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miR-99a Suppresses the Metastasis of Human Non-Small Cell Lung Cancer Cells by Targeting AKT1 Signaling Pathway

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ABSTRACT

MicroRNAs (miRNAs) play an important role in the development and progression of non-small cell lung cancer (NSCLC). Recently, several studies have shown that miR-99a is downregulated in various cancers, which can affect tumor initiation and maintenance. Herein, we found that miR-99a was downregulated in NSCLC tissues and suppressed tumor metastasis of NSCLC cells. Down-regulation of miR-99a is significantly associated with last-stage and tumor metastasis in NSCLC patients. Further functional experiments found that overexpression of miR-99a inhibit cell proliferation, migration, and invasion of NSCLC cells in vitro and tumor metastasis of NSCLC in vivo. In addition, we also found that *AKT1* is directly involved in miR-99a-mediated tumor suppression. Restored the expression of AKT1 partially abolished the suppressive effects miR-99a on proliferation and invasion of NSCLC cells. Collectively, our data suggest that miR-99a plays an important role in the tumorigenesis and metastasis of NSCLC and may serve as a therapeutic target to avoid dissemination of NSCLC cells. J. Cell. Biochem. 116: 268–276, 2015.

KEY WORDS: microRNA; NON-SMALL CELL LUNG CANCER; miR-99a; *AKT1*; MATRIX METALLOPROTEINASE 2

Human lung cancer is the most commonly diagnosed cancer and the leading cause of cancer mortality worldwide, with nearly 1.4 million deaths each year [Jemal et al., 2010]. Lung cancer is a malignant tumor with high level of heterogeneity, among of which non-small cell lung cancer (NSCLC) accounts for almost 70–85% of this disease. Although great efforts have been exerted in the systematic therapies of NSCLC patients, the 5-year overall survival rate still remains less than 15% [Heist and Engelman, 2012]. One of the major causes of cancer death is the tumor metastasis, as 40% stage I NSCLC patients are dying of distant metastasis within 5 year after curative tumor resection, and the number raises to 60% in stage II patients [Strauss, 2005]. Metastasis has been a consistent problem in tumor prognosis and therapy, while the knowledge about it remains poor. Hence, identification of new molecules involved in tumor metastasis is of crucial importance to reduce morbidity and mortality of this devastating disease.

Nowadays, microRNAs (miRNAs), which are a kind of endogenous, small, non-coding RNAs, are considered to be important components of cancer signaling network and are emerging as novel biomarkers of the disease [Kong et al., 2012; Cao et al., 2013]. By partially complementing with the 3′-untranslated region (3′UTR) of specific messenger RNAs (mRNAs), miRNAs can modulate gene expression by regulating translational efficiency or cleavage of target mRNAs [Bartel, 2004], and be involved in various physiologically and/or pathologically biological progresses [Chen and Rajewsky, 2007].

Currently, the role of dysregulated miRNAs in the initiation and progression of lung carcinogenesis has been widely studied [Zhang et al., 2013]. Many miRNAs have been reported to be associated with the tumor metastasis of NSCLC, such as Arora et al. profiled the miRNAs in NSCLC patients with and without brain metastasis, and found that expression of miR-328 and miR-330–3p was able to correctly classify NSCLC patients with brain metastasis or not. Overexpression of miR-328 has a role in conferring migratory potential to NSCLC cells through targeting protein kinase C alpha (*PRKCA*) [Arora et al., 2011]; MiR-335 was reported to inhibit small cell lung cancer cells bone metastases through suppressing the IGF-1R and RANKL signaling pathways [Gong et al., 2014]; Moreover,

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let-7c was down-regulated in NSCLC tissues and ectopic let-7c significantly impairs migratory and invasive capacity of NSCLC cells in vitro [Zhao et al., 2014]. Thus, identifying the dysregulated miRNAs during NSCLC metastasis may provide new avenues for the diagnosis and therapy of NSCLC in the future.

MiR-99a is a newly identified tumor suppressor, the suppressive effects of which have been reported in a variety of human cancers, including breast, oral, and hepatocellular cancers [Hu et al., 2014; Yen et al., 2014; Zhang et al., 2014]. Especially, the role of miR-99a in tumor migration and metastasis has been widely investigated. In normal murine mammary gland cells, exogenous transfection of miR-99a induced the epithelial to mesenchymal transition (EMT) through modulating the expression of transforming growth factor-β (TGF-β) signaling pathway [Turcatel et al., 2012]. During EMT, epithelial cells lose epithelial features and express mesenchymal cell markers, which correlated with increased cell migration and invasion [Kiesslich et al., 2012]. Through inhibiting myotubularin-related protein 3 (MTMR3) expression, increased miR-99a expression reduced cell matrigel invasion and transendothelial migration of oral cancer cell lines, HSC-3 and OEC-M1 [Kuo et al., 2014]. Otherwise, miR-99a was found to be significantly reduced during the dermal wound healing, which composed of a complex series of biological events containing cell proliferation, migration and angiogenesis, and *AKT1* was involved in miR-99a-mediated regulation [Jin et al., 2013].

AKT1 belongs to the *AKT* family, which consists of *AKT1*, *AKT2,* and *AKT3*. Among these genes, *AKT1* is an intriguing member, which locates on chromosome 14q32.32 and has been reported to be a recurrent activating mutation in many cancers, such as breast, colorectal, and ovarian cancers [Carpten et al., 2007]. Due to each protein′s specific expression and function in various tissues, their effects on biological phenotypes are totally dissimilar. Homozygous knockdown of *AKT1* results in partial embryonic lethality and surviving mice are approximately 20% smaller in size than wild-type littermates [Cho et al., 2001b]. In contrast, insulin resistance and a diabetes type II-like phenotype was observed in *AKT2* knockdown mice, and knockdown of *AKT3* in mice resulted in reduction in overall brain size of mice, respectively [Cho et al., 2001a; Easton et al., 2005]. For NSCLC patients, *AKT1* polymorphisms were significantly associated with survival outcomes [Kim et al., 2012]. Moreover, siRNA-induced knockdown of AKT1 significantly enhanced the chemosensitivity of NSCLC cell line H460 to cisplatin, and decreased colony formation and migration. But *AKT2* siRNA had no significant effects on these parameters [Lee et al., 2011]. These studies provide a solid foundation for *AKT1* is positively related with NSCLC carcinogenesis. Thus, we hypothesized that *AKT1* might be involved in miR-99a-mediated acquisition of metastasis characteristics of NSCLC.

In this study, we have revealed that the expression of miR-99a was suppressed in primary NSCLC tissues, and it was highly associated with metastasis and clinical stage of NSCLC patients. Further analyses showed that ectopic miR-99a expression suppressed NSCLC cell growth and metastasis in vitro and in vivo, through suppressing the *AKT1* signaling pathway. These results indicate a molecular pathway with might relieve the malignant biological behaviors of NSCLC, and provide a potential therapy strategy for NSCLC patients by targeting miRNA expression.

MATERIALS AND METHODS

PATIENTS AND TISSUE SPECIMENS

105 paired samples of human NSCLC and their matched adjacent noncancerous tissues were collected at the time of surgery between 2012 and 2013 at the Department of Pulmonary Disease, the First Affiliated Hospital of Harbin Medical University. The matched normal tissues were obtained 5 cm distant from the tumor margin, which were further confirmed by pathologists. Upon resection, human surgical specimens were immediately frozen in liquid nitrogen and stored at −80°C in the refrigerator. All patients did not undergo any therapy before recruitment to this research. The use of the tissue samples for all experiments was approved by the Ethics Committee of the instruction.

CELL CULTURE AND TRANSFECTION

Human NSCLC lines (A427, A549, NCI-H520, NCI-H1299, and NCI-H1437) or human bronchial epithelial cell line (16HBE) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in Dulbecco′s modified Eagle medium (16HBE, A427, and A549) (DMEM, Gibco, Life Technologies, Darmstadt, Germany), RMPI-1640 (NCI-H520, NCI-H1299, and NCI-H1437) supplemented with 10% fetal bovine serum (FBS; PAA, Pasching, Austria), streptomycin (100 μg/ml), and penicillin (100 U/ml). Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C. MiR-99a/scramble mimics were purchased from Dharmacon (Austin, TX). Among these cell lines, A549 and NCI-H1299 were picked up for their highly metastatic capacity. According to manufacturer′s instructions, all oligonucleotides were transfected into NSCLC cells to a final concentration of 50 nM by Dhamafect 1 (Dharmacon, Lafayette, CO). Cells were collected for further experiments 48 h post-transfection.

RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR

For analysis the expression of miR-99a in NSCLC, total RNA was isolated from human surgical specimens and cells according to the protocol of Recover All Total Nucleic Acid Isolation Kit (Ambion, Austin, TX). Following gel electrophoresis verification of RNA integrity, total RNA was reverse transcribed using a First-Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA) with specific primers. The expression of small nuclear U6 was used as internal control. Then, quantitative real-time PCR was performed to quantify relative expression of miR-99a using the Quanti-Tect SYBR Green PCR mixture on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Carlsbad, CA). For analysis of mRNA expression, the expression of GAPDH was used as internal control and Oligo (dT) was used as the primer for reverse transcription. PCR efficiencies were calculated with a relative standard curve derived from a complementary DNA mixture and gave regression coefficients >0.95. The relative expression levels were evaluated using the $2^{-\Delta\Delta Ct}$ method. All experiments repeated five times. The primers mentioned above were summarized in Table I.

CCK-8 ASSAY

Before analysis of cell proliferation, NSCLC cells were seeded into 24-well plates at a concentration of 5×10^3 cells/well. The Cell

TABLE I. Oligonucleotide Primer Sequences for PCR or Reverse Transcription

Gene	Primer sequence
Primers for real-time PCR	
miR-99a-sense	5′-CGGAACCCGTAGATCCGAT-3′
miR-137-antisence	5′-CAGTGCAGGGTCCGAGGT-3′
U6-sense	5′-CTCGCTTCGGCAGCACATATACT-3′
U6-anti-sense	5′-ACGCTTCACGAATTTGCGTGTC-3′
AKT1-sense	5′-ACTCTTTCCAGACCCACGAC-3′
AKT1-antisense	5′-CCAGGGCTGACACAATCTCA-3′
GAPDH-sense	5′-TCAACGACCACTTTGTCAAGCTCA-3′
GAPDH-antisense	5′-GCTGGTGGTCCAGGGGTCTTACT-3′
Primers for reverse transcription	
miR-99a	5′-GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGCACTGGCACAAG-3′
U6	5′-AAAATATGGAACGCTTCACGAATTTG-3′
AKT1	5′-TTTTTTTTTTTTTTTTT-3′(Oligo(dT))
GAPDH	5′-TTTTTTTTTTTTTTTTT-3′(Oligo(dT))
Primers for AKT1 PCR amplifying	
AKT1-F	5′-ATGAGCGACGTGGCTATTGTGAAGGA-3′
AKT1-R	5′-TGCCGCTGGCCGAGTAGGAGAACTG-3′
Primers for AKT1 luciferase reporter	
AKT1-F	5′-ATTTGAGAGAAGCCACGCT-3′
AKT1-R	5′-CCCAGAGAGATGACAGATAGC-3′

F, forward; R, reverse.

Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was added to the wells at 0, 24, 48, and 72 h posttransfection and cells were diluted in normal culture medium at 37°C until visual color conversion occurred. The absorbance values in each well were measured with a microplate reader set at 450 and 630 nM. All experiments were performed four times and the average percentages of cells are shown.

CELL MIGRATION AND INVASION ASSAYS

Migration assays were carried out in modified Boyden chambers (BD Biosciences, San Jose, CA) with 8 μm pore filter inserts in 24-well plates. Twenty-four hour after transfection, 2×10^5 cells suspended in serum-free medium were added to the upper chamber. Medium containing 20% FBS were added to the lower chambers as a chemoattractant. After 24 h transfection, the cells have not pass through the chambers were gently removed with a cotton swab, while the passed cells which located on the lower side of the chamber were stained with crystal violet, air-dried and photographed. For analysis of invasive capacity, the transwell migration chambers were coated with Matrigel (BD Biosciences) and incubated at 37°C for 3 h, allowing it to solidify. After 24 h of transfection, 4×10^5 cells suspended in serum-free medium were added to the upper chamber. The remaining steps were the same as migration assays. Three independent experiments were performed.

LUCIFERASE REPORTER ASSAYS

To show that miR-99a regulates the expression of the human *AKT1* gene by directly targeting its 3′UTR, the complete *AKT1* 3′UTR was amplified from genomic DNA and cloned into the pGL-3-vector (Promega, San Luis, CA). The QuickChange Site-Directed Mutagenesis kit (Stratagene, Santa Clara, CA) was used to mutate the *AKT1* 3′UTR. A luciferase reporter construct containing the miR-99a consensus target sequence served as a positive control. About 1×10^5 cells/well were seeded into 24-well plates and grown for 24 h prior to transfection. Cells were transfected with the pGL-3 firefly luciferase reporter (50 ng/well), pRL-TK Renilla luciferase reporter (10 ng/well), and the miR-99a mimics (50 nM). The pRL-TK vector served as an internal control. All transfections were carried out in triplicate using Lipofectamine 2000 (Invitrogen). Cell lysates were prepared using Passive Lysis Buffer (Promega) 48 h after transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega) and normalized to Renilla luciferase activity.

PLASMID CONSTRUCTION

The full length *AKT1* gene open reading frame (ORF) were amplified by PCR reaction and cloned into pCDNA3.1 construct to generate the pCDNA3.1_AKT1 construct. The empty pCDNA3.1 construct was used as control. NSCLC cells were first transfected with miR-99a mimics or scramble mimics (60 nM) in 6-well plates. After 24 h of culture, these NSCLC cells were then co-transfected with miR-99a mimics (30 nM) and 2.0 μg of either pcDNA3.1_AKT1 or pcDNA-3.1 constructs. Cells were harvested at predetermined intervals and assayed as necessary. Sequences of primers used for PCR amplification are summarized in Table I.

IMMUNOBLOT ANALYSIS

For the Immunoblot assay, cells were harvested in ice-cold PBS 48 h after transfection and lysed on ice in cold-modified radioimmunoprecipitation buffer supplemented with protease inhibitors. Protein concentration was determined by the BCA Protein Assay Kit (Bio-Rad, CA) and equal amounts of protein were analyzed by SDS-PAGE. Gels were electroblotted onto nitrocellulose membranes (Millipore, WI). After blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 2 h, membranes were incubated at 4 °C over night with primary antibody. Primary antibodies used were anti-AKT, anti-p-AKT, anti-MMP-2 (Cell Signaling) and GAPDH (Zhong-Shan JinQiao, China). Then, membranes were incubated with respective second antibodies and detected by peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system (ECL) (Millipore, WI). The experiment was repeated three times.

LENTIVIRAL VECTOR CONSTRUCTION

To analysis the effect of miR-99a on the tumor metastasis of NSCLC cells in vivo, NCI-H1299 cells with miR-99a stable expression was constructed. The self-inactivating transfer vector pMIRNA1, control plasmid, and the packaging kit System Biosciences were used according to the manufacturer's instructions. DNA fragments 500 bp in size containing the miR-99a were inserted under the CMV promoter in pMIRNA1. After virus packaging, the recombinant lentivirus particles were harvested and titrated. The NCI-H1299 cells were infected with lentivector supernatants (lenti-miR-99a and lenti-control) at an MOI of 50 in the presence of polybrene (5 μg/ml). The cells were washed the next day with PBS and liquid cultures.

IN VIVO METASTASIS ASSAYS

Five-week-old BALB/C-nu/nu nude male mice were used for animal studies, and all animals were maintained in the pathogen-free (SPF) conditions at our institution. For the in vivo tumor metastasis assay,

2×10^6 NCI-H1299 cells with stable miR-99a expression were injected into the lateral tail veins of nude mice (12 per group). After 30 days, all mice were killed and the livers and lungs were removed and fixed in 10% neutral phosphate-buffered formalin. The fixed samples were embedded in paraffin and stained with hematoxylin and eosin. All animal experiments were housed and performed according to institute guidelines.

STATISTICAL ANALYSIS

Data were expressed as the mean ± standard deviation of at least three independent experiments. Statistical analysis was carried out using SPSS 15.0 software. The Student's *t*-test was used for comparisons between two groups, and analysis of variance was used for comparisons among three groups. The χ-squared test was used for occurrence analysis. *P* values of less than 0.05 were considered to be significant.

RESULTS

miR-99a EXPRESSION IS DOWNREGULATED IN NSCLC TISSUES AND ASSOCIATED WITH TUMOR INVASION AND METASTASIS

Although miR-99a has previously been implicated in tumor metastasis, its exact role in NSCLC remains unclear. In an attempt to explore the expression and significance of miR-99a in NSCLC progression and metastasis, we detected the expression of miR-99a in 105 pairs of NSCLC tissues and the adjacent normal tissues. As compared with the adjacent normal tissues, 71 cases of tumor tissues exhibited decreased miR-99a expression (67%, 71 of 105, Fig. 1A). Further statistical analysis identified the expression of miR-99a in NSCLC tissues is much lower relative to normal lung tissues (Fig. 1B). The relationship between the miR-99a expression level and clinicopathological characteristics of NSCLC patients is summarized in Table II. Although the results showed that no statistically significant correlations were observed between the miR-99a expression and age, gender, tumor size, differentiation and local invasion, interestingly, a statistically significant association was observed between the expression of miR-99a and NSCLC clinical stage as well as metastasis. The patients with lower levels of miR-99a expression seemed to be associated with late-stage tumors (stage III and IV) (1.0377 ± 0.6430 vs. 1.6892 ± 0.8462, $P < 0.001$) (Fig. 1C). Otherwise, when the patients were stratified on the basis of lymph node and distal metastases, we observed that miR-99a was significantly downregulated in NSCLC tissues with lymph node or distal metastasis (1.1910 ± 0.8045 vs. 1.5277 ± 0.7853, $P < 0.05$) (Fig. 1D). The generality of this observation was further confirmed in NSCLC cell lines. Comparing with the human bronchial epithelial cell line 16HBE, the expression of miR-99a is consistently downregulated in five NSCLC cell lines (A427, A549, NCI-H520, NCI-H1299, and NCI-H1437) (Fig. 1E). These data suggested that alteration of miR-99a is a frequent event in human NSCLC and has a pivotal role in the aggressiveness of NCSLC.

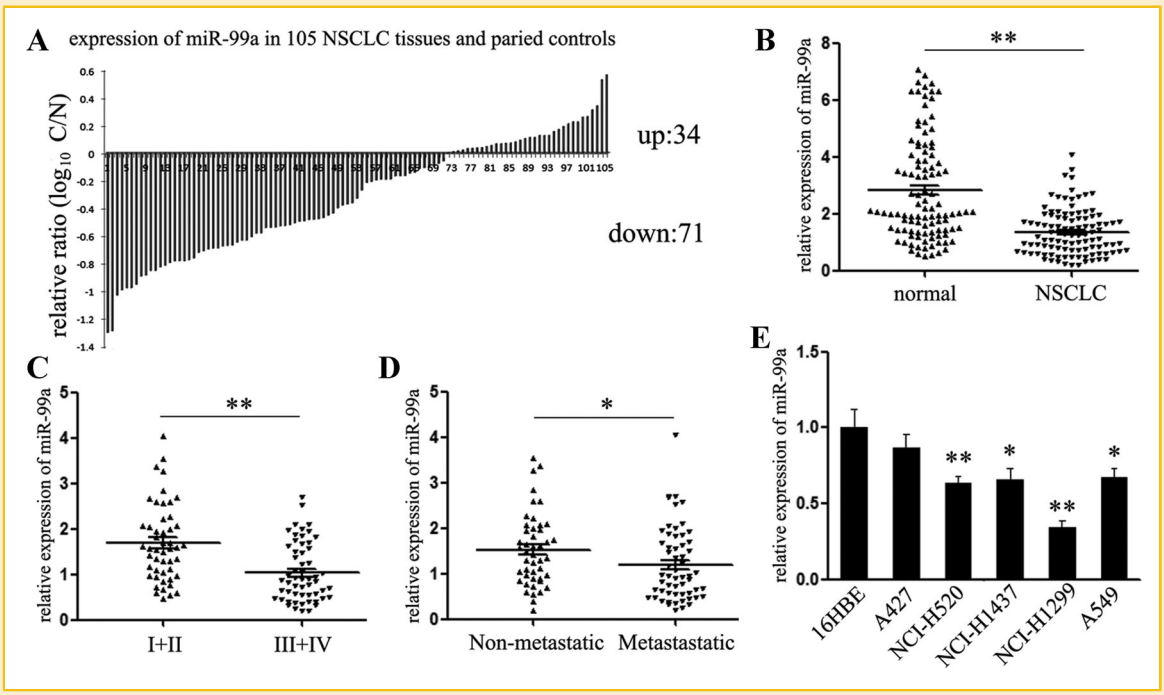

Fig. 1. Expression levels of miR-99a in NSCLC specimens and cell lines. A: The expression of miR-99a in 105 pairs of NSCLC tissues and compared normal tissues was detected by quantitative RT-PCR. Data are shown as $\log_{10}$ of relative ratio change of NSCLC tissues relative to adjacent normal tissues. B: Statistical analysis of relative miR-99a expression levels in NSCLC tissues and compared normal tissues. C: Statistical analysis of relative miR-99a expression levels in NSCLC tissues of stage I + II and stage III + IV. D: Statistical analysis of relative miR-99a expression levels in NSCLC tissues with or without metastasis. E: Using quantitative RT-PCR analysis, the expression of miR-99a in five NSCLC cell lines (A427, A549, NCI-H520, NCI-H1299, and NCI-H1437) was analyzed relative to normal human bronchial epithelial cell line 16HBE. The data are representative of three independent experiments. Error bars represent s.e.m. *$P < 0.05$, **$P < 0.01$.

TABLE II. Relationship Between miR-99a Expression and Their Clinicopathological Parameters in 105 NSCLC Patients

Clinicolpathological parameters	Number of cases	Expression of miR-99a	*P*-value
Age (years)			
<60	39	1.4382 ± 0.9186	0.3504
≥60	66	1.2847 ± 0.7397	
Gender			
Male	63	1.3545 ± 0.8726	0.8447
Female	42	1.3226 ± 0.7152	
Tumor size (cm)			
≤3	47	1.3089 ± 0.7212	0.6115
>3	58	1.3683 ± 0.8805	
Degree of differentiation			
Well and moderately	37	1.2614 ± 0.6662	0.2366
Poorly	68	1.3854 ± 0.8798	
Local invasion			
T1 + T2	72	1.4264 ± 0.8061	0.1139
T3 + T4	33	1.1570 ± 0.7990	
TNM stage			
Stage I + II	49	1.6892 ± 0.8462	0.0001**
Stage III + IV	56	1.0377 ± 0.6430	
Metastasis			
No	47	1.5277 ± 0.7853	0.0335*
Yes	58	1.1910 ± 0.8045	

TNM, tumor node metastasis. *P*-value represents the probability from a Student's *t*-test for miR-99a expression between variable subgroups. *$P < 0.05$ and **$P < 0.01$ were considered to have a significant difference.

ECTOPIC EXPRESSION OF miR-99a SUPPRESSES CELL PROLIFERATION, MIGRATION AND INVASION OF NSCLC CELLS IN VITRO

To further measure the effects of miR-99a on NSCLC metastasis, we established miR-99a overexpression models in NSCLC cell lines, A549 and NCI-H1299, by transiently transfection with miR-99a mimics (Fig. 2A). Then, we investigated the effects of miR-99a overexpression on the proliferation, migration and invasion of both cell lines, respectively. As shown in Figure 2B, overexpression of miR-99a significantly suppressed the growth rate of NSCLC cells. Transwell assays without Matrigel showed that restored expression of miR-99a in A549 and NCI-H1299 cells resulted in a significant reduction of cells passed through the chambers compared with scramble group (148 ± 23 cells vs. 62 ± 15 cells in A549 and 123 ± 30 cells vs. 35 ± 17 cells in NCI-H1299, respectively) (both $P < 0.05$) (Fig. 2C). The similar result was observed in Matrigel invasion assays. As shown in Figure 2D, miR-99a overexpression could significantly suppress cells passed through the chambers (130 ± 29 cells vs. 45 ± 17 cells in A549 and 95 ± 20 cells vs. 35 ± 15 cells in NCI-H1299, respectively) (both $P < 0.05$). These data indicated that miR-99a could efficiently repress cell motility and invasiveness of NSCLC cells in vitro.

ECTOPIC EXPRESSION OF miR-99a IMPAIRS THE FORMATION OF METASTASES IN VIVO

Previous in vitro studies indicated the suppressive effects of miR-99a on the migratory and invasive capacity of NSCLC cells. Thus, we further investigated whether miR-99a affects the NSCLC cell metastasis in vivo. The NCI-H1299 cells with stable miR-99a expression (NCI-H1299-miR-99a group) or empty lentiviral construct only (NCI-H1299-control group) were injected into the lateral tail veins of nude mice. At the termination of experiments, animals in both groups showed evidence of visual macrometastases in the lung (Fig. 3A). However, no significant evidence of visual macrometastases in the livers was found (data not shown). The number of lung metastasis nodules was significantly decreased in the NCI-H1299-miR-99a group compared with the NCI-H1299-control group

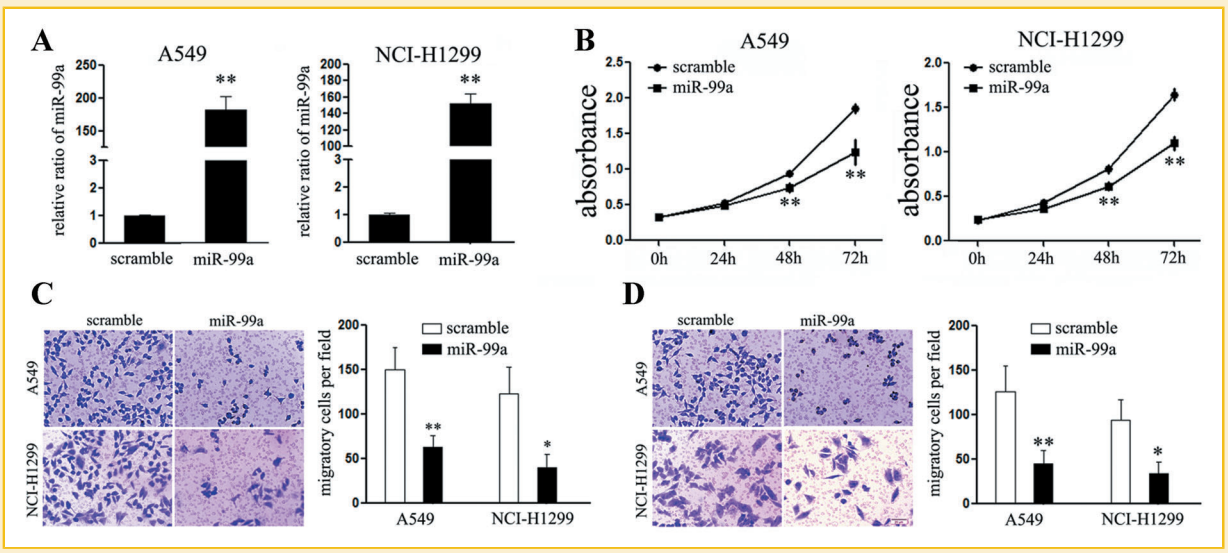

Fig. 2. miR-99a suppresses NSCLC cell proliferation, migration and invasion in vitro. A: RT-PCR was performed to detect the expression of miR-99a in NSCLC cell lines (A549 and NCI-H1299) upon transfection with miR-99a mimic. B: CCK-8 was performed to analyze the effect of miR-99a on cell proliferation of both cell lines. C,D: The effects of miR-137 on cell migration and invasion were detected using transwell chamber assays. Panel C showed the results on migration (×400); Panel D showed the results on invasion (×400). The chambers have been coated with Matrigel, which functions as the extracellular cell matrix. Ectopic expression of miR-99a inhibited cells passing through the membrane. The data are representative of three independent experiments. Error bars represent s.e.m. *$P < 0.05$; **$P < 0.01$.

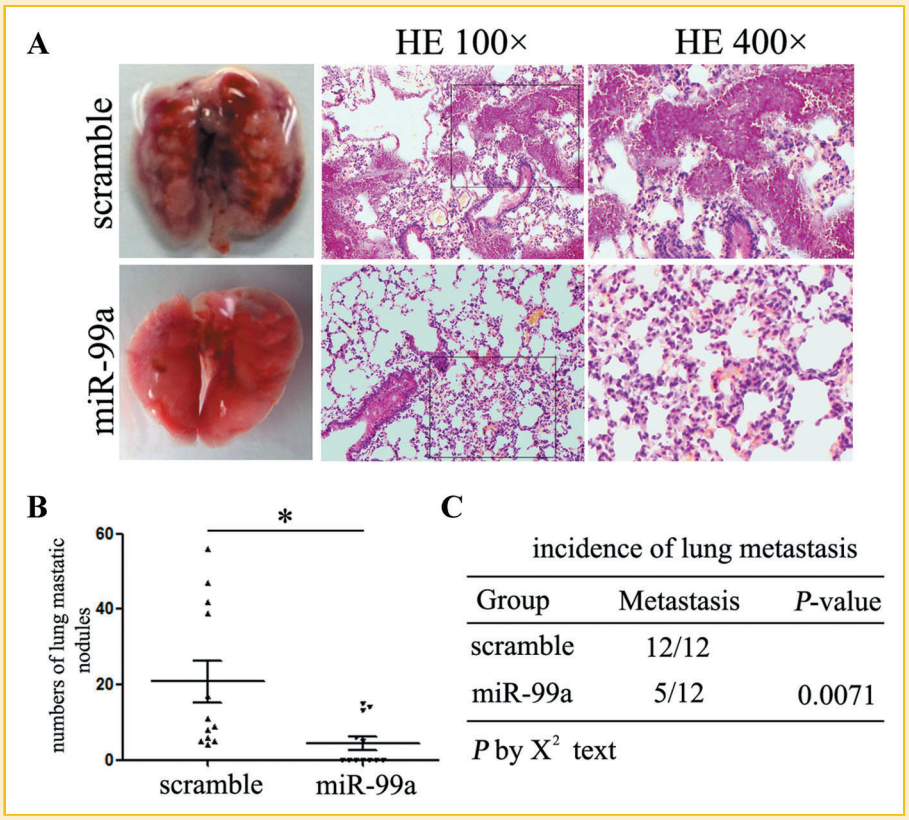

Fig. 3. miR-99a suppresses the lung metastasis of NSCLC cells in vivo. A: Representative images of mouse lungs and histological inspection of mouse lungs for the presence of macrometastases and microscopic lesions after tail vein injection with NCI-H1299 cells stably expressing miR-99a or scramble mimic lentiviral vector (×400). B: Quantification of lung metastasis nodules in lungs of both groups. C: The incidence of metastasis in mouse after intravenous tail injections is shown in the table. The data are representative of three independent experiments. Error bars represent s.e.m. $*P < 0.05$.

(Fig. 3B). In addition, the ratio of lung metastasis in the miR-99a overexpression group was markedly lower than that of the controls (Fig. 3C). Taken together, these results suggested that miR-99a is a negative regulator of NSCLC metastasis in vivo.

miR-99a DIRECTLY REGULATED THE AKT1 GENE IN NSCLC CELLS

To identify the putative genes involved in miR-99a-mediated tumor suppression, targets of miR-99a were searched using the target prediction programs, PicTar and miRanda [Krek et al., 2005; Betel et al., 2008]. Among these genes being searched, AKT1 was picked out for its important role in phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway [Scheid and Woodgett, 2003], which mediates multiple biological processes in eukaryotes, especially tumor metastasis [Cheung and Testa, 2013]. Sequence analysis revealed a putative miR-99a-binding site in the 3′UTR of the *AKT1* promoter (Fig. 4A). To further explore whether this binding site performs function, luciferase reporter assays were used. As shown in Figure 4B, miR-99a overexpression inhibited the transcriptional activity of a luciferase reporter containing the *AKT1* 3′UTR, but did not affect the activity of a mutated reporter construct lacking the miR-99a binding site (Fig. 4B). Moreover, ectopic miR-99a expression significantly suppressed the mRNA and protein levels of AKT1 in both A549 and NCI-H1299 cells (Figs. 4C and D). Previous studies indicated that AKT signaling functions through activating the transcription of matrix metallo preteinases-2 (*MMP-2*) to regulate cell invasion [Tian et al., 2010]. MMP-2 is a member of the matrix metalloproteinase family, which mediates the degradation of extracellular matrix (ECM) and inducing tumor metastasis [Ataie-Kachoie et al., 2013]. We found that ectopic miR-99a expression significantly reduced the expression of phosphorylation of AKT (p-AKT), which represents the activation of AKT, and MMP-2 in both A549 and NCI-H1299 cells. These data suggest that overepxression of miR-99a impairs the AKT signaling pathway in NSCLC.

AKT1 IS INVOLVED IN miR-99a MEDIATED SUPPRESSION OF TUMOR METASTASIS

To clarify the biological role of AKT1 in miR-99a mediated tumor suppression in NSCLC cells, we adopted a "rescue" methodology to examine the functional relevance of miR-99a/AKT1 interaction in NCI-H1299 cells. We generated a new construct containing the full ORF of AKT1 gene (pcDNA3.1_AKT1). As expected, the level of AKT1 and its downstream genes were rescued when pcDNA3.1_AKT1 was transfected into NCI-H1299 cells that had been treated with miR-99a mimics for 24 h (Fig. 5A). In agreement with the restored expression of target proteins, increased cell growth, accompanied with invasive capacity (cells transfected with pcDNA3.1_AKT1 and miR-99a mimic vs cells transfected with pcDNA_empty and miR-99a mimic)

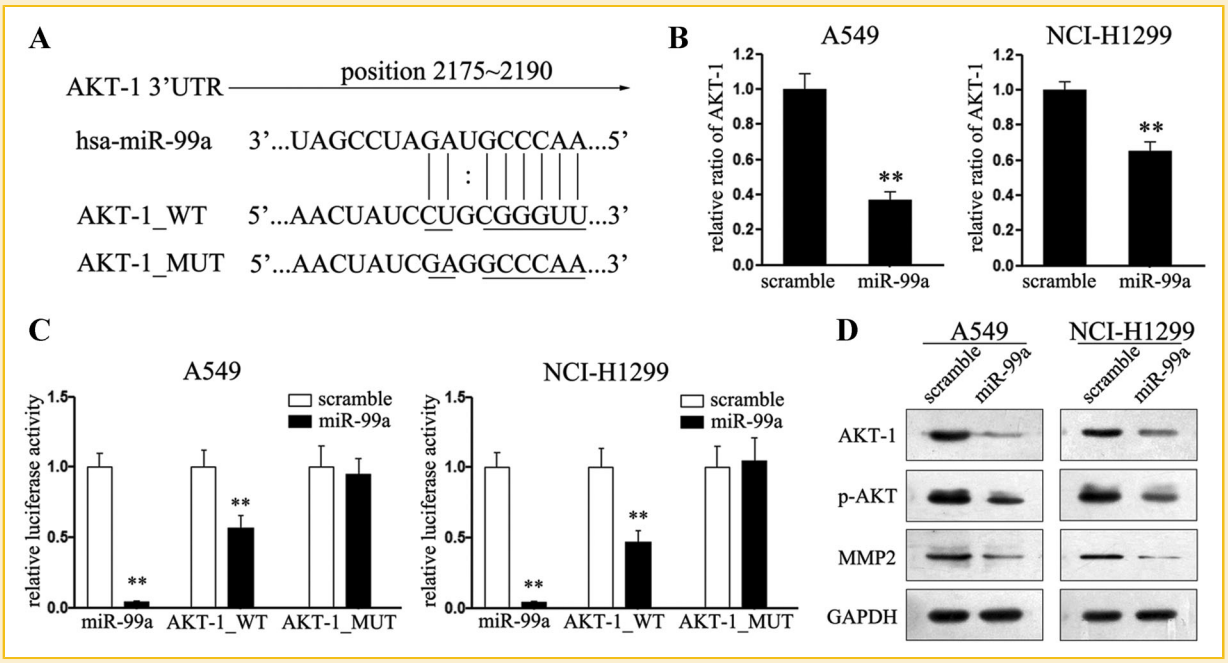

Fig. 4. miR-99a targets *AKT1* gene in NSCLC cells. A: Schematic representation of *AKT1* 3′UTR showing putative miR-99a target site. B: Quantitative RT-PCR assays were performed to detect the expression of *AKT* upon trasnfection with miR-99a mimic or scramble mimic. C: Relative luciferase activity of the indicated AKT1 reporter construct in A549 and NCI-H1299 cells, co-transfected with miR-99a mimic or scramble mimic, is shown. D: Western blot analysis showed the expression levels of AKT, p-AKT, and MMP2 proteins in NSCLC cells treated with miR-99a mimic. $^{**}P < 0.01$.

(both $P < 0.05$) (Figs. 5B and C) were also observed in NSCLC cells transfected with pcDNA3.1_AKT1 construct following the treatment of miR-99a mimics, suggesting that AKT1 and its downstream genes are involved in the miR-99a-mediated suppression of NSCLC cells metastasis. These data provided further evidence that AKT1 is a direct and functional target of miR-99a in NSCLC cells.

DISCUSSION

Although miR-99a has been reported to be a tumor suppressor in many cancers, its definite function in NSCLC metastasis has not been described yet. In the paper, we found that the expression of miR-99a was reduced in NSCLC tissues, especially the one with last-stage and metastasis. Further in vitro and in vivo experiments identified that overexpression of miR-99a suppresses tumor metastasis of NSCLC cells through targeting AKT signaling pathway.

It was reported that miR-99a gene was located at chromosome 21, trisomy of which is one of the most common chromosomal aneuploidy in live born infants (Centers for Disease Control and Prevention [CDC], 2006). Consistently, Nagayama et al. [2007] performed a genome scanning in 43 human lung cancer cell lines, and found that three miRNA genes, let-7c, miR-99a, and miR-125b-2, were mapped in a region with homozygous deletion (HD) at 21q11-q21. HD is a genetic event causing inactivation of tumor suppressive genes, suggesting that the miR-99a might be reduced in lung cancers and function as a tumor suppressor. Our paper is in accord with this hypothesis. We found that miR-99a is downregulated in NSCLC tissues, especially the one with last stage or metastasis. Moreover, we explored the effects of miR-99a on migration and metastasis of NSCLC cells. As expected, overexpression of miR-99a significantly suppressed NSCLC cell migration and invasion in vitro and tumor metastasis to lung in vivo. Interestingly, we did not find significant evidence of visual macro-metastases in the liver. It might because the observation time upon tail veins injection was not long enough. Our experiment is partially consistent with Gu and colleagues, who reported that low miR-99a expression was closely correlated with advanced clinical stage and lymph node metastasis of lung adenocarcinoma, and overexpression of miR-99a suppressed proliferation and induced apoptosis of SPC-A1 cells [Gu et al., 2013]. However, they did not investigate the role of miR-99a in tumor metastasis. Our paper consummates the effects of miR-99a on the tumor metastasis of NSCLC cells, all of which suggested the tumor suppressor role of miR-99a in NSCLC consistently.

To further investigate the mechanisms of miR-99a-mediated tumor suppression in NSCLC, we predicted putative targets of miR-99a by prediction programs. Among these genes, the *AKT1* was picked out, as *AKT1* has been shown to be closely related to the carcinogenesis and metastasis of various cancers [Cheng et al., 2013; Shin et al., 2013]. The target role of *AKT1* was identified by the luciferase reporter assays, as transfection of miR-99a caused a substantial reduction of luciferase activity by the luciferase expression constructs carrying the target *AKT1* fragment, and ectopic expression of miR-99a reduced the mRNA and protein levels of *AKT1* at the same time.

The AKT/PKB (protein kinase B) family of kinases are known to be central components in the signaling pathway composed of upstream PI3K and phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) [Cheung and Testa, 2013]. A large body of literature has

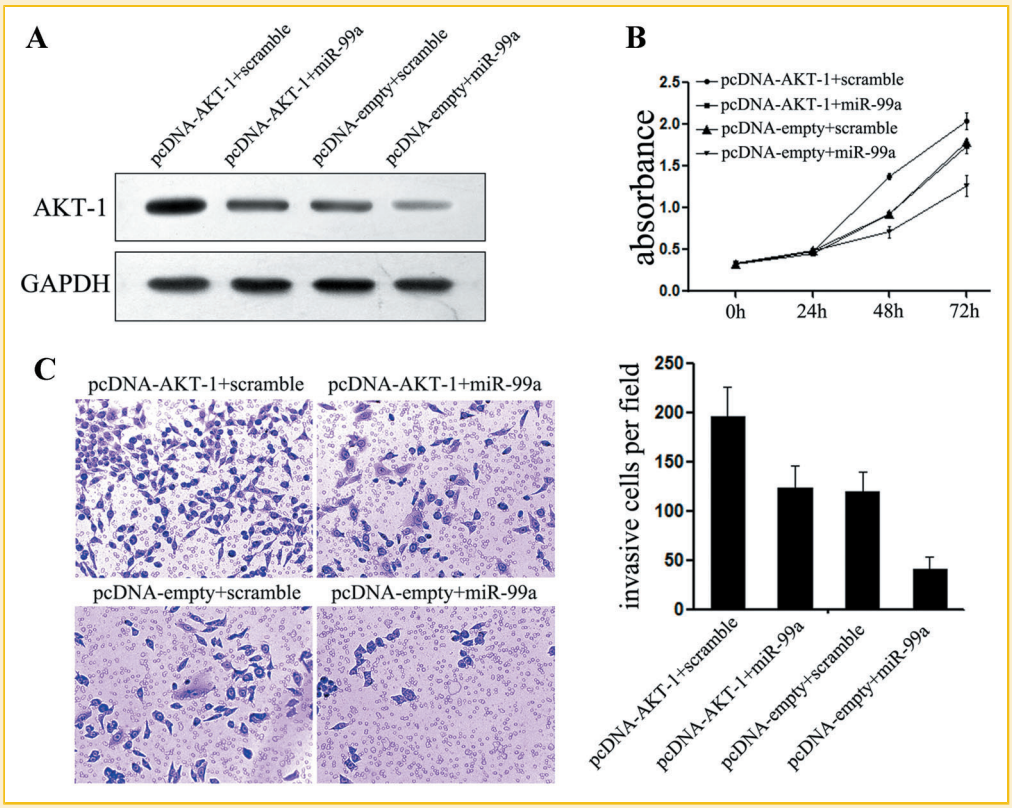

Fig. 5. AKT1 is involved in miR-99a-mediated suppression of cell proliferation and invasion of NSCLC cells. A: Upon transfection with *AKT1* construct, we rescued the expression of *AKT* in both NSCLC cell lines. B: CCK-8 assays were used to detect to explore the effects of AKT on proliferation of NSCLC cells that has been treated with miR-99a before. C: Transwell assays were performed to detect the effects on cell invasion of A549 and NCI-H1299 cells treated as described in B (×400). Restored the expression of AKT partially abolished the suppressive effects of miR-99a on proliferation and invasion of NSCLC cells. (cells transfected with pcDNA3.1_AKT1 and miR-99a mimic *vs* cells transfected with pcDNA_empty and miR-99a mimic) (both $P < 0.05$); The data are representative of three independent experiments. Error bars represent s.e.m.

documented hyperactivated AKT signaling in human solid tumors and hematological malignancies [Bellacosa et al., 2005]. *AKT1* is an important member of *AKT* family, homozygous knockout of which results in partial embryonic lethality, and surviving mice are approximately 20% smaller in size than wild-type littermates [Cho et al., 2001b]. In a cohort of 140 skin cancer patients, Barrette et al. demonstrate a significant upregulation of EMT in the invasive cutaneous squamous cell carcinoma (SCC) versus normal and/or SCC in situ, which is paralleled by AKT activation. The expression of p-AKT was significantly increased in invasive SCC tissues compared with normal skin, and suppression of it inhibited the EMT process of SCC cells [Barrette et al., 2014]; Moreover, AKT was also reported to be involved in leukemia inhibitory factor (*LIF*)-mediated tumor initiation, as inhibiting the AKT through wortmannin largely abolished the promoting effect of *LIF* on tumorigenesis and metastasis of breast cancer [Li et al., 2014]. Herein, we further investigated the expression of p-AKT and its downstream gene, MMP-2 [Tian et al., 2010], upon transfection with miR-99a. Consistent with the expression of total AKT, the expression of p-AKT and MMP-2 were significantly reduced upon transfection, which demonstrated that overexpression of miR-99a suppressed the AKT/MMP-2 signaling pathway in NSCLC.

Further investigation shows that AKT1 is directly involved in miR-99a-mediated tumor suppression. Restored the expression of AKT1 in NCI-H1299 cells partially abolished the suppressive effects miR-99a on proliferation and invasion of NSCLC cells. These findings demonstrate that miR-99a suppresses tumorigenesis and metastasis of NSCLC through AKT1/MMP-2 signaling pathway. However, it is unclear that whether other members of AKT family were also involved in miR-99a-mediated tumor suppression. Thus, further research is warranted.

In conclusion, our findings demonstrate that miR-99a plays a vital role in suppressing growth and metastasis of NSCLC. This function of miR-99a in NSCLC is partially mediated by the AKT1 signaling pathway. This finding not only helps us understand the molecular mechanism of NSCLC carcinogenesis, but also provides a solid foundation for NSCLC utilization of miR-99a as an important biomarker for prognosis and anticancer therapy in future.

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