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Original Articles

EZH2 promotes tumor progression via regulating VEGF-A/AKT signaling in non-small cell lung cancer

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ABSTRACT

Enhancer of Zeste Homologue 2 (*EZH2*) accounts for aggressiveness and unfavorable prognosis of tumor. We investigated the mechanisms and signaling pathways of *EZH2* in non-small cell lung carcinoma (NSCLC) progression. Increased expression of EZH2, vascular endothelial growth factor-A (VEGF-A) and AKT phosphorylation correlated with differentiation, lymph node metastasis, size and TNM stage in NSCLC. There was a positive correlation between EZH2 and VEGF-A expression and high EZH2 expression, as an independent prognostic factor, predicted a shorter overall survival time for NSCLC patients. The expression of VEGF-A and phosphorylated Ser^{473}-AKT, cell proliferation, migration and metastasis were enhanced in *EZH2*-overexpressing A549 cells, but inhibited in parental H2087 cells with *EZH2* silencing or GSK126 treatment. AKT activity was enhanced by recombinant human VEGF-165 but suppressed by bevacizumab. An AKT inhibitor MK-2206 blocked VEGF-A expression and AKT phosphorylation in parental H2087 and *EZH2*-overexpressing A549 cells. EZH2 activity was not affected by either VEGF-A stimulation/depletion or MK-2206 inhibition. These results demonstrate that *EZH2* promotes lung cancer progression via the VEGF-A/AKT signaling pathway.

Introduction

Non-small cell lung carcinoma (NSCLC), mainly adenocarcinoma and squamous cell carcinoma, accounts for 80% of all lung cancer cases. Being the leading cause of cancer-related death worldwide, lung cancer exhibits a high mortality and morality rate. Approximately 70% of newly diagnosed lung cancer patients present with metastatic lesions or local recurrence after resection. Undoubtedly, elucidating mechanisms of progression and metastasis of lung cancer is crucial for the treatment of the disease.

Enhancer of Zeste Homologue 2 (*EZH2*) is required for the stable transmission of gene expression patterns to progeny cells throughout development. Being a key member of the Drosophila Polycomb group (PcG), *EZH2* plays a crucial role in gene expression regulation, maintenance of cell identity, stem cell renewal and oncogenesis. Recent studies have shown that EZH2 overexpression predicts poor patient prognosis and is a frequent event in various cancers, such as breast cancer [1], malignant prostate cancer [2], and hepatocellular carcinoma [3]. There are reports depicting the expression and oncogenic role of *EZH2* in lung tumorigenesis [4,5] and *EZH2* has been suggested to modulate several pathophysiological processes by promoting cell proliferation and cell cycle progression [6], accelerating cell infiltration and metastasis [7], and inhibiting apoptosis [8]. *EZH2* also activates tumor angiogenesis by silencing vasohibin 1 in ovarian cancer [9]. However, its relationship with vascular endothelial growth factor-A (VEGF-A), a critical player in angiogenesis, has not been comprehensively investigated, though VEGF-A has been implicated in the carcinogenesis of multiple cancers including lung cancer [10,11].

The phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway has been documented in angiogenesis and lung cancer progression [12] and its activation stimulates VEGF-A expression [13]. However, whether *EZH2* promotes tumor progression via VEGF-A/AKT signaling pathway warrants validation.

Abbreviations: EZH2, Enhancer of Zeste Homologue 2; VEGF-A, vascular endothelial growth factor-A; NSCLC, non-small cell lung carcinoma; PcG, Drosophila polycomb group; PI3K/AKT, phosphatidylinositol 3-kinase/protein kinase B; RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum; EGFP, enhanced green fluorescence protein; PBS, phosphate-buffered saline; SEM, scanning electron microscope; siRNA, small interfering RNA; PI, propidium iodide; H&E, hematoxylin and eosin; RT-PCR, real-time reverse transcription–polymerase chain reaction; MMP-2, matrix metalloproteinase-2; HIF-1, hypoxia inducible factor-1.

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To clarify these important issues we thus designed the study to elucidate whether *EZH2* regulated VEGF-A expression and tumor progression via PI3K/AKT signaling pathway in NSCLC. We analyzed correlations between EZH2 expression and clinical pathological characteristics and lung cancer patients' overall survival and prognosis.

Materials and methods

Patients and tumor specimens

A total of 195 NSCLC patients who underwent complete tumor resection in Nanfang Hospital in Guangzhou China from 2003 to 2013 were selected for this study. Written informed consent was obtained from each patient and tissue specimens were processed according to the protocols of the Southern Medical University Ethnics Committee. The overall survival time after tumor removal was 52.6 months (range 8–121 months). At the time of censoring the data, the follow-up information of 48 (25%) patients was lost because of death or other reasons. Specimens from these patients were obtained from the Department of Pathology and the Department of Thoracic Surgery in Nanfang Hospital. Forty snap frozen fresh tumor samples and matched normal lung tissues (10 cm from the tumor) obtained from among the 195 specimens were also available for the study. The clinical pathological characteristics of the patients were based on the World Health Organization criteria [14,15], as was described in Table 1. Details are described in the Supplementary Materials and Methods.

Immunohistochemistry and immunofluorescence microscopy

Immunohistochemical staining and immunofluorescence co-labeling were carried out according to previously published procedures [16]. The immunostained slides were examined under a 40× objective lens using a BX51 light microscope (Olympus, Tokyo) with appropriate filters. Details are described in the Supplementary Materials and Methods.

Cell line and culture

A549 and H2087 lung adenocarcinoma cells were maintained in RPMI 1640 supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) in 5% CO_2, 37 °C cell culture incubator for analysis.

Enhanced green fluorescence protein (EGFP)/EZH2-mediated overexpression of EZH2

Constructs of recombinant EGFP/EZH2 and EGFP-N1 plasmid were obtained from Thermo Scientific (Beijing). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for transfecting A549 lung cancer cells according to the manufacturer's procedures. Details are described in the Supplementary Materials and Methods.

Table 1
Clinicopathological characteristics of patients with lung cancer.

Characteristics	All patients (N = 195) (%)
Gender	
Male	79 (41%)
Female	116 (59%)
Smoking status	
Nonsmoker	94 (48%)
Smoker	101 (52%)
Tumor location	
Central	92 (47%)
Peripheral	103 (53%)
Tumor subtypes	
Adenocarcinoma	96 (46%)
Squamous cell carcinoma	99 (54%)
Differentiation	
High	97 (50%)
Moderate–poor	98 (50%)
LN metastasis	
Yes	98 (50%)
No	97 (50%)
Tumor size	
>3 cm	96 (49%)
<3 cm	99 (51%)
TNM stage	
I–II	89 (46%)
III–IV	106 (54%)

Small interfering RNA-mediated gene silencing of EZH2

Parental H2087 cells, with high endogenous EZH2 expression, were plated at a density of 0.5×10^5 cells/ml and cultured for 24 hours before experimentation. *EZH2* was knocked down with small interfering RNA (siRNA) duplexes specifically targeting human EZH2 mRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA and protein were extracted and analyzed respectively. Details are described in the Supplementary Materials and Methods.

Drug treatment

Recombinant human VEGF-165 (2 ng/ml, rhVEGF165, Millipore, Billerica, MA), bevacizumab (2.5 μM, VEGF-A inhibitor, Sigma-Aldrich, St. Louis, MO) and MK-2206 (100 nM, AKT inhibitor, sc-364537, Santa Cruz, CA) were used to treat parental H2087 and *EZH2*-overexpressing A549 cells. An EZH2 inhibitor, GSK126 (100 nM, 1346574-57-9, MedKoo Biosciences, Inc., Chapel Hill, NC), was applied to parental H2087 cells. Each group of cells was treated for 72 hours and harvested for further analysis.

Cell proliferation assay

Cell proliferation analysis was performed in triplicate using a CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI) following the manufacturer's protocols. Details are described in the Supplementary Materials and Methods.

In vitro *plate-colony formation assay*

Cells were plated at a density of 200 cells per well in a six-well tissue culture plate. After two weeks, colonies with ≥50 cells were counted and plate colony formation efficiency was evaluated according to the following formula: (number of colonies/number of cells inoculated) × 100%. Wells with tissue culture medium but no cells inoculated were used as negative controls. Triplicate samples from each group of cells were examined and colonies were counted by two individuals (JG and XL).

Cell cycle analysis using flow cytometry

Fixed cells (1×10^6 cells/ml) were stained with propidium iodide (PI, Sigma, MO) and the fluorescence of DNA-bound PI in cells was measured with a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ). The cell cycle was examined and results were analyzed with the ModFit 3.0 software (Verity Software House, Topsham, ME). All experiments were repeated in triplicate.

In vitro *migration assay*

Cells in serum-free medium (1×10^6 cells/ml) were added to the top chamber of 24-well transwell plates (8 μm pore size; Corning Star, Cambridge, MA) and 600 μl of complete medium with 10% FBS into the bottom chamber. The assembled chamber was incubated at 37 °C in a humidified, 5% CO_2 cell culture incubator for 24 hours, followed by fixing with 10% formalin and staining with hematoxylin and eosin (H&E) for visualization. Details are described in the Supplementary Materials and Methods.

Quantitative real-time reverse transcription–polymerase chain reaction analysis

Quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis was performed in triplicate with Power PCR SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) using the ABI PRISM 7500 FAST Real-TIME PCR System (Applied Biosystems, Carlsbad, CA) with results normalized to β-actin expression. The relative expression was calculated using the $\Delta\Delta C_T$ method. Primer sequences used in RT-PCR are listed in Table 2.

Western blot analysis

Cell lysates were used for Western blot analysis to demonstrate changes in the protein level of EZH2, VEGF-A, AKT and phosphorylated Ser^{473}-AKT. Details are described in the Supplementary Materials and Methods.

In vivo *studies of tumorigenicity*

Male balb/c nude mice were kept in the Animal Center of Nanfang Hospital, Guangzhou, China according to the policies of the Committee for Animal Usage. A549/EGFP-EZH2 and A549/EGFP-N1 cells (2×10^6 cells/ml) were respectively injected subcutaneously into the left flanks of ten balb/c nude mice. GSK126 was administered to mice generated with A549/EGFP-EZH2 cells to analyze the inhibitory effect of EZH2 on tumor growth. *In vivo* tumor growth and tumor volume (mm^3) were evaluated. Details are described in the Supplementary Materials and Methods.