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MicroRNA-217 alleviates development of non-small cell lung cancer by inhibiting AKT3 via PI3K pathway

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Abstract. – OBJECTIVE: To explore the role of microRNA-217 in non-small cell lung cancer (NSCLC) and its underlying mechanism.

PATIENTS AND METHODS: MicroRNA-217 expression in 48 NSCLC tissues and paracancerous tissues were detected by qRT-PCR (quantitative Real-time polymerase chain reaction). The relationship between microRNA-217 expression and prognosis of NSCLC patients was analyzed. Target gene of microRNA-217 was predicted by bioinformatics method and further verified by luciferase reporter gene assay. Cell proliferation, cell cycle and apoptosis were detected after altering microRNA-217 expression in NSCLC cells. The effect of microRNA-217 on regulating PI3K pathway was detected by Western blot.

RESULTS: MicroRNA-217 was downregulated in NSCLC tissues than that of paracancerous tissues. Shorter overall survival (OS) was observed in NSCLC patients with lower expression of microRNA-217 than those with higher expression. Overexpressed microRNA-217 remarkably inhibited proliferation and cell cycle, whereas induced apoptosis of NSCLC cells. AKT3 was screened out to be the target gene of microRNA-217. Western blot results demonstrated that microRNA-217 upregulated AKT3 and PI3K pathway-related genes.

CONCLUSIONS: Downregulated microRNA-217 promotes the occurrence and progression of NSCLC through upregulating AKT3 *via* PI3K pathway.

Key Words:
MicroRNA-217, NSCLC, AKT3, PI3K pathway.

Introduction

Lung cancer is the most common primary pulmonary cancer. It is also known as bronchial lung cancer, reflecting the origin from bronchial epithelium. Lung cancer could classified into two types based on the cell morphology, namely non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC)[1], of which about 80-85% of lung cancers belong to NSCLC. The most frequent types of NSCLC are adenocarcinoma and squamous cell carcinoma[2]. According to the latest data released by the World Health Organization in September 2011, mortality and morbidity of NSCLC ranked the first among malignancies throughout the world[3]. The death number of lung cancer exceeds the total number of deaths from breast cancer, prostate cancer and colorectal cancer, which has been a serious public health problem. High incidence, rapid growth, high mortality, and poor prognosis are prominent features of NSCLC[4]. It is of great significance to explore the potential mechanism of NSCLC, so as to better improve clinical outcomes of NSCLC patients.

MicroRNAs are a kind of small, non-coding, single-stranded RNAs with 18-25 nt in length. MicroRNAs degrade or inhibit translation of mRNA *via* binding to target mRNAs at post-transcriptional level. A wide range of biological behaviors could be precisely regulated by microRNAs, such as cell proliferation, apoptosis, migration and immune responses[5,6]. At present, it has been found that the dysregulation of certain microRNAs participate in tumor development[7]. For example, microRNA-217 has been confirmed to exert an important role in the pathogenesis of breast cancer, pancreatic cancer, gastric cancer and lung cancer[8-11]. We have already discovered that microRNA-217 is downregulated in lung cancer tissues than that of paracancerous tissues. The

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specific mechanism of microRNA-217 in regulating NSCLC, however, remains unclear.

PI3K is a heterodimer composed of a regulatory subunit p85 and a catalytic subunit p110. PI3K is classified into Class I, II and III depending on the structure and specificity of the substrate. Among them, Class I PI3K has been frequently studied, which can be activated by cell surface receptors. Class I PI3K exerts a crucial role in tumor development and progression[12,13]. On the one hand, PI3K is activated by interaction with growth factor receptors or connexins, thereafter altering conformational change of the dimer. On the other hand, PI3K could be activated through directly binding to Ras and p110. Activated PI3K leads to the production of PIP3, which further binds to AKT containing intracellular PH domain-and phosphoinositide-dependent kinase (PDK1). Subsequently, protein structure of AKT is altered and translocated to cell membrane, where phosphorylates PDK21 and PDK-2. The phosphorylated Ser308 in PDK1 eventually leads to AKT activation[14]. AKT could regulate cell proliferation, apoptosis and differentiation *via* targeting downstream genes, including Bad, Caspase-9, NF-κB and GSK23[15]. Researches have found aberrantly expressed AKT3 in human breast, prostate, and thyroid cancers[16-18], indicating the potential role of AKT3 in regulating tumor development. This study aims to elucidate the specific function of microRNA-217 in NSCLC and its underlying mechanism.

Patients and Methods

Patients

48 pairs of NSCLC tissues and paracancerous tissues were surgically resected and preserved in liquid nitrogen. Clinical data of enrolled NSCLC patients were collected. This work was approved by our Hospital Ethic Committee and patients were informed consent.

Cell Culture and Transfection

BEAS-2B, A549, HCC823, NCL-H23 and NCL-H358 cells were cultured in RPMI-1640 (Roswell Park Memorial Institute-1640) containing 10% FBS (fetal bovine serum), 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone, South Logan, UT, USA). Cells were maintained in a 5% CO_2 incubator at 37°C. For cell transfection, cells were washed with PBS (phosphate-buffered saline) when the confluence was up to 50-60%. MicroRNA-217 mimic or inhibitor was transfected based on the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), respectively. Corresponding plasmids used in the study were constructed by Gene Pharma (Shanghai, China).

CCK-8 (Cell Counting Kit-8) Assay

Transfected HCC823 cells were seeded into 96-well plates at a density of 1×10^4/μL. 10 μL of CCK-8 solution (Cell Counting Kit-8, Dojindo, Kumamoto, Japan) was added in each well after cell culture for 0, 24, 48 and 72 h, respectively. The absorbance at 450 nm of each sample was measure by a microplate reader (Bio-Rad, Hercules, CA, USA). Each group had 5 replicates.

Transwell Assay

Transfected cells were centrifuged, washed with PBS for three times and resuspended in serum-free medium. Cell density was adjusted to 2×10^5 /mL. For Transwell assay, 100 μL of cell suspension and 600 μL of RPMI-1640 were added in the upper and lower chamber, respectively. After cell culture for 48 h, cells were washed with PBS, fixed with ethanol and stained with crystal violet. 6 randomly selected fields were observed and captured using an inverted microscope (Nikon, Tokyo, Japan).

Cell Cycle Detection

Cells were collected and cell density was adjusted to 1×10^5/mL. Subsequently, cells were fixed with pre-cooled ethanol overnight, washed with PBS twice and incubated with 100 μL of RNaseA at 37°C in dark. 25 min later, cells were stained with 400 μL of PI (propidium iodide). Cell cycle was detected using flow cytometry (Partec AG, Arlesheim, Switzerland) at the wavelength of 488 nm. Each experiment was performed in triplicate.

Cell Apoptosis Detection

Cells were digested with Ethylene Diamine Tetraacetic Acid (EDTA)-free trypsin and cell density was adjusted to 1×10^5/mL. After resuspended with 1×Annexin, cells were labeled with 5 μL of Annexin V and stained with 1 μL of propidium iodide (PI) at room temperature in dark. 15 min later, cell apoptosis was detected using flow cytometry. Each experiment was repeated in triplicate.

Luciferase Reporter Gene Assay

The binding site of microRNA-217 and AKT3 was predicted by TargetScan. Wild-type AKT3 (AKT3 WT) and mutant-type AKT3 (AKT3

MUT) were constructed based on the sequences downloaded online. Cells were co-transfected with AKT3 WT or AKT3 MUT and microRNA-217 mimic or inhibitor, respectively. After co-transfection for 48 h, luciferase activity was detected and average value was recorded from three independent detections.

RNA Extraction and qRT-PCR (Quantitative Real-Time Polymerase Chain Reaction)

Total RNA in treated cells was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan), with 3 replicates in each group. The specific qRT-PCR reaction parameters were: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. Primers used in the study were as follows: AKT3, F: 5'-TGTGGATTTACCTTATCCCCTCA-3', R: 5'-GTTTGGCTTTGGTCGTTCTGT-3'; GAPDH (glyceraldehyde 3-phosphate dehydrogenase), F: 5'-AGCCACATCGCTCAGACAC-3', R: 5'-GCCCAATACGACCAAATCC-3'; U6, F: 5'-CTCGCTTCGGCAGCACATATA-3', R: 5'-AAATATGGAACGCTTCACGA-3'.

Colony Formation Assay

HCC823 cells in logarithmic phase were collected and cell density was adjusted to 1×10^4/L. Cells were seeded in the 6-well plates with 200 cells per well. After culturing for 1-2 weeks, cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for another 30 min. Colony formation was observed and captured using a light microscope (Olympus, Tokyo, Japan).

Western Blot

Total protein was extracted from treated cells by RIPA (radioimmunoprecipitation assay) solution (Beyotime, Shanghai, China). Protein sample was separated by electrophoresis on 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and then transferred to PVDF (polyvinylidene fluoride) membrane (Millipore, Billerica, MA, USA). After membranes were blocked with skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then washed with TBST (Tris-buffered Saline with Tween 20) and followed by the incubation of secondary antibody at room temperature for 1 h. The protein blot on the membrane was exposed by chemiluminescence.

Statistical Analysis

SPSS (Statistical Product and Service Solutions) 20.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. GraphPad Prism 7 (La Jolla, CA, USA) was introduced for figure editing. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and compared using the *t*-test. $p<0.05$ considered the difference was statistically significant.

Results

MicroRNA-217 was Downregulated in NSCLC

MicroRNA-217 was downregulated in NSCLC tissues compared with that of paracancerous tissues (Figure 1A). Clinical data of NSCLC patients indicated that shorter OS was observed in NSCLC patients with lower level of microRNA-217 than those with higher level (Figure 1B and 1C). Furthermore, we detected microRNA-217 expression in NSCLC cell lines and normal lung epithelial cell line. Compared with BEAS-2B cells, microRNA-217 was remarkably downregulated in A549, HCC823, NCL-H23 and NCL-H358 cells (Figure 1D). Among them, HCC823 cells expressed the lowest level of microRNA-217, which were selected for the following experiments.

Overexpressed microRNA-217 Inhibited Proliferation and Migration of HCC823 Cells

We first constructed microRNA-217 mimic and inhibitor. Transfection efficacy was verified by qRT-PCR (Figure 2A). Overexpressed microRNA-217 remarkably decreased proliferative and migratory abilities of HCC823 cells (Figure 2B and 2C). Besides, cell apoptosis was increased after microRNA-217 mimic transfection (Figure 2D). Cell cycle was arrested in G2 phase induced by microRNA-217 overexpression (Figure 2E). Colony formation assay demonstrated that clonogenic capacity of HCC823 cells was suppressed after microRNA-217 overexpression (Figure 2F).

Overexpressed microRNA-217 Directly Downregulated AKT3

Luciferase reporter gene assay demonstrated that microRNA-217 could bind to AKT3 (Figure

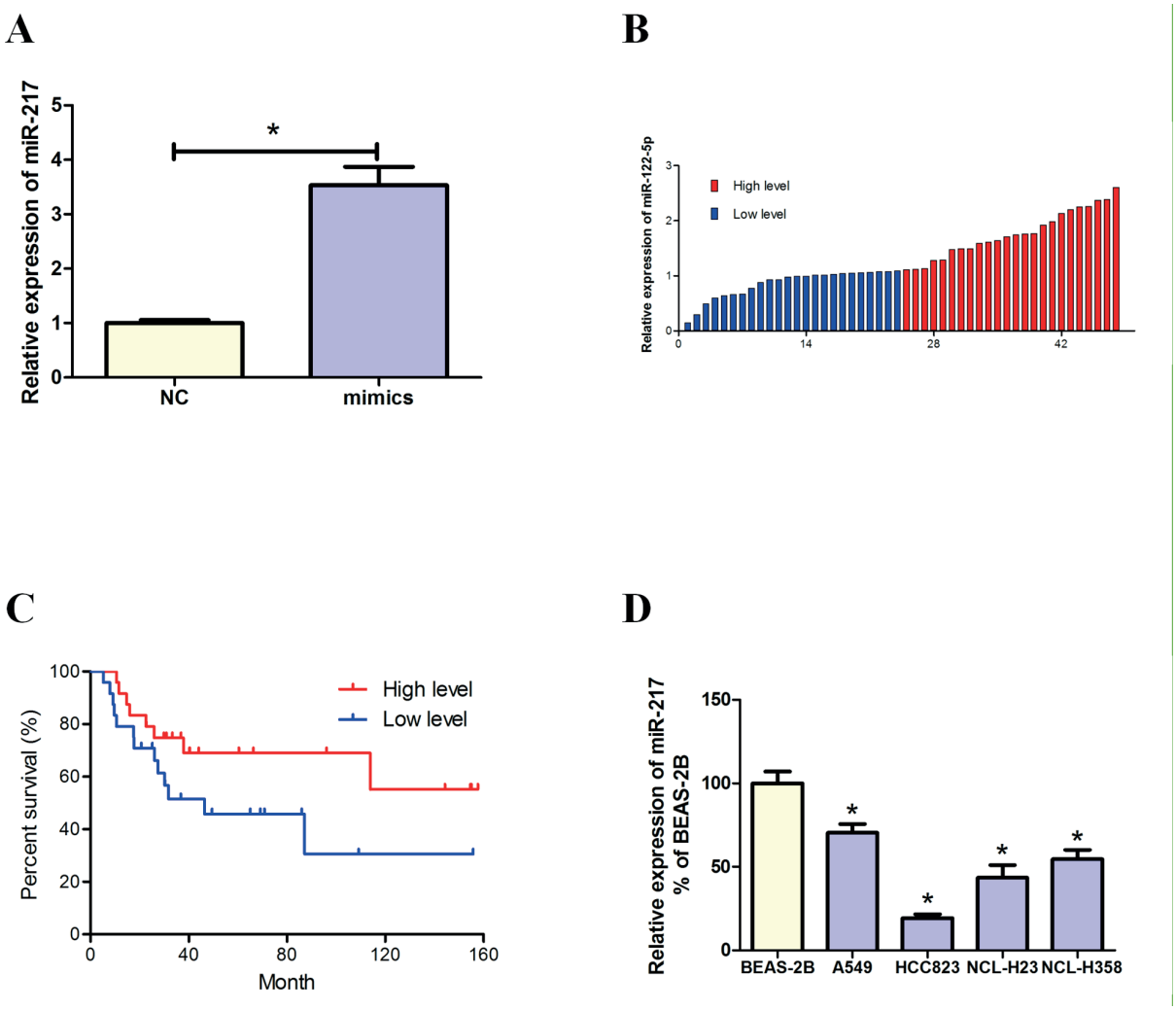

Figure 1. MicroRNA-217 was downregulated in NSCLC. ***A,*** MicroRNA-217 was downregulated in NSCLC tissues compared with that of paracancerous tissues. ***B, C,*** Shorter overall survival was observed in NSCLC patients with lower level of microRNA-217 than those with higher level. ***D,*** Compared with BEAS-2B cells, microRNA-217 was remarkably downregulated in A549, HCC823, NCL-H23 and NCL-H358 cells.

3A). Overexpressed AKT3 has already been reported to participate in the development of multiple tumors[19]. Our data showed that both mRNA and protein levels of AKT3 were reduced after microRNA-217 overexpression (Figure 3B and 3C), indicating that microRNA-217 could directly downregulate AKT3.

Overexpressed microRNA-217 Inhibited PI3K Pathway

After transfection of microRNA-217 mimic in HCC823 cells, phosphorylation level of AKT was markedly reduced (Figure 4A and 4B). Similarly, phosphorylation level of PI3K was also downregulated after microRNA-217 overexpression (Figure 4C and 4D). The above results demonstrated that microRNA-217 regulates NSCLC development by targeting AKT3 *via* PI3K pathway.

Discussion

Accumulating evidence[20] has shown that microRNAs are greatly involved in maintaining normal cell growth and function. Differentially expressed microRNAs are closely related to various types of tumors. It has been observed that microRNAs are capable of regulating tumorigenesis *via* modulating proliferation and migration of tumor cells[21]. Disordered microRNAs could

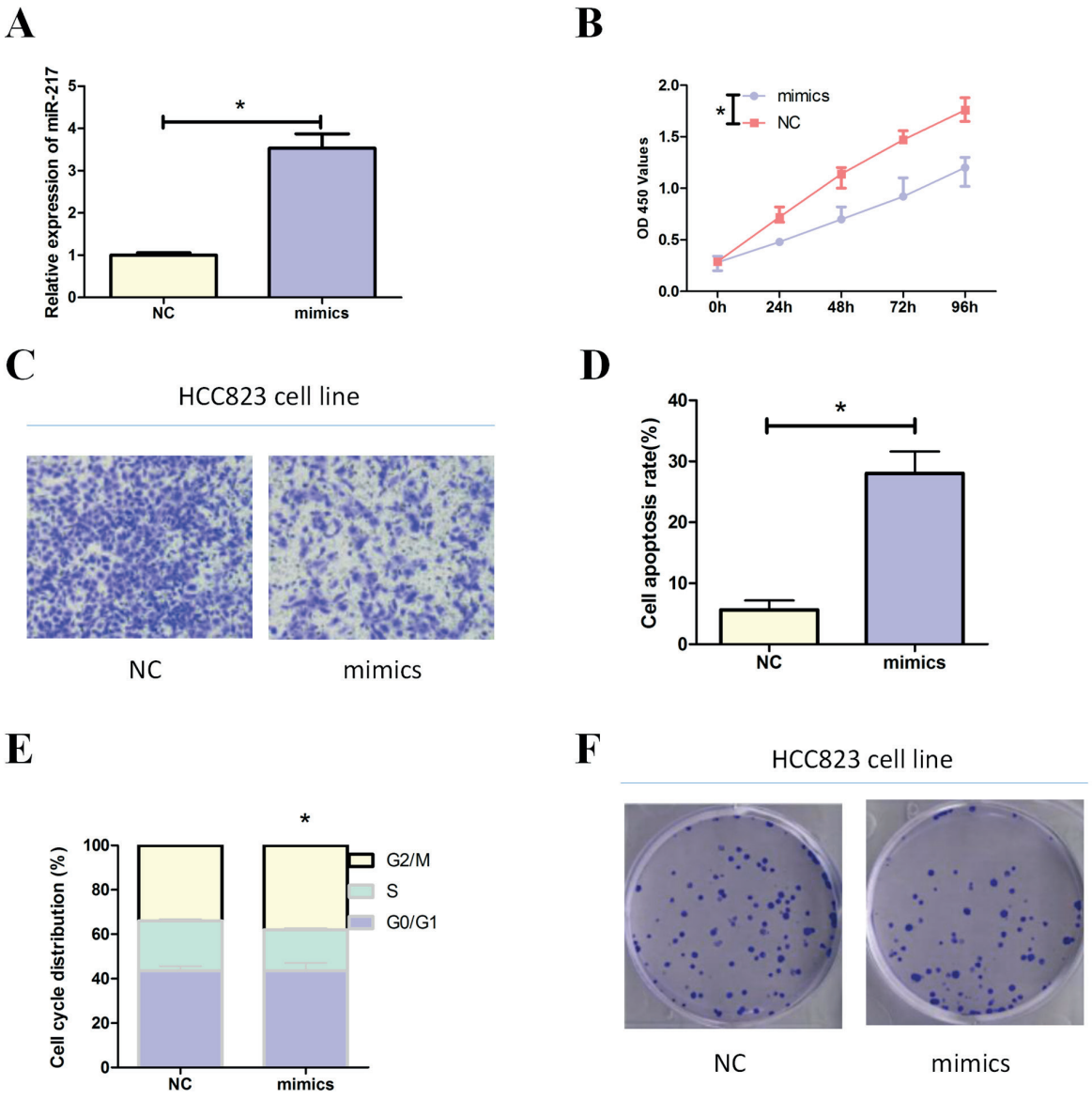

Figure 2. Overexpressed microRNA-217 inhibited proliferation and migration of HCC823 cells. ***A,*** Transfection efficacies of microRNA-217 mimic and inhibitor were verified by qRT-PCR. ***B, C,*** Overexpressed microRNA-217 remarkably decreased proliferative (***B***) and migratory (***C***) abilities of HCC823 cells. ***D,*** Cell apoptosis was increased after microRNA-217 mimic transfection. ***E,*** Cell cycle was arrested in G2 phase induced by microRNA-217 overexpression. ***F,*** Colony formation assay demonstrated that clonogenic capacity of HCC823 cells was suppressed after microRNA-217 overexpression.

markedly affect cell homeostasis. In this study, we analyzed the role of microRNA-217 in NSCLC through downloading GEO database. Subsequent experiments were carried out to further explore the potential mechanism of microRNA-217 in regulating occurrence and progression of NSCLC. Our study aims to provide theoretical basis for improving clinical outcomes of NSCLC patients.

Through online prediction using REGRNA and TargetScan, AKT3 was screened out to be the target gene of microRNA-217. AKT, also known as PKB or Rac, is important in maintaining normal cellular functions. Multiple factors could activate AKT pathway, including insulin and inflammatory factors. Previous investigations[22] have already confirmed the correlation between AKT3 and tumor development. Some other studies[23, 24] also elucidated that AKT3 exerts its biological function *via* binding to microRNAs. In our study, AKT3 was screened out to be the target gene of microRNA-217, which was further verified by luciferase reporter gene assay. AKT3 expression was negatively regulated by microRNA-217.

Currently, there are a great number of researches have pointed out that PI3K pathway participates in NSCLC development[25]. PI3K is a heterodimer composed of a regulatory subunit p85 and a catalytic subunit p110. The regulatory subunit contains SH2 and SH3 domains where target proteins containing the corresponding binding sites are interacted. There are four catalytic subunits, namely p110α, β, δ and γ[26]. AKT, an important

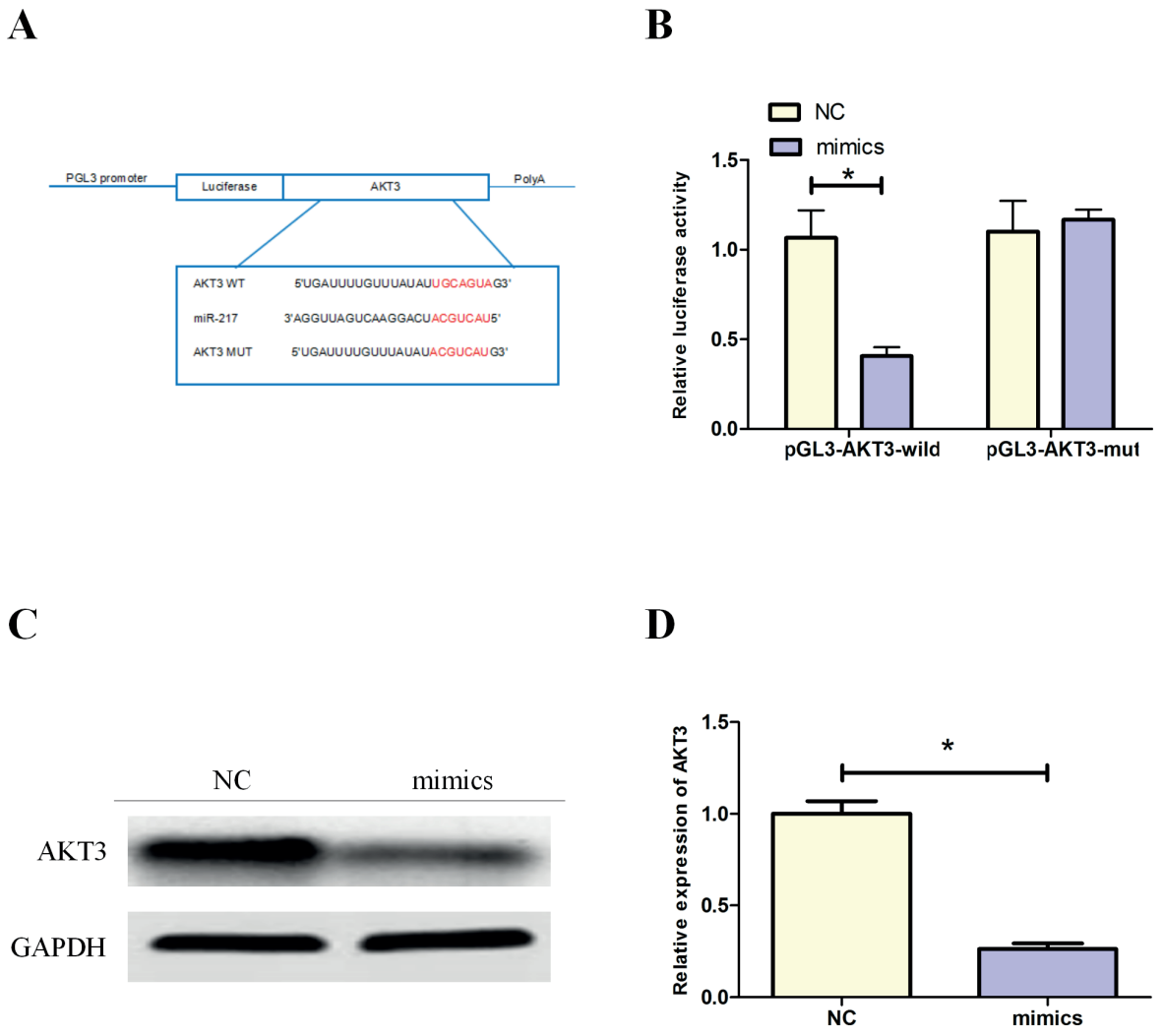

Figure 3. Overexpressed microRNA-217 directly downregulated AKT3. ***A,*** Luciferase reporter gene assay demonstrated that microRNA-217 could bind to AKT3. ***B-C,*** The mRNA (***B***) and protein (***C***) levels of AKT3 were reduced after microRNA-217 overexpression.

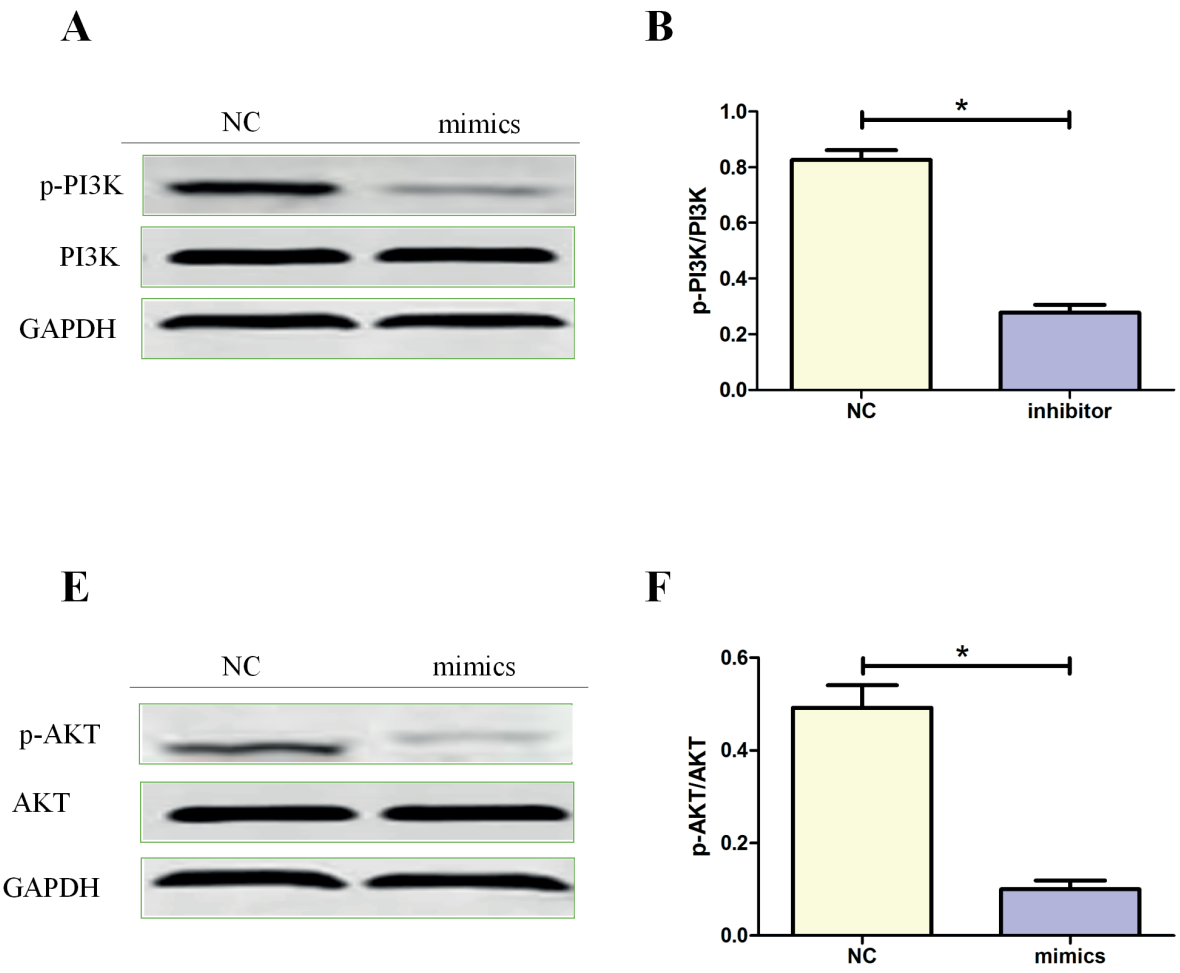

Figure 4. Overexpressed microRNA-217 inhibited PI3K pathway. ***A-B,*** Phosphorylation level of AKT was markedly reduced after transfection of microRNA-217 mimic in HCC823 cells. ***C-D,*** Phosphorylation level of PI3K was downregulated after microRNA-217 overexpression.

downstream molecule of PI3K pathway, includes at least three forms, namely Class I, II and III PI3K. They exert very important roles in the regulation of cell growth, proliferation, migration, survival, and glucose metabolism[27]. Our work suggested that PI3K pathway is activated by microRNA-217 induction, thereby participating in NSCLC development.

Conclusions

We found that the downregulated microRNA-217 promotes the occurrence and progression of NSCLC through upregulating AKT3 *via* PI3K pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Nanavaty P, Alvarez MS, Alberts WM. Lung cancer screening: advantages, controversies, and applications. Cancer Control 2014; 21: 9-14.
2) Wakelee H, Kelly K, Edelman MJ. 50 Years of progress in the systemic therapy of non-small cell lung cancer. Am Soc Clin Oncol Educ Book 2014: 177-189.
3) Warth A. [Diagnosis, prognosis, and prediction of non-small cell lung cancer. Importance of mor-

phology, immunohistochemistry and molecular pathology]. Pathologe 2015; 36 Suppl 2: 194-200.

4) See KC, Lee P. Advances in the diagnosis of pleural disease in lung cancer. Ther Adv Respir Dis 2011; 5: 409-418.

5) Liu Q, Yang W, Luo Y, Hu S, Zhu L. Correlation between miR-21 and miR-145 and the incidence and prognosis of colorectal cancer. J BUON 2018; 23: 29-35.

6) Wojtas B, Ferraz C, Stokowy T, Hauptmann S, Lange D, Dralle H, Musholt T, Jarzab B, Paschke R, Eszlinger M. Differential miRNA expression defines migration and reduced apoptosis in follicular thyroid carcinomas. Mol Cell Endocrinol 2014; 388: 1-9.

7) Di Leva G, Garofalo M, Croce CM. MicroRNAs in cancer. Annu Rev Pathol 2014; 9: 287-314.

8) Zhang Q, Yuan Y, Cui J, Xiao T, Jiang D. MiR-217 promotes tumor proliferation in breast cancer via targeting DACH1. J Cancer 2015; 6: 184-191.

9) Rachagani S, Macha MA, Menning MS, Dey P, Pai P, Smith LM, Mo YY, Batra SK. Changes in microRNA (miRNA) expression during pancreatic cancer development and progression in a genetically engineered KrasG12D;Pdx1-Cre mouse (KC) model. Oncotarget 2015; 6: 40295-40309.

10) Liu YP, Sun XH, Cao XL, Jiang WW, Wang XX, Zhang YF, Wang JL. MicroRNA-217 suppressed epithelial-to-mesenchymal transition in gastric cancer metastasis through targeting PTPN14. Eur Rev Med Pharmacol Sci 2017; 21: 1759-1767.

11) Kim J, Lim NJ, Jang SG, Kim HK, Lee GK. MiR-592 and miR-552 can distinguish between primary lung adenocarcinoma and colorectal cancer metastases in the lung. Anticancer Res 2014; 34: 2297-2302.

12) Yap TA, Bjerke L, Clarke PA, Workman P. Drugging PI3K in cancer: refining targets and therapeutic strategies. Curr Opin Pharmacol 2015; 23: 98-107.

13) Faes S, Dormond O. PI3K and AKT: Unfaithful partners in cancer. Int J Mol Sci 2015; 16: 21138-21152.

14) Franke TF, Kaplan DR, Cantley LC. PI3K: downstream AKTion blocks apoptosis. Cell 1997; 88: 435-437.

15) Gold MR, Ingham RJ, McLeod SJ, Christian SL, Scheid MP, Duronio V, Santos L, Matsuuchi L. Targets of B-cell antigen receptor signaling: the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase-3 signaling pathway and the Rap1 GTPase. Immunol Rev 2000; 176: 47-68.

16) Chin YR, Yoshida T, Marusyk A, Beck AH, Polyak K, Toker A. Targeting Akt3 signaling in triple-negative breast cancer. Cancer Res 2014; 74: 964-973.

17) Lin HP, Lin CY, Huo C, Jan YJ, Tseng JC, Jiang SS, Kuo YY, Chen SC, Wang CT, Chan TM, Liou JY, Wang J, Chang WS, Chang CH, Kung HJ, Chuu CP. AKT3 promotes prostate cancer proliferation cells through regulation of Akt, B-Raf, and TSC1/TSC2. Oncotarget 2015; 6: 27097-27112.

18) Boufraqech M, Zhang L, Jain M, Patel D, Ellis R, Xiong Y, He M, Nilubol N, Merino MJ, Kebebew E. MiR-145 suppresses thyroid cancer growth and metastasis and targets AKT3. Endocr Relat Cancer 2014; 21: 517-531.

19) Madhunapantula SV, Robertson GP. Targeting protein kinase-b3 (akt3) signaling in melanoma. Expert Opin Ther Targets 2017; 21: 273-290.

20) Liu W, Ma R, Yuan Y. Post-transcriptional regulation of genes related to biological behaviors of gastric cancer by long noncoding RNAs and MicroRNAs. J Cancer 2017; 8: 4141-4154.

21) Zhang H, Liu A, Feng X, Tian L, Bo W, Wang H, Hu Y. MiR-132 promotes the proliferation, invasion and migration of human pancreatic carcinoma by inhibition of the tumor suppressor gene PTEN. Prog Biophys Mol Biol 2017 Sep 20. pii: S0079-6107(17)30203-1. doi: 10.1016/j.pbiomolbio.2017.09.019. [Epub ahead of print]

22) Hers I, Vincent EE, Tavare JM. Akt signalling in health and disease. Cell Signal 2011; 23: 1515-1527.

23) Li Y, Cai B, Shen L, Dong Y, Lu Q, Sun S, Liu S, Ma S, Ma PX, Chen J. MiRNA-29b suppresses tumor growth through simultaneously inhibiting angiogenesis and tumorigenesis by targeting Akt3. Cancer Lett 2017; 397: 111-119.

24) Zhu LP, Zhou JP, Zhang JX, Wang JY, Wang ZY, Pan M, Li LF, Li CC, Wang KK, Bai YP, Zhang GG. MiR-15b-5p regulates collateral artery formation by targeting AKT3 (Protein kinase b-3). Arterioscler Thromb Vasc Biol 2017; 37: 957-968.

25) Zhu Y, Luo G, Jiang B, Yu M, Feng Y, Wang M, Xu N, Zhang X. Apolipoprotein M promotes proliferation and invasion in non-small cell lung cancers via upregulating S1PR1 and activating the ERK1/2 and PI3K/AKT signaling pathways. Biochem Biophys Res Commun 2018; 502: 520-526. doi: 10.1016/j.bbrc.2018.05.029.

26) Wymann MP, Pirola L. Structure and function of phosphoinositide 3-kinases. Biochim Biophys Acta 1998; 1436: 127-150.

27) Summers SA, Birnbaum MJ. A role for the serine/threonine kinase, Akt, in insulin-stimulated glucose uptake. Biochem Soc Trans 1997; 25: 981-988.