Biomedicine & Pharmacotherapy 103 (2018) 399–405

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

CDK16 overexpressed in non-small cell lung cancer and regulates cancer cell growth and apoptosis via a p27-dependent mechanism

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ARTICLE INFO

Keywords:
Lung cancer
NSCLC
Cyclin-dependent kinase 16
CDK16
Cyclin-dependent kinase inhibitor 1B

ABSTRACT

Cyclin-dependent kinase 16 (CDK16, PCTAIRE1) expression is upregulated in a wide variety of human malignancies. However, the function(s) of CDK16 in non-small cell lung cancer (NSCLC) remain unknown. Therefore, here we investigated the role of CDK16 in NSCLC. From 43 NSCLC tumors and matching healthy control lung tissues, immunohistochemistry revealed significantly greater CDK16 and phospho-p27^{Ser10} staining levels in NSCLC samples relative to healthy controls. The NSCLC cell line EKVX was transfected with a control siRNA, a CDK16-siRNA, or CDK16-siRNA + p27-siRNA. We found significantly decreased proliferation levels and significantly increased apoptosis levels in CDK16-silenced NSCLC cells. However, these effects were abrogated in cells treated with both the CDK16-siRNA and the p27-siRNA. In CDK16-silenced NSCLC cells, we found upregulated p27 and downregulated phospho-p27^{Ser10} protein expression but downregulated ubiquitinated p27 and ubiquitinated phospho-p27^{Ser10} protein expression. Cycloheximide-treated CDK16-silenced NSCLC cells displayed a much milder reduction in p27 protein expression over time relative to untreated CDK16-silenced NSCLC cells. In summary, CDK16 is significantly upregulated in human NSCLC tumor tissue and plays an oncogenic role in NSCLC cells via promoting cell proliferation and inhibiting apoptosis in a p27-dependent manner. Moreover, CDK16 negatively regulates expression of the p27 via ubiquination and protein degradation.

1. Introduction

Lung cancer is one of the most common type of cancer and the leading cause of death among malignancies worldwide [1–3]. Non-small cell lung cancer (NSCLC) is the most common lung malignancy, accounting for over 80% of all lung cancer cases [1]. Unfortunately, the mortality rates associated with NSCLC have remained unchanged over the last 30 years, indicating a pressing need for a better understanding of this devastating condition [1–3].

Cyclin-dependent kinases (CDKs) are key enzymes that regulate cell cycle transitions in eukaryotic cells [4]. Many cancers, including NSCLC, exhibit features of cell cycle dysregulation, and numerous chemotherapeutics targeting CDK activity (e.g., CDK4 and CDK6) in cancer cells have been introduced over the past few decades [4,5]. One lesser-known CDK – cyclin-dependent kinase 16 (CDK16, PCTAIRE1, PCTK1) – has been shown to be critically involved in neuronal vesicular transport and spermatogenesis [6–8]. The central kinase domain of CDK16 is similar to other CDKs; however, the N- and C-terminal regions are unique, and their functions remain unknown. That being said, structural analysis suggests that CDK16 may be involved in the phosphorylation of other kinases [9]. Through yeast two-hybrid screening, researchers have identified that CDK16 interacts directly with cyclin-dependent kinase inhibitor 1B (CDKN1B, p27, Kip1), a key cell cycle regulator and tumor suppressor [10–12]. Specifically, CDK16 negatively regulates p27 through phosphorylation of the Ser10 residue on p27, an action facilitating p27 degradation [13,14]. These findings suggest that CDK16 may play an important role in cell cycle regulation and oncogenesis.

With respect to cancer, CDK16 expression is significantly upregulated in a wide variety of human malignancies, including breast cancer, ovarian cancer, prostate cancer, hepatocellular carcinoma (HCC), colon cancer, brain cancer, melanoma, and squamous cell carcinoma (SCC) [11,12,15,16]. Furthermore, significant positive correlations between CDK16 expression and tumor invasiveness (or aggressive phenotype) have been observed in ovarian cancer, prostate cancer, and HCC [10,12,16]. Despite this previous evidence linking CDK16 to

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https://doi.org/10.1016/j.biopha.2018.04.080
Received 29 November 2017; Received in revised form 2 April 2018; Accepted 10 April 2018

oncogenesis, the function(s) of CDK16 in NSCLC remain unknown. Therefore, here we investigated the role of CDK16 in NSCLC using both primary human NSCLC tumor cells and the NSCLC cell line EKVX.

2. Materials and methods

2.1. Ethics statement

The Institutional Review Board (IRB) of the First Affiliated Hospital of Bengbu Medical College (Bengbu, China) approved the protocols of this study. All patients have provided informed consent prior to inclusion in this study.

2.2. Patients and specimens

Surgical pneumonectomy or lobectomy were performed at the Department of Surgery at the First Affiliated Hospital of Bengbu Medical College. The paraffin-embedded tissue samples of lung tumor cores and matching healthy control lung tissue were stored at −80 °C immediately after surgical resection. Clinical records (Department of Pathology, the First Affiliated Hospital of Bengbu Medical College) for all lung tumor resection cases performed between January 2013 and December 2015 were screened. After screening, a total of 43 NSCLC cases (mean age: 56 years, range: 45–70 years; sex: 30 male and 13 female) were finally enrolled in this study.

2.3. EKVX cell line and siRNA transfection

The NSCLC cell line EKVX was obtained from the Cell Bank at the Chinese Academy of Sciences (Shanghai, China). As recommended, EKVX cells were grown in complete growth medium and cultured in a humidified incubator (5% CO_2) at 37 °C.

Pre-designed small interfering RNAs (siRNAs) against human CDK16 and p27 as well as a non-coding scrambled siRNA were purchased from Life Technologies (Carlsbad, CA, USA). For transfection, EKVX cells were seeded in six-well plates at a density of 5×10^5 cells per well, cultured overnight, and then transfected with the CDK16-siRNA alone or a combination of the CDK16-siRNA plus the p27-siRNA. The non-coding scrambled siRNA served as a negative control. According to the manufacturer's protocol, cells were transfected with 20 nM of each siRNA with the Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Validation of CDK16 silencing and p27 silencing in EKVX cells was validated by immunoblotting (Supplementary Fig. S1, Supplementary Information).

2.4. Immunohistochemistry and immunoreactivity scoring

Specimens had been fixed in 10% formalin and embedded in paraffin wax immediately after resection. Three-micrometer sections were then cut from the paraffin blocks for immunohistochemical (IHC) analysis. The sections were stained with an anti-human CDK16 antibody (diluted 1:500, Invitrogen, Carlsbad, CA, USA) or an anti-human phospho-$p27^{Ser10}$ antibody (diluted 1:50, Invitrogen) at 4 °C overnight. Following application of the secondary antibody, the immunoreactions were visualized with the avidin-biotin-peroxidase complex method according to the manufacturer's recommendations (Vectastain Elite ABC Kit, Vector Labs, Burlingame, CA, USA). Secondary-only negative controls were used to control for non-specific binding.

Immunoreactivity scoring in IHC samples was performed as previously described by Fromowitz et al. with minor modifications [17]. Briefly, the formula for the immunoreactivity score was as follows: immunoreactivity score = staining intensity (SI) + percentage of positive cells (PP). SI was tabulated as follows: negative staining = 0; weak staining = 1; moderate staining = 2; and strong staining = 3. PP was tabulated as follows: 0% = 0; 1–20% positive cells = 1; 21–50% positive cells = 2; and 51–100% positive cells = 3. Ten visual fields were randomly chosen from different areas of each specimen for immunoreactivity scoring, and the average immunoreactivity score derived from the ten visual fields was calculated as the final value for each patient.

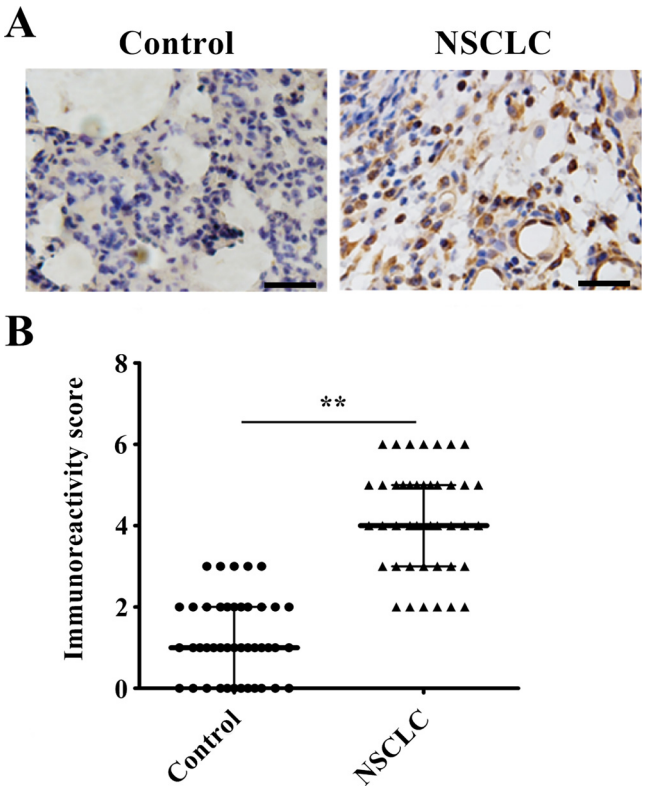

Fig. 1. CDK16 Upregulated in NSCLC Tumor Cells.
CDK16 protein expression was assessed by immunohistochemical analysis of tissue samples. (A) Representative images of healthy control lung tissue samples (left panel) and matching NSCLC tumor tissue samples (right panel) are provided. Scale bars, 50 μm. (B) CDK16 immunoreactivity scores were determined through blinded analysis. Box plots display the median (center line) and the interquartile range (top and bottom lines). $^{**}p < 0.01$ versus control group.

2.5. GFP fluorescence proliferation assay

Analysis of proliferation was determined using a Thermo Scientific Cellomics ArrayScan HCS reader (Thermo Fisher Scientific) with the Spot detector V3 Cellomics Bioapplication (Cellomics, Pittsburgh, PA, USA). GFP-positive cells were detected using the FITC channel at a 20× magnification, and images were acquired on a high-resolution charge-coupled device (CCD) camera on days one, two, three, four, and five (GRAS-14S5M; Point Grey, Richmond, Canada). Cells were identified based on the presence of nuclei and GFP fluorescence intensity. Cell count values were reported as the fold-change in cell count from day one using ArrayScan software (Thermo Fisher Scientific). A total of 1×10^3 cells were counted per sample, and experiments were repeated three times.

2.6. MTT proliferation assay

The capacity for cellular proliferation was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A total of 3×10^3 cells were seeded in 96-well culture plates for one, two, three, four, and five days. The cells were then incubated with 20 μl of MTT (5 mg/ml) for four hours at 37 °C, and 150 μl of DMSO was added to solubilize the crystals for 20 min at room temperature. The optical density was determined with a spectrophotometer (Shanghai