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Original Article

The microRNA-520a-3p inhibits proliferation, apoptosis and metastasis by targeting MAP3K2 in non-small cell lung cancer

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Abstract: Growing evidence indicates that miR-520a was involved in the complement attack and migration of tumor cells, but nonetheless, the role of miR-520a-3p in non-small cell lung cancer (NSCLC) is not clear. Mitogen-activated protein kinase kinase kinase 2 (MAP3K2) is a kinase belonging to the serine/threonine protein kinase family. To develop potential therapy targeting MAP3K2, we studied the roles of miR-520a-3p in the proliferation, apoptosis and metastasis of NSCLC. The expression levels of miR-520a-3p were quantified in tumor tissues of NSCLC by qRT-PCR, and the mimics and inhibitors were used to verify the function of miR-520a-3p. The cell proliferation was evaluated by MTT assay, and the migration and invasion was evaluated by transwell assay. The athymic mice subcutaneous injection was used to research NSCLC cell tumor formation. The bioinformatics tools and luciferase assay was applied to detect the relationship between miR-520a-3p and its target. Protein levels of miR-520a-3p target was determined by western blot analysis. MiR-520a-3p expression was decreased in the NSCLC tissues compared with their normal counterparts and lower expression of miR-520a-3p in NSCLC tissues was associated with a higher clinical stage, NSCLC metastasis and poor prognosis. Inhibition of expression of miR-520a-3p can reduce in vitro NSCLC cell migration and invasion as well as in vivo metastasis. MAP3K2 mRNA contains a binding site for miR-520a-3p in the 3'UTR. MAP3K2 is one of target of miR-520a-3p. Together, our data demonstrated that miR-520a-3p inhibits proliferation, apoptosis and metastasis in NSCLC by targeting MAP3K2, and miR-520a-3p may be used as a prognosis marker for NSCLC in clinical research.

Keywords: MiR-520a-3p, non-small cell lung cancer, metastasis, prognosis, MAP3K2

Introduction

Nowadays, as the most common malignant tumor, lung cancer is defined by the World Health Organization as the most serious disease that threatens human health in this century. Scholars reported that the incidence of lung cancer increases by an average of 16% to 69% in various countries in the near half century [1, 2]. Treatment of lung cancer in recent years has made some progress, though, the overall prognosis is still poor, with invasion and metastasis found in a majority of patients when diagnosed [3, 4]. Thus, to explore the mechanism of occurrence and development of NSCLC is significant for diagnosis and prevention of NSCLC. Present study suggests that non-coding RNAs (ncRNAs) may be involved in the pathogenesis of NSCLC, which has provided a new perspective for the research.

MicroRNAs (miRNAs) are a class of small non-coding RNA approximately 18-25 nucleotides in length that suppress gene expression by post-transcriptional mechanisms by targeting 3' untranslated regions (UTR) of gene mRNAs [5-7]. Emerging evidence has shown that miRNA can act as both oncogenes and tumor suppressors and play an important role in gene regulation, apoptosis, the maintenance of cell differentiation and tumorigenesis [8, 9]. Thus, identification of tumor-associated microRNAs as well as exploration of role of microRNAs in the development of tumors has become an important issue in cancer research. Multiple tumor-suppressive miRNAs have been identified, such as miR-7, miR-218, and miR-143 in NSCLC [10-12]. MiR-520 has been characterized as a tumor suppressor in several human cancers, such as breast cancer, hepatocellular carcinoma, pancreatic cancer and so on [13,

Table 1. Correlation of the expression of miR-520a-3p with clinicopathologic features

Clinicopathologic features	N (%)	Relative expression of miR-520a-3p[a]	*P*-value[b]
Gender			P = 0.813
Male	35 (71.4)	11.38	
Female	14 (28.6)	10.19	
Site of tumor			P = 0.746
Left lung	23 (46.9)	8.35	
Right lung	26 (53.1)	9.67	
Differentiation			P = 0.329
Poor	30 (61.2)	12.42	
High/moderate	19 (38.8)	7.29	
Lymph node Metastasis			P = 0.006
N0	6 (12.2)	1.69	
N1	11 (22.5)	2.73	
N2	17 (34.7)	18.16	
N3	15 (30.6)	28.7	

14]. Inhibition of expression of miR-520 can promote metastasis of breast cancer. On the contrary, the overexpression of miR-520 can inhibit cancer cell invasion and proliferation, change cell cycle progression and induct apoptosis, which has shown that miR-520 is playing an important role in cancer occurrence and progress [15]. Nevertheless, little is known about the role of miR-520a-3p on NSCLC carcinogenesis or metastasis.

To further clarify the role of miR-520a-3p in NSCLC development and progression, we analyze the targets of miR-520a-3p. MAP3K2, a kinase belonging to the serine/threonine protein kinase family, had been predicted as a target of miR-520a-3p, and this kinase preferentially activates other kinases involved in the MAP kinase signaling pathway and it's frequently overexpressed in human cancers [16-18]. However, evidence on the relationship between miR-520a-3p and MAP3K2 in NSCLC metastasis are scarce. we investigated the expression level of miR-520a-3p in NSCLC tissues and analyzed the relationship between miR-520a-3p and clinical pathological features. We used both vitro and vivo experiments to explore the molecular mechanisms of miR-520a-3p in changing NSCLC cell phenotype, and bioinformatics analysis was used to forecast miR-520a-3p target in NSCLC, and then bioinformatics tools and luciferase reporter assay was applied to detect the relationship between miR-520a-3p and its target, thus the role of miR-520a-3p in the pathogenesis of NSCLC can be probed from the perspective of target.

Materials and methods

Tissue collection

49 pairs of NSCLC and adjacent tissues were obtained from patients who were diagnosed with NSCLC (stage II, III and IV) and underwent surgery in Daping Hospital, Third Military Medical University from 2007 to 2008. None of these patients underwent local or systemic therapy before surgery. This research was approved by the Research Ethics Committee of Third Military Medical University, China. and all patients signed the written informed consent.

Cell culture conditions and transfection

NSCLC cell lines SPC-A1, A549, NCI-H358, and normal GNHu27 cells were purchased from ATCC. Cells were cultured in the DMEM (GIBCO-BRL) and supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma, USA), at 37°C, in 5% CO_2 incubator (Memmert, Germany). MiR-520a-3p mimics and inhibitors were purchased from Scirince (Scirince, China). Cell lines were transfected with mimics and inhibitors with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was carried out using SYBR PremixExTaq™ (Takara, Japan). The total RNA was isolated using Trizol reagent (Invitrogen, USA) following the instructions. SYBR Green qRT-PCR was performed on BIO-RAD IQ5 (USA). All primer sequences are listed in **Table 2**. The qRT-PCR results were analyzed and the relative CT (threshold cycle) values were converted into expression fold alterations. The cell, in which the expression level of miR-520a-3p differ from GNHu27 cells will be used for follow-up study.

Cell proliferation assays

The Cell Proliferation Reagent Kit I (MTT) (Roche, USA) was used to evaluated the cell

Table 2. Primer sequences of products expression

Gene name	Primer name	Primer sequence
miR-520a-3p	RT primer	5'CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACAGTCCAAA3'
	Forward primer	5'ACACTCCAGCTGGGAAAGTGCTTCCC3'
	Reverse primer	5'CTCAACTGGTGTCGTGGA3'
U6	RT primer	5'GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATGGAAC3'
	Forward primer	5'CTCGCTTCGGCAGCACA3'
	Reverse primer	5'AACGCTTCACGAATTTGCGT3'
MAP3K2	RT primer	Radom primer
	Forward primer	5'CCCCAGGTTACATTCCAGATGA3'
	Reverse primer	5'GCATTCGTGATTTTGGATAGCTC3'
GAPDH	RT primer	Radom primer
	Forward primer	5'TGGGTGTGAACCACGAGAA3'
	Reverse primer	5'GGCATGGACTGTGGTCATGA3'
MAP3K2-3'UTR	Forward primer	CCCTCGAGGTCATCAGTGGAGAAGGCTA
	Reverse primer	TTGCGGCCGCTCTCTCTGAACCACATCAGT

proliferation. A549 cells transfected with miR-520a-3p mimics, miR-520a-3p inhibitors were grown in the 96-well plates (4000 cells/well). and Cell proliferation was recorded every 24 h according the manufacturer's protocol. For each treatment group wells were assessed in triplicate.

Flow-cytometric analysis of apoptosis

The cells transiently transfected with miR-520a-3p mimics or miR-520a-3p inhibitors, were harvested 48 h after transfection by trypsinization. Cells double stained by FITC and PI underwent flow cytometry for apoptosis detection. All cells were divided into living cells, early apoptotic dead cells and late apoptotic cells. All samples were tested three times.

Cell migration and invasion assays

Cell invasion assays were performed using 24-well transwell (8 mm pore size, Millipore, USA) coated with matrigel (1 mg/ml, BD Sciences) 48 h after transfection. The cells (104/well) were seeded in the upper chambers of the wells in 200 μl FBS-free medium, and the lower chambers were filled with 500 μl 10% FBS medium to induce cell migration. Cells were incubated for 24 h, the cells on the filter surface were fixed with 4% formaldehyde, stained with 0.5% crystal violet, and took image with inverted microscope (Nikon, Japan), calculate the number of cells in each field, count samples of three batches for each group.

Bioinformatics methods and luciferase assay

TargetScan and PicTar software were used to forecast miR-520a-3p target genes, the target genes predicted by two software which turned out to be the same were screened out. The target gene MAP3K2 was screened preliminarily. The wild-type of MAP3K2 3'-UTR was amplified from a human cDNA library with the primers in **Table 2**. Mutations of miR-520a-3p binding site was introduced by site-directed mutagenesis using a fast mutation kit (NEB, US). The PCR fragment was cloned into psiCHECK-2 vector downstream of the firefly luciferase coding region within XhoI and NotI (Takara, Japan). psiCHECK-2-control was used as internal control.

Animal assay

Male athymic mice (5 weeks old), purchased from the Animal Center of Southern Medical University (Guangzhou, China), were acclimated for one week under sterile conditions. Collected cells transfected with miR-520a-3p mimics or miR-520a-3p inhibitors from the 6 well plates, and washed the cells with PBS rendering resuspended cells of 2×10^7 cells/ml. Subcutaneously inject 0.1 ml of suspension cells to mice, 10 mice per group. 8 weeks later the mice were sacrificed, and the tumors were dissected, photographed and weighed, and then were immediately snap-frozen in liquid nitrogen and stored at -80°C until use. The study was approved by the Ethics Committee of

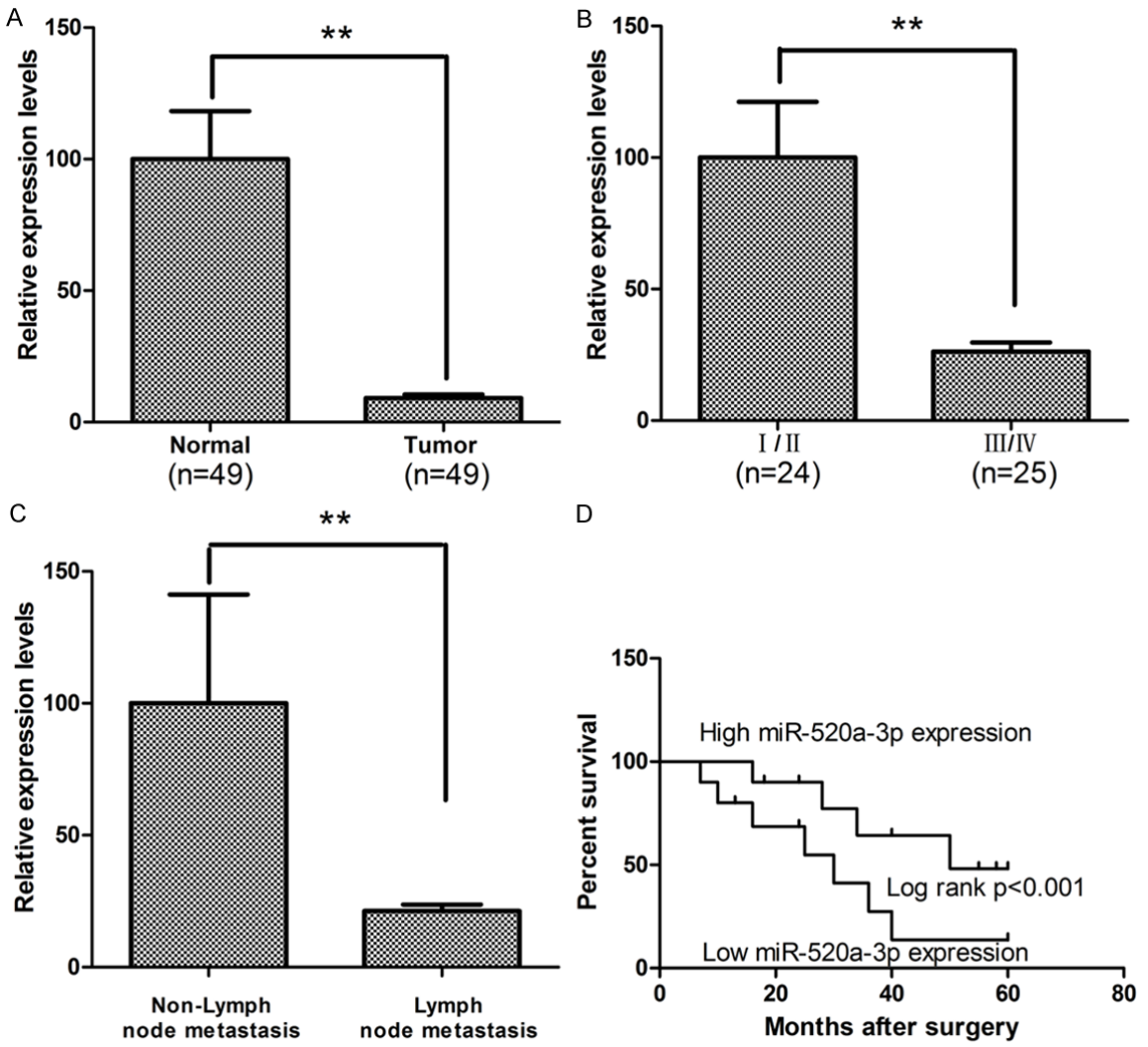

Figure 1. Relative miR-520a-3p expression levels in NSCLC tissues and its clinical significance. A. miR-520a-3p was detected in 49 pairs of NSCLC tissues by qRT-PCR. Data are presented as fold change in tumor tissues relative to normal tissues. B. miR-520a-3p expression was significantly lower in patients at advanced pathological stages. C. miR-520a-3p expression was significantly lower in patients with lymph node metastasis than in patients with non-lymph node metastasis. D. Patients with high levels of miR-520a-3p expression showed increased survival times compared with patients with low levels of miR-520a-3p expression ($P < 0.001$, log-rank test). **$P < 0.01$.

the Third Military Medical University. In order to minimize the suffering of mice, all surgery were conducted under sodium pentobarbital anesthesia [19].

Western blot analysis

The samples were collected and lysed using mammalian protein extraction reagent RIPA (Beyotime, Haimen, China) supplemented with protease inhibitors cocktail (Roche) and PMSF (Roche). Approximately 50 μg of the protein extraction was separated by 10% SDS-PAGE, then transferred to 0.22 mm nitrocellulose membranes (Sigma) and incubated with specific antibodies. The antibody was purchased from Abcam (Abcam, USA); Quantity One v4.4.0 software (Bio-Rad, USA) was used to assay optical density of the MAP3K2 bands. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control, and results were normalized to the expression of GAPDH.

Statistical analysis

Data are expressed as mean ± SEM. The difference among groups was determined by ANOVA analysis and comparison between two groups

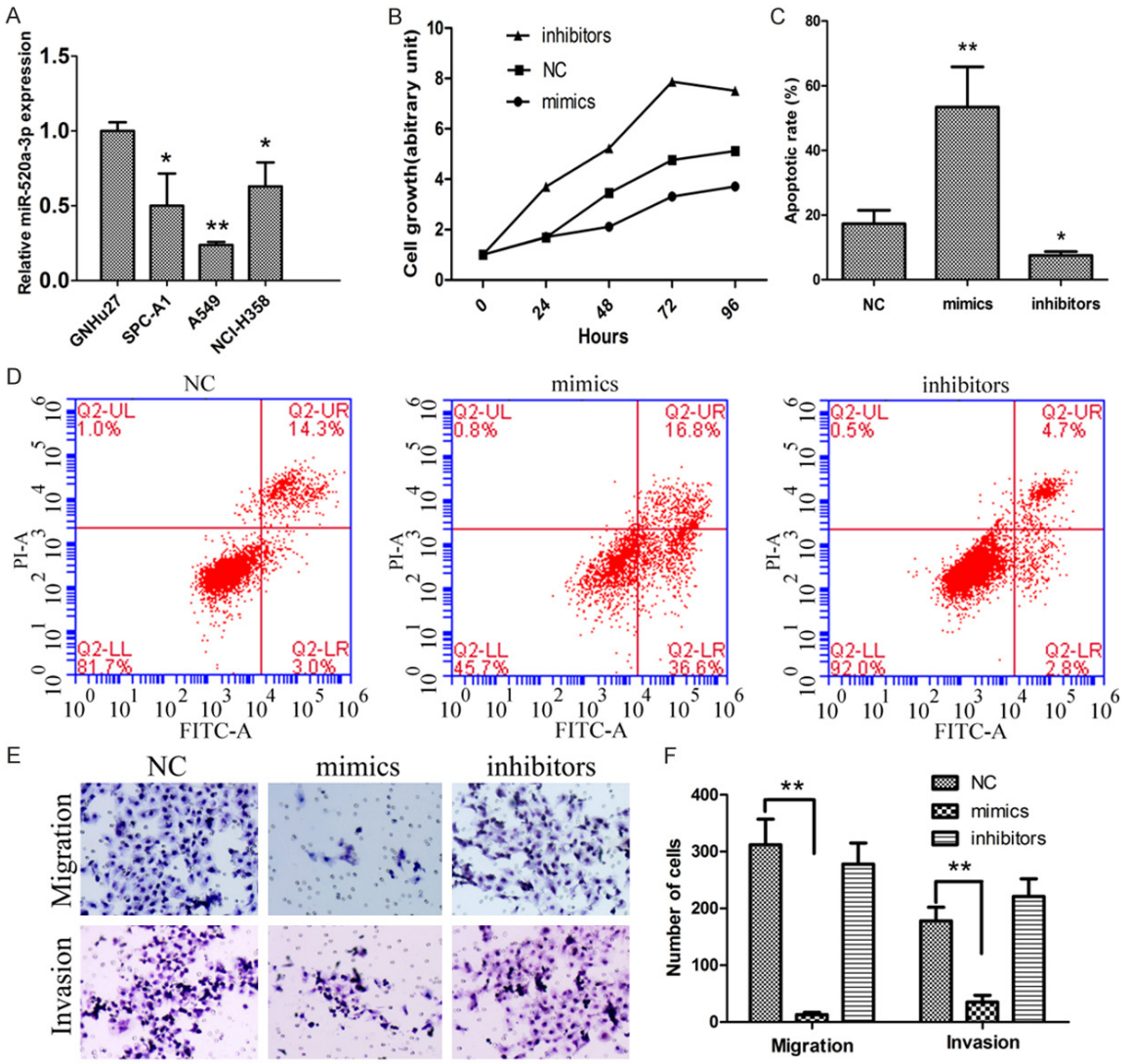

Figure 2. Effect of miR-520a-3p on cell proliferation, apoptosis, migration and invasion. A. The expression of miR-520a-3p were detected in SPC-A1, A549, NCI-H358, and normal GNHu27 cells. B. A549 cells was transfected with miR-520a-3p mimics or miR-520a-3p inhibitors, and MTT assays were performed to determine the proliferation in A549 cells. Data represent the mean ± S.D. from three independent experiments. C, D. Apoptotic rates were detected by flow cytometry in A549 cells. UL, necrotic cells; UR, terminal apoptotic cells; LR,early apoptotic cells. $^{**}P < 0.01$. E, F. Transwell assays were performed to investigate the migratory and invasive ability in A549 cells.

was analyzed by the Student's t-test using GraphPad software version 5.0 (GraphPad Software, CA). *P* value less than 0.05 was considered statistically significant.

Results

MiR-520a-3p is downregulated in NSCLC

Setting adjacent tissues as control, expression levels of miR-520a-3p in NSCLC tissue among 49 cases were assayed with qRT-PCR, miR-520a-3p expression in cancer tissues was significantly reduced (**Figure 1A**) in comparison to the adjacent tissues. The relationship between expression of miR-520a-3p and pathological feature of NSCLC patients reveals a close and significant association (**Figure 1B**, **1C**) between miR-520a-3p expression changes and lymph node metastasis in NSCLC. However, miR-520a-3p expression change has no relation to patient's tumor location or sex (**Table 1**). To further assess the relevance of miR-520a-3p expression and prognosis of NSCLC, a postoperative Kaplan-Meier survival analysis was conducted. The Kaplan-Meier survival profile indi-

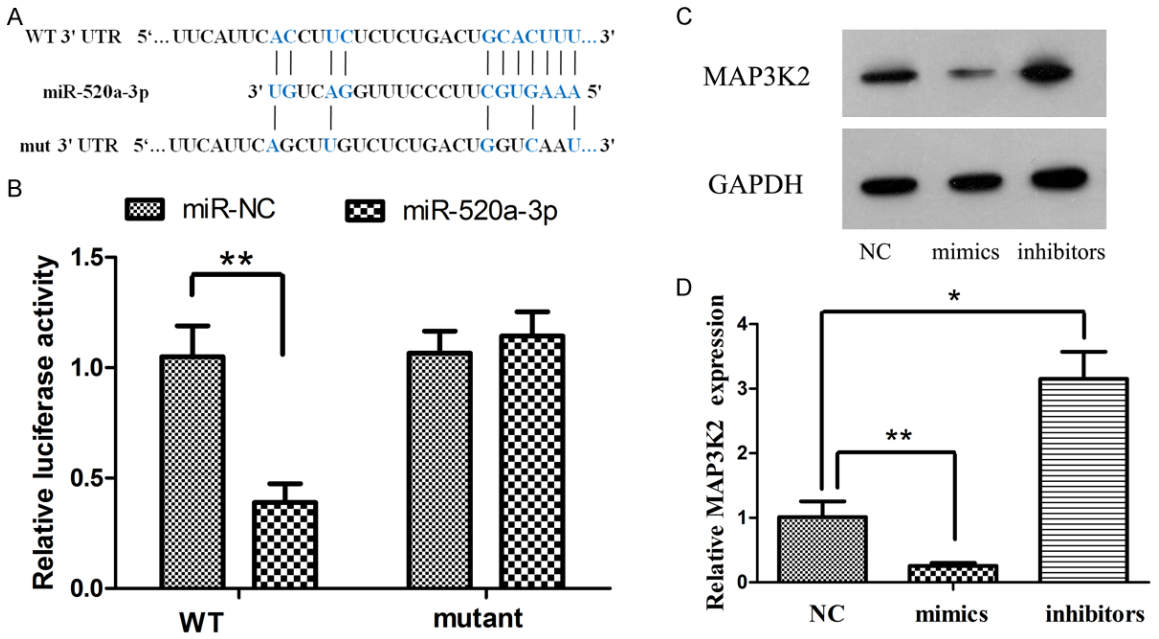

Figure 3. MAP3K2 is a direct target of miR-520a-3p in NSCLC cells. A. Wild type MAP3K2-untranslated regions (3'-UTR) (WT) or Mutated MAP3K2 3'-UTR (Mut) of MAP3K2 sequence. B. HEK-293T cells were co-transfected with miR-520a-3p/miR-NC with WT/Mut 3'-UTR of MAP3K2. Relative luciferase activity was evaluated. C & D. A549 cells transfected with miR-520a-3p mimics or miR-520a-3p inhibitors, and the expression level of MAP3K2 was detected by western blot. GAPDH was used as a control. Experiments were performed in triplicate. $*p < 0.05$, $**p < 0.01$ compared with control.

cated that patients with low miR-520a-3p expression level had a shorter survival time than those of high level ($P < 0.001$) (**Figure 1D**). These findings support the hypothesis that downregulated miR-520a-3p may play a key role in the occurrence and development of NSCLC.

MiR-520a-3p suppress cell proliferation and induce cell apoptosis in vitro

To assess the role of miR-520a-3p in NSCLC, we examined effect of miR-520a-3p mimics and miR-520a-3p inhibitors on cell proliferation and apoptosis. The A549 cell was chosen for the follow-up experiments because of the low miR-520a-3p expression level (**Figure 2A**). MTT assay showed that growth of A549 cells was affected by the transfection of miR-520a-3p mimics and miR-520a-3p inhibitors (**Figure 2B**). A549 cell was cultured in 6-well plates for apoptosis assay. The flow cytometry was used to assay the apoptosis of cells transfected by miR-520a-3p mimics and miR-520a-3p inhibitors. The results shown in **Figure 3C**, **3D** demonstrated a significantly higher percentage of apoptotic cells for miR-520a-3p mimics-treated cells compared to NC, and a significantly lower percentage of apoptotic cells for miR-520a-3p inhibitors-treated cells compared to NC. These results indicate that miR-520a-3p can suppress the growth and induce apoptosis in A549 cell.

MiR-520a-3p suppress cell metastasis in vitro

Cell migration and invasion is an important aspect of cancer metastasis, involving dissolution of extracellular matrix proteins and migration of tumor cells to adjacent tissue. We further investigated whether miR-520a-3p can inhibit migration and invasion of NSCLC, and found that transfection of miR-520a-3p inhibitors increased the A549 cell migration capability about 2.4 times than that of the NC, and in contrast, transfection of miR-520a-3p mimics inhibited A549 cell migration and invasion capacity by 58% (**Figure 2D**, **2E**).

MAP3K2 is a target of miR-520a-3p

Bioinformatics analysis show that MAP3K2 was predicted to be a target of miR-520a-3p (**Figure 3A**). The luciferase activity of wild-type

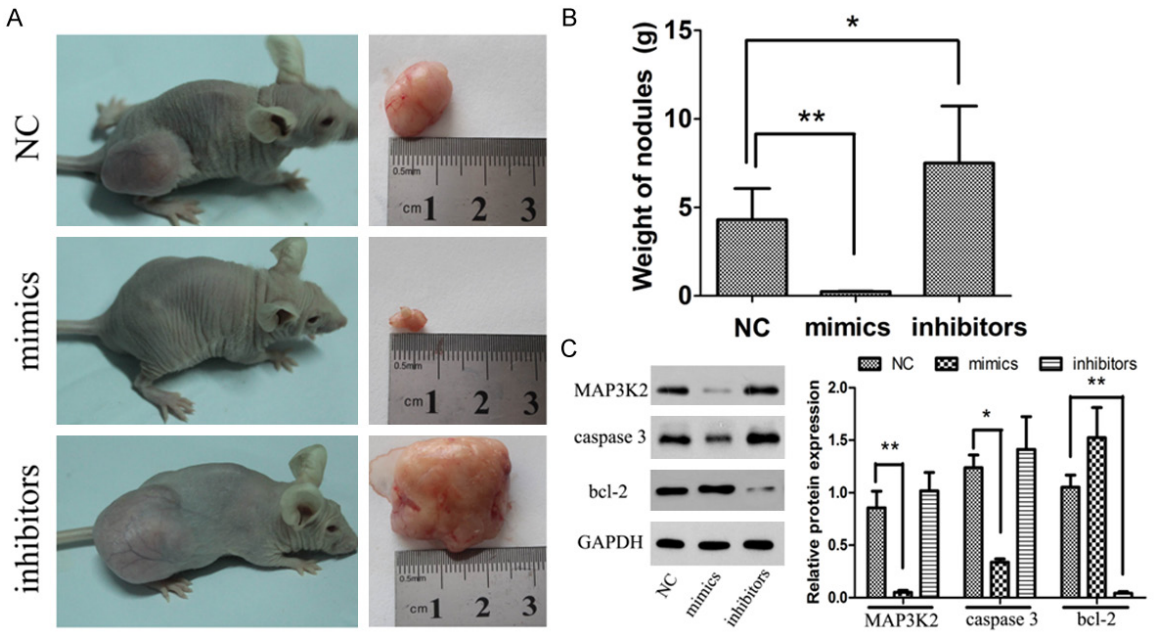

Figure 4. The effect of miR-520a-3p on tumorigenesis in vivo. A. miR-520a-3p mimics or miR-520a-3p inhibitors was transfected into A549 cells, which were injected in male athymic mice (n = 10), respectively. B. Tumor weights were calculated after injection 8 weeks. Bars indicate S.D. C. Western blot was performed to detect the expression of MAP3K2, caspase3, bcl-2 in tumor nodule. GAPDH protein was used as an internal control. *P < 0.05; **P < 0.01.

decreased 10.8 times compared to mutation in HEK-293 cells (**Figure 4B**). The fluorescein signal values of the transfection groups were removed of the background signal of the blank control, and then the sea cucumber signals were uniformized based on the firefly luciferase signal. The result (**Figure 3B**) shown that miR-520a-3p suppressed the luciferase activity of the wild type but not the mutant 3'-UTR in HEK-293 cells. Moreover, treatment with the miR-520a-3p mimics decreased the protein level of MAP3K2, and in contrast, treatment with the miR-520a-3p inhibitors promoted the protein level of MAP3K2 in A549 cell (**Figure 3C**). The results showed that MAP3K2 is a direct target genes of miR-520a-3p.

MiR-520a-3p inhibits tumor growth in athymic mice

To verify the effect of miR-520a-3p inhibiting on NSCLC cell metastasis in vivo, we injected male athymic mice with miR-520a-3p mimics and miR-520a-3p inhibitors transfected A549 cells and weighed the tumor 8 weeks later. Inhibition of miR-520a-3p significantly increased the tumor volume and weight compared with the control group, and on the contrary, promotion of miR-520a-3p significantly decreased the tumor volume and weight compared with the control group (**Figure 4A**, **4B**), suggesting that miR-520a-3p can inhibit metastasis of A549 cells in vivo.

MiR-520a-3p suppress expression of MAP3K2, caspase3 and bcl-2 in vivo

To explore molecular mechanisms by which miR-520a-3p contributes to the proliferation, apoptosis and metastasis of NSCLC cells, the expression of MAP3K2, caspase3 and bcl-2 was detected by western blot. MAP3K2 is a potential target involves in tumor proliferation, apoptosis and metastasis, and both caspase3 and bcl-2 play important role in cell apoptosis, cell differentiation and tumorigenesis. The results suggested that miR-520a-3p mimics can significantly inhibit protein expression of MAP3K2 and caspase3, and promote the expression of bcl-2. On the contrary, miR-520a-3p inhibitors could significantly promote protein expression of MAP3K2 and caspase3, and decrease the expression of bcl-2 (**Figure 4C**). These data indicated that miR-520a-3p may affect the proliferation, apoptosis and metastasis potential of NSCLC cells by altering MAP3K2, caspase3 and bcl-2 protein expression.

Discussion

The development and progression of malignant tumor is a very complex process, affected by many factors including biological characteristics of the tumor cells themselves. NSCLC, one of the most common and deadly malignant disease, holds metastasis accountable for its poor prognosis [20-29]. The mechanism inducing and stimulating metastasis is very complex and still unknown although in recent years more and more evidences have confirmed that non-coding RNA may render the NSCLC metastases. Researchers have identified a number of non-coding RNA as NSCLC metastasis and prognosis markers but the mechanism is far from clear [30-36].

In this study, we investigated the role of miR-520a-3p, a non-coding RNA deemed associated with a variety of human cancers, in NSCLC carcinogenesis and metastasis. In early stage of this study, we found that miR-520a-3p participated in the invasion and metastasis in NSCLC tissues and cells, which may help us to make a breakthrough.

To further evaluate the role of miR-520a-3p in carcinogenesis and metastasis of NSCLC, we designed experiments in which we changed the expression level of miR-520a-3p so as to detect the various aspects of biology of NSCLC. Our research showed that miR-520a-3p expression was significantly downregulated in NSCLC compared with normal tissues. Then, we increased the expression of miR-520a-3p, the invasion and metastasis capacity of NSCLC decreased significantly and apoptosis in NSCLC cells was induced. These results indicate miR-520a-3p plays a crucial role in the progress of NSCLC and may become a new biomarker for NSCLC prognosis.

In order to explore molecular mechanism of miR-520a-3p in the invasion and metastasis of NSCLC, we investigated the miR-520a-3p's potential target in A549 cell. MAP3K2 is an important protein in cell proliferation, differentiation, and cell migration. Bioinformatics analysis and luciferase activity assay demonstrated that MAP3K2 is a direct target genes of miR-520a-3p. The miR-520a-3p mimics can significantly decreased protein expression level of MAP3K2 in the NSCLC cells.

In summary, our studies show that miR-520a-3p can inhibit proliferation, apoptosis and metastasis of NSCLC by regulating the expression level of MAP3K2 partially, which have laid us a theoretical basis for further understanding the pathogenesis of NSCLC and conducting diagnosis and treatment.

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

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