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Original article

LncRNA CCAT2 promotes tumorigenesis by over-expressed Pokemon in non-small cell lung cancer

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ABSTRACT

Background: Non-small cell lung cancer (NSCLC) remains one of the most important death-related diseases, with poor effective diagnosis and less therapeutic biomarkers. LncRNA colon cancer-associated transcript 2 (CCAT2) was identified as an oncogenic lncRNA and over-expressed in many tumor cells. The aims of this study were to detect the correlation between CCAT2 and its regulatory genes and then explore the potential mechanism between them in NSCLC.
Methods: In this study, qRT-PCR was used to detect CCAT2, Pokemon and p21 expression. Western-blot was used to detect protein levels of Pokemon and p21. CCK-8 assay and Transwell chambers were used to assess cell viability and invasion.
Results: CCAT2 and Pokemon were over-expressed in NSCLC tissue and cells. In NSCLC cells, CCAT2 knockdown significantly decreased cell viability and invasion as well as Pokemon expression, but increased the expression of p21; then CCAT2 overexpression revealed an opposite result. In addition, over-expressed Pokemon reversed the results that induced by si-CCAT2, while down-regulation of Pokemon significantly reversed the results that induced by CCAT2 overexpression.
Conclusion: The results indicated that CCAT2 promotes tumorigenesis by over-expression of Pokemon, and the potential mechanism might relate to the Pokemon related gene p21.

1. Introduction

Lung cancer has been reported as the leading cause of cancer-related deaths worldwide and was a high incidence disease in any age [1]. Non-small cell lung cancer (NSCLC) is a category of lung cancer that is different from small cell lung cancer (SCLC), which accounting for about 85% of all lung cancer cases [2]. Since most NSCLC patients were diagnosed at advanced stages, which increased the mortality rate. Despite several improvements have made in chemotherapy, radiotherapy and surgical methods for NSCLC therapy over the past years, the 5 year alive was still no more than 15% [3,4]. Thus, reliable and adequate prognostic biomarkers combining with targeted molecular therapies are essential for NSCLC therapy.

Long non-coding RNA (LncRNA) is a set of RNAs that without the function of encoding protein, but can regulate gene expression at chromatin modification, transcriptional or posttranscriptional level [5]. Several reports have verified the biological functions of lncRNA in several cancers, such as ovarian cancer [6], renal cell cancer [7], gastric cancer [8], prostate cancer [9], hepatocellular carcinoma [10] as well as in lung cancer [11–13]. Moreover, the dysregulation of lncRNA was harmful in human health, which was found to participate in tumor genesis and progression [14].

Colon cancer-associated transcript 2 (CCAT2) is a novel lncRNA that located in 8q24 gene desert and was first discovered in colon cancer in 2013 [15]. Reports have investigated that CCAT2 might considered as a metastatic biomarker and play a vital role on tumor genesis and growth. For example, Ling et al. suggested that the expression level of CCAT2 was increased in colorectal cancer, which also affected tumor growth and metastasis [15]; another study revealed that over-expressed CCAT2 resulted in improvement of cell migration in breast cancer [16]. In NSCLC, CCAT2 was considered as a lung adenocarcinoma-specific lncRNA that promoted invasion of NSCLC, and acted as an important biomarker for lymph node metastasis [17].

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POK erythroid myeloid ontogenic factor (Pokemon), also known as transcription factor poken, is located at 19p13.3. Pokemon belongs to POK family, and play an important role in tumor genesis. Data has proved that Pokemon was over-expressed in many tumors, such as breast cancer [18], liver cancer [19], malignant glioma [20]. Whether Pokemon regulated by CCAT2 plays an important role on tumorigenesis of NSCLC, and the potential mechanism between them was still obscure.

In the present study, lncRNA CCAT2 combining with Pokemon was investigated in NSCLC, our aims were to explore the role of Pokemon regulated by lncRNA CCAT2 in NSCLC, and the potential mechanism in regulating tumorigenesis.

2. Materials and methods

2.1. Patients and tissue samples

Twenty non-small cell lung cancer patients were collected at the First Affiliated Hospital of Harbin Medical University. Clinical NSCLC tissues and adjacent normal tissues obtained from the NSCLC patients were immediately frozen in liquid nitrogen, and then used for RNA extraction. The study was approved by Research Ethics Committee, and all participants signed informed consents.

2.2. Cell lines and cell culture

NSCLC cell lines Pc-9,H358,H1975 and normal cell line HBE were purchased from Procell Biotechnology company, Wuhan, China. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing of 10% fetal bovine serum, 100IU/mL penicillin, and 100 mg/mL streptomycin with an atmosphere of 5% CO2 at 37 °C.

2.3. Cell transfection

For transfection, cell line H1975 was transfected with si-CCAT2, si-control, si-CCAT2 + pcDNA and si-CCAT2 + pcDNA-Pokemon individually, and cell line Pc-9 was transfected with pcDNA, pcDNA-CCAT2, pcDNA-CCAT2 + si-control, pcDNA-CCAT2 + si-Pokemon, individually. Lipofectamine 2000(Invitrogen, Shanghai, China) was used for the transfecting according to the manufacturer's instruction.

2.4. RNA extraction and quantitative RT-PCR

Total RNA was extracted from tissues and cell lines by using of TRIzol regent (Invitrogen, USA) according to the manufacturer's protocol. Nanodrop 2000 (Eppendorf) was used for quantifying RNA quality. Then, 2 μg RNA was taken out for synthesis of cDNA with a manufacture of RevertAidHMinus First Strand cDNA synthesis kit (Fermentas, USA). Real-time PCR was carried out on ABI 7900 system (Applied Biosystems, Foster City, CA, USA). GAPDH was regarded as internal control. All mRNA levels were calculated using the $2^{-D\Delta Ct}$ method. Each experiment was performed in triplicate. The results were analyzed by Mx3000P real-time PCR software version 2.00.

2.5. Westernblot

Cell protein was extracted using RIPA protein extraction reagent (Beyotime, China) according to the manufacture's instruction. The quantitation was determined by a Bio-Rad protein assay kit. 10% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) was used for separating protein extracts, and the protein was then transferred to the PVDF membranes (Sigma) and incubated with specific antibodies for 24 h, after that, a peroxidase-linked secondary antibody was added onto the PVDF membranes and incubated for another 2 h. Enhanced chemiluminescence was used to visualize the bands and the NIH ImageJ software was used to quantify the protein levels. β-actin was recognized as the internal control.

2.6. Cell viability

Cell Counting Kit-8(CCK-8, sigma, USA) assay was used to determine cell viability after transfection according to the manufacturer's instructions. A total of 1×10^5Cells were seeded in a 96-well plate and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS in humidified 5% CO_2 atmosphere at 37 °C for 48 h. The absorbance values were measured at OD = 450 nm with an ELx-800 Universal Microplate Reader. Each experiment was repeated in triplicate.

2.7. Cell invasion

Transwell chambers (8-μm pore size, Corning Life Sciences) were used for detecting of cell invasion assay. Briefly, a total of 2×10^5 cells was planted in the upper chamber with 200 μL of serum-free medium, while the lower chamber was filled with 800 μL of the medium supplemented with 20% FBS. After culturing for 24 h, the cells were fixed and stained, a phase-contrast inverted microscope was used for the following photograph.

2.8. Statistical analysis

Data was expressed as mean ± SD. Statistical analysis was carried out using SPSS 18.0 software. One-way analysis of variance (ANOVA) was used to determine the difference between groups. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. The expression of CCAT2 and Pokemon was over-expressed in NSCLS tissue

To investigate whether CCAT2 and Pokemon were involved in NSCLS, the relative mRNA and protein expression were detected in both NSCLC tissue (n = 20) and adjacent normal tissue(n = 20). As a result, significantly overexpressed CCAT2 and Pokemon mRNA were observed in NSCLC tissue rather than in adjacent normal tissue (Fig. 1A and B). Then the protein level of Pokemon was also detected, and the results were consistent with its mRNA level (Fig. 1C). The results indicated that the expression level of CCAT2 and Pokemon may be concerned with NSCLS.

3.2. Expression profile of CCAT2 and Pokemon in NSCLC cell lines

To determine whether CCAT2 and Pokemon were expressed in NSCLC, NSCLC cell lines of Pc-9, H358, H1975 and normal bronchial epithelial cell line HBE were used to detect the expression of CCAT2 and Pokemon. According to the normalized HBE, the expression of CCAT2 and the mRNA level of Pokemon were significantly up-regulated in NSCLC cell lines, and the cell line H1975 showed the highest expression level, while Pc-9 showed the lowest expression level (Fig. 2A and B). The protein expression of Pokemon in the four cell lines was accordance with the mRNA expression(Fig. 2C). The results indicated that the expression levels of CCAT2 and Pokemon were elevated in NSCLC cell lines. To reduce the errors among different cell lines, we chose H1975 and Pc-9 for the following experiments.