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Original article

Long non-coding RNA BANCR promotes proliferation and migration of lung carcinoma via MAPK pathways

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ABSTRACT

Lung carcinoma (LC) is one of the most mortal malignant tumors, and is becoming one of most lethal threat to human health and life. LncRNAs, emerging non-coding RNAs but poorly understood, are involved in the proliferation, metastasis, infiltration and apoptosis of LC. In this study, an lncRNA BANCR in LC cells was chosen to investigate the effect on LC cells, and clarify the possible mechanism. The results showed that BANCR levels were downregulated in LC cells. When BANCR expression was improved by tranfection with pcDNA-BANCR vector, tumor growth was suppressed. Vise versa, when BANCR was knockdown by si-BANCR, cell proliferation and migration of LC were remarkably promoted. We further found that MAPK pathways were involved in the BANCR-mediated cell proliferation and migration of LC. Moreover, BANCR was found to regulate LC proliferation and migration via not ERK MAPK, but p38 MAPK and JNK inactivations. These findings not only suggested that BANCR may be a new target for LC chemotherapy in future, but also will help us to fully understand the oncogenesis of LC.

1. Introduction

Lung carcinoma (LC) occurs in epithelia of tunica mucosa bronchiorum, which is also called bronchiolar carcinoma. It is one of the malignant tumors that with the fastest growth rate of morbidity and mortality, and it is becoming one of most lethal threat to human health and life [1]. In recent 50 years, morbidity of lung cancer is reported to rise greatly in many countries, especially in male patients, for whom lung cancer morbidity has been the first in all types of cancers [2,3]. Although its pathogenesis has not been fully understood, long-term heavy smoking has been manifested to be the most dominant pathogenic factor according to mass data [2,4].

Non-coding RNAs are those RNAs that don't encode any proteins, including rRNA, tRNA, snRNA, snoRNA, microRNA and many other RNAs with already-known functions, and long non-coding RNAs (lncRNAs) with unknown functions [5]. What the characteristics they share is that these RNAs are able to be transcripted, but cannot be translated into proteins, but they exert their respective biological functions at RNA level [6]. The length of the lncRNA doesn't exceed 200 nt long, and doesn't encode proteins as well. They are different from tRNA, rRNA and other housekeeper RNAs, and their functional mechanisms are distinct from those of microRNAs as well. According to their relationships with mRNA, lncRNAs are divided into five categories:

- sense;
- antisense;
- bidirectional;
- intronic;
- intergenic [7].

LncRNAs are involved in the genesis, development, proliferation, metastasis, infiltration and apoptosis of tumors [8]. It is reported that an lncRNA BANCR had close relations with tumor metastasis in LC and MAPK pathways in melanoma [9,10]. However, the significance of BANCR in LC is unknown and whether the cell proliferation and migration are associated with MAPK signaling in LC are still not clear. And inspired by the literature [9,10], we chose BANCR in LC cells to investigate its effect on cell proliferation and migration of LC cells and the relations with MAPK cascades, and then clarify the possible mechanism.

2. Materials and methods

2.1. Tissues

Twelve paired LC tissues and their adjacent non-tumor tissues from the patients who underwent surgery at the Fourth

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Affiliated Hospital of China Medical University between 2012 and 2013. The patients were diagnosed with SCLC (extensive stage) or NSCLC (stage II). No local or systemic treatment was performed on the patients before operation. The tissue samples were collected and instantly snap-frozen in liquid nitrogen and stored in a −80 °C refrigerator until needed. Written informed consents from all patients were obtained. This research was approved by the Research Ethics Committee of China Medical University.

2.2. Cell lines and culture

Two types of SCLC cell lines (NCI-H1688 and NCI-H446), two types of NSCLC cell lines (A549 and SPC-A1) and normal human bronchial epithelial cell line 16HBE were purchased from American Type Culture Collection. They were maintained and cultured in RPMI-1640 medium with 10% fetal bovine serum (Hyclone, USA) and 100 IU penicillin and 100 μg/ml streptomycin (Hyclone, USA) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. RNA extraction and real-time quantitative PCR

Total RNA was extracted from tissues or cells using RNeasy Plus Mini Kit (Qiagen, Germany) according the manufacturer's protocol. BANCR expression levels were determined by SYBR Premix Ex Taq (Takara, China) in the light of the manufacturer's instructions. Results were normalized to the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Referring to the previous report by Sun et al. (2014) [9], primer sequences were designed as follows:

- BANCR;
- forward: 5′-ACAGGACTCCATGGCAAACG-3′;
- reverse: 5′-ATGAAGAAAGCCTGGTGCAGT-3′;
- GAPDH;
- forward: 5′-GGGAGCCAAAAGGGTCAT-3′;
- reverse: 5′-GAGTCCTTCCACGATACCAA-3′.

The data were processed using 2-DDCT method and normalized to GAPDH expression.

2.4. Plasmid construction and cell transfection

The BANCR sequence was subcloned into the pcDNA3.1 vector (Invitrogen, USA). BANCR Ectopic expression was achieved through pcDNA3.1-BANCR transfection using lipofectamine 2000 (Invitrogen, USA), with an empty pCDNA3.1 vector used as a control. The expression levels of BANCR were measured by quantitative PCR.

Plasmid vectors (pcDNA3.1-BANCR and pcDNA3.1) for transfection were extracted using Midiprep kits (Qiagen, Germany), and respectively transfected into NCI-H446 or SPC-A1 cells. The siRNAs (si-BANCR and si-NC) were respectively transfected into NCI-H446 or SPC-A1 cells. According to the manufacturer's instructions, fusion and transfection of NCI-H446 or SPC-A1 cells by lipofectamine 2000 (Invitrogen, USA) were performed when the cells were cultivated on six-well plates. After transfection for 48 h, cells were collected for cell proliferation and migration assays, and lysed for quantitative PCR or Western Blot analysis.

2.5. Protein extraction and Western Blot

Cells were lysed in RIPA (radioimmunoprecipitation assay) lysis buffer with protease inhibitors and phosphatase inhibitors. The concentration of proteins was determined using a protein assay kit (Bio-Rad, USA). The protein extracts were loaded onto a 10% sodium SDS-PAGE (dodecyl sulfate-polyacrylamide gel electrophoresis) gel and transferred to a PVDF (polyvinylidene fluoride) membrane. The blots were probed with primary antibodies (anti-p-p38, p-38, p-JNK, JNK, p-Raf-1, Raf-1, p-Erk1/2, Erk1/2 and β-actin antibodies, Cell Signaling Technology, USA,) followed by the horseradish peroxidase (HRP)-conjugated secondary antibody (BGI, China). The bands were visualized using EasyBlot ECL (enhanced chemiluminescence) kit (Shanghai Sangon Biotech, China). Beta-actin served as the loading control. Primary antibodies (1/1000 diluted) against p-p38, p-38, p-JNK, JNK, p-Raf-1, Raf-1, p-Erk1/2, Erk1/2 and β-actin.

2.6. Cell proliferation and migration assays

The proliferation of the cells was measured using a CCK-8 assay kit purchased from Beyotime (Shanghai, China). In each 96-well plate, the cells (1×10^5) were seeded and cultivated for 24 h, treated with or without JNK inhibitor SP600125 or p38 inhibitor SB203580, and further incubated for 24, 48 and 72 h respectively. After the addition of 10 μl CCK-8 reagent to each well, the plate was reincubated for 1 h, the absorbance was detected at a wavelength of 450 nm. In each treatment group, five wells were measured for cell proliferation, and all independent groups were performed in triplicate.

For the migration assay, 5×10^4 transfected or non-transfected cells suspended in serum-free media were put into the upper chamber of an 8-μm-pore insert (Nalge Nunc International, Rochester, NY) coated with Matrigel (BD Biosciences, San Diego, CA, USA). Complete medium containing 10% FBS was added into the lower chamber. After the cells were incubated for 48 h, cells migrated to the other side of the membrane were stained with methanol and 0.1% crystal violet. The cells remaining on the upper side were removed with cotton swab. The stained cells were then imaged, and counted with optical microscopy at 100 × magnification. All experiments were performed in triplicate.

2.7. Tumorigenicity assay

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Our protocol was approved by the Committee on the Ethics of Animal Experiments of the Fourth Affiliated Hospital of China Medical University (Permit Number: 201403). All operations on the mice were performed under sodium pentobarbital anesthesia (0.3%, 50 ∼ 60 mg/kg), and no effort was spared to minimize suffering.

Forty-five-week-old male BALB/c nude mice (20 ∼ 22 g), which were obtained from Animal Center of the Chinese Academy of Science (Shanghai, China), were randomly divided into 8 groups and grew under specific-pathogen-free conditions in the Animal Care Facility Service (the Fourth Affiliated Hospital of China Medical University). The mice were injected subcutaneously with 5×10^6 cells (NCI-H446 and SPC-A1 cells transfected with empty vector pcDNA3.1/plasmid pcDNA3.1-BANCR/si-NC/si-BANCR) into right flanks. Four weeks later, all the mice were sacrificed by 1% overdosed pentobarbital and exsanguination according to American Veterinary Medical Association guidelines on euthanasia, and the tumor mass was weighed.

2.8. Statistical analysis

All values were expressed as mean ± SD and processed by the DPS software (version 6.55). Differences among the groups were assessed by Student's *t*-test, and they were considered statistical significance if $P < 0.05$.