Blocking VRK2 suppresses pulmonary adenocarcinoma progression via ERK1/2/AKT signal pathway by targeting miR-145-5p

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Abstract. – OBJECTIVE: The incidence of pulmonary adenocarcinoma locates first in all the malignant tumors in the world. At present, there are many diagnostic methods for pulmonary adenocarcinoma, but there are a few methods that are mature or have ideal application prospects. We aim to explore the role of VRK2 in the occurrence and development of pulmonary adenocarcinoma and its possible regulatory mechanism.

PATIENTS AND METHODS: Western blot and qRT-PCR were performed to assess the expression of VRK2. Flow cytometry, Western blot, and Caspase-3 colorimetric assay Kit were used to evaluate the apoptosis level. The proliferation, migration, and invasion ability were measured via cell cycle assay, wound healing, and transwell invasion assay. Luciferase assay verified the relationship between VRK2 and miR-145-5p. The effect of FGD5-AS1 on the tumorigenesis of glioma was detected by the xenograft nude mice model.

RESULTS: VRK2 was significantly increased in tumor tissues and cell lines. Loss of VRK2 promoted apoptosis and inhibited the proliferation, migration, and invasion in A549 cells via regulating the ERK1/2/AKT signal pathway. Luciferase assay reported that VRK2 could bind with miR-145-5p. The level of miR-145-5p was negatively correlated with the expression of VRK2. Downregulation of VRK2 in regulating tumor progression showed that the silencing of VRK2 inhibited tumorigenesis via targeting ERK1/2/AKT pathway.

CONCLUSIONS: Knockdown of VRK2 inhibits the development of pulmonary adenocarcinoma by regulating the ERK1/2/AKT signal pathway and targeting miR-145-5p, which provides some novel experimental basis for clinical treatment of pulmonary adenocarcinoma.

Key Words: Pulmonary adenocarcinoma, VRK2, MiRNA, Proliferation, Metastasis.
circRNA CEP128 could sponge miR-145-5p in accelerating the development of bladder cancer by controlling SOX116 and Myd88/MAPK signal pathway. MiR-145-5p regulates the differentiation progression of gastric cancer via KLF5. LncRNA SNHG1 acts as a sponge of miR-145-5p promoting the development of NSCLC via promoting MTDH expression. MiR-145-5p also inhibits EMT progression via MAP3K/JNK signaling pathway. In this research, we observed that VRK2 was a target of miR-145-5p, and positively correlated with miR-145-5p, which regulates the pathways of EKR 1/2 and AKT to affect the process of pulmonary adenocarcinoma.

**Patients and Methods**

**Clinical Samples**

The tumor tissue samples and adjacent normal tissue samples were collected from 60 NSCLC patients at Affiliated Cancer Hospital of Zhengzhou University. The present study was supported by the Ethics Committee of Affiliated Cancer Hospital of Zhengzhou University and also has been carried out in accordance with the World Medical Association Declaration of Helsinki. The subjects had been informed the objective. Certain, written consents were signed by every subject in the present study.

**Cell Culture**

Beas-2B, H1299, H1975, SPC-A-1, PG49, and A549 cell lines were purchased from the Science Cell Laboratory. NSCLC cell lines were cultured in PRIM 1640 (Thermo Fisher Scientific, MA, USA) with 10% FBS (Thermo Fisher Scientific, MA, USA) and 100 μL/mL penicillin and streptomycin (Beyotime, Shanghai, China) and placed at 37°C with 5% CO2.

**Cell Transfection**

Si-RNA (si-VRK2) was produced by Ribobio Co., Ltd. (Guangdong, China). Si-NC (negative control) was indicated as control (si-NC). About 5×10⁵ cells per well were seeded in 6 well plates, Regens (20 nmol/L) were transfected into the cells with Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA) for 48 h.

**qRT-PCR**

RNA isolation, reverse transcription, and quantitative expression were carried out according to the manufacturer’s instructions. RNA was collected and transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Qiagen, Hilden, Germany). The cDNA and PCR program were run in triplicate. The data were analyzed using the 2-ΔΔCt method. The primer sequences were as follows: VRK2: Forward: 5′-GCAAGGUUCUGGAUGAUAU3′; MiRNA-145-5p mimics: 5′-ATCGTCACGCGCTGAGTCTCTC3′; Reverse: 5′-CAAGAGGTCTTCTGAGAGCT-3′; GAPDH: Forward: 5′-GGTCTTACTCCTTGAGGCCATGTG-3′; Reverse: 5′-ACCTAACCACATGTTTACATGGT-3′; miRNA-145: Forward: 5′-CTCGCTTCGCTGCAGCTGAGTTG-3′; Reverse: 5′-AACGCTTCGCAATTGCGT-3′.

**Western Blot**

Total protein was collected from tissues and cells and cells with RIPA lysis Mix (Beyotime, Shanghai, China). Briefly, 40-60 μg protein extraction was loaded via SDS-PAGE and transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA), the membranes were incubated in 5% non-fat blocking solution for 1 h. After incubation with primary antibodies for 1 h at room temperature, and then, plated at 4°C for one night. After incubation with secondary antibodies, the membranes were scanned using an Odyssey, and data were analyzed with Odyssey software (LI-COR, Lincoln, NE, USA); p-ERK (sc-7383, 1:200) was purchased from Proteintech (Rosemont, IL, USA); p-AKT (ab-7838, 1:200) was purchased from Abcam (Cambridge, UK); and GraphPad (60004-1-Ig, 1:2000) was used as an internal control.
VRK2 expression and lung cancer via miR-145-5p/ERK1/2/AKT axis

*CCK-8 Assay*
Cells were cultured in 96-well cell plates and added CCK-8 buffer (MedChemExpress, Monmouth Junction, NJ, USA) at 0, 24, 48, and 72 h. 2 h later, measure 450 OD value with an MKJ3ELISA photometer (Thermo Fisher Scientific, Waltham, MA, USA).

*Matrigel Invasion Assay*
Cells in the logarithmic growth phase were adjusted to 2 × 10^5 cells/well of medium (without serum) and plated 1μg/μl Matrigel into the upper chamber. The lower chamber was added with 500 μL of the medium, and then incubate the plate at 37°C for 48 h. Then, the invading cells were visualized by the crystal violet and inverted microscope.

*In Vivo Tumor Growth Assay*
Nude mice were purchased from the Beijing Charles River. Stable VRK2 knockdown cell lines were constructed, A549 cells (5 × 10^6) were subcutaneously injected in the right lower limb of the nude mice. Tumor size was measured every five days. After another 15 d of injection, the tumor was removed for follow-up study. The animal study was approved by the Animal Clinical Committee of Affiliated Cancer Hospital of Zhengzhou University. Nude mice were euthanized and operated strictly in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health.

*Cell Apoptosis Assay*
The A549 cells were collected, about 5×10^5 cells/mL. Then, 1 mL cells were centrifuged, 1000 rpm, 10 min, 4°C, and the medium was throw away. The cells were washed with PBS and dropped medium. Then, the cells were resuspended and avoided light for 15 min, 200 μL Binding Buffer with 10 μL Annexin V-FITC and 10 μL PI. Flow cytometry was used to measured apoptosis rate.

*Cell Cycle Assay*
The A549 cells were collected with 1 ml trypsin for 2 min, suspension the cell with 5 ml PBS, centrifuged at 1000 RPM for 5 min at 4°C. 10 ml PBS buffer was used to re-washed and dropping medium. Then, the cells were fixed with 70% ethanol overnight. The next day, the cell medium was filtered with a 300-mesh sieve, centrifuged at 1000 RPM at 4°C for 5min, and the supernatant was discarded. The cells were avoided light and fixed with 1ml PI solution and stated at 4°C for 30 min. Flow cytometer was used to evaluate the cell cycle.

*Luciferase Assay*
HEK293T cells were co-transfected with 20 mmol/L miRNA mimic or miR-NC together with VRK2-WT or VRK2-mutation. Luciferase activity was measured with Dual-Luciferase Reporter Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) on GloMax20/20 at 48 hr after transfection.

*Immunohistochemistry (IHC)*
The tumor tissue was cut into 5-μm-thick sections. The sections were dewaxed and deparaaffinized, rehydrated in gradient alcohol solutions. Sections were heated in the Tris-EDTA buffer for 30 min to extract antigens. The samples were incubated with primary antibodies for ki-67 (27309-1-AP, 1:100), VEGF (19003-1-AP, 1:20), p-ERK (sc-7383, 1:20), p-AKT (66444-1-Ig, 1:50), cleaved-caspase3 (ab2302, 1:100, Abcam, Cambridge, UK), and HRP-labeled streptavidin (A0303, 1:200, Beyotime, Shanghai, China) secondary antibodies. Sections were avoided light with hematoxylin, then dehydrated and secured. Photos were scanned with an Olympus camera.

*Statistical Analysis*
All values are expressed as the mean ± SEM. Statistical significances were measured by Student’s t-test and ANOVA. A two-tailed value of p < 0.05 was indicated as a statistically significant difference. Data statistics were used the GraphPad 7.0 (La Jolla, CA, USA).

**Results**

**VRK2 Was an Upregulation in Pulmonary Adenocarcinoma Tissue and Cells**
To detect the role of VRK2 in pulmonary adenocarcinoma, we collected tumor and para-cancer tissue from 60 pulmonary adenocarcinoma patients. By performing qRT-PCR assays, we found VRK2 was an upregulation in tumor tissue comparing with normal tissues (Figure 1A). We also detected the level of VRK2 in different human pulmonary adenocarcinoma cell lines (H1299, H1975, SPC-A-1, PG49, and A549); the Beas-2B cell line was indicated as control. The
results showed that VRK2 was increased in different of human pulmonary adenocarcinoma cell lines, moreover; the expression increased more evidently in A549 cells (Figure 1B and C).

Knockdown of VRK2 Promotes Apoptosis of A549

Next, we would verify the function of VRK2 in pulmonary adenocarcinoma. We constructed siRNA to knockdown expression of VRK2. Si-VRK2/si-NC were transfected into A549 cells; then, flow cytometry performed that the apoptosis rate was significantly increased in the si-VRK2 group compared with si-NC (Figure 2A). The result revealed significantly increased G0/G1 cells and decreased S cells after downregulation of VRK2 (Figure 2B). Furthermore, we detected caspase3 activity and apoptosis-associated proteins, cleaved-caspase3, cleaved-caspase8, cleaved-caspase9 (Figure 2C&D), the results showed that knockdown of VRK2 could facilitate apoptosis of A549 cell.

Knockdown of VRK2 Prevents Proliferation and Metastasis of A549

Then CCK8 assay was carried out to evaluate the function of VRK2 on NSCLC cell proliferation after 72 h, si-VRK2 significantly inhibited cell proliferation (Figure 3A). To further link VRK2 expression to cell invasion, we applied the chamber-transwell invasion assay. The results showed that VRK2 downregulation could block cell invasion in cells (Figure 3B). Figure 3C showed that si-VRK2 inhibited A549 migration at 24 h and 48 h. Downregulation of VRK2 inhibited the activation of AKT and ERK1/2. (Figure 3D).

VRK2 Could Interact With MiR-145-5p

Bioinformatics sites showed that VRK2 was negatively correlated with miRNA-145-5p and that miRNA-145-5p could bind with the 3 'UTR region of VRK2 (Figure 4A&B). To verify the forecast, we created miR-145-5p mimics/miR-NC and performed Luciferase assay; the report confirmed the link between VRK2 and miRNA-145-5p (Figure 4C). We transfected si-VRK2/si-NC, miR-145-5p mimics/AMO-145-5p/miR-NC into A549 cells to measure the expression of miR-145-5p and VRK2, there was a negative correlation between VRK2 and miRNA (Figure 4D&E). Meanwhile, AMO-145-5p could block the inactivation of AKT and ERK1/2 induced by si-VRK2 (Figure 4F). In summary, interacting with miR-145-5p, VRK2 participated in the proliferation
VRK2 expression and lung cancer via miR-145-5p/ERK1/2/AKT axis

Figure 2. Knockdown of VRK2 promotes apoptosis in A549 cells. A, The apoptosis ratio was measured by flow cytometry. n=6, *p < 0.05. B, The effect of VRK2 on cell cycle. n=5, *p < 0.05. C, Si-VRK2 induced Caspase-3 activation in A549 cells. n=6, *p < 0.05. D, The protein level of apoptosis-associated protein (Cleaved-PARP, Bcl2, Cleaved Caspase3, Cleaved Caspase8, Cleaved Caspase9) was detected in A549 after si-VRK2/si-NC transfection. n=8, *p < 0.05.

Figure 3. Knockdown of VRK2 inhibits proliferation and metastasis via AKT/ERK1/2 signal pathway in A549 cells. A, CCK8 assay was performed to detect the proliferation ability. n=4, *p < 0.05. B, Cell invasion ability was detected in A549 after si-VRK2/si-NC transfection (magnification×100). n=6, *p<0.05. C, Representative images (left) and histogram (right) from wound-healing assays using A549 cells. n=4, *p < 0.05. D, The protein level of VRK2, AKT, p-AKT, ERK1/2, p-ERK1/2 were identified by Western blot. n=6, *p<0.05.
Knockdown of VRK2 Prevents Tumor Growth In Vivo

For further confirmation, we constructed a stable low expression of VRK2 A549 cell (si-VRK2-A549), the normal level of VRK2 A549 cell as a control (si-NC-A549). Two groups of A549 cells were randomly and subcutaneously injected in the right lower limb of the nude mice. Tumor size was measured every five days. Si-VRK2-A549 injection group significantly reduced tumor volume and weight (Figure 5A-C). The tumor tissues of the si-VRK2-A549 injection group showed a higher level of miR-145-5p and a lower level of VRK2 compared with si-NC-A549 (Figure 5D&E), the phosphorylation of AKT and ERK1/2 were also decreased in si-VRK2-A549 injection mice (Figure 5F). The tumor tissue was sectioned for immunohistochemical staining (Figure 5G). The Ki-67 staining performed that VRK2 knockdown significantly inhibited the proliferation of tumor, which got similar results in vitro.

Si-VRK2-A549 decreased the level of VEGF, p-ERK, and p-AKT, accompanied by increased expression of cleaved-Caspase3.

**Discussion**

Until now, the commonly used tumor markers such as carcinoembryonic antigen (CEA) have a specific value in the diagnosis of the tumor; their sensitivity and specificity are not high enough.
The early diagnosis of pulmonary adenocarcinoma is severely difficult due to its weak expression in patients, the late diagnosis is a challenge. Furthermore, the pulmonary adenocarcinoma incidence rate is lower than other types of pulmonary adenocarcinoma, the onset age is smaller, and the lump growth is slow, so it is more accessible to misdiagnosis. Although there have been huge bioinformatics analysis studies on pulmonary adenocarcinoma, a large number of reports have been conducted in the development, metastasis, drug resistance, and prognostic molecular biology of pulmonary adenocarcinoma. Early diagnosis of pulmonary adenocarcinoma is a critical step in improving the diagnosis and survival rate of pulmonary adenocarcinoma. In previous studies, VRK2 was an abnormal expression in patients with mental and neurological disorders and epilepsies. In an earlier study, VRK2 was identified as a potential mental and neurological disorder, but few studies have reported on tumors, especially in pulmonary adenocarcinoma. It was reported that VRK2 could regulate the hypoxia stress response induced by TAK1. VRK1 and VRK2 were both described as an indicator of rectal adenocarcinoma response to neoadjuvant chemoradiation therapy28. Some shortcomings of this study are that appropriate VRK2 specific inhibitors were not used to inhibit its activity; we choose siRNA to inhibit VRK2, which may have differential results. In our study, after quantitative detection of VRK2 level in pulmonary adenocarcinoma patients, it was
found that VRK2 expression in pulmonary adenocarcinoma tissues was significantly higher than adjacent tissues, the similar results were performed in pulmonary adenocarcinoma cell lines. Knockdown of VRK2 could promote apoptosis and press proliferation, metastasis of A549 cells. Further results showed that miR-145-5p could bind with VRK2 involved in pulmonary adenocarcinoma progression via regulating AKT/ERK1/2 signal pathway. In recent years, the research on the function of miRNA has become one of the breakthroughs in life science, especially in the mechanism of occurrence and development of malignant tumors. MiRNA is a kind of non-coding small molecule RNA, which is widely involved in the regulation of life activities. The results show that most of the genes in human and other mammals are regulated by miRNA, and each miRNA can regulate hundreds of target genes. With the deepening of the study of miRNA, it has been reported that the expression of miRNA has significant tissue specificity, and its expression profile analysis can be used to identify the source of tumor, and has higher accuracy than the classification methods of mRNA expression profile, and can more directly reflect the level of gene function. Some studies have also shown that changes in the expression level of miRNA can cause changes in a series of oncogenes and tumor suppressor genes, so they can be used as effective targets for tumor treatment by specifically inhibiting the activity of carcinogenic miRNA. Using miRNA microarray to detect the differentially expressed miRNA, the pulmonary adenocarcinoma and paracancerous tissues are compared to systematically study the interaction between genes and miRNA, and then provide a new idea for the treatment of non-small cell lung cancer. The development of this malignant tumor, and provide reliable help for the prevention, diagnosis and treatment of pulmonary adenocarcinoma. Conclusions For the first, we established the correlation between VRK2 and miR-145-5p and explored the role and underlying mechanism of VRK2 in pulmonary adenocarcinoma. Although the research is still insufficient, it also can provide a specific theoretical basis for clinical research.

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