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mTOR up-regulation of BEX4 promotes lung adenocarcinoma cell proliferation by potentiating OCT4

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ABSTRACT

Previously, BEX family members have been reported to participate in cancer development. However, little is known about the role of BEX4 in lung adenocarcinoma (LAC). Here, we found that BEX4 was over-expressed in LAC tissues compared with adjacent tissues. LAC tissues from metastatic patients exhibited higher expression of BEX4 comparing to those from non-metastatic ones. *In vitro*, BEX4 ectopic expression accelerated the proliferation of both A549 and H1975 cells. By contrast, knockdown of BEX4 suppressed the proliferation of A549 and H1975 cells. BEX4 positively regulated the expression of OCT4, silencing of which reduced the proliferation of A549 and H1975 cells with over-expressed BEX4. Additionally, mTOR activation, which is frequently observed in LAC, potentiated BEX4 depending on mTORC1 but not mTORC2. BEX4 abundance dictated the sensitivity of A549 and H1975 cells to rapamycin treatment. Our findings reveal that BEX4 is an oncogene in LAC and may contribute to the hyper-active mTOR-induced LAC development.

1. Introduction

Lung cancer is the leading cause of cancer-related death worldwide [1]. The malignant types of lung cancer are generally comprised of non–small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Lung adenocarcinoma (LAC) is the commonest histologic subtype of NSCLC. For the past decades, numerous studies have identified critical driver genes for this disease. Small molecular inhibitors such as tyrosine kinase inhibitors (TKIs) of EGFR, have been used to treat this deadly disease [2–4]. However, the clinical outcomes of these inhibitors are far from satisfactory.

Brain-expressed X-linked (BEX) 4, one of the BEX family members, is abundant in heart, skeletal muscle, and liver [5]. Even though some studies have reported BEX2 as an oncogene in glioma, breast cancer and colorectal cancer [6–8], the role of BEX4 in tumorigenesis remains controversial. Over-expression of BEX4 causes increased proliferation and growth potential of tumors [9]. However, evidences have shown that BEX4 may act as a tumor suppressor in ovarian cancer and oral squamous cell carcinoma [10,11]. Still, the role of BEX4 in LAC remains unclear.

mTOR is a 290 kDa serine/threonine kinase that presents as a member of mTORC1 and mTORC2 [12]. It plays an important role in regulating cell metabolism, growth and proliferation [13,14]. Activation of mTOR signaling pathway is commonly found in various cancers, such as hepatocellular carcinoma and LAC [15] [16], mainly due to dys-regulation of upstream tumor suppressors or oncogenes. Although numerous downstream targets have been identified to participate mTOR-induced tumorigenesis, the correlation between mTOR and BEX4 remains unclear.

In this study, we identified BEX4 as an oncogene in LAC. BEX4 was over-expressed in LAC tissues. Using knockdown or over-expressing strategy, we found that BEX4 promoted the proliferation and growth of LAC cells through up-regulation of OCT4. BEX4 was stimulated by mTOR activation and its abundance dictated the sensitivity of LAC cells to rapamycin treatment. Our study revealed the mTOR/BEX4/OCT4 cascade in LAC.

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2. Materials and methods

2.1. Patients

In this study, all the LAC patients were enrolled from 2014 to 2017 at Cancer Hospital of Chinese Academy of Medical Science and Peking Union Medical College (CAMS&PUMC). The LAC and adjacent tissues were used for Western blot and qRT-PCR analysis of BEX4. The tumor tissues from patients with or without metastasis were subjected to qRT-PCR analysis of BEX4. A written form of consent for this study was provided from all the patients. This study was approved by the Clinical Research Ethics Committee of CAMS&PUMC.

2.2. Cell culture

Human lung epithelial cells HBE, LAC cells A549 and H1975 and lung cancer cells NCI–H520 were obtained from American Type Culture Collection (Manassas, VA, USA). All the cells were cultured Dulbecco modified Eagle's medium (DMEM), supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were maintained in a 37 °C incubator containing 5% CO_2.

2.3. BEX4 over-expression

The coding sequence of BEX4 were synthesized from Shanghai Generay Biotech Company and inserted into pCDH lentivirus vector. Empty control (Ctrl) and BEX4 pCDH vector were co-transfected with packaging vectors (PSPAX2 and PDM2G) into 293T cells. 72 h later, lentivirus supernatants were collected from the culture medium and filtered through a 0.45 μm filter. Then the virus was used to infect A549 and H1975 cells. The infection efficiency was determined by qRT-PCR and Western blot assays.

2.4. Knockdown assay

pLL3.7 lentivirus system was used for knocking down indicated genes in LAC cells. The shRNA sequences were as follow: BEX4 forward: 5′- TGCACTATATGCGCTTCCAATTCAAGAGATTGGAA GCGCATATAGTGCTTTTTTC -3′, BEX4 reverse: 5′- TCGAGAAA AAAGCACTATATGCGCTTCCAATCTCTTGAATTGGAAGCGCATATAGT GCA -3'. TSC2 forward: 5′-TGCTCTCTGCTCCATGCTTTTTCAAGA-GAAAAGCATGGAGCAGAGAGCTTTTTTC-3′, TSC2 reverse: 5′-TCGA-GAAAAAAGCTCTCTGCTCCATGCTTTTCTCTTGAAAAAGCATGGAGCA-GAGAGCA-3'. pLL3.7-shRNA and packaging vectors (VSVG, REV, and pMDL) were co-transfected into 293T cells. 48 h after transfection, virus supernatants were collected, filtered with a 0.45 μm filter and then used for infecting indicated cells. The infection efficiency was analyzed by the expression of green fluorescent protein and mRNA or Protein abundance of indicated genes.

2.5. Small RNA interference

The siRNA oligonucleotides against Raptor, Rictor, OCT4 and negative control were synthesized from GenePharma (Shanghai, China). Indicated cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen). The siRNA sequences were as follow: Negative Control (NC): 5′–UUCUCCGAACGUGUCACGU-3'; Raptor: 5′-GGAUCGAUCCUCUGUCGAU-3'; Rictor: 5′–UCAGGAGCU UAAGACCUAC-3'; OCT4: 5′-AAGGAUGUGGUCCGAGUGUGG-3'.

2.6. Cell proliferation analysis

CCK kit was used for cell proliferation analysis. In brief, a total of 3000–5000 indicated cells were in triplicate seeded into 96-well plates containing 200 μl culture medium. 0, 24, 48 or 72 h after seeding, 20 μl CCK solution was added into each well and incubated at 37 °C for 2 h. Then the spectrometric absorbance at 450 nm was analyzed on a microplate reader. The cell number at 0 h was recognized as 100. The cell number of indicated time was calculated as OD value of indicated time/OD value of 0 h * 100.

2.7. Colony formation assay

Equal number of control or BEX4 over-expressed A549 cells were seeded in triplicate in 10 cm plates. 12 days after culture, the culture medium was removed and the plates were washed with PBS for three times. Then the plates were fixed with methanol for 30 min and stained with 0.1% crystal violet for 20 min. The colonies were photographed by a Nikon camera.

2.8. Western blot

RIPA buffer (Beyotime) containing proteinase inhibitor and phosphatase inhibitor cocktail (Roche) were used for protein extraction from indicated cells. The concentration of the proteins was determined by BCA protein assay kit (Thermo Fisher). A total of 40–60 μg proteins were separated on a 12% odium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membranes. After blocking with 5% skim milk for 1 h at room temperature, the membranes were incubated with primary antibodies at 4 °C overnight. The membranes were washed with PBST for three times and incubated with HRP-conjugated secondary antibodies at room temperature for 2 h and then subjected to chemiluminesence analysis using the ECL-Plus kit (Amersham Biosciences). Antibodies against BEX4, OCT4 and GAPDH were from Abcam. Antibodies against TSC2, p-mTOR, mTOR, p-S6, S6, Raptor, Rictor were from Cell Signaling. β-actin primary antibody and all the secondary antibodies were from Santa Cruz.

2.9. Total RNA isolation and quantitative real-time PCR

Total RNA of indicated cells was isolated using Trizol reagent (Invitrogen) and the RNeasy Mini kit (QIAGEN), following the manufacturer's protocol. A total of 0.5–1 μg RNA was subjected to reverse transcription using M-MLV reverse transcriptase (Promega). mRNA expression of indicated genes were determined by quantitative real-time PCR using TransStart Top Green qPCR SuperMix (TransGen Biotech). The primer sequences are as follows: BEX4 forward, 5′-AAAGAGGAACTAGCGGCAAAC-3′, and reverse, 5′–CCAAATGGCGGGATTCTTCTTC-3'; OCT4 forward, 5′- CAAAGCA-GAAACCCTCGTGC-3′, and reverse, 5′-AACCACACTCGGACCACTCG-3'; and β-actin forward, 5′-CATGTACGTTGCTATCCAGGC-3′, and reverse, 5′-CTCCTTAATGTCACGCACGAT-3'. β-actin serves as an internal control.

2.10. Statistical analysis

All the statistical data as shown were mean ± standard error of mean (SEM) of at least three independent repeats. The