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miR-582-5p is a potential prognostic marker in human non-small cell lung cancer and functions as a tumor suppressor by targeting MAP3K2

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Abstract. – OBJECTIVE: Emerging evidence has shown that microRNAs (miRNAs) play important roles in tumor development and progression. The aim of the present study was to investigate the role of miR-582-5p in non-small cell lung cancer (NSCLC) and to determine the molecular mechanisms underlying its action.

PATIENTS AND METHODS: Using quantitative RT-PCR, we detected miR-582-5p expression in NSCLC cell lines and primary tumor tissues. The association of miR-582-5p expression with clinicopathological factors and prognosis was statistically analyzed. The effect of miR-582-5p on proliferation was evaluated by CCK-8. Cell migration and invasion were assessed by transwell assay. miR-582-5p target genes were confirmed using luciferase activity, RT-PCR and Western blot assays.

RESULTS: We found that miR-582-5p expression was significantly downregulated in NSCLC cell lines and clinical specimens. Low miR-582-5p expression was significantly associated with lymph node metastasis ($p = 0.012$) and advanced TNM stage ($p = 0.004$). Kaplan-Meier assay showed that patients with low expression of miR-582-5p had a shorter overall survival than those with high expression of miR-582-5p ($p = 0.0033$). Functional experiments demonstrated that overexpression of miR-582-5p suppressed the proliferation, migration and invasion of NSCLC cells in vitro. We identified MAP3K2 as a direct target gene of miR-582-5p in NSCLC cells. In addition, ectopic expression of MAP3K2 restored the effects of miR-582-5p on NSCLC cell proliferation, migration and invasion.

CONCLUSIONS: We showed that miR-582-5p inhibits NSCLC metastasis by targeting MAP3K2 expression and could be used as an independent prognostic biomarker for patients with NSCLC.

Key Words:
miR-582-5p, NSCLC, Prognosis, MAP3K2, Proliferation, Migration, Invasion.

Introduction

Lung cancer remains a leading cause of cancer-related mortality and morbidity, resulting in 1.4 million deaths annually worldwide[1]. Non-small cell lung cancer (NSCLC) accounts for about 80% of all lung cancer cases, and patients usually present advanced disease at initial diagnosis[2,3]. Despite the enormous improvements made in chemotherapy and radiotherapy over the past few decades, the prognosis of this malignant tumor remains unfavorable, with a 5-year overall survival rate of 15% only[4]. Understanding the molecular mechanisms for lung carcinogenesis is very important to develop effective therapies for NSCLC. MicroRNAs (miRNAs) are is a class of small non-coding RNAs (19-25 nucleotides) involved in modulating gene expression through specifically binding to the 3'untranslated region (3'UTR) of target mRNAs[5]. Growing biological evidence shows that miRNAs act as important regulators in various physiological and pathophysiological process such as cell proliferation, differentiation, metastasis and apoptosis[6,7]. It has been proposed that dysregulation of miRNAs expression contributes to the initiation and progression by serving as tumor suppressors or promoters in various human cancers, including NSCLC[8-10]. For instance, Liang et al[11] reported that miR-433 expression was significantly down-regulated in cervical cancer and its overexpression by miR-433 mimics suppressed cervical cancer cell proliferation and invasion via regulating the AKT and β-catenin signalling pathways. Kawano et al[12] found that miR-301a was significantly highly expressed in osteosarcoma and its knockdown significantly suppressed tumor growth *in vivo* by targeting PTEN. Importantly, miR-652-3p expression was higher in NSCLC tissues and cell lines,

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and associated with poor prognosis of NSCLC patients. *In vitro* assay[13] revealed that suppression of miR-652-3p inhibited the proliferation and metastasis by directly targeting Lgl1. MiR-582-5p, a tumor-related miRNA, has been reported to be dysregulated in several tumors[14,15]. However, its expression pattern and biological function in NSCLC has not been investigated. In this work, we investigated the potential roles of miR-582-5p in NSCLC. We examined the expression of miR-582-5p in human NSCLC cells and tissues and tested its effects on cells proliferation, migration and invasion. Besides, the prognostic value of miR-582-5p in NSCLC patients was also determined. Mitogen-activated protein kinase kinase kinase 2 (MAP3K2), also known as MEKK2, was a member of the MAPK signaling pathway[16]. Previous reseraches[17] showed the tumor-promotive role of MAP3K2 in NSCLC. In order to explore the potential mechanism by which miR-582-5p exerted its tumor-suppressive role in NSCLC, our attention focused on the association between miR-582-5p and MAP3K2. Our findings firstly showed that miR-582-5p suppressed the proliferation, migration and invasion by targeting MAP3K2.

Patients and Methods

Patients and Specimens

Paired NSCLC and adjacent normal lung tissues were obtained from 122 patients who received curative resection of NSCLC in Heilongjiang Province Hospital between July 2010 and February 2013. All the patients with NSCLC were pathologically confirmed and diagnosed. These tissues were flash-frozen in liquid nitrogen immediately after resection and stored at -80°C until use. No previous local or systemic treatment had been conducted on these patients before the operation or biopsy. Clinicopathological characteristics were available for all samples (Table II). This study protocol was approved by the Institutional Research Ethics Committee of Heilongjiang Province Hospital, and informed consent was obtained from all NSCLC patients.

Cell Culture and Transfection

NSCLC cell lines (A549, SPC-A-1, H1299, SK-MES-1 and 95D) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Normal lung cells HELF were obtained from Doctor Wang (Peking University, Haidian, Beijing, China). All above cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) supplemented with 10% fetal bovine serum (FBS, Longhu Technology, Beijing, China), 100 U/ml penicillin and 100 microg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified incubator containing 5% CO_2. For miR-582-5p overexpression, miR-582-5p mimics and its negative controls (NC) were designed and synthesized by GenePharma Company (Xuhui, Shanghai, China). The MAP3K2 cDNA was cloned into pcDNA3.1 to construct the MAP3K2 expression plasmid. For transfection, A549 and H1299 cells were seeded in 24-well plates at a density of 30% and cultured for 24 h. Next, cells at approximately 70% confluence were transfected with miR-582-5p mimics and NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection efficiencies were evaluated in every experiment by RT-qPCR 24 h post-transfection.

Real-Time PCR

Total RNA was isolated from tissue or cells by using the mirVana miRNA isolation kit (Ambion, Haidian, Beijing, China) according to the manufacturer's protocol. The purity and concentration of the total RNA were determined using NanoDrop 2000 Spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA). cDNA was generated via reverse transcription of total RNA using miR-582-5p-specific stem-loop primers. MAP3K2 mRNA and miR-582-5p expression levels were detected by qPCR with Applied Biosystems 7500 Fast Real-Time PCR System (Invitrogen, Carlsbad, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal controls for miRNA and mRNA detection. Quantifications were measured by a Bio-Rad PCR instrument (Hercules, CA, USA), using the method of $2^{-\Delta\Delta CT}$. The sequences of primers were shown in Table I.

Cell Proliferation Assay

To quantify proliferation, the Cell Counting Kit-8 (CCK-8; Solarbio, Haidian, Beijing, China)

Table I. Primer sets used in the present study.

Gene names	Sequence (5′-3′)
miR-582-5p (F)	GCGGTTACAGTTGTTCAACC
miR-582-5p (R)	CTCAACTGGTGTCGTGGA
MAP3K2 (F)	CCCCAGGTTACATTCCAGATGA
MAP3K2 (R)	GCATTCGTGATTTTGGATAGCTC
GAPDH (F)	GGAGCGAGATCCCTCCAAAAT
GAPDH (R)	GGCTGTTGTCATACTTCTCATGG

was performed. Briefly, A549 and H1299 cells were seeded into 96-well culture plates at a density of 3 ×10^3 cells in 300 µl/well and incubated at 37°C for 24 h. Then, 5 µl Cell CCK-8 reagent was added to each well and incubated at 37°C for 1 h. The absorbance was measured at 490 nm with a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Three individual experiments were performed.

Migration and Invasion Assays

Twenty-four well Millipore transwell chambers (Millipore Corporation, Billerica, MA, USA) were used to perform the migration and invasion assays. For migration assays, A549 and H1299 cells suspended in serum-free medium were seeded in the top chamber of a 24-well transwell insert (Millipore Corporation, Billerica, MA, USA). For invasion assays, the insert was precoated with Matrigel (Sigma-Aldrich, St. Louis, MO, USA). Medium containing 20% fetal bovine serum (FBS) was added to the lower chamber. After 24 hours, the non-migrated or non-invading cells were removed with cotton wool. Then, the cells invaded through the membrane were stained with 0.1% crystal violet. Finally, the cells from five randomly selected fields were counted under a light microscope.

Dual Luciferase Assay

The interaction between miR-582-5p and MAP3K2 was determined by dual luciferase assay. The pmirGLO-MAP3K2-3'UTR WT and pmirGLO-MAP3K2-3'UTR MUT luciferase reporter vectors were synthesized and purified by GenePharma (Pudong, Shanghai, China). For transfection, A549 and H1299 cells were seeded in 24-well plates (2 × 10^4 cells/well) 24 h before transfections were performed. Then, cells were co-transfected with 200 ng of pmirGLO-MAP3K2-WT or pmirGLO-MAP3K2-MUT vector and mir-582-5p mimics or NC. Twenty-four hours later, cells were collected, and the firefly and renilla luciferase activities were detected using a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Western Blot Analysis

Total proteins were extracted and quantified using a protein assay (Biosystems, Foster City, CA, USA). The proteins (60 µg/sample) were separated in 10% sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Thermo-Fisher Scientific, Waltham, MA, USA). The transferred protein on the membrane was confirmed by Ponceau staining solution. Next, the membrane was blocked by 3% fat-free milk in tris-buffered saline (TBS) and blotted with primary antibodies MAP3K2 and GAPDH at 4°C overnight. Those antibodies were purchased from Dajiang Technology (Haidian, Beijing, China). The membranes were then incubated for 1 h at room temperature with horseradish peroxidase(HRP)-conjugated

Table II. Clinicopathological correlation of miR-582-5p expression in human NSCLC.

			miR-582-5p expression		
Parameters	Group	Total	Low	High	*p*-value
Gender	Male	62	32	30	NS
	Female	60	27	33	
Age	< 60	64	35	29	NS
	≥ 60	58	24	34	
Tumor size	< 4 cm	47	20	27	NS
	≥ 4 cm	75	39	36	
Histologic type	SCC	54	24	30	NS
	AD	68	35	33	
Smoking	No	75	32	43	NS
	Yes	45	27	20	
Family history	No	102	50	52	NS
	Yes	20	9	11	
Surgery margins	Free	81	36	45	NS
	Not free	41	23	18	
Lymph node metastasis	Negative	76	30	46	0.012
	Positive	46	29	17	
TNM stage	I-II	70	24	44	0.004
	III-IV	52	33	19	

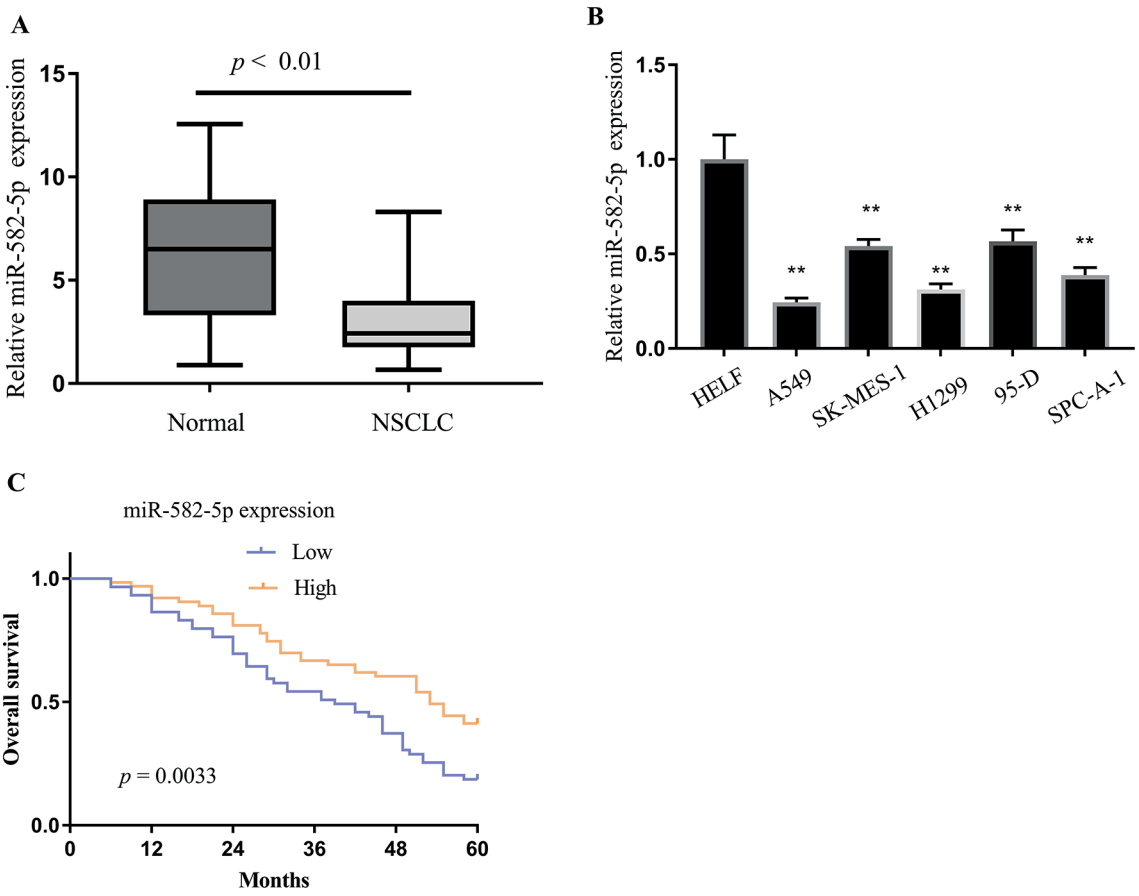

Figure 1. Relative miR-582-5p expression in NSCLC tissues and its clinical significance. ***(A)*** The expression of miR-582-5p in NSCLC tissues was significantly lower than that in adjacent normal lung tissues. ***(B)*** miR-582-5p expression was down-regulated in NSCLC cell lines (A549, SPC-A-1, H1299, SK-MES-1 and 95D) compared with that in HELF. ***(C)*** Patients with low expression of miR-582-5p had significantly worse overall survival rates compared with those who had cancers with high miR-582-5p expression. $^{*}p < 0.05$, $^{**}p < 0.01$.

goat anti-rabbit IgG. Bands were visualized using Amersham ECL Prime (GE Healthcare, Pittsburgh, PA, USA) according to the manufacturer's instructions.

Statistical Analysis

All the statistical analyses were performed using SPSS13.0 for Windows (SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed using Student's t-test. The multi-group comparison was performed using one-way analysis of variance. The paired comparison was performed by Student-Newman-Keuls (SNK) method. The relationship between miR-582-5p level and clinical and pathological variables was analyzed using x^2-test. Overall survival curves were plotted using the Kaplan-Meier method and were evaluated for the statistical significance using a log-rank test. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-582-5p Was Downregulated in NSCLC Tissues and Cell Lines

For the purpose of determining whether miR-582-5p was dysregulated in NSCLC, 122 NSCLC tissues and matched tumor-adjacent tissues were detected by qRT-PCR for miR-582-5p expression. The results were shown in Figure 1A; we found that miR-582-5p expression was decreased in NSCLC tissues compared with that in adjacent normal tissues ($p < 0.01$). After that, we further determined the levels of miR-582-5p in five NSCLC cell lines and normal lung cells HELF. As shown in Figure 1B, we observed that miR-582-5p

was lowly expressed in several NSCLC cell types, including A549, SPC-A-1, H1299, SK-MES-1 and 95D, compared with HELF cells. Our results indicated that miR-582-5p expression may be down-regulated in both NSCLC tissues and cell lines. Because miR-582-5p showed relatively low levels in A549 and H1299 cells, we chose them for following *in vitro* assay.

Association of miR-582-5p Expression With Clinical Characteristics and Prognosis of NSCLC Patients

To identify the clinical relevance of miR-582-5p expression in NSCLC, the 122 human NSCLC tissues was classified into high miR-582-5p group (n=63) and low miR-582-5p group (n = 59) according to the median miR-582-5p expression level. Subsequently, x^2-test was used to analyze association between miR-582-5p expression and clinicopathological parameters. As shown in Table II, we found that low miR-582-5p expression levels were correlated with lymph node metastasis (p = 0.012) and advanced TNM stage (p = 0.004). However, there was no association between miR-582-5p expression and other clinical features, such as gender, age, tumor size, histologic type, smoking, family history and surgery margins (p > 0.05). In order to further explore the prognostic value of miR-582-5p expression in NSCLC patients, we performed Kaplan-Meier analysis in NSCLC patients who received five years follow-up. As shown in Figure 1C, our results suggested that a trend toward poorer overall survival was observed in patients with lower miR-582-5p expression compared to those with higher miR-582-5p expression (p = 0.0033). Taken together, our findings indicated that low miR-582-5p was associated with advanced clinical progression and poor prognosis of NSCLC patients.

miR-582-5p Acted as An Anti-oncogenic Role in Regulating NSCLC Cells Proliferation, Migration and Invasion

In order to generate stably infected cells, A549 and H1299 cells were transfected with miR-582-5p mimics or NC. RT-PCR showed that the expression of miR-582-5p was remarkably upregulated in A549 and H1299 cells after transfection (Figure 2A). Next, we performed CCK-8 to test whether overexpression of miR-582-5p in A549 and H1299 influence the cells proliferation. As shown in Figure 2B and 2C, A549 and H1299 cells with increased miR-582-5p expression proliferated at a slower rate than did control cells.

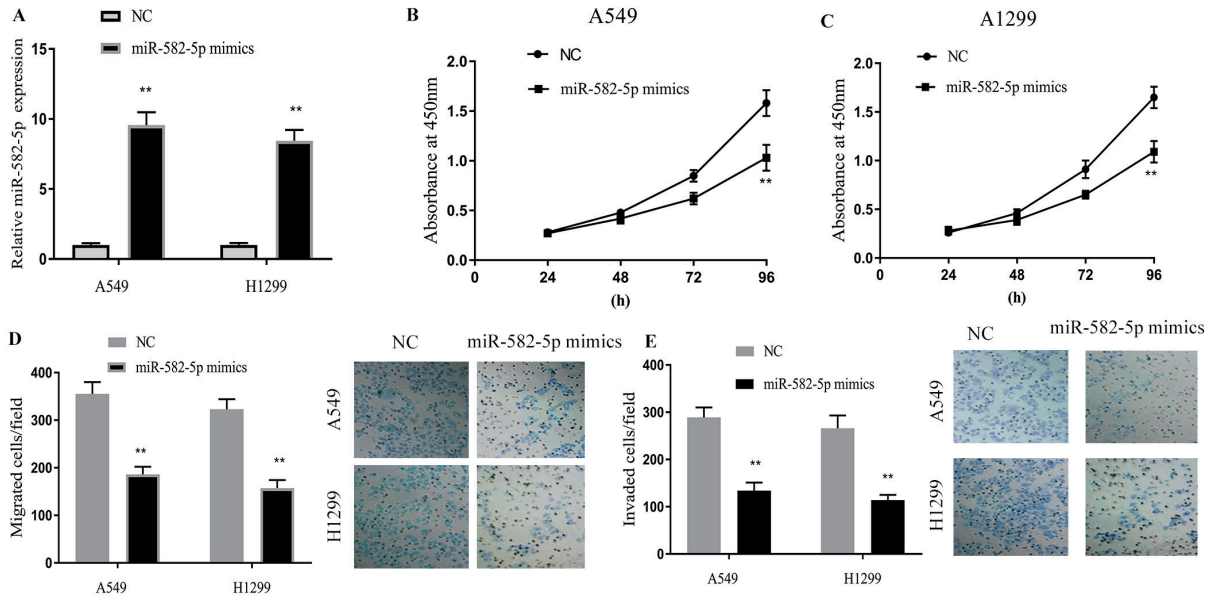

Figure 2. miR-582-5p upregulation inhibited NSCLC cells proliferation, migration and invasion. ***(A)*** After transfection with miR-582-5p mimics or NC, miR-582-5p expression in A549 and H1299 cells was detected by qRT-PCR. ***(B, C)*** CCK-8 assays of miR-494 mimics and negative control-transfected cells. ***(D)*** Transwell assay was used to determine A549 and H1299 cells migratory capability. ***(E)*** Transwell assay was used to determine A549 and H1299 cell invasive capability. $^{*}p < 0.05$, $^{**}p < 0.01$.

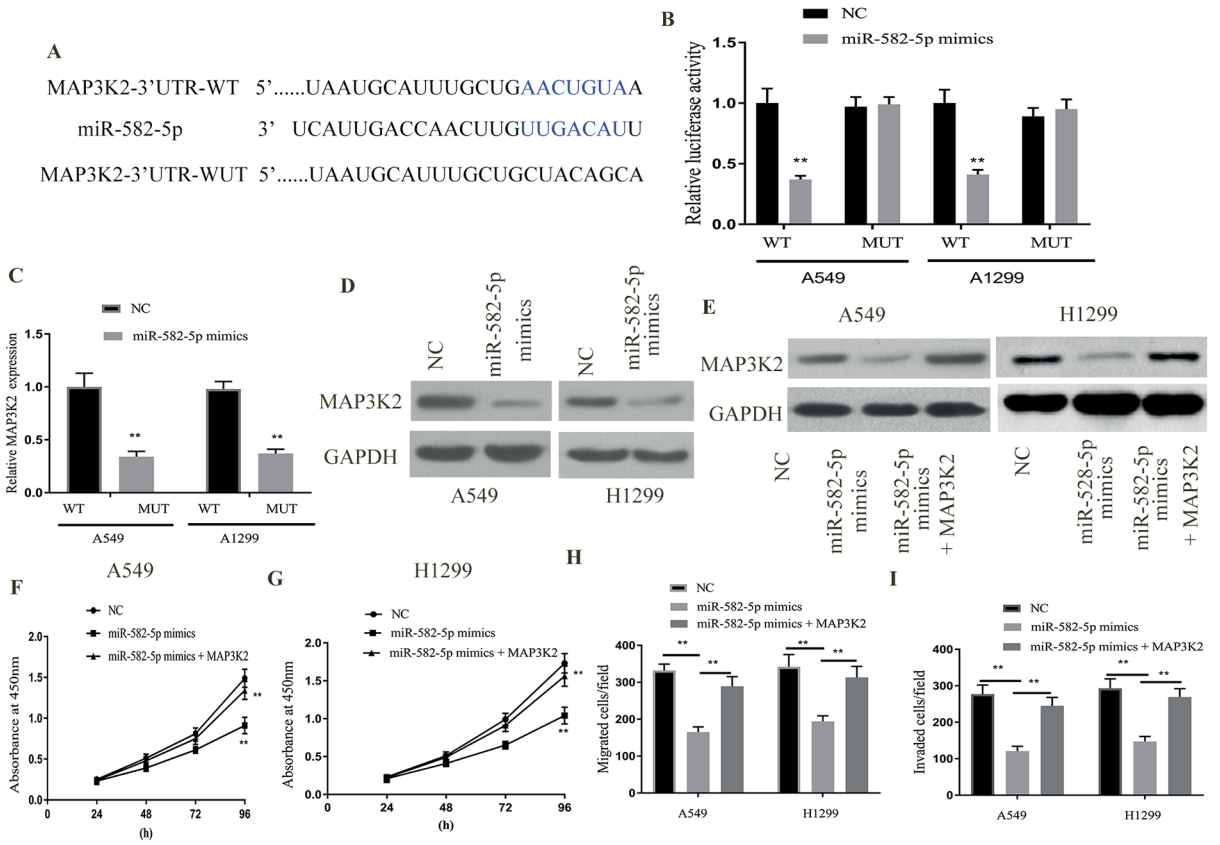

Figure 3. MAP3K2 was a direct target gene of miR-582-5p in NSCLC. ***(A)*** Algorithms predicted MAP3K2 contains miR-582-5p binding site on its 3'-untranslated regions. ***(B)*** Overexpression of miR-582-5p significantly inhibited the luciferase activity that carried WT 3'-UTR of MAP3K2 but had no obvious influence on MUT 3'-UTR of MAP3K2in both A549 and H1299 cells. ***(C)*** Upregulation of miR-582-5p significantly suppressed MAP3K2 mRNA levels in both A549 and H1299 cells via RT-PCR. ***(D)*** Upregulation of miR-582-5p significantly suppressed MAP3K2 protein levels in both A549 and H1299 cells via Western blot. ***(E)*** MAP3K2 protein expression in A549 and H1299 cells transfected with NC, miR-582-5p, or miR-582-5p plus MAP3K2 plasmid. ***(F, G)*** CCK-8 was used to determine the effect of MAP3K2 on A549 and H1299 cells proliferation. (H, I) Transwell assay was used to determine the effect of MAP3K2 on A549 and H1299 cells migration and invasion. $*p < 0.05$, $**p < 0.01$.

Besides, statistical analysis conformed this difference. To further study the effect of miR-582-5p on A549 and H1299 cells, transwell assay was performed to determine the migration and invasion ability. As shown in Figure 2D and 2E, our findings showed that over-expression of miR-582-5p inhibited the migration and invasion ability of A549 and H1299 cells. Taken together, our results indicated that miR-582-5p may function as a tumor suppressor in progression of NSCLC.

miR-582-5p Directly Targets MAP3K2

In order to investigate the mechanism of miR-582-5p in the development of NSCLC, we found that miR-582-5p targets MAP3K2 using bioinformatics software tools (Figure 3A). To further confirm the predicted results, we constructed a luciferase reporter vector with the putative MAP3K2 3'UTR target site for the miR-582-5p downstream of the luciferase gene. Dual-luciferase assay was used to analyze the role of miR-582-5p on the luciferase activity. As shown in Figure 3B, transfection of miR-582-5p mimics significantly inhibited the luciferase activity of MAP3K2 3'UTR WT, whereas transfection of miR-582-5p mimics had no inhibition effect on the mutant MAP3K2-3'UTR reporter activity in A549 and H1299 cells. Then, we performed qRT-PCR and Western blot analyses to further study whether miR-582-5p could regulated the expression of MAP3K2. Our findings showed that overexpression of miR-582-5p significantly suppressed the expression of MAP3K2 mRNA (Figure 3C). Similar, overexpression of miR-582-5p led to a significant reduction in miR-582-5p protein expression in both A549 and H1299

cells (Figure 3D). Overall, results suggest that MAP3K2 is a direct target gene of miR-582-5p in NSCLC.

Alteration of MAP3K2 Expression Influences the Effects of miR-582-5p on NSCLC Cells

To further confirm that MAP3K2 is a functional target of miR-582-5p, a specific MAP3K2 plasmid was transfected into miR-582-5p up-regulating A549 and H1299 cells. The expression of MAP3K2 was confirmed by Western blotting (Figure 3E). The results of *in vitro* assay showed that up-regulation of MAP3K2 partially abrogated miR-582-5p-induced inhibition of A549 and H1299 cell proliferation, migration, and invasion (Figure 3F-3I). These data further supported the result that MAP3K2 was a direct and functional target of miR-582-5p in NSCLC.

Discussion

As important regulators in tumor progression, miRNAs become a research hotspot in developing new therapeutic target[18]. In this study, we firstly determined the expression of miR-582-5p in NSCLC tissues and cells. Our results revealed that miR-582-5p was lowly expressed in NSCLC tissues and cells. Subsequently, clinical significance of miR-582-5p was analyzed and the results showed that low miR-582-5p expression was significantly associated with lymph node metastasis and advanced TNM stage, suggesting miR-582-5p as a negative regulator in development and progression of NSCLC. Further five years follow-up research confirmed that low miR-582-5p was associated with poorer overall survival, suggesting that miR-582-5p may be used as a prognostic biomarker for NSCLC patients. However, because sample size is small, a more in-depth and larger scale study remains to confirm our results. On the other hand, functionally, we found that miR-582-5p overexpression by miR-582-5p mimics could suppress the proliferation, migration and invasion of NSCLC cells, suggesting that miR-582-5p may serve as a tumor suppressor in tumorigenesis. Since the expression and function of miR-582-5p was firstly reported in bladder cancer, several studies have reported the tumor-suppressive role of miR-582-5p in various tumors[19,20]. For instance, Wang et al[21] reported that up-regulation of miR-582-5p suppressed invasion and migration of salivary adenoid cystic carcinoma cells by targeting FOXC1. Zhang et al[22] found that the expression levels of miR-582-5p was significantly down-regulated in hepatocellular carcinoma tissues and cell lines. *In vitro* experiment revealed that forced miR-582-5p expression suppressed cellular proliferation, and arrested cell cycle in G0/G1 phase by targeting CDK1 and AKT3. Jin et al[23] suggested that miR-582-5p was lowly expressed in gastric cancer and its overexpression attenuated cell proliferation and viability capacities by targeting AKT3. Zhang et al[24] showed miR-582-5p as an anti-carcinogenic miRNA which could inhibits cell proliferation and invasion by targeting Rab27a in human colorectal carcinoma. All above findings indicated that miR-582-5p served as a tumor suppressor in various tumors. However, up to date, the expression pattern and biological function remains unknown. Our data also showed miR-582-5p functioned as a tumor suppressor in NSCLC. These findings were in line with previous studies. In order to explore the molecular mechanism by which miR-582-5p suppressed NSCLC cells proliferation, migration and invasion, we used TargetScan and found MAP3K2 as a direct target of miR-582-5p. MAP3K2 has been reported to have the ability to activate a number of downstream MAPK pathways, including ERK1/2, JNK, p38, and ERK5[25]. MAPK pathways were critical signal pathways involved in tumor development and progression[26]. Huang et al[17] reported that MAP3K2 promoted NSCLC cells proliferation, migration and invasion. To validate the prediction experimentally, luciferase reporter assay was employed and the results confirmed that MAP3K2 is a target gene of miR-582-5p. In addition, RT-PCR and Western blot showed that overexpression of miR-582-5p can suppress the levels of MAP3K2 in A549 and H1299 cells. More importantly, rescue experiments showed that restoration of MAP3K2 abrogated miR-582-5p -induced inhibition of NSCLC cell proliferation, migration, and invasion. Taken together, these findings suggested that miR-582-5p suppressed the proliferation, migration and invasion by regulating MAP3K2 in human NSCLC cells.

Conclusions

We firstly showed that miR-582-5p was down-regulated in both NSCLC tissues and cell lines, and that its ectopic expression inhibited cell pro-

liferation, migration and invasion. In addition, miR-582-5p functioned as a tumor suppressor by targeting MAP3K2. The expression levels of miR-582-5p were positively associated with overall survival, indicating that miR-582-5p could serve as a new prognostic biomarker in NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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