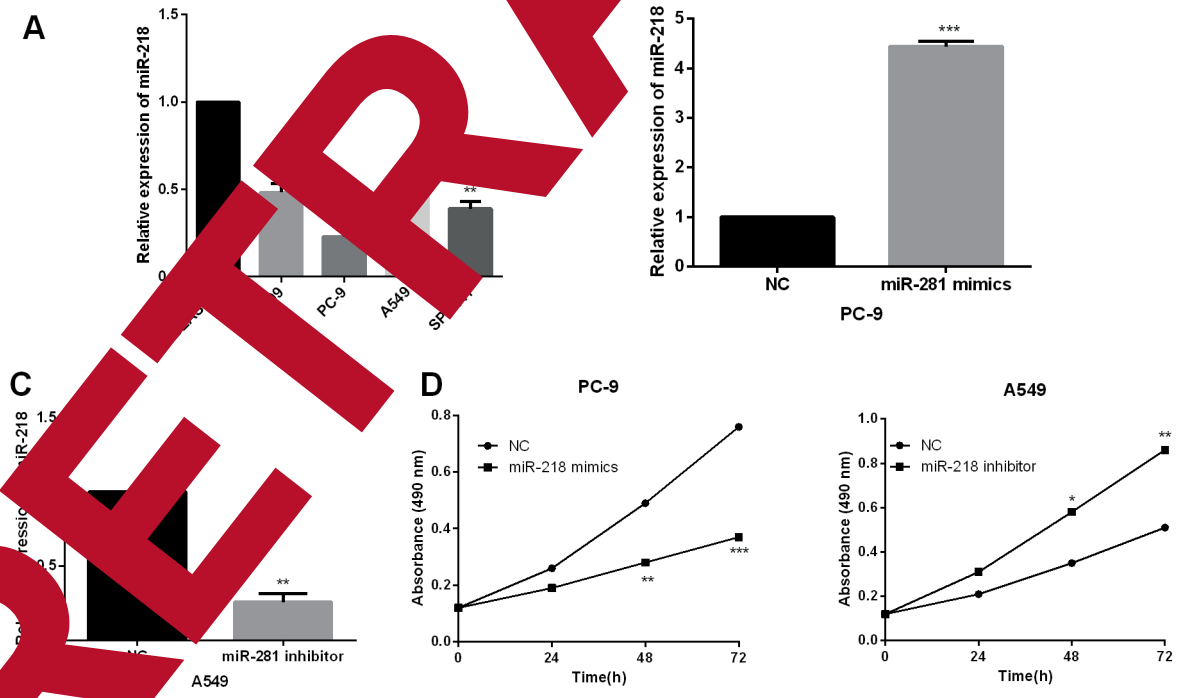


Figure 2. miR-218 overexpression inhibited LA cell proliferation. **A,** qRT-PCR analysis was utilized to measure miR-218 expressions in LA cells and normal pulmonary epithelial cell BEAS–2B. **B,** miR-218 expressions in PC-9 with transfections of miR-218 mimics. **C,** miR-218 expressions in A549 with transfection of miR-218 inhibitor. **D,** Cell proliferation was observed by MTT assays in PC-9 or A549 cells treated with miR-218 mimics or inhibitor. ***p<0.001, **p<0.01, *p<0.05.

218 inhibition in A549 cells led to significant increase of p-PI3K, p-Akt, N-cadherin and vimentin expressions and decrease of E-cadherin expressions. Above data indicated that miR-218 inhibited LA progression by regulating EMT and PI3K/Akt signaling pathway.

miR-218 Suppressed the Tumor Growth of LA in Vivo

The functions of miR-218 in tumor growth *in vivo* were further investigated. A549 cells stably transfected with lentiviral miR-218 (lenti-miR-218) or the negative lentiviral miR-controls (lenti-control) were subcutaneously injected into nude mice and the tumor sizes were detected every 3 days. Results showed that tumors of mice in lenti-miR-218 group grew more slowly and were smaller than control groups (Figure 6A and 6B).

Discussion

LA remains one great challen[illegible] health and contemporary oncol[illegible] around the world. Although existing diagn[illegible]nd therapic modalities are still innovating, th[illegible]osis for LA patients remains poor [illegible]h the i[illegible] sur vival rate is 15%[26]. The[illegible]e, develop[illegible] LA have heightened th[illegible]ed for[illegible]ore rese es on the mechanis[illegible] provi[illegible]eoretical basis for further LA [illegible]ies[illegible]er the [illegible] few decades, our un[illegible]stan[illegible] LA p[illegible]genesis has greatly. Th[illegible] regulati[illegible]vari[illegible]miRNAs has been de[illegible]ed in LA, fu[illegible]g as either a suppres[illegible]oncogene in [illegible]mor progression[27]. F[illegible]exam[illegible]iao *et al*[28] proposed that miR-1290 facilitated[illegible]ell proliferation and inv[illegible]*a* targeting [illegible]4; Xu *et al*[29] indi-

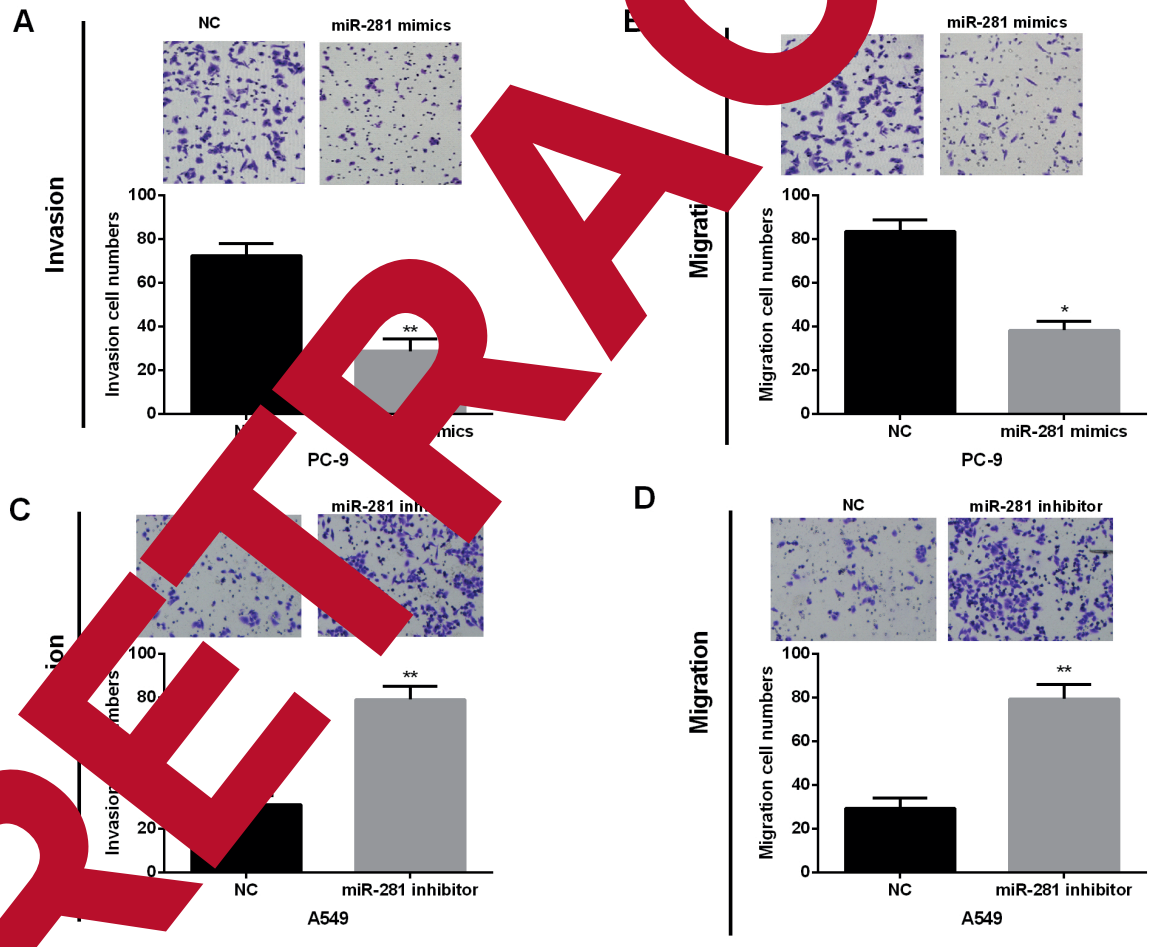


Figure 3. miR-218 overexpression suppressed LA cell invasion and migration. **A,** Cell invasion and **B,** migration abilities were [illegible]sed by Transwell assay in PC-9 with transfections of miR-218 mimics. **C,** Cell invasion and **D,** migration abilities were [illegible]rved by Transwell assay in A549 with transfection of miR-218 inhibitor. $^{**}p<0.01$, $^{*}p<0.05$.

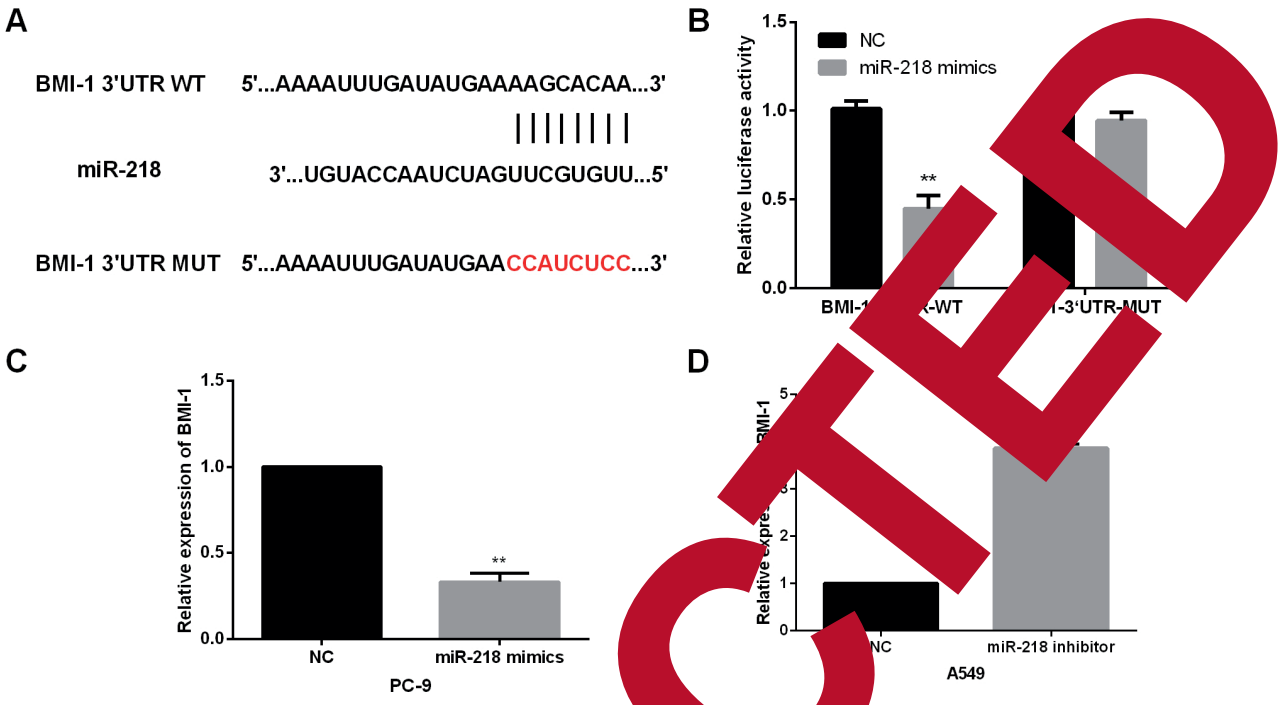


Figure 4. BMI-1 was a direct target of miR-218 in LA cells. **A,** Putat and mutant (MUT) miR-218 binding sites in the 3'-UTR of BMI-1. **B,** Relative luciferase activities were anal ls co-transfected with miR-218 mimics and WT or MUT reporter plasmids. **C-D,** BMI-1 ex s in PC-9 or A549 cells treated with miR-218 mimics or inhibitor respectively. $^{**}p<0.01$.

cated that miR-31 exerted anti-tumor functi in LA mainly *via* regulating HuR; Zhao *et a* reported that miR-15b regula ll metas- tasis and cisplatin resistance arget PEBP4. However, the functional cts of 218 on LA progression remain unc has been verified to b abn xpressed in various malignanci playing functions. For instance, Zha *al*[31] reporte miR-218 inhibited gastri angiogenesis a regu- lation of RO He found that miR-218 modulated breast cancer atin chemosensi- tivity *via* eting BRCA1; dinejad *et al*[33] demon ed that decreasedmiR-218 expression in hu breas cinoma was associated with ma t fe s. In rent study, the func- tiona d pot mechanism of miR- 218 in LA inve ted. It was observed that tissue patients exhibited signif- ican crease R-218 expressions. Further- mor iR-218 overexpression caused prominent su A cell proliferation, invasion and ation by regulating PI3K/Akt signaling path- and EMT. In addition, decreased miR-218 in confirmed to be related to worse clinico- path gical features and poorer prognosis. miR-218 overexpression was found to suppress the tumor growth rate and tumor size of LA mice. These data together demonstrated that miR-218 may serve as a tumor-suppressor in LA. Previous studies demonstrated that BMI-1 is closely associated with cancer progression[34,35], playing vital clinical function. Given the abilities of BMI-1 in regulating multiple oncogenic processes, we investigated the roles of BMI-1 in LA to further our understanding of the mechanisms underlying LA progression. Briefly, in this study, BMI-1 was identified as a direct target of miR-218 and was up-regulated in LA. In addition, high BMI-1 expressions indicated poorer prognosis of LA patients. Consistent with our findings, Meng *et al*[36] found that BMI-1 knockdown inhibited LA cell metastasis. Taken together, the present study indicated that miR-218 expressions were remarkably reduced in LA tissues and was related to poor prognosis and worse clinicopathological features of LA patients. miR-218 overexpression prominently suppressed LA cell proliferation, invasion and migration by regulating PI3K/Akt pathway and EMT. Moreover, miR-218 overexpression could suppress the tumor growth rate and tumor size of LA mice. Furthermore, BMI-

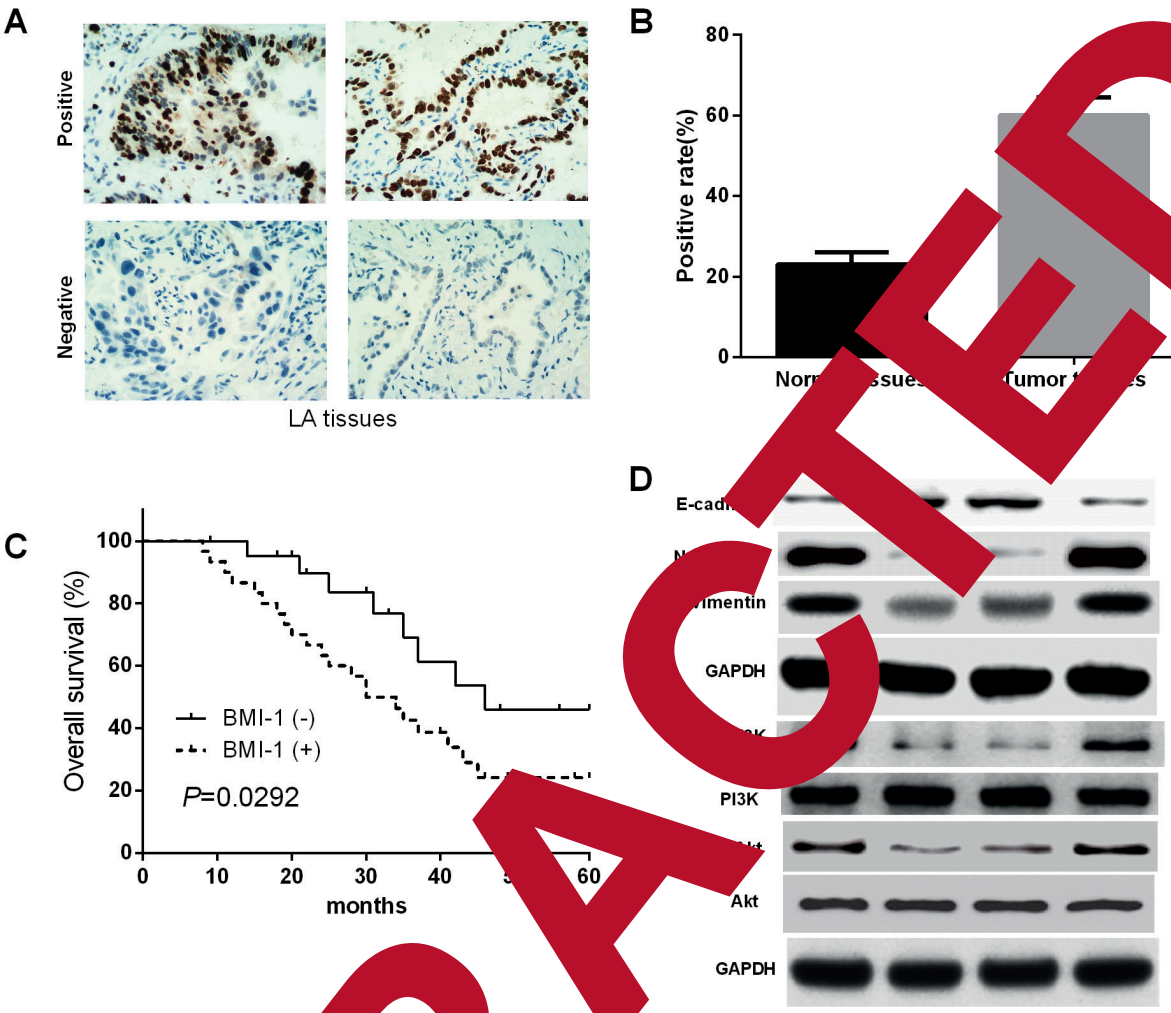
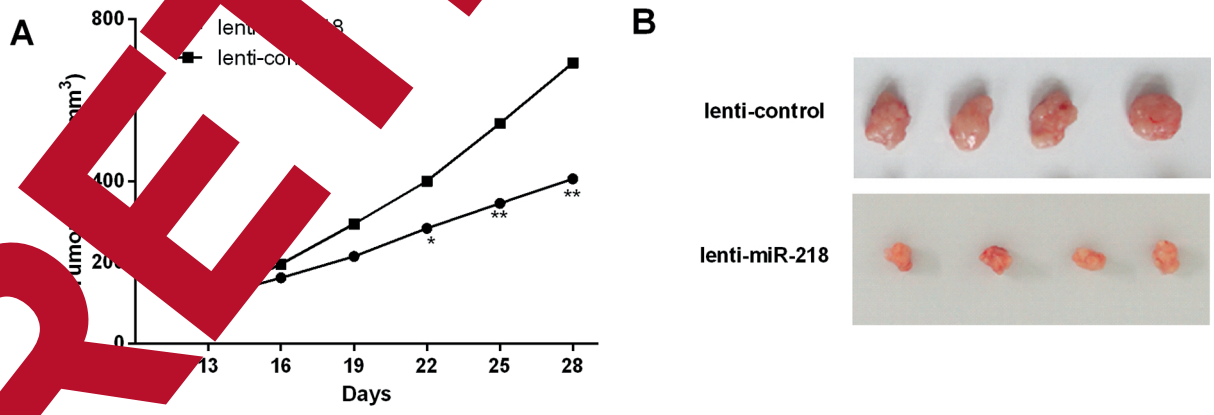


Figure 5. miR-218 regulated P AKT sign pathway and EMT in LA cells. **A-B,** BMI-1 expressions were determined by IHC in LA tissue samples. Representa C assays and the IHC scores. **C,** Survival analysis of LA patients with different BMI-1 expre d by Kap -Meier survival analysis. **D,** The effects of miR-218 on expressions of EMT markers and PI3 T sig hway were determined. **$p<0.01$.



6. miR-218 inhibited LA tumor growth in vivo. **A,** Tumor volumes were calculated every 3 days after inoculation from d 28. **B,** Compared with the lenti-control group, the tumor sizes in lenti-miR-218 group were significantly decreased. **$p<$ *$p<0.05$.

1 was considered as a direct functional target of miR-218 and overexpressed in LA tissues, indicating a poor prognosis.

Conclusions

The suppressive functions of miR-218 in LA were partially regulated by BMI-1, which may provide a novel insight into tumorigenesis and the basis for the development of miRNA-targeting therapies against LA.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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