Knockdown of microRNA-126 Suppresses Non-Small-Cell Lung Cancer via Inhibiting PI3K-AKT-mTOR Pathway

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Research

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Abstract

Background

As one of the most common cause of cancer death in the world, lung cancer causes approximate 1.6 million deaths annually. Among, NSCLC accounts approximately 85% of patients in whole lung cancer patients. As one of miRNAs, miR-126 closely involves in pathogenesis of several types of cancers including colorectal, prostate, bladder and gastric cancer, and so on. Thus, the present study aims to investigate effects of miR126 on pathogenesis of NSCLC. In the study, two lung cancer cell lines including A549 and H1650 were used.

Results

It was found that expression of miR126 was decreased in PBMC of NSCLC patients compared to healthy control. Expression of miR126 was decreased in cancer tissue compared to paracancerous tissues in NSCLC patients. MiR-126 KD remarkably increased expression of apoptosis genes including caspase3 and capsae9, and decreased cell viability in lung cancer cells including A549 and H1650 cells. Interesting, *In Silico* analysis indicated that miR-126 could target PI3K signaling pathway, which was confirmed by WB assay. KD of PI3KR2 compromised promotion of miR-126 on cell apoptosis. Similarly, it was found that KD of mTOR compromised promotion of miR-126 on cell apoptosis.

Conclusions

Thus, the present study confirmed that miR-126 plays an important role in apoptosis of lung cells via mTOR signaling pathway. miR-126 might be a potential therapeutic target for developing novel drugs against NSCLC.

Introduction

Lung cancer is the most common cause of cancer death all over the world, which annually causes approximate 1.6 million deaths [1]. There are two distinct categories of lung cancer including non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) according to histological classification [2]. Among, NSCLC accounts approximately 85% of patients in whole lung cancer patients [2]. The pathogenesis of lung cancer is multiple, mainly including tobacco smoking (accounting for more than 80% of cases), environmental pollution, genetics, and so on [3]. Lung cancer is also molecularly heterogeneous disease, which makes it difficult to fully understand its biology [3]. The empirical use of cytotoxic therapy based on a physician's preference is the traditional therapy to treat lung cancer, which has moved to personalized medicine according to the genetic alterations of their tumour and the status of programmed death ligand-1 (PD-L1), which predict for benefit from targeted therapies or immune

checkpoint blockers (ICBs) [4]. Better understanding about the diseases plays an important role in developing effective therapies to treat lung cancer.

In China, lung cancer is one of most severe cancer types causing a large population of deaths. In fact, the peak of morbidity and mortality caused by lung cancer has never fallen. the number of new cases of lung cancer is about 326,600 and the death number due to lung cancer is about 569,400 in a year in China [5]. Similar to other regions in the world, NSCLC accounts for 85% of all lung cancer cases [6].

Micro-RNAs are a great family of small non-condign ribonucleic acid molecules (RNA), among, as an important member of this family, microRNA-126 (miRNA-126, miR-126) is encoded by 7th intron of the EGFL7 gene in human chromosome 9q34.3 and expressed in many humans cells such as cardiomyocytes, endothelial and lung cells [7]. miR-126 is reported to have several important physiological functions, for example, it is found to bind directly the DNA, preventing the transcription, translation and degradation of mRNA [8]. miR-126 plays vital roles in several immune-related diseases [9]. MiR-126 is reported to mediate brain endothelial cell exosome treatment-induced neurorestorative effects after stroke in type 2 diabetes mellitus Mice [8]. MiR-126 can facilitate vascular remodeling and decline fibrosis, thus is considered to be an important factor in the pathogenesis of cardiovascular diseases and cerebral stroke [7]. Importantly, miR-126 closely involves in pathogenesis of cancers. The expression of miR-126 is found to be reduced in colorectal, prostate, bladder and gastric cancer [10]. Similar phenomenon was found in lung cancer cell lines [11]. Thus, miR-126 may highly involve in pathogenesis of lung cancer.

The PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase)-Akt (protein kinase B)-mTOR (mammalian target of rapamycin) signaling pathway is a signal transduction pathway involved in the regulation of multiple cellular functions including cell proliferation, survival, differentiation, adhesion, motility and invasion, which is one of the most frequently dysregulated pathways in human cancers [12]. PI3Ks are a family of related intracellular signal transducer enzymes capable of phosphorylating the 3 position hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns) [13]. As a member of the phosphatidylinositol 3-kinase-related kinase family of protein kinases, mTOR plays an important role in cancer [14]. PI3K/mTOR pathways are important in pathogenesis of NSCLC. In fact, inhibitors of PI3K signaling have been suggested as potential therapeutic agents in NSCLC [15]. Interestingly, it is found that miR-126 can improve viability, colony formation, and migration of keratinocytes HaCaT cells by regulating PI3 K/AKT signaling pathway [16].

In the present study, we investigated the expression of miR-126 in healthy and NSCLC patients, and effects of miR-126 on growth of lung cancer cells. It was also investigate the underling mechanism of effects of miR-126 on lung cancer cells.

Results

The expression of miR-126 was down-regulated in PBMC and lung tissue of NSCLC patients

To investigate whether miR-126 involves in pathogenesis of NSCLC, expression of miR-126 in PBMC of NSCLC patients (n = 81) and healthy control (n=81). It was indicated that the expression of miR-126 was significantly lower in NSCLC compared to health control ($P < 0.001$, Fig. 1A). To further explore whether miR-126 involves in pathogenesis of NSCLC. The expression of miR126 in lung cancer needle biopsies and paracancerous tissues, which indicated that expression of the miR-126 was significantly higher in lung cancer tissues than lung paracancerous tissues ($P < 0.001$, Fig. 1B). Thus, it was confirmed that expression of miR-126 was higher in PBMC of NSCLC patients than health controls, and it was higher in lung cancer tissue than in paracancerous tissues in NSCLC patients.

Knockdown (KD) of miR-126 induced apoptosis of A549 and H1650 cells

To investigate effects of miR-126 on growth of lung cancer cells. Two miR-126 vectors (#1 and #2) and control vector (siRNA-scramble) were transfected in A549 cells, which indicated that both miR-126 vectors significantly increased expression of the caspase3 in A549 cells ($**P < 0.01$, n = 4, Fig. 2A). Moreover, it was found that both miR-126 vectors significantly increased expression of the caspase9 in A549 cells ($**P < 0.01$, n = 4, Fig. 2B). In addition, it was found that two miR-126 vectors significantly decreased growth of A549 cells ($***P < 0.001$, n = 6, Fig. 2C). To further validate effects of miR-126 on growth of lung cancer cells, another NSCLC cell line named H1650 was used. Two miR-126 vectors (#1 and #2) and control vector were transfected in H1650 cells, which indicated that both miR-126 vectors significantly increased expression of the caspase3 in H1650 cells ($**P < 0.01$, $***P < 0.001$, n = 4, Fig. 2D). It was also found that both miR-126 vectors significantly increased expression of the caspase9 in H1650 cells ($***P < 0.001$, n = 4, Fig. 2E). The MTT assay indicated that two miR-126 vectors significantly decreased growth of H1650 cells ($***P < 0.001$, n = 6, Fig. 2F). Collectively, it was confirmed that KD of miR-126 induced apoptosis of A549 and H1650 cells.

miR-126 targets PIK3R2 and PI3K/mTOR signaling in lung cancer cells

The functions of miRNAs are elucidated with analyses of the genes they target. PIK3R2 was identified as a putative target of miR-126 using target prediction programs (Targetscan 7.2, http://www.targetscan.org/vert_72/) (Fig. 3A). To further investigate the interaction of miR-126 and PI3K/mTOR signaling, effects of miR-126 on elements of mTOR signaling were studied using WB. It was found that KD of miR-126 could increase protein level of phosphorylated PI3K and mTOR in both A549 (Fig. 3B) and H1650 cells (Fig. 3C), while KD of miR-126 did not change protein level of total PI3K and mTOR in both A549 (Fig. 3B) and H1650 cells (Fig. 3C). Thus, we confirmed that miR-126 could interact with mTOR signaling in lung cancer cells.

Blocking PI3K compromised increase of miR-126 KD on apoptosis in lung cancer cells

To investigate the mechanism of action (MOA) of effects of miR-126 KD on apoptosis in lung cancer cells, two si-PI3KR2 vectors and si-scramble vectors were transfected in A549 cells, which indicated that two si-PI3KR2 vectors significantly decreased mRNA expression of PI3KR2 in A549 cells ($**P < 0.01$, $***P < 0.001$, n = 4, Fig. 4A). Interestingly, it was found that KD of PI3KR2 compromised increase of miR-126

KD on increase of caspase3 mRNA expression in A549 cells (**P < 0.01, n = 4, Fig. 4B). In parallel, it was found that KD of PI3KR2 compromised increase of miR-126 KD on increase of caspase9 mRNA expression in A549 cells (**P < 0.01, ***P < 0.001, n = 4, Fig. 4C). Moreover, it was found that KD of PI3KR2 compromised increase of miR-126 KD on decrease of cell viability in A549 cells (**P < 0.01, ***P < 0.001, n = 4, Fig. 4D). To further investigate, two si-PI3KR2 vectors and si-scramble vectors were transfected in H1650 cells, which indicated that two si-PI3KR2 vectors significantly decreased mRNA expression of PI3KR2 in H1650 cells (**P < 0.01, ***P < 0.001, n = 4, Fig. 4E). Interestingly, it was found that KD of PI3KR2 compromised increase of miR-126 KD on increase of caspase3 mRNA expression in H1650 cells (**P < 0.01, ***P < 0.001, n = 4, Fig. 4F). In parallel, it was found that KD of PI3KR2 compromised increase of miR-126 KD on increase of caspase9 mRNA expression in H1650 cells (**P < 0.01, n = 4, Fig. 4G). KD of PI3KR2 compromised increase of miR-126 KD on decrease of cell viability in H1650 cells (**P < 0.01, ***P < 0.001, n = 4, Fig. 4H). Collectively, it was demonstrated that blocking PI3K compromised increase of miR-126 KD on apoptosis in lung cancer cells.

Blocking mTOR compromised increase of miR-126 KD on apoptosis in lung cancer cells

To further explore effects of mTOR signaling on increase of miR-126 KD on apoptosis in lung cancer cells, two si-mTOR vectors and si-scramble vectors were transfected in A549 cells, which indicated that two si-mTOR vectors significantly decreased mRNA expression of PI3KR2 in A549 cells (**P < 0.01, ***P < 0.001, n = 4, Fig. 5A). It was found that KD of mTOR compromised increase of miR-126 KD on increase of caspase3 mRNA expression in A549 cells (*P < 0.05, **P < 0.01, n = 4, Fig. 5B). Moreover, it was found that KD of mTOR compromised increase of miR-126 KD on increase of caspase9 mRNA expression in A549 cells (*P < 0.05, **P < 0.01, ***P < 0.001, n = 4, Fig. 5C). It was also found that KD of mTOR compromised increase of miR-126 KD on decrease of cell viability in A549 cells (*P < 0.5, **P < 0.01, n = 4, Fig. 5D). To further investigate, two si-mTOR vectors and si-scramble vectors were transfected in H1650 cells, which indicated that two si-mTOR vectors significantly decreased mRNA expression of mTOR in H1650 cells (**P < 0.01, n = 4, Fig. 5E). Interestingly, it was found that KD of mTOR compromised increase of miR-126 KD on increase of caspase3 mRNA expression in H1650 cells (*P < 0.05, **P < 0.01, n = 4, Fig. 5F). In parallel, it was found that KD of mTOR compromised increase of miR-126 KD on increase of caspase9 mRNA expression in H1650 cells (*P < 0.05, n = 4, Fig. 5G). KD of mTOR compromised increase of miR-126 KD on decrease of cell viability in H1650 cells (**P < 0.01, ***P < 0.001, n = 4, Fig. 5H). Collectively, it was demonstrated that mTOR closely involved in increase of miR-126 KD on apoptosis in lung cancer cells.

Discussion

Increasing evidence indicates that the population of NSCLC patients is increasing in the world. Better understanding regarding NSCLC promotes discovery of effective therapies treating the disease. To investigate role of the miR-126 on pathogenesis of NSCLC. It was first investigate miR-126 expression in NSCLC patients. Two NSCLC cells including A549 and H1650 cell lines were used. It was found that expression of miR126 was decreased in PBMC of NSCLC patients compared to healthy control.

Expression of miR126 was decreased in cancer tissue compared to paracancerous tissues in NSCLC patients. miR-126 KD remarkably increased expression of apoptosis genes including caspase3 and capsae9, and decreased cell viability in lung cancer cells including A549 and H1650 cells. Interesting, *In Silico* analysis indicated that miR-126 could target PI3K signaling pathway, which was confirmed by WB assay. KD of PI3KR2 compromised promotion of miR-126 on cell apoptosis. Similarly, it was found that KD of mTOR compromised promotion of miR-126 on cell apoptosis. Thus, the present study confirmed that miR-126 plays an important role in apoptosis of lung cells via mTOR signaling pathway.

According to the Global Cancer Observatory (GCO), lung cancer is the deadliest form of cancer in the world, which causes a large population deaths [17]. Understanding the pathogenesis of the disease plays an important role in discovery of effective drugs to treat NSCLC. Seeking effective biomarker is important to reduce incidence rate of NSCLC. Several genes including bromodomain PHD finger transcription factor (BPTF) [18], Serum thrombospondin-2 [19], CTAPIII/CXCL7 [20], c-MET [21], and so on. Accumulating evidence indicates that miRNAs can be biomarker of NSCLC. For example, Sui et al found that MiRNA-30 plays an important role in NSCLC and might be a biomarker for NSCLC [22]. Luo et al confirmed that increased plasma miRNA-30a could be a biomarker for NSCCLC [23]. Circulating microRNA-590-5p was found to serve as a liquid biopsy marker in NSCLC [24]. Interestingly, the present study found that expression of miR126 was decreased in PBMC of NSCLC patients compared to healthy control. Expression of miR126 was decreased in cancer tissue compared to paracancerous tissues in NSCLC patients (Fig. 1). Moreover, it was found that miR-126 KD remarkably increased expression of apoptosis genes including caspase3 and capsae9, and decreased cell viability in lung cancer cells including A549 and H1650 cells in our study (Fig. 2). In parallel, Santarelli et al found that exosomal miR-126 was a circulating biomarker and closely involved in regulating cancer progression in NSCLC [25]. Zheng et al found that bone marrow mesenchymal stem cell-derived exosomal microRNA-126-3p inhibited the growth and process of pancreatic cancer by targeting ADAM9 [26]. Zhu et al found that down-regulated serum miR-126 was closely linked to aggressive progression and poor prognosis of gastric cancer [27]. Collectively, miR126 plays an essential role in diagnosis of NSCLC.

miR126 was confirmed to involve in multiple signaling pathways such as angiogenic signaling, AKT/Rac1 signaling pathway, PI3K/AKT signaling pathway, SIRT1/Nrf2 signaling pathway and so on [28]. PI3K/ATK/mTOR pathway was confirmed to involve in pathogenesis of NSCLC [29]. In the present study, the bioinformatics found that PI3KR2 was the target gene of miR126 (Fig. 3) Moreover, it was found that miR126 vectors increase phosphorylation of PI3K and mTOR in A549 and H1650 cells (Fig. 3). Similarly, Cheng et al found that LncRNA-XIST/microRNA-126 could involve in cell proliferation and glucose metabolism via the IRS1/PI3K/Akt pathway in glioma [30]. Lin et al found that miR-126 played important role in pathogenesis of glioma via regulating PTEN/PI3K/Akt and MDM2-p53 pathways [31]. Yang et al found the maturation of miR-126-5p promotes ovarian cancer progression could aid ovarian cancer progression, which was through PTEN-mediated PI3K/Akt/mTOR pathway [32]. Of note, it was found that KD of PI3KR2 compromised promotion effects of miR-126 on apoptosis of A549 and H1650 cells (Fig. 4). Similarly, we also found that KD of mTOR compromised promotion effects of miR-126 KD

on apoptosis of A549 and H1650 cells (Fig. 5). Thus, it is clearly confirmed that miR-126 can regulate pathogenesis of lung cancer cells via controlling PI3K/mTOR signaling pathway.

Conclusion

In conclusion, the present study confirms that expression of the miR126 is reduced in lung cancer patients. KD of miR126 could induce apoptosis of lung cancer cells. Importantly, it is found that miR126 could target PI3KR2, and miR126 could increase protein level of phosphorylated PI3K and mTOR. KD of PI3KR2 and mTOR weakens promotion of miR126 KD on apoptosis of A549 and H1650 cells. In summary, the study confirms that miR126 closely regulates growth of lung cancer cells, which might be developed to a the therapeutic targets against lung cancer.

Materials And Methods

Serum and tissue samples

NSCLC patients (n = 81) and healthy controls (n = 81) were recruited from the Tianjin First Central Hospital. After obtaining written informed consent from these subjects, blood samples were collected to obtain serum samples by centrifugation. The serum samples in cryopreservation tubes were treated with liquid nitrogen and stored at – 80 °C conditions. For lung tissue samples, there 30 paracancerous and cancer samples were collected from NSCLC patients using needle biopsy method. This study design obtained the approval of the Ethics Committee of Tianjin First Central Hospital (TJFCH 200101-TJ).

Cell lines

Human NSCLC cells including A549 and H1650 were purchased from Procell Biotechnology (Wuhan, China). A549 and H1650 were cultured according to the manufacturer's instruction. Briefly, A549 and H1650 cells were cultured in RPMI1640 (Thermo Fisher, Catalog number: 61870036) supplemented with 10% exosomes-free FBS (Thermo Fisher, Catalog number: 10082147) at 37 °C incubators containing 5% CO₂. Cells were sub-cultured when confluence reaches more than 90%.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from A549 and H1650 cells using the Beyozol total RNA isolation reagent (Beyotime, Beijing, China). Briefly, 500 µL of each sample was incubated with 500 µL Beyozol total RNA isolation reagent and incubated for 2 min at room temperature, followed by vigorously mixed. Then, samples were added to RNA isolation column according to the protocol. Finally, RNA was dissolved in 20 µL RNase-free water. The quality of the RNA was determined by NanoDrop 2000 (Thermo Fisher Scientific). Then, cDNA was transcribed from 500 ng of total RNA using BeyoRT™ II cDNA synthesis kit (Beyotime, Catalog number: D7170M, Beijing, China).

For qRT-PCR, it was performed on the Applied Biosystems real-time PCR ecosystem (Thermo Fisher, USA) using BeyoFast™ SYBR Green qPCR Mix (2X) (Beyotime, Catalog number: D7260-1ml, Beijing, China). The assay was carried out in a total volume of 25 µL reaction mixture containing 12.5 µL BeyoFast™ SYBR Green qPCR Mix, 2 µL of cDNA, 1 µL of each primer and 8.5 µL RNase-free water. The optimized cycling conditions were as follow: initial denaturation at 95°C for 10 s, followed by 40 cycles of denaturation at 95°C for 10 s, primer annealing and extension at 56°C for 30 s. Fluorescence signals were recorded at the end of each cycle. A melt curve analysis was measured following amplification to confirm the specificity of the amplified products. Melting curve analysis consisted of 65°C for 5 s, and followed by increase in temperature to 95°C for 5 s with continuous fluorescence reading. RNase-free water, 5 × reaction buffer, dNTP, RNasin Ribonuclease inhibitor, BeyoFast™ SYBR Green qPCR Mix. The relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method with GAPDH as the internal reference. The primers sequences were exhibited in table 1.

Western blot

The protein level of several genes were quantified by western blot. Briefly, total proteins were extracted using RIPA Lysis (Beyotime, Catalog number: P0013C, Beijing, China) according to the manufacturer's protocols. Then, proteins were isolated by 10% SDS-PAGE and electro-transferred on PVDF membranes (Bio-Rad, Hercules, CA, USA). Subsequently, the membranes containing proteins were seriatim incubated with the primary and secondary antibodies for indicated time, including Phospho-PI3K p85/p55 (Tyr458, Tyr199) Monoclonal Antibody (PI3KY458-1A11, Thermo Fisher), PI3K p85 alpha Monoclonal Antibody (A3-D0) (Catalog # MA5-32917, Thermo Fisher), mTOR Antibody (#2972, Cell Signaling Technology), Phospho-mTOR (Ser2448) (D9C2) XP® Rabbit (mAb #5536, Cell Signaling Technology) and anti-GAPDH (ab8245; Abcam). The second antibodies are goat Anti-Mouse/Rabbit (ab205719 and ab205718; Abcam). The protein signals were visualized using the ECL Western Blotting Substrate (Bio-Rad) and quantified using Image J software (NIH, Bethesda, MA, USA).

β

MTT assay

A549 and H1650 cells were plated into a 96-well plate at the density of 5×10^3 cells/well and cultured in an incubator at 37°C supplemented with 5% CO₂. After culturing for 48 h, 10 µL MTT solution (Catalog number: C0009S, Beyotime, Beijing, China) was added into each well, incubating for another 2 h. Then, DMSO was added to remove formazan. The value of optical density (OD) in each well at 570 nm was measured using a microplate reader (Thermo Fisher Scientific; Waltham, MA, USA).

siRNA transfection

24-well plates were used for seeding cells that were subjected to transfection with either siRNA (0.4 µg) vectors including miR-126 mimics, miR-126-siRNA, PIK3R2-siRNA (silencing vector for PIK3R2, p-GPU6-PIK3R2-shRNA), mTOR-siRNA, and siRNA control (scramble). Transfections were performed according to

the instructions for Lipofectamine 2000 (Invitrogen, USA), and 48 hours later, further analysis was performed.

Statistical analysis

The data were analyzed using GraphPad Prism software (v8.0; GraphPad Prism, La Jolla, CA, USA) and shown as mean $\pm$ Standard Error of Mean. Data comparison was conducted by using Student's t test or analyses of variance (ANOVA) with Tukey post hoc test. P value less than 0.05 was regarded as statistically significant.

Abbreviations

NSCLC: non-small cell lung cancer;

SCLC: small cell lung cancer;

PD-L1: programmed death ligand-1;

ICBs: immune checkpoint blockers;

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinas;

Akt: protein kinase B;

mTOR: mammalian target of rapamycin;

KD: Knockdown;

MOA: mechanism of action;

GCO: Global Cancer Observatory;

BPTF: bromodomain PHD finger transcription factor;

qRT-PCR: quantitative real-time polymerase chain reaction;

OD: optical density;

Declarations

Ethical Approval and Consent to participate

This study design obtained the approval of the Ethics Committee of Tianjin First Central Hospital (TJFCH 200101-TJ). All patients involved in this study received a REB-approved Letter of Information and provided a signed Letter of Informed Consent before entering into the study.

Consent for publication

I, the undersigned, give my consent for the publication of identifiable details, which can include photograph(s) and/or videos and/or case history and/or details within the text (“Material and Methods”) to be published in the above Journal and Article.

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Competing interests

All authors have no conflict of interests.

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Authors' contributions

Conceptualization by P.C. and M.Y.; data curation by P.J., X.Y., and M.Y.; formal analysis by P.C. and M.Y.; methodology by P.C. and M.Y.; project administration by P.C.; supervision by P.C.; validation by P.C. and M.Y.; writing—original draft by P.J., X.Y., and M.Y.; and, writing—review & editing by P.C.

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Tables

Table 1. Primers used in the study.

Genes		Sequence	GC%	
PI3KR2	Sense	GAGACCAGTACCTCGTGTGG	119	60
	Anti-sense	ATCGTCCTCGTCCTCCATGA		55
mTOR	Sense	CTTAGAGGACAGCGGGGAAG	111	60
	Anti-sense	TCCTTTAATATTGCGCGGCGC		50
miR-126	Sense	CGCTGGTGATGGGACATTATT	84	48
	Anti-sense	GCTGTGGACAGCGCATTATT		50

Figures

Figure 1

The expression of miR-126 was down-regulated in PBMC and lung tissue of NSCLC patients. (A) the expression of miR-126 was significantly lower in NSCLC compared to health control ($P < 0.001$, $n = 81$); (B) expression of the miR-126 was significantly higher in lung cancer tissues than lung paracancerous tissues ($P < 0.001$, $n = 30$).

Figure 2

Knockdown (KD) of miR-126 induced apoptosis of A549 and H1650 cells. (A) Two miR-126 vectors (#1 and #2) significantly increased expression of the caspase3 in A549 cells (** $P < 0.01$, $n = 4$); (B) both miR-126 vectors significantly increased expression of the caspase9 in A549 cells (** $P < 0.01$, $n = 4$); (C) two miR-126 vectors significantly decreased growth of A549 cells (*** $P < 0.001$); (D) both miR-126 vectors significantly increased expression of the caspase3 in H1650 cells (** $P < 0.01$, *** $P < 0.001$, $n = 4$); (E) both

miR-126 vectors significantly increased expression of the caspase9 in H1650 cells (**P<0.001, n=4); (F) two miR-126 vectors significantly decreased growth of H1650 cells (**P<0.001, n=6).

Figure 3

miR-126 targets PIK3R2 and PI3K/mTOR signaling in lung cancer cells. (A) The functions of miRNAs are elucidated with analyses of the genes they target. PIK3R2 was identified as a putative target of miR-126; (B) KD of miR-126 could increase protein level of phosphorylated PI3K and mTOR, but not protein level of total PI3K and mTOR in A549 cells; (C) KD of miR-126 could increase protein level of phosphorylated PI3K and mTOR, but not protein level of total PI3K and mTOR in H1650 cells.

Figure 4

Blocking PI3K compromised increase of miR-126 KD on apoptosis in lung cancer cells. (A) two si-PI3KR2 vectors significantly decreased mRNA expression of PI3KR2 in A549 cells (** $P < 0.01$, *** $P < 0.001$, $n = 4$); (B) KD of PI3KR2 compromised increase of miR-126 KD on increase of caspase3 mRNA expression in A549 cells (** $P < 0.01$, $n = 4$); (C) KD of PI3KR2 compromised increase of miR-126 KD on increase of caspase9 mRNA expression in A549 cells (** $P < 0.01$, *** $P < 0.001$, $n = 4$); (D) KD of PI3KR2 compromised increase of

miR-126 KD on decrease of cell viability in A549 cells (**P<0.01, ***P<0.001, n=4); (E) two si-PI3KR2 vectors and si-scramble vectors were transfected in H1650 cells, which indicated that two si-PI3KR2 vectors significantly decreased mRNA expression of PI3KR2 in H1650 cells (**P<0.01, ***P<0.001, n=4); (F) KD of PI3KR2 compromised increase of miR-126 KD on increase of caspase3 mRNA expression in H1650 cells (**P<0.01, ***P<0.001, n=4); (G) KD of PI3KR2 compromised increase of miR-126 KD on increase of caspase9 mRNA expression in H1650 cells (**P<0.01, n=4); (H) KD of PI3KR2 compromised increase of miR-126 KD on decrease of cell viability in H1650 cells (**P<0.01, ***P<0.001, n=4).

Figure 5

Blocking mTOR compromised increase of miR-126 KD on apoptosis in lung cancer cells. (A) two si-mTOR vectors significantly decreased mRNA expression of PI3KR2 in A549 cells (**P<0.01, ***P<0.001, n=4); (B) KD of mTOR compromised increase of miR-126 KD on increase of caspase3 mRNA expression in A549 cells (*P<0.05, **P<0.01, n=4); (C) KD of mTOR compromised increase of miR-126 KD on increase of caspase9 mRNA expression in A549 cells (*P<0.05, **P<0.01, ***P<0.001, n=4); (D) KD of mTOR compromised increase of miR-126 KD on decrease of cell viability in A549 cells (*P<0.5, **P<0.01, n=4); (E) two si-mTOR vectors significantly decreased mRNA expression of mTOR in H1650 cells (**P<0.01, n=4); (F) KD of mTOR compromised increase of miR-126 KD on increase of caspase3 mRNA expression in H1650 cells (*P<0.05, **P<0.01, n=4); (G) KD of mTOR compromised increase of miR-126 KD on increase of caspase9 mRNA expression in H1650 cells (*P<0.05, n=4); (H) KD of mTOR compromised increase of miR-126 KD on decrease of cell viability in H1650 cells (**P<0.01, ***P<0.001, n=4).