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Original Article

miR-126 enhances the sensitivity of non-small cell lung cancer cells to anticancer agents by targeting vascular endothelial growth factor A

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Increasing evidence suggests that hsa-miR-126 (miR-126) is down-regulated in non-small cell lung cancer (NSCLC) cell lines and the restoration of miR-126 impairs tumor cell proliferation, migration, invasion, and survival by targeting specific molecules. Here, we reported for the first time that miR-126 was involved in regulating the response of NSCLC cells to cancer chemotherapy. After transfected A549 cells with miR-126 mimic or inhibitor, we found that an elevated level of miR-126 was significantly associated with a decreased half maximal inhibitory concentration of adriamycin (ADM) and vincristine, an increased accumulation of ADM, down-regulation of vascular endothelial growth factor A (VEGFA) and multidrug resistance-associated protein 1 (MRP1), and inactivation of the Akt signaling pathway. Furthermore, enhanced expression of miR-126 suppressed the growth of A549 xenograft and inhibited the expression of VEGFA and MRP1. miR-126 could efficiently down-regulate VEGFA expression through the interaction with the *VEGFA* 3′-untranslated region, whereas restoration of VEGFA could partially attenuate the suppression of MRP1 by miR-126. However, LY294002, an inhibitor of the PI3K/Akt signaling pathway, diminished this effect, suggesting that enhanced expression of miR-126 increased the sensitivity of NSCLC cells to anticancer agents through negative regulation of a VEGF/PI3K/Akt/MRP1 signaling pathway.

Keywords miR-126; vascular endothelial growth factor A; multidrug resistance-associated protein 1; PI3K/Akt signaling pathway

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Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, with non-small cell lung cancer (NSCLC) accounting for at least 80% of cases. Due to the poor prognosis of this malignancy, NSCLC is considered to be a chemotherapy-refractory malignancy, but the mechanisms remain rather obscure [1]. Multidrug resistance-associated protein 1 (MRP1) plays a critical role in the development of multidrug resistance (MDR) in tumor cells and its expression is strongly associated with the decreased cellular drug accumulation of a broad spectrum of widely used range of antineoplastic drugs including anthracyclines, vinca alkaloids, etoposide, docetaxel, paclitaxel, and cisplatin [2–4]. MRP1 was expressed in a high proportion of NSCLC cell lines and 32%–100% of clinical NSCLC tumor specimens [5,6], and it is also highly expressed in several non-P-glycoprotein (P-gp)-expressing multidrug-resistant cells including several lung cancer cells, leukemic cells, and breast cancer cells (such as GLC4/ADR, H69/AR, HL60/ADM, and MCF-7/VP) [2,7–10]. A better understanding of MRP1 regulation might inspire strategies to reverse the MDR phenotype of cancer cells to enhance their susceptibility to chemotherapeutic agents and decrease patient mortality.

Previous studies indicated that microRNAs (miRNAs) acted as crucial modulators in cancer progression by targeting mRNAs through cleavage or transcriptional repression [11]. However, the correlation between miRNA expression and drug sensitivity in cancer cells has only been recently investigated. miR-126, derived from a common precursor structure located within the epidermal growth factor-like domain 7 (*EGFL7*) gene, has been shown to act as a suppressor of tumor formation in leukemia as well as cancers of the breast, lung, cervix, bladder, prostate, colon, esophagus, and gastric [12–20]. A recent study has reported that, miR-126 levels are decreased ~3 folds in docetaxel-resistant breast cancer cells compared with docetaxel-sensitive breast cancer cells [21]. This observation raises the possibility that miR-126 may act as an MDR-associated molecule, but the biological mechanisms remain unexplored.

The vascular endothelial growth factor A (*VEGFA*) oncogene has been revealed as a direct target of miR-126

[13,20]. Down-regulation of VEGF reduces the drug sensitivity of some leukemic cells [22]. The expression levels of VEGF and its receptor FLT-1 are markedly higher in cells resistant to adriamycin (ADM) compared with those sensitive to ADM [23]. Moreover, the VEGFA-induced MDR phenotype that renders human dermal microvascular endothelial cell insensitive to cisplatin, mitomycin, VP-16, and paclitaxel has been attributed to the overexpression of MRP1 and lung resistance-related protein (LRP) [24]. Notably, our previous studies showed that an anti-VEGFA antibody reduced the levels of both MRP1 mRNA and protein in K562/A02 cells in a dose-dependent manner and VEGFA expression at both mRNA and protein levels are closely related to MRP1 expression [25].

By binding to and triggering the receptor tyrosine kinase activity of VEGF receptors, VEGF activates the downstream PI3K/Akt signaling pathway, which is a critical regulator of cellular growth, differentiation, and metabolism [26]. Like VEGF, PI3K/Akt has been linked to the regulation of MRP1, as the specific PI3K inhibitor, wortmannin, reduces MRP1 expression by inhibiting Akt phosphorylation [27]. Here we sought to test the hypothesis that the restoration of miR-126 could overcome the resistance of NSCLC cells to antineoplastic drugs through inhibition of a VEGF–PI3K/Akt signaling pathway that resulted in the down-regulation of MRP1.

Materials and Methods

Cell culture

Human NSCLC cell line A549 was obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI 1640 containing 10% fetal calf serum (Gibco BRL, Grand Island, USA) at 37°C with 5% CO_2.

Quantitative real-time PCR analysis for miRNA

A549 cells were reverse transfected with miR-126 mimic (A549 + mimic), miR-126 inhibitor (A549 + INH), mimic control (A549 + mimic-NC), or inhibitor control (A549 + INH-NC) (Genepharma, Shanghai, China) in six-well plates (6×10^5 cells/well) with a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. The sequence of the miR-126 mimic, miR-126 inhibitor, and their negative controls are shown in **Table 1**. Forty-eight hours after transfection, total RNA was prepared using Trizol (Invitrogen), then reverse transcribed with the First strand cDNA synthesis kit (Fermentas, Glen Burnie, USA), and quantified by the SYBR green real-time PCR kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The cycle number at which the reaction crossed an arbitrarily placed threshold (C_t) was determined for each gene, and the relative amount of each miRNA to U6 snRNA was calculated using the equation $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = (C_{t\ \text{miRNA}} - C_{t\text{U6}\ \text{snRNA}})_{\text{transfected}} - (C_{t\ \text{miRNA}} - C_{t\text{U6}\ \text{snRNA}})_{\text{control}}$. The primers used for stem-loop reverse transcriptase-polymerase chain reaction (RT-PCR) for miR-126 are listed in **Table 2**.

Table 1 The sequence of the miR-126 mimic, inhibitor, and their negative control

miRNAs	Sequence (5′ →3′)
miR-126 mimic-NC	UUCUCCGAACGUGUCACGUTT
	ACGUGACACGUUCGGAGAATT
miR-126 mimic	UCGUACCGUGAGUAAUAAUGCG
	CAUUAUUACUCACGGUACGAUU
miR-126 INH-NC	CAGUACUUUUGUGUAGUACAA
miR-126 INH	CGCAUUAUUACUCACGGUACGA

In vitro drug sensitivity assay

A549 cells were reverse transfected as described and seeded into 96-well plates (5×10^3 cells/well) after 24 h of transfection. Then cells were treated with serial dilutions of ADM and vincristine (VCR). Following 48 h of treatment, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml; Sigma, St Louis, USA) was added into each well. After an additional 4 h at 37°C, the culture medium was removed and 200 μl of dimethyl sulfoxide was added into each well. The absorbance in each well was read at 490 nm by an automated microplate reader (Bio-Rad, Hercules, USA).

Intracellular ADM concentration analysis

A549 cells were reverse transfected as described earlier. The fluorescence intensity of intracellular ADM was determined by flow cytometry (FCM). Briefly, cells were seeded into six-well plates (1×10^6 cells/well) and cultured overnight at 37°C. After adding ADM to a final concentration of 3 μg/ml, cells were cultured for an additional 1 h before harvesting for detection of ADM accumulation. The cells were washed with phosphate-buffered saline and the mean fluorescence intensity (MFI) of the intracellular ADM was detected by FCM.

Dual-luciferase activity assay

The putative target sites of the human *VEGFA* 3′-untranslated region (UTR) segments for miR-126 were amplified using Pyrobest DNA polymerase (Fermentas) and cloned into the *Xba*I site of pGL3 control (Promega, Madison, USA). The mutated putative miR-126 binding site in the *VEGFA* 3′-UTR was generated using the Quick

Table 2 The primers used for stem-loop RT-PCR for miR-126 and U6

Primer	Sequence (5′ →3′)
U6 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACGATT
U6 forward	CCTGCGCAAGGATGAC
U6 reverse	GTGCAGGGTCCGAGGT
miR-126 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGCATT
miR-126 forward	GGCTCGTACCGTGAGTAAT
miR-126 reverse	GTGCAGGGTCCGAGGT

change site-directed mutagenesis kit (Stratagene, Cedar Creek, USA) according to the manufacturer's protocol. A549 cells were plated into 24-well plates (5×10^4 cells/well). One day later, cells were co-transfected with 800 ng luciferase vector, including the 3′-UTR of *VEGFA*, and miR-126 mimic or mimic control at a final concentration of 50 nM with Lipofectamine 2000. Luciferase assays and activity were performed by using the dual-luciferase reporter assay system (Promega) 48 h after transfection.

Western blot analysis

Forty-eight hours after transfection, recombinant human VEGF (PeproTech, Rocky Hill, USA) was added at a concentration of 32 ng/ml [28] either alone or in combination with LY294002 (50 mM; Cell Signaling, Beverly, USA) to miR-126 mimic-transfected cells. After incubation for 2 h, proteins from each group were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked and probed with antibodies against VEGFA, MRP1, P-gp, Akt, phospho-Akt, ERK, phospho-ERK (Santa Cruz Biotechnology, Santa Cruz, USA), and β-actin (Sigma). After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by super-enhanced chemiluminiscence detection reagent (Applygen, Beijing, China).

Lung cancer xenograft

According to the previous method [7,29], A549 cells (5×10^7) were transfected with 200 nM miR-126 mimic or mimic control for 24 h before respective subcutaneous injection into the left and right flanks of 10 nude BALB/c mice (SLAC Laboratory, Shanghai, China). Tumor volumes were calculated using the formula: Tumor volume = length × width^2/2. Tumor growth was monitored weekly and the tumors were harvested 6 weeks after the cells were injected.

Statistical analysis

Data were presented as the mean ± SD of three independent experiments. Statistical analyses were performed using SPSS version 10.1 (Statistical Package for Social Sciences, Chicago, USA). Statistical significance was determined by Student's *t*-test with $P < 0.05$ considered statistically significant.

Results

miR-126 enhances the cytotoxic effect of ADM and VCR on A549 cells

To study the potential role of miR-126 in the cytotoxic effect of ADM and VCR on A549 cells, we transfected A549 cells with miR-126 mimic or inhibitor. After transfection, miR-126 expression was detected by qRT-PCR. As shown in **Fig. 1(A)**, miR-126 expression was significantly increased in the miR-126 mimic-transfected cells (A549 + mimic) compared with the control group (A549 + mimic-NC) ($P < 0.05$). To investigate the significance of miR-126 expression in NSCLC cells, we performed an *in vitro* drug sensitivity assay. Enhanced miR-126 expression led to a significant decrease in the inhibitory concentration (IC50) of ADM and VCR in A549 cells (**Table 3**). To confirm these findings, we measured the intracellular levels of ADM, which is fluorescent and thus easily monitored by FCM. An increased accumulation of ADM was observed in mimic-transfected cells compared with the control cells ($P < 0.05$) [**Fig. 1(B–D)**], indicating that miR-126 expression might contribute to the drug-resistant phenotype of NSCLC cells.

miR-126 suppresses the expression of VEGFA and MRP1

The enhanced drug sensitivity observed in miR-126 mimic-transfected cells may depend on the down-regulation of MDR molecules. Therefore, after transfection of A549 cells with the miR-126 mimic, the expression of two well-characterized drug transporters, P-gp and MRP1, was examined. We also detected the expression of VEGF, which has been reported not only to be a target of

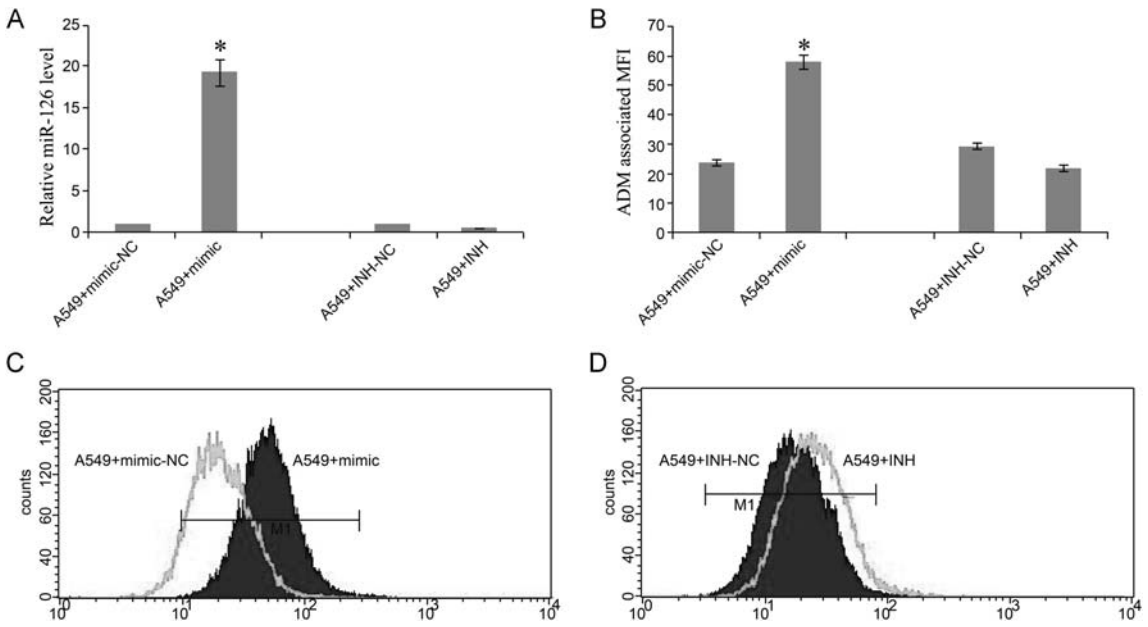

Figure 1 Effects of miR-126 transfection on A549 cells The expression of miR-126 in A549 cells was measured by qRT-PCR. (A) After transfection, the relative amount of miR-126 was normalized to U6 snRNA. (B) The mean fluorescence intensity (MFI) of ADM in A549 cells was determined by FCM. (C, D) We detected an increased accumulation of ADM in mimic-transfected cells compared with the control cells. The inhibitor-transfected cells were not significantly affected. *$P < 0.05$.

Table 3 IC50 values of ADM and VCR for A549 cells after transfection

A549	IC50 (μg/ml)	
	ADM	VCR
A549 + mimic-NC	4.380 ± 0.112	1.209 ± 0.021
A549 + mimic	1.217 ± 0.104*	0.607 ± 0.005*
A549 + INH-NC	4.493 ± 0.190	1.196 ± 0.027
A549 + INH	4.787 ± 0.201	1.208 ± 0.012

*$P < 0.05$ vs. control.

miR-126 but also to affect the drug sensitivity of cancer cells [13,20,22]. As shown in **Fig. 2(A)**, the relative expression of VEGFA and MRP1 was markedly decreased in mimic-transfected cells compared with control cells. However, no obvious difference in P-gp expression was observed, suggesting that the classical MDR molecule MRP1 is specifically involved in miR-126-related MDR.

VEGFA is a direct target of miR-126

Freely accessible algorithms including PicTar, miRBase, miRanda, Bibiserv, and Targetscan were used to identify and analyze potential targets of miR-126. We found that *VEGFA*, but not *MRP1*, was a potential target of miR-126. VEGFA was clearly down-regulated by enhanced miR-126 expression, consistent with previous reports [13,20]. We then performed luciferase reporter assays to explore whether *VEGFA* was a direct target gene of miR-126. The sequence alignment between miR-126 and the targeted *VEGFA* 3′-UTR is shown in **Fig. 2(B)**. In A549 cells, the relative luciferase activity was decreased when the *VEGFA* 3′-UTR was co-transfected with the miR-126 mimic compared with the mimic control group. However, no decrease was observed in A549 cells transfected with a mutant *VEGF* 3′-UTR [**Fig. 2(B)**], suggesting that *VEGFA* is a direct target gene of miR-126.

Regulation of MRP1 by miR-126 requires VEGFA

Based on our previous studies [28], we suspected that the miR-126-dependent suppression of MRP1 in A549 cells was due to VEGFA down-regulation. To confirm this hypothesis, we evaluated MRP1 levels after the addition of recombinant human VEGFA to mimic-transfected A549 cells. Down-regulation of MRP1 by miR-126 is impaired by the VEGFA restoration, indicating that VEGFA plays a role in the regulation of MRP1 by miR-126 [**Fig. 2(C)**].

The PI3K/Akt signaling pathway is involved in the regulation of MRP1 by VEGFA

In addition to promoting cellular survival, the PI3K/Akt signaling pathway has been implicated in the MDR of cancer cells. Previous studies have suggested that inhibitors of the PI3K/Akt signaling pathway can significantly increase the susceptibility of cancer cells to chemotherapeutic drugs [27]. As shown in **Fig. 2(D,E)**, miR-126 reduced the activation of Akt, but no effect was observed on the

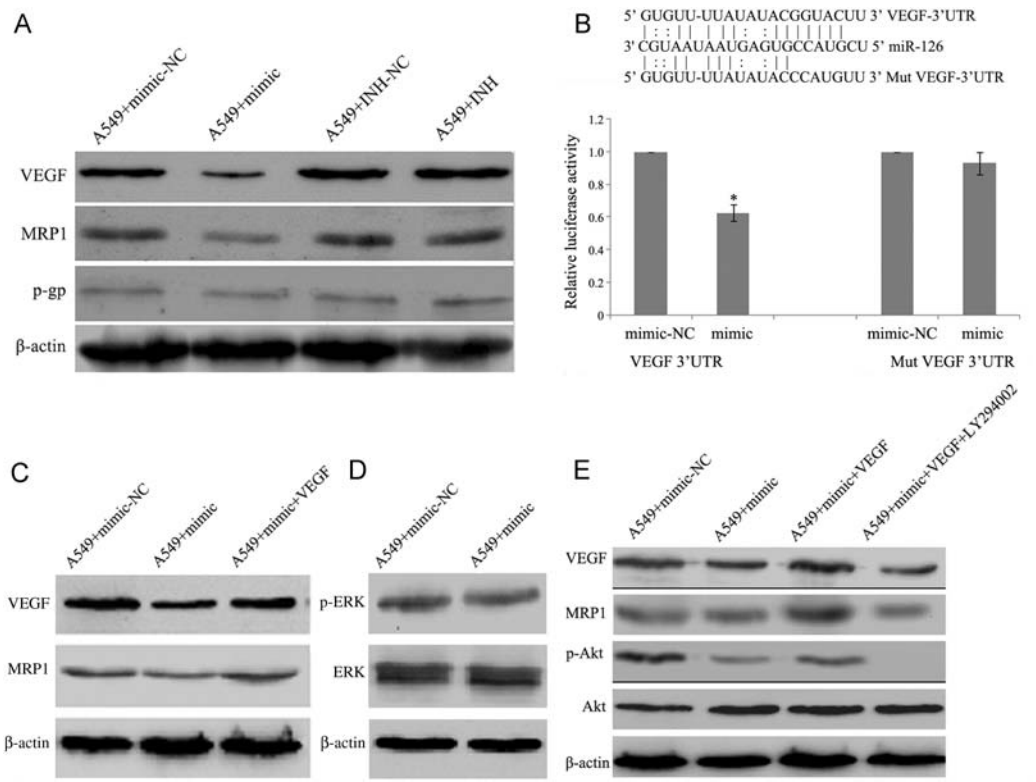

Figure 2 Regulation of P-gp, MRP1, VEGFA, and PI3K/Akt expression by miR-126 in A549 cells (A) Western blot analysis of P-gp, MRP1, and VEGFA in A549 cells. Decreased expression of VEGF and MRP1 was detected in the mimic-transfected cells compared with the control group. However, P-gp did not show any significant change. (B) Sequence alignment between miR-126 and the VEGFA 3′-UTR. The luciferase reporter assay showed a significant decrease in VEGFA 3′-UTR relative luciferase activity in mimic-transfected A549 cells. (C) The restoration of VEGFA can rescue MRP1 expression that is down-regulated by miR-126 transfection. (D) Phosphorylated ERK was analyzed, but it showed no obvious difference after miR-126 transfection. (E) LY294002, a specific inhibitor of the PI3K/Akt signaling pathway, could diminish this effect of VEGFA on MRP1. β-Actin was detected as the loading control.

activation of ERK. LY294002, an inhibitor of the PI3K/Akt signaling pathway, was used to explore the effects of this pathway on the regulation of MRP1 by VEGFA. In the presence of LY294002, up-regulation of MRP1 by VEGFA was eliminated [**Fig. 2(E)**], implicating the PI3K/Akt signaling pathway in the regulation of MRP1 by VEGFA, consistent with previous reports [25,28].

miR-126 inhibits tumorigenicity in nude mice

We then investigated whether miR-126 could inhibit NSCLC tumor formation *in vivo*. As shown in **Fig. 3(A,B)**, the tumors generated from miR-126 mimic-transfected cells were much smaller than those produced by mimic control-transfected cells, demonstrating that miR-126 inhibits tumor growth *in vivo*. After harvesting the tumors, VEGFA and MRP1 expression levels were checked. We found that VEGFA and MRP1 expression levels were reduced compared with the control group [**Fig. 3(C)**], suggesting that miR-126 regulates VEGFA and MRP1 *in vivo*.

Discussion

Despite growing evidence indicating that miRNAs are novel classes of cancer-relevant molecules that regulate gene expression at the post-transcriptional level, only a few publications have reported on the involvement of miRNAs in the development of MDR in cancers. miR-451 and miR-27 have been shown to regulate expression of P-gp and promote the resistance of tumor cells to cytotoxic drugs [30]. miR-328 is down-regulated in an MDR breast cancer cell line, MCF-7/MX100, and modulated expression of breast cancer resistance protein [31], and that elevated levels of miR-326 can sensitize MCF-7/VP MDR cells to cytotoxic drugs by down-regulating expression of MRP1 [32].

miR-126, a widely studied human miRNA, has been identified as a suppressor of tumor formation in various types of cancer by targeting well-known oncogenes such as VEGFA, PI3K regulatory subunit beta (p85β), HOX9A, CRK, IRS-1, and VCAM1 [14–22]. Down-regulation of miR-126 in cancerous vs. non-cancerous tissues has been

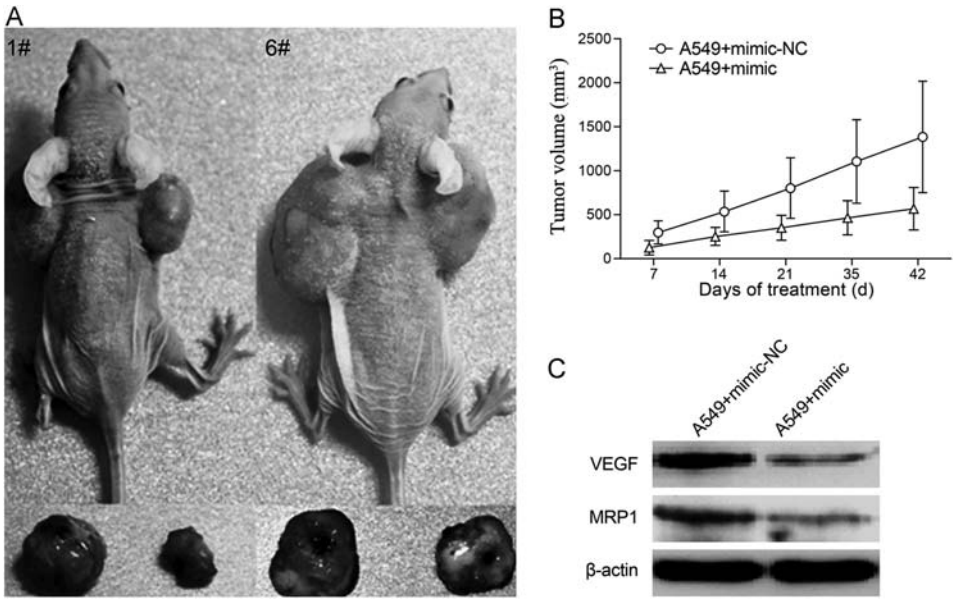

Figure 3 Restoration of miR-126 suppresses tumor growth in nude mice A549 cells transfected with the miR-126 mimic or the mimic-NC were subcutaneously injected into nude mice and tumor growth was monitored for 6 weeks. (A) Typical mice and tumor sizes. (B) Enhanced expression of miR-126 decreased tumor growth compared with the negative control. $n = 10$. (C) Proteins were extracted from tumors and subjected to western blot analysis to determine VEGF and MRP1 expression levels.

reported in cancers via largely unknown mechanisms. Li *et al*. [33] demonstrated that v-Src-induced oncogenic transformation caused a decrease in miR-126 and the treatment of breast cancer cell lines with the Src kinase inhibitor PP2 resulted in an increase of miR-126 expression, leading to decreased tumorigenicity. It has been shown that the A24G single nucleotide polymorphism in the pri-miR-126 gene results in dramatically reduced mature miR-126 expression [34]. Conversely, emerging evidence suggests that miR-126 may also play a supportive role in the progression of cancer. It has been reported that miR-126 inhibits SOX2 expression and contributes to gastric carcinogenesis [35] and that enhanced expression of miR-126 inhibits apoptosis and increases the viability of acute myeloid leukemia cells [36]. Although the role of miR-126 in cancer remains controversial so far, we were intrigued by the findings of Wang *et al*. [37], who found that miR-126 promoted irradiation-induced apoptosis of NSCLC cells through the PI3K/Akt pathway, thus sensitizing NSCLC cells to radiotherapy. However, the role of miR-126 in MDR has not yet been reported. Here, we address for the first time the relationship between miR-126 expression and the response to cancer chemotherapy.

To understand the role of miR-126 in chemosensitivity, we used the intrinsically drug-resistant lung adenocarcinoma cell line A549, which displayed the highest levels of MRP1 expression according to the results of Giaccone *et al*. [5]. We observed that exogenous miR-126 increased the sensitivity of A549 cells to VCR and ADM, two common substrates for the MDR molecules MRP1 and P-gp/MDR1. To characterize the association of MRP1 and P-gp/MDR1 with miR-126, we transfected A549 cells with miR-126 mimic and observed that miR-126 specifically regulated the expression of MRP1 but not P-gp. A further analysis of the regulatory mechanisms revealed that miR-126 interacted with the *VEGFA* 3′-UTR to block the activation of the PI3K/Akt signaling pathway, which subsequently resulted in the down-regulation of MRP1. These observations are an extension of previous reports that VEGF activates the PI3K/Akt signaling pathway [38], the PI3K/Akt signaling pathway up-regulates MRP1, and LY294002 treatment antagonizes MRP1 [39]. This study is the first to demonstrate that miR-126 orchestrates a novel and efficient chemosensitivity regulatory mechanism. The restoration of tumor suppressive miR-126 can overcome the cellular resistance to chemotherapeutics in A549 cells partially by eliciting a VEGFA and MRP1 inhibitory response involving the PI3K/Akt signaling pathway. Further analyses of the biological actions of miR-126 in MDR in NSCLC as well as in other cancers are warranted.

Due to the development of various methods of miRNA delivery [40], miRNAs are being developed for new therapeutics. VEGF is expressed in ~50%–95% of NSCLC patients and is related to poor prognosis and metastatic potential. Bevacizumab, a monoclonal antibody against VEGF, showed a significant survival benefit in combination with other antitumor drugs in the treatment of patients with NSCLC. An enhanced risk of treatment-related death caused by pulmonary hemorrhage and toxic effects such as febrile neutropenia due in part to the disruption of the

normal vascular integrity has been observed [41], so a new approach to evade adverse effects of anti-VEGF treatments would be welcome. Therefore, in the near future, endogenous miR-126 might be useful compared with monoclonal antibody-based anti-VEGF therapies, for it not only maintains the integrity of the normal vascular system [42,43], but also inhibits tumor cell growth and sensitizes tumor cells to chemotherapeutics.

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