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Original Article

MicroRNA-126 is down-regulated in human esophageal squamous cell carcinoma and inhibits the proliferation and migration in EC109 cell via PI3K/AKT signaling pathway

Zheng-Chao Nie[1*], Wen-Hao Weng[1*], Yu-Shan Shang[2], Yin Long[3], Jing Li[1], Yan-Ting Xu[1], Zhi Li[3]

[1]Department of Clinical Laboratory, Shanghai Tenth People's Hospital, Tongji University, Shanghai 200072, China; [2]Department of Clinical Laboratory, Chuzhou Second People's Hospital, Chuzhou, China; [3]Department of Clinical Laboratory, Yangpu Hospital, Tongji University, Shanghai 200090, China. [*]Equal contributors.

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Abstract: MicroRNA-126 (miR-126) was found down-regulated in different types of cancer including esophageal squamous cell carcinoma (ESCC). However, the onco-genetic role of miR-126 in ESCC still remains unknown. In the present study, we found the relative expression of miR-126 in ESCC was significant decreased in ESCC tissues compared to adjacent normal tissues. Overexpression of miR-126 in EC109 cells resulted in significant decrease in cell proliferation, colon formation and migration. PI3K regulatory subunit p85 beta (PIK3R2), a member of PI3K/AKT signaling pathway was found upregulated in ESCC tissues and there is a negative relation between expression of PIK3R2 and miR-126. Restoration of miR-126 in EC109 cells induced a reduction in PIK3R2 protein levels, accompanied with a substantial reduction in phosphorylated AKT levels in EC109 cells, suggesting impairment in PI3K/AKT signaling pathway. The luciferase reporter assay confirmed that PIK3R2 was a direct target of miR-126. Furthermore, we also indicated overexpression of miR-126 suppresses G_2/M transition in EC109 cells. Taken together, our study suggests that miR-126 functions as a potential tumor suppressor in ESCC progression via regulating PI3K/AKT signaling pathway partly by targeting PIK3R2, and targeting of miR-126 may provide a novel strategy for the diagnosis and treatment of ESCC.

Keywords: miR-126, ESCC, PIK3R2, AKT, cell cycle arrest

Introduction

Esophageal cancer is the eighth most common malignant cancer and the sixth most common cause of cancer death worldwide [1]. There are two main types of esophageal cancer: esophageal squamous cell carcinoma (ESCC) and adenocarcinoma [2]. Adenocarcinoma is more common in western countries while ESCC is predominant in East Asia [3, 4]. Although surgical resection can apparently improve the prognosis of patients with ESCC, but the recurrence rate is high, five-year survival rate of patients after surgery is still low [5]. Therefore, it is needed to look for sensitive molecules as therapeutic targets for ESCC.

MicroRNAs (miRNAs) are a class of small non-coding RNAs with 18-25 nucleotides that negatively regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of their target gene mRNAs [6]. miRNAs play a vital role in almost all physiology and pathophysiology process including cell differentiation, proliferation, apoptosis [7] and cell cycle distribution [8]. Many miRNAs are found to be aberrantly expression in various cancers, suggesting that miRNAs may function as a tumor suppressor genes or oncogenes [9]. miR-126 (also called miR-126-3p) is encoded by intron 7 of the epidermal growth factor-like domain 7 (EGFL7) gene which resides on human chromosome 9 [10]. Expression analyses revealed that there is a high level of miR-126 in highly vascularized tissues such as heart, liver, lung or human umbilical vein endothelial cells, playing a crucial role in angiogenesis and maintaining vascular integrity

[11]. Recently, the role of miR-126 in tumor progression has been widely studied. It was commonly decreased in multiple cancer and shows tumor suppressive properties. For example, low miR-126 expression was observed in gastric carcinoma and the expression of miR-126 was correlated with the survival rate of the patients with gastric carcinoma [12]. Expression of miR-126 was lost in the majority of primary breast tumors from patients who relapse, and the loss of expression of miR-126 was associated with poor distal metastasis-free survival [13]. Li N et al. found miR-126 expression was down-regulated in colon cancer cell lines with highly invasive ability and miR-126 functions as a tumor suppressor by inhibiting RhoA/ROCK signaling pathway in colon cancer [14]. However, the functions of miR-126 in ESCC and relative mechanisms are poorly understood.

In the present study, we found that the expression of miR-126 in ESCC tissues was obvious lower than that of adjacent normal tissues. Further, cell functional studies showed that miR-126 functions as a tumor suppressor by regulating the PI3K/AKT signaling pathway in EC109 cells. Finally, we validated that overexpression of miR-126 can induce cell cycle G_2/M phase arrest in EC109 cells.

Materials and methods

Tissue specimens, cell lines and cell culture

30 pairs ESCC tissues and matched adjacent normal tissues were obtained from surgical specimens from Anyang Tumor Hospital (Anyang, Henan, China). All samples were snap frozen in liquid nitrogen. None of the patients received radiotherapy or chemotherapy before surgery. All samples used in this study were approved by Ethics Committee of Anyang Tumor Hospital. The Human ESCC cell line EC-109 and Human embryonic kidney cells HEK293T were purchased from the Chinese Science Institute (Shanghai, China). EC-109 cells were maintained in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA). HEK293T were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA), both mediums were supplemented with 10% fetal bovine serum (FBS; Gibco, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were incubated at 37°C with 5% CO_2.

RNA extraction and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the tissues or cultured cells using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. To quantitate the miR-126 expression, small nuclear U6 was used as internal control. The First-Strand cDNA was synthesized with M-MLV Reverse Transcriptase (Premega, Madison, WI, USA). Using stem-loop method designed miR-126 and U6 reverse transcription primers: miR-126: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCGCATTAT-3'; U6: 5'-AAAATATGGAACGCTTCACGAATTTG-3'. Then qPCR was performed using the KAPA SYBR FAST qPCR kit (KAPA, USA) on an ABI 7900 Fast Real-Time PCR system (Applied Biosystems, CA, USA). The primers for qPCR were used as follows: 5'-ACACTCCAGCTGGGTCGTACCGTGAGTAAT-3' (forward) and 5'- TGGTGTCGTGGAGTCG -3' (reverse) for miR-126; 5'-CTCGCTTCGGCAGCACA-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse) for U6.

For analysis of mRNA expression, the expression of GAPDH was used as internal control and Oligo (dT) was used as the primer for reverse transcription. Then qPCR performed with the primers: 5'-GCACCACGAGGAACGCACTT-3' (forward) and 5'-CGTCCACTACCACGGAGCAG-3' (reverse) for PIK3R2; 5'-TGGGTGTGAACCATGAGAAGT-3' (forward) and 5'-TGAGTCCTTCCACGATACCAA-3' (reverse) for GAPDH. All samples were normalized to GAPDH as internal controls, and the relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments repeated three times.

Construction of miR-126 expression Lentiviral and transfection

The following amplified primer pair was designed based on the cDNA sequence of the Homo sapiens miR-126 precursor, included restriction enzyme cut sites and protect bases: 5'-CGGGTACCGGTCATATCAGCCAAGAAGGCAG-3' (AgeI) and 5'-CAGGCTAGGGGCTATGCCGCCTAAGTAC-3' (NheI). Non-specific sequence as control: 5'-CGGGTACCGGTAGCGTTCACTCCCAACCTG-3' (AgeI) and 5'-CAGGCTAGCAGGTTGGGAGTGAACGCT-3' (NheI). They were then cloned into GV310 constructs to generate lentiviral vector of human miR-126 mimics oligonucleotides and non-specific oligonucleotides. The

recombinant plasmid was confirmed by DNA sequencing. The lentiviral expression vectors were then co-transfected with packaging plasmid into HEK293T cells. After 48 h, lentiviral particles of hsa-miR-126 mimics (lv-miR-126) and non-specific miRNA (lv-NC) were harvested and filtered through a 0.45 μm filter. For transfection, EC109 cells were plated at 40-50% confluency the day before transfection. The lv-miR-126 and lv-NC were added to the cells with 10 μg/mL polybrene.

Cell proliferation [3-(4.5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)] assay

EC109 cells transfection with lv-miR-126 and lv-NC were planted in 96-well plates (BD Biosciences, USA) with 2000 cells per well. We then assessed cell proliferation at 24 h, 48 h, 72 h, 96 h and 120 h respectively, and then 20 μl MTT was added to each well. After incubated at 37°C for 4 h, the supernatant was removed and 150 μl DMSO (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. After 10 min, the absorbance at 490 nm of each well was measured by microplate reader (BioTek, USA). The experiment was performed in triplicate.

Cell colony formation assay

500 cells were added to each well of a 12-well culture plate and incubated at 37°C for 7-10 days. After washing twice with PBS, the cells were fixed with 70% ethanol for 15min and stained with 0.5% crystal violet. The number of colonies was calculated with Image J software. The experiment was performed in triplicate.

Transwell migration assay

The migration ability of resulted EC109 cells after lentiviral transfection was examined in chambers (Corning, USA) with 8 μm pore filter inserts in 24-well plates. 5×10^5 cells suspended in 200 μl serum-free RPMI1640 were added to the upper chamber, and RPMI1640 containing 10% FBS were added to the lower chamber as a chemoattractant. Plates were incubated at 37°C with 5% CO_2. After 20 h, the non-filtered cells were gently removed with a cotton swab. The cells migrated through the membranes and located on the lower side of the chamber were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, washed for three times with running water, air-dried and photographed. Membrane-binding crystal violet was dissolved with 300 μl 33% glacial acetic acid, and then absorbance at 573 nm was measured by microplate reader.

Dual-luciferase reporter assay

The 3'-UTR sequence of PIK3R2 or a mutation in the 3'-UTR of PIK3R2 gene with miR-126 target sites was inserted into the XhoI and NotI of psi-CHECK-2 report plasmid (Promega, Madison, WI, USA). HEK293T cells were seeded in 24-well plates for 12 h before transfection. Cells were transfected with the PIK3R2 3'-UTR wild type (WT) plasmid or the PIK3R2 3'-UTR mutant (MUT) plasmid in the presence of either lv-miR-126 or lv-miR-NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, cells were washed twice with PBS and lysed with passive lysis buffer (PLB) for 15 min. The Firefly luciferase reporter was measured immediately after adding Luciferase Assay Reagent II. Then 100 ul Stop & Glo reagent was added to initiate the Renilla luciferase. Results were normalized to the Renilla luciferase. Luciferase activity = Firefly luciferase/Renilla luciferase. All experiments were performed three times.

Cell cycle analysis

EC109 cells transfected with lv-miR-126 and lv-NC were seeded in 60-mm plates with 2×10^5 cells per plate. After 48 h, cells were harvested, washed with cold PBS for three times and fixed in 70% ethane at 4°C overnight. Then cells treated with 10 μg/ml RNase and stained with 50 μg/ml propidium iodide for 30 min at room temperature in the dark. The cell cycle was then analyzed by a flow cytometer (FACS Canto™ II, BD Biosciences).

Western blot analysis

Cell protein was extracted from transfected EC109 cells using modified RIPA buffer in the presence of proteinase inhibitor cocktail. Protein concentration was determined by the BCA Protein Assay Kit (Beyotime, China) and each sample with 50 μg protein was denatured with 5× sodium dodecyl sulfate (SDS) loading buffer at 95°C for 5 min. The proteins were separated by 10% SDS-PAGE gels and transferred to 0.45 μm nitrocellulose membranes. Membranes were blocked with 5% non-fat milk for 1 h, and incubated overnight at 4°C with the

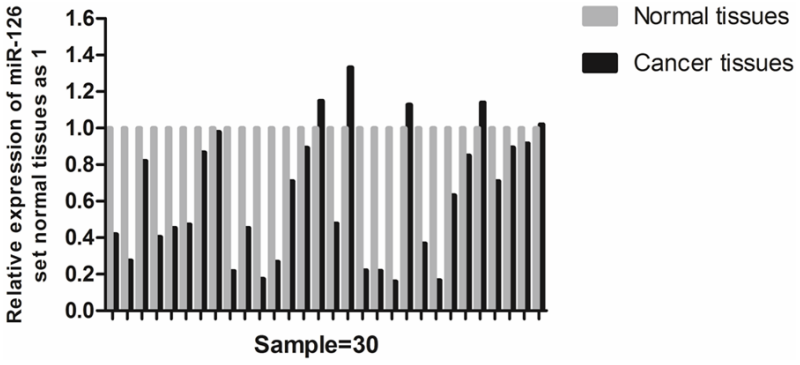

Figure 1. miR-126 was significantly downregulated in the ESCC tissues. The expression of miR-126 was significantly decreased in 30 ESCC tissues compared to paired adjacent normal tissues by qPCR (**P<0.01).

specific primary antibodies at the dilutions specified by the manufacturer. The membranes were then washed and incubated for 1 h with HRP-conjugated secondary antibodies. The bands were detected with Odyssey scanning system (LI-COR, Lincoln, NE, USA). Primary antibodies were as follows: Antibodies against AKT, phosphor-AKT (Ser473), Cdc2, phosphor-Cdc2 (Tyr[15]), p53, Wee1, Cdc25C, Myt1, cyclin B1 were obtained from Cell Signaling Technology. Antibody against PIK3R2 was purchased from R & D. β-actin (Proteintech, USA) was used as an internal control.

Statistical analysis

The SPSS 18.0 software was used for statistical analysis. All data are expressed as the mean ± standard deviation values of at least three independent experiments. Student's *t*-test (two-tailed) and One-way ANOVA were performed to analyze the data. *P* values <0.05 and *P* values <0.01 were considered to be statistically significant.

Results

miR-126 was downregulated in ESCC tissues

We measured the mRNA expression of miR-126 in 30 pairs of ESCC tissues and paired adjacent normal tissues by qPCR. Compared with the adjacent normal tissues, miR-126 was markedly decreased in ESCC tissues (**Figure 1**, **P<0.01).

Overexpression of miR-126 in EC109 cells inhibited cell proliferation, colony formation and migration.

As miR-126 significantly decreases in ESCC tissues, we sought to compensate for its loss through transfection with lv-miR-126 to upregulate miR-126 expression in EC109 cells, lv-NC was used as negative control. The transfection efficiency in EC109 cells was detected by qPCR analysis. The intracellular level of miR-126 was about 73-fold higher in EC109 cells transfected with lv-miR-126 relative to the lv-NC (**Figure 2A**, **P<0.01). Then we detected cell proliferation by MTT assay. We found that overexpression of miR-126 significant decreases cell proliferation of EC109 cells (**Figure 2B**, *P<0.05, **P<0.01). The capacity of colony formation was evaluated on EC109 cells transfected with lv-miR-126. Colony number of lv-miR-126 transfected group (62.33±4.33) was significantly lower than that of lv-NC group (113.7±7.45), indicated that overexpression of miR-126 both the number and the size of the colonies were suppressed (**Figure 2C**, **P< 0.01).

The cell migratory ability of EC109 cells was detected by Transwell migration assay. Absorbance at 573 nm showed that tumor cells migrating out of chamber in lv-miR-126 group (0.321±0.024) were markedly reduced than lv-NC group (0.413±0.013). (**Figure 2D**, **P< 0.01)

PIK3R2 expression was increased in ESCC tissues

We measured the mRNA expression level of PIK3R2 in 30 pairs of ESCC tissues and paired adjacent normal tissues by qPCR. As shown in **Figure 3A**, the expression level of PIK3R2 was significantly upregulated in ESCC tissues compared to the adjacent normal tissues (**P<0.01), and more than half of the ESCC tissues exhibited up-expression of PIK3R2 (**Figure 3B**). Moreover, we found that there is a negative relation between expression of PIK3R2 and miR-126 (r=-0.706, **P<0.01) (**Figure 3C**).

miR-126 repressed PI3K/AKT signaling pathway by targeting PIK3R2 in EC109 cell

miRNAs regulate gene expression by targeting the 3'UTR of relative mRNAs and accelerate mRNA degration or to repress the translation.

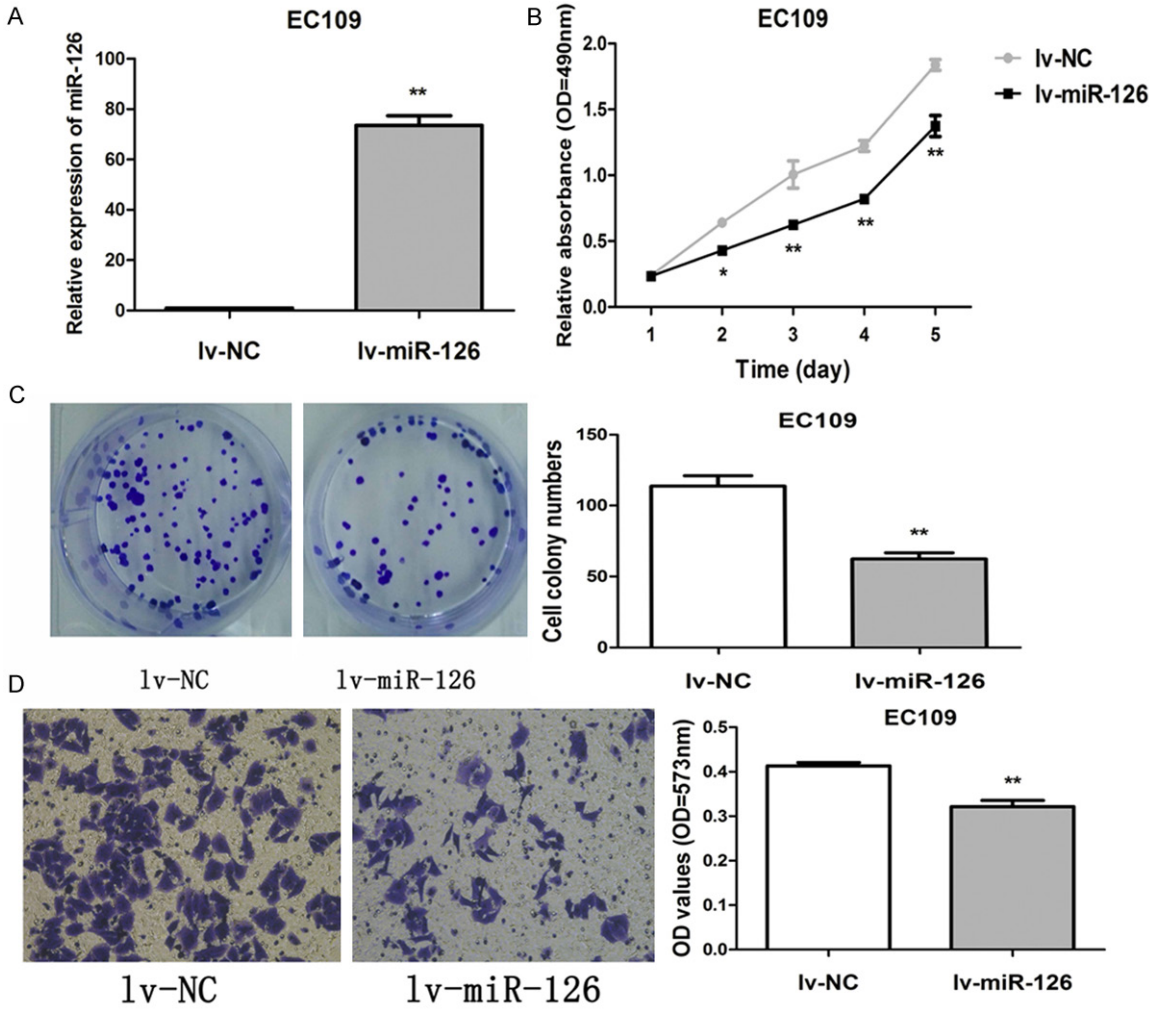

Figure 2. Overexpression of miR-126 inhibited ESCC cell proliferation, colony formation and migration. EC109 cells were transfected with lv-miR-126 and lv-NC. A. The expression of miR-126 in EC109 cells transfected with lv-miR-126 was detected by qPCR. Cells transfected with lv-NC was used as an internal control (**P<0.01). B. Cell growth was measured by MTT assay for 5 days (*P<0.05; **P<0.01). C. Colony formation ability of EC109 cells transfected with the lv-miR-126 and lv-NC (**P<0.01). The right rectangular graph is the quantization of the number of crystal violet stained colonies in the EC109 cells. D. Cell migration ability was measured by Transwell chamber assay. The left representative images of crystal violet stained EC109 migratory cells. Images were captured using an inverted microscope with x100 magnification. The right rectangular graph is the quantification of the migratory cells by solubilization of crystal violet and spectrophotometric reading at OD 573 nm (**P<0.01).

Through bioinformatic analyses using PicTar, miRanda, and MicroCosm, we found that PIK3R2 was a potential target gene of miR-126. The 3'-UTR of PIK3R2 contained a binding site for miR-126 (**Figure 4A**). We then performed a luciferase assay to confirm that miR-126 was directly targeting PIK3R2 in EC109 cells. The WT 3'UTR or the MUT 3'UTR of PIK3R2 gene was amplified and cloned into the reporter. The result showed that compared with lv-NC group, the relative luciferase activity of the reporter that contains WT 3'UTR was significantly decreased when lv-miR-126 was cotransfected. In contrast, the activity of MUT 3'UTR construct group was unaffected by transfection with lv-miR-126 compared with lv-NC group (**Figure 4B**, **P<0.01). The result indicated that miR-126 suppressed the transcription activity of the PIK3R2 by targeting the 3'-UTR of PIK3R2.

We performed qPCR and western blot to further verify whether miR-126 was directly regulating PIK3R2 in EC109 cells. We found that overexpression of miR-126 caused a significant decrease both in mRNA (**Figure 4C**) and protein (**Figure 4D**). Collectively, these findings suggest that miR-126 regulate the expression of PIK3R2

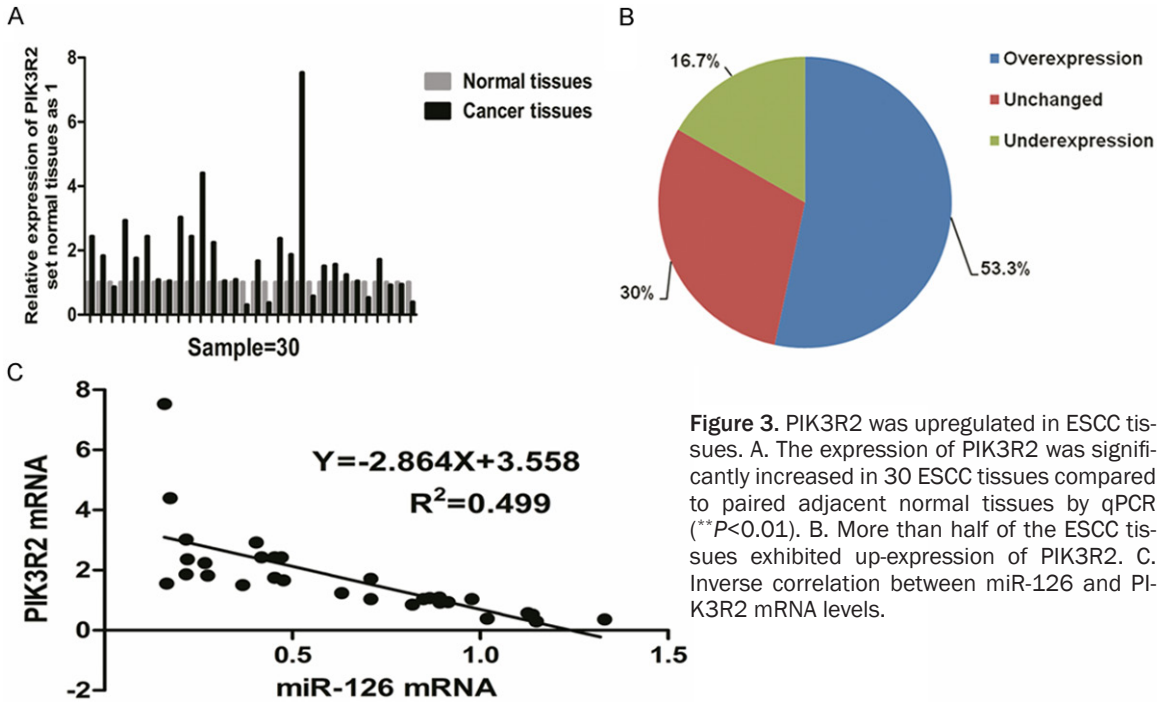

Figure 3. PIK3R2 was upregulated in ESCC tissues. A. The expression of PIK3R2 was significantly increased in 30 ESCC tissues compared to paired adjacent normal tissues by qPCR (**P<0.01). B. More than half of the ESCC tissues exhibited up-expression of PIK3R2. C. Inverse correlation between miR-126 and PIK3R2 mRNA levels.

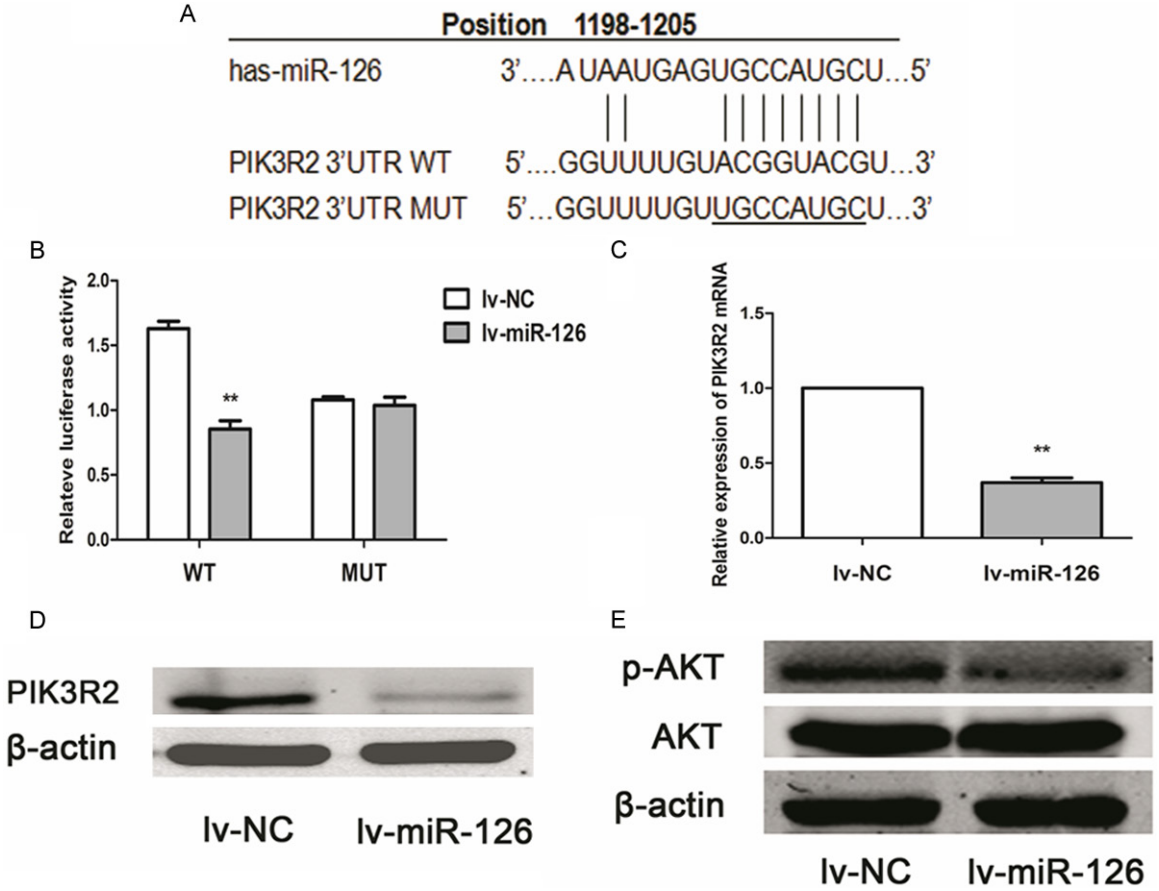

Figure 4. Overexpression of miR-126 repressed PI3K/AKT signaling pathway by targeting PIK3R2. A. The predicted binding site of miR-126 on 3'UTR of wild-type (WT) PIK3R2 was shown. The mutated nucleotides on 3'UTR of PIK3R2 (MUT) are underlined. B. Dual luciferase assay. The WT or MUT 3'UTR of PIK3R2 luciferase reporter plasmids were cotransfected with lv-NC or lv-miR-126 in HEK293T cells. The luciferase activities were normalized to lv-NC

(**P<0.01). C. The expression levels of PIK3R2 in EC109 cells transfected with lv-miR-126 or lv-NC were measured by qPCR and GAPDH was used as an internal control (**P<0.01). D. Western blotting was used to examine the protein expression of PIK3R2 in EC109 cells after transfection with lv-NC or lv-miR-126. β-actin was used as an internal control (**P<0.01). E. The protein expression of p-AKT and AKT was examined by western blotting. β-actin was used as an internal control (**P<0.01).

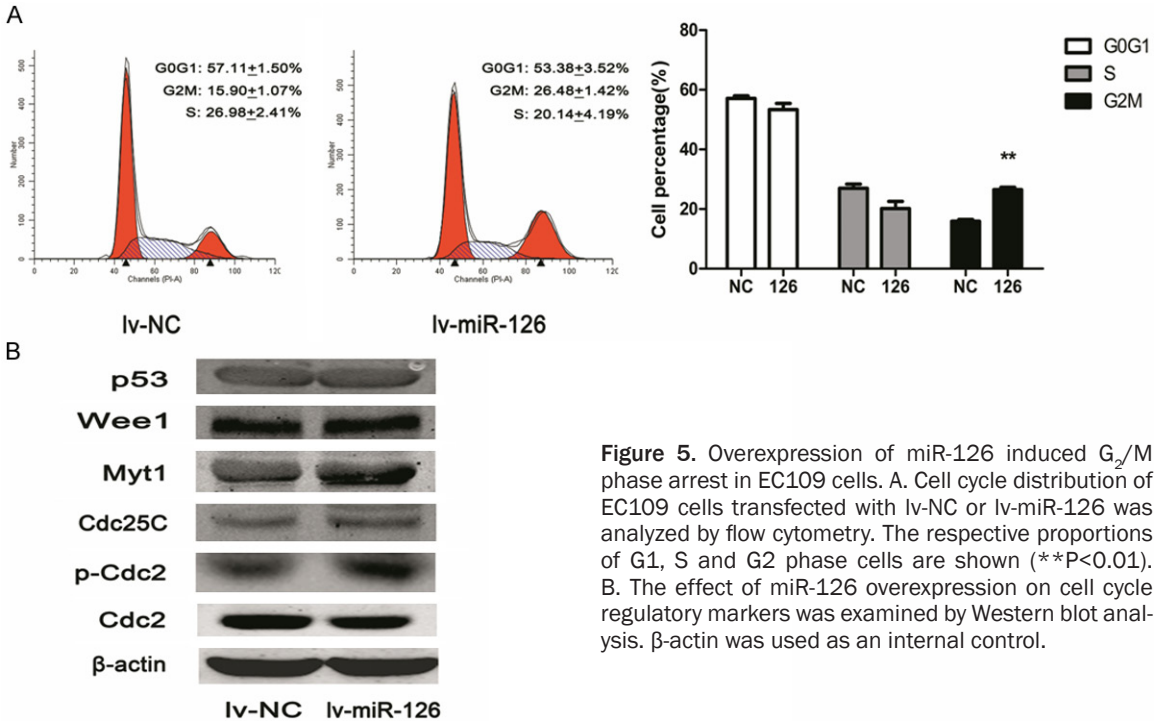

Figure 5. Overexpression of miR-126 induced G_2/M phase arrest in EC109 cells. A. Cell cycle distribution of EC109 cells transfected with lv-NC or lv-miR-126 was analyzed by flow cytometry. The respective proportions of G1, S and G2 phase cells are shown (**P<0.01). B. The effect of miR-126 overexpression on cell cycle regulatory markers was examined by Western blot analysis. β-actin was used as an internal control.

post-transcriptionally. Phosphatidylinositol 3-kinase (PI3K) is composed of a heterodimer of a regulatory unit p85 and a catalytic subunit p110. PIK3R2 is one of the PI3K p85 subunit family members. To determine whether miR-126 regulates the PI3K/AKT signaling pathway in EC109 cells, we examined the levels of phospho-AKT (p-AKT) and total AKT in EC109 cells transfection with lv-miR-126 or lv-NC. As revealed in **Figure 4E**, miR-126 significantly reduced the levels of p-AKT, without altering total AKT protein. It is therefore likely that miR-126 targeting PIK3R2 normally provides a negative regulation of the PI3K-AKT signaling pathway. These results indicated that miR-126 exerts suppression effects in esophageal cancer at least partly through repressing PI3K/AKT signaling pathway.

Overexpression of miR-126 suppressed G_2/M transition in EC109 cells

The effect of miR-126 overexpression on the cell cycle was analyzed by flow cytometry. As shown in **Figure 5A**, cell cycle analysis showed that transfection with the lv-miR-126 increased the percentage of cells in G_2-M phase compared to transfection with the lv-NC (**P<0.01). This result indicates that over-expression of miR-126 could cause a G_2/M phase arrest in EC109 cells.

Cdc2/Cyclin B complex is a pivotal regulator of the cell cycle progression from the G_2 to M phase. The activation of the Cdc2/Cyclin B complex is maintained through dephosphorylation at threonine 14(Thr^{14}) and tyrosine 15(Tyr^{15}) of Cdc2. During G_2, the Cdc2/Cyclin B complex is kept inactive through phosphorylation of Cdc2 by the kinases Wee1 and Myt1. At M phase entry, Cdc2/Cyclin B complex is activated through dephosphorylation by Cdc25C phosphatase. Therefore, to explore the molecular mechanisms by which miR-126 inhibits the G_2/M transition, we examined the expression of p53, Wee1, Myt1, Cdc2, p-Cdc2 (Tyr^{15}), Cyclin B1 and Cdc25C that involved in G_2/M phase transition. As shown in **Figure 5B**, the expressions of Myt1 and p-Cdc2 were increased while the levels of Cdc2 showed a decrease in EC109

cells transfection with lv-miR-126. These results indicated that overexpression of miR-126 induced a G_2/M cell cycle arrest, at least partially, by decreasing the levels of Cdc2 and increasing levels of Myt1 and p-Cdc2 in EC109 cells.

Discussion

miRNAs negatively regulate gene expression by binding to the 3'UTR of target mRNAs and inhibit gene expression by either blocking translation or inducing degradation of mRNAs. Over 30% of human genes are regulated by miRNAs [15]. Numerous studies have showed that aberrant expression of miRNAs is closely associated with the development, proliferation, invasion and prognosis of human cancers [16-18]. Both losses and gains of miRNAs function have been shown to contribute to cancer development through a range of mechanisms [19]. A lot of studies have showed that miR-126 plays a significant role in different physiological and pathological processes, including inflammation, blood vessel growth as well as cancer [20]. miR-126 was markedly decreased in various tumor tissues and downregulation of miR-126 induces cancer cell proliferation, migration, and invasion via targeting specific oncogenes. Therefore, miR-126 was considered as a tumor suppressor gene.

Although using miRNA microarrays, Liu and colleagues reported that the expression of miR-126 was reduced in esophageal cancer tissues compared with the matched normal tissues [21], the function and relevant mechanisms of miR-126 in ESCC have not been identified. In this study, we detected the expression of miR-126 in 30 paired ESCC tissues and matched adjacent normal tissues. As expected, the expression of miR-126 in ESCC tissues was lower than that of adjacent normal tissues, which was consistent with Liu. To further investigate the function and potential mechanisms of miR-126 in ESCC, the miR-126 lentiviral and NC lentiviral as control were transfected into EC109 cells. Restored expression of miR-126 significantly inhibited proliferation and colony formation ability of EC109 cells. Moreover, cell migration ability was also markedly suppressed by overexpression of miR-126 in EC109 cells. These results strongly suggested possible tumor suppressive role of miR-126 in ESCC.

To explore why miR-126 exhibited these effects on the cell function, we investigated putative targets of miR-126 and confirmed the gene coding for PIK3R2 was a direct target gene of miR-126 by using a luciferase reporter assay. Overexpression of miR-126 also caused a significant decrease both in mRNA and protein in EC109 cells. These findings indicated that PIK3R2 was a direct target of miR-126 in ESCC. PIK3R2 is one of the PI3K p85 subunit family members. PI3K is a second messenger that is composed of a heterodimer of a regulatory unit p85 and a catalytic subunit p110. One of its major effectors is AKT [22]. In addition to inducing cell proliferation and tumor development, the PI3K/AKT pathway also plays a pivotal role in regulating other cellular functions including angiogenesis, invasion, metastasis and survival [23]. In our study, we evaluated the activity of the PI3K/AKT pathway by analyzing the status of phosphorylated AKT in the EC109 cells after transfection of lv-miR-126. The result showed that overexpression of miR-126 decreased the phospho-AKT expression without changing total AKT protein. It's likely that miR-126 reduced PIK3R2 expression offers an inhibitory effect of PI3K/AKT signaling pathway. This observation was consistent with studies exploring the mechanism of miR-126 function in colon cancer [24] and non-small cell lung cancer [25]. Our study suggested that anti-tumorigenicity effect of miR-126 in EC109 cells may be partly mediated through PI3K/AKT pathway.

More and more evidences have revealed that deregulation of cell cycle control is an essential step in carcinogenesis [26]. Previous studies have reported that miR-126 mainly suppress cell cycle progression from G_0/G_1 to S phase in gastric cancer [27], breast cancer [28] and lung cancer [29]. Our data rather indicate that miR-126 induced a G_2/M arrest in ESCC cells. Further mechanism was explored. The cell cycle blockade was associated with the deregulation of Myt1 and p-cdc2 activity. Activation of the Cdc2/Cyclin B kinase is a pivotal step of the eukaryotic cell cycle [30]. During G2-phase, the Cdc2/Cyclin B complex remains inactive through phosphorylation on Thr14 and Tyr^{15} of Cdc2 by the kinases Wee1 family [31, 32]. At M-phase entry, Cdc2/Cyclin B is activated through dephosphorylation Cdc2 on both Thr14 and Tyr^{15} by Cdc25C [33, 34]. Myt1 is a mem-

ber of the Wee1 family of protein kinases, it phosphorylates Cdc2 on Thr14 and Tyr[15], thereby maintaining Cdc2/Cyclin B complexes in an inactive state throughout interphase [35]. Repression the activity of Cdc2/Cyclin B kinase enforces the G_2/M arrest. Inhibitory phosphorylation of Cdc2 is essential for the p53-independent G2 arrest that occurs in response to DNA damage, and is dependent on the protein kinases Atm and Atr. Atm and Atr inactivate Cdc2 by increasing phosphorylation of the residues Thr[14] and Tyr[15], a major p53-independent mechanism that causes G_2 arrest in response to DNA damage [36]. We found that overexpression of miR-126 in EC109 cells did not change the expression of p53, which indicated that p53-independent G_2 arrest may be the main mechanism in ESCC cells cycle block. In this study, neither Wee1 nor Cdc25 was activated or inhibited, while the expression of Myt1 and p-Cdc2 was increased, suggesting that the deregulation of Cdc2 activity was induced maybe by activating Myt1/Cdc2 pathway in EC109 cells. Moreover, AKT was reported to inhibit Myt1 through AKT-dependent phosphorylation and downregulation at the G_2/M transition [37]. In the present study, both p-AKT (Ser473) and Myt1 expression were reduced in EC109 cells after overexpression of miR-126, indicating that the inhibition of AKT/Myt1 may contributes to the G_2/M arrest. Further studies will be required to support these assumptions and to determine the role of upstream or downstream events in ESCC.

In summary, our data suggest that miR-126 was generally downregulated in ESCC tissues. Overexpression of miR-126 inhibited the proliferation, colony formation and migration by interaction with the target gene PI3KR2 to regulate the PI3K/AKT signaling pathway. Moreover, restoration of miR-126 can inhibit the G_2/M phase transition. Therefore, our data may help to identify miR-126 as a potential diagnostic and therapeutic target for a miRNA-based ESCC therapy.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhi Li, Department of Clinical Laboratory, Yangpu Hospital, Tongji University, Shanghai 200090, China. E-mail: hao-123lz@126.com

References

[1] Matsushima K, Isomoto H, Kohno S, Nakao K. MicroRNAs and esophageal squamous cell carcinoma. Digestion 2010; 82: 138-144.

[2] Holmes RS, Vaughan TL. Epidemiology and pathogenesis of esophageal cancer. Semin Radiat Oncol 2007; 17: 2-9.

[3] Brown LM, Devesa SS, Chow WH. Incidence of adenocarcinoma of the esophagus among white Americans by sex, stage, and age. J Natl Cancer Inst 2008; 100: 1184-1187.

[4] Su M, Liu M, Tian DP, Li XY, Zhang GH, Yang HL, Fan X, Huang HH, Gao YX. Temporal trends of esophageal cancer during 1995-2004 in Nanao Island, an extremely high-risk area in China. Eur J Epidemiol 2007; 22: 43-48.

[5] Enzinger PC, Mayer RJ. Esophageal cancer. N Engl J Med 2003; 349: 2241-2252.

[6] Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215-233.

[7] Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM. bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. Cell 2003; 113: 25-36.

[8] Liang LH, He XH. Macro-management of microRNAs in cell cycle progression of tumor cells and its implications in anti-cancer therapy. Acta Pharmacol Sin 2011; 32: 1311-1320.

[9] Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 2009; 10: 704-714.

[10] Ebrahimi F, Gopalan V, Smith RA, Lam AK. miR-126 in human cancers: clinical roles and current perspectives. Exp Mol Pathol 2014; 96: 98-107.

[11] Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Richardson JA, Bassel-Duby R, Olson EN. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev Cell 2008; 15: 261-271.

[12] Li X, Zhang Y, Zhang Y, Ding J, Wu K, Fan D. Survival prediction of gastric cancer by a seven-microRNA signature. Gut 2010; 59: 579-585.

[13] Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD, Gerald WL, Massaque J. Endogenous human microRNAs that suppress breast cancer metastasis. Nature 2008; 451: 147-152.

[14] Li N, Tang A, Huang S, Li Z, Li X, Shen S, Ma J, Wang X. MiR-126 suppresses colon cancer cell

proliferation and invasion via inhibiting RhoA/ROCK signaling pathway. Mol Cell Biochem 2013; 380: 107-119.
[15] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.
[16] Yamasaki T, Seki N, Yamada Y, Yoshino H, Hidaka H, Chiyomaru T, Nohata N, Kinoshita T, Nakaqawa M, Enokida H. Tumor suppressive microRNA138 contributes to cell migration and invasion through its targeting of vimentin in renal cell carcinoma. Int J Oncol 2012; 41: 805-817.
[17] Ma S, Tang KH, Chan YP, Lee TK, Kwan PS, Castilho A, Nq I, Man K, Wong N, To KF, Zheng BJ, Lai PB, Lo CM, Chan KW, Guan XY. miR-130b Promotes CD133(+) liver tumor-initiating cell growth and self-renewal via tumor protein 53-induced nuclear protein 1. Cell Stem Cell 2010; 7: 694-707.
[18] Yuan X, He J, Sun F, Gu J. Effects and interactions of MiR-577 and TSGA10 in regulating esophageal squamous cell carcinoma. Int J Clin Exp Pathol 2013; 6: 2651-2667.
[19] Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 2009; 10: 704-714.
[20] Meister J, Schmidt MH. miR-126 and miR-126*: new players in cancer. ScientificWorldJournal 2010; 10: 2090-2100.
[21] Liu SG, Qin XG, Zhao BS, Qi B, Yao WJ, Wang TY, Li HC, Wu XN. Differential expression of miRNAs in esophageal cancer tissue. Oncol Lett 2013; 5: 1639-1642.
[22] Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov 2005; 4: 988-1004.
[23] Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002; 2: 489-501.
[24] Guo C, Sah JF, Beard L, Willson JK, Markowitz SD, Guda K. The noncoding RNA, miR-126, suppresses the growth of neoplastic cells by targeting phosphatidylinositol 3-kinase signaling and is frequently lost in colon cancers. Genes Chromosomes Cancer 2008; 47: 939-946.
[25] Yang J, Lan H, Huang X, Liu B, Tong Y. MicroRNA-126 inhibits tumor cell growth and its expression level correlates with poor survival in non-small cell lung cancer patients. PLoS One 2012; 7: e42978.
[26] Deshpande A, Sicinski P, Hinds PW. Cyclins and cdks in development and cancer: a perspective. Oncogene 2005; 24: 2909-2915.
[27] Feng R, Chen X, Yu Y, Su L, Yu B, Li J, Cai Q, Yan M, Liu B, Zhu Z. miR-126 functions as a tumour suppressor in human gastric cancer. Cancer Lett 2010; 298: 50-63.
[28] Zhang J, Du YY, Lin YF, Chen YT, Yang L, Wang HJ, Ma D. The cell growth suppressor, mir-126, targets IRS-1. Biochem Biophys Res Commun 2008; 377: 136-140.
[29] Liu B, Peng XC, Zheng XL, Wang J, Qin YW. MiR-126 restoration down-regulate VEGF and inhibit the growth of lung cancer cell lines in vitro and in vivo. Lung Cancer 2009; 66: 169-175.
[30] Nigg EA. Mitotic kinases as regulators of cell division and its checkpoints. Nat Rev Mol Cell Biol 2001; 2: 21-32.
[31] Krek W, Nigg EA. Differential phosphorylation of vertebrate p34cdc2 kinase at the G1/S and G2/M transitions of the cell cycle: identification of major phosphorylation sites. EMBO J 1991; 10: 305-316.
[32] Parker LL, Atherton-Fessler S, Piwnica-Worms H. p107wee1 is a dual-specificity kinase that phosphorylates p34cdc2 on tyrosine 15. Proc Natl Acad Sci U S A 1992; 89: 2917-2921.
[33] Sebastian B, Kakizuka A, Hunter T. Cdc25M2 activation of cyclin-dependent kinases by dephosphorylation of threonine-14 and tyrosine-15. Proc Natl Acad Sci U S A 1993; 90: 3521-3524.
[34] Strausfeld U, Labbe JC, Fesquet D, Cavadore JC, Picard A, Sadhu K, Russell P, Doree M. Dephosphorylation and activation of a p34cdc2/cyclin B complex in vitro by human CDC25 protein. Nature 1991; 351: 242-245.
[35] Nakamizo A, Inamura T, Inoha S, Amano T, Ochi H, Ikezaki K, Fukui M. Suppression of Cdc2 dephosphorylation at the tyrosine 15 residue during nitrosourea-induced G2M phase arrest in glioblastoma cell lines. J Neurooncol 2002; 59: 7-13.
[36] Taylor WR, Stark GR. Regulation of the G2/M transition by p53. Oncogene 2001; 20: 1803-1815.
[37] Okumura E, Fukuhara T, Yoshida H, Hanada Si S, Kozutsumi R, Mori M, Tachibana K, Kishimoto T. Akt inhibits Myt1 in the signalling pathway that leads to meiotic G2/M-phase transition. Nat Cell Biol 2002; 4: 111-116.