IncRNA BANCR suppresses cell viability and invasion and promotes apoptosis in non-small-cell lung cancer cells in vitro and in vivo

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Background: As a leading cause of deaths worldwide, lung cancer is a collection of diseases with diverse etiologies which includes non-small-cell lung cancer (NSCLC). Increasing evidence reported that aberrant expression of BRAF activated non-coding RNA (BANCR) was involved in the tumorigenesis and progression of various malignancies.

Purpose and methods: However, its role in NSCLC has not been completely clarified. In the present study, we identified the role of BANCR in the regulation of NSCLC cell viability, invasion, and apoptosis. Down-regulation of BANCR expression was significantly observed in different NSCLC cell lines (A549, H1299, H1650, H1975, SPC-A1, and PC-9), tumor tissue from NSCLC mouse model and 30 human NSCLC tissues compared with adjacent normal tissues.

Result: Overexpression of BANCR in these six NSCLC cell lines attenuated the cell viability and invasion. An increased apoptotic level caused by BANCR overexpression was also detected and displayed a reversed influence on Bcl-2 and Bax expression in mRNA and protein level. Furthermore, we identified the effect of BANCR overexpression on tumor growth in NSCLC mouse model. The restoration of BANCR expression inhibits NSCLC.

Conclusion: Taken together, our findings shed an insight on the novel molecular mechanisms of lung NSCLC oncogenesis and provide the information for new therapeutic approaches on the disease.

Keywords: NSCLC, non-coding RNA, BANCR, apoptosis, tumor growth

Introduction

Non-small-cell lung cancer (NSCLC) including adenocarcinomas and squamous cell carcinomas, ranks among the top 10 list with high prevalence and mortality worldwide. NSCLC was a highly malignant tumor with severe poor prognosis, distant invasion and migration.1 Although the effect of invasion and migration has been increasingly concerned, it was still the overwhelming cause of mortality in patients with NSCLC.2 A comprehensive understanding of the mechanisms and relative signal pathways activated in NSCLC cells was thus essential for the development of novel anti-cancer therapeutic strategy on tumor-associated treatment.

Non-coding RNA (ncRNA), which does not encode any proteins, is a group of RNAs including rRNA, tRNA, snRNA, microRNA and many other RNAs with extensively known functions and long non-coding RNAs (lncRNAs) with unexplored functions.3 It was once thought that lncRNAs were merely a matter of transcriptional noise, and did not involve in the cellular life circles.4 However, accumulating reports showed that lncRNAs
participated in various cellular biological processes and might contribute in tumor development through promoting or suppressing cell proliferation, invasion, metastasis, apoptosis, and differentiation.\textsuperscript{5-9} Owing to their impact on certain cancers, lncRNAs can be divided into two categories: oncogenes and tumor suppressor. Overexpression of those oncogenic lncRNAs in normal epithelial cells might result in carcinogenesis,\textsuperscript{10,11} in contrast, down-regulation of tumor suppressor lncRNAs led to tumor formation.\textsuperscript{12,13} It is apparent that the tissue-specific expression phenomenon of \textit{BRAF-activated non-coding RNA} (BANCR) existed in various human cancers. Down-regulation of BANCR was associated with the progression of cancers and capable of modifying the tumor volume and weight, clinical stage and TNM stage of cancer patients. Notably, BANCR was responsible for viability, proliferation, migration, invasion, and apoptosis of cancer cells.

The aim of the study was to probe the effect of \textit{BANCR} on NSCLC development in vitro and in vivo. Furthermore, we displayed that BANCR expression change in different NSCLC cells presented an influence on their viability, metastasis, and apoptosis. An alteration of E-cadherin, N-cadherin, Vimentin, Bcl-2 and BAX at protein and RNA level, confirmed the function of BANCR on metastasis and apoptosis of NSCLC cells. Our study expanded the understanding of the role of BANCR as a NSCLC suppressor and might facilitate the development of lncRNA-targeted cancer diagnostics and therapeutics.

\textbf{Materials and method}

\textbf{Patients}

Twenty-seven cases of NSCLC patients in this study ranged from 25- to 65-year-old, with a mean of 53-year-old. These patients were diagnosed with NSCLC (stages I, 8 cases; II, 16 cases; and III, 3 cases.) based on histopathological evaluation. Clinicopathological characteristics, including TNM staging, were recorded. No local or systemic treatment was conducted in these patients before surgery. All collected tissue samples were immediately snap-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until required. The corresponding NSCLC para-neoplastic tissues were acquired at least 1.00 cm\textsuperscript{3} from the neoplastic tissue. All cases were initial pneumonectomies and randomly chosen from the pneumonectomies performed over a 1–2 years duration in the Hongqi Hospital, Mudanjia Medical college, P.R. China between April 2012 and April 2016. The experimental protocol was approved by the local Ethical Committee of the Hongqi Hospital, Mudanjia Medical college, P.R. China. Patients tissue samples were conducted in accordance with Declaration of Helsinki. Written informed consent for sample usage in this research was obtained from both the patients and clinicians. All samples were reviewed and diagnosed by two pathologists independently.

\textbf{Cells}

Six NSCLC adenocarcinoma cell lines (A549, SPC-A1, H1299, H1650, H1975, and PC-9) normal human pneumocytes 16HBE were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium or DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C and 5% CO\textsubscript{2}.

\textbf{Construction of expression vector for BANCR}

The \textit{BANCR} sequence was constructed into the pcDNA3.0 vector. Ectopic expression of BANCR was achieved through pcDNA3.0-BANCR transfection using lipofectamine-2000, with an empty pcDNA3.0 vector was served as a negative control (NC). The expression levels of BANCR were measured by real-time quantitative PCR.

\textbf{In vivo tumorigenesis in NSCLC mouse model}

Forty male BALB/c nude mice (20–22 g) were purchased from Animal Center of the Chinese Academy of Science. For mouse model establishment and tumor growth assay, a total number of $2 \times 10^6$ SPC-A1 cells transfected with empty vector pcDNA3.0 or plasmid pcDNA3.0-BANCR were firstly resuspended in PBS, and then subcutaneously injected into the right flank of nude mice (n=6 per group). Tumor length and width were measured every 3 days after injection. Four weeks later, tumor volume was calculated as length $\times$ (width\textsuperscript{2}/2). Mice were sacrificed at 28 days after the injection, and the tumors were weighed.

The animal experiment was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Scientific Ethics Committee of Mudanjia Medical college (permit number: MDJMC20160708005).

\textbf{Immunohistochemistry staining (IHC)}

Tumor tissues were embedded in paraffin and subjected to IHC staining. Tissues were deparaffinized and hydrated and then permeabilized in 0.5% Triton X-100 in PBS for 10 mins.
IHC staining for BRAF-antibody (1:250) was performed using the indirect avidin biotin-enhanced horseradish peroxidase method according to the manufacturer’s instructions (Vector Laboratories, Burlingame, CA, USA). After developing, all sections were observed by microscope (20x) and analyzed using the Image-Pro Premier software offline (v.9.0) program (Media Cybernetics).

CCK-8 assay
Cell viability of NSCLC cell lines was determined by Cell Counting Kit CCK-8/WST-8 assay, which was performed following standard procedure in a 96-well plate as manufacturer’s instructions. In brief, cells with a quantity of $5 \times 10^5$ cells/well were seeded in a 96-well plate and grown to 80% confluence. Then, either BANCR or its empty controls were transfected into NSCLC cell lines. At 0, 24, 48 and 72 hrs post-transfection, CCK-8 reagent was added into each well. After 1 hr of incubation, cellular viability was determined by measuring the absorbance of the converted dye at 450 nm.

BrdU immunofluorescence assay
A549, SPC-A1, H1299, H1650, H1975, and PC-9 cells were seeded on cover glasses placed in a 6-well plate. After transfection with BANCR vector or empty control for 48 hrs, BrdU stock solution at 10 mg/mL in PBS was diluted 1000 x in the culture medium and incubated for 60 mins. After washing with PBS, cells were fixed in 4% paraformaldehyde for 20 mins and permeabilized with 0.3% Triton X-100 for 10 mins. After blocking with 10% FBS for 1 hr, cells were incubated with a primary rabbit antibody against BrdU (1:200) overnight at 4°C, and then incubated with the corresponding secondary antibody coupled to a fluorescent marker, Cy3, at room temperature for 2 hr. After DAPI stain and PBS washing, the coverslips were mounted on to glass slides and visualized using a fluorescence microscope (Olympus 600 auto-biochemical analyzer; Olympus Corporation, Tokyo, Japan) with Image-Pro Plus software for image analysis, and 10 microscopic fields were taken for BrdU calculating.

Transwell migration assays
After 24 hrs of transfection, NSCLC cells were harvested by trypsinization and washed once with D-Hanks solution. To measure cell invasion, 8-µm pore size culture or Matrigel inserts were placed into the wells of 24-well plates. In the lower chamber, 400 µL F-12 containing 10% FBS and 20 ng/mL of HGF was added. Then, $1 \times 10^5$ cells were added to the upper chamber. After 20 hrs of incubation, the cells that migrated through the pores were stained with crystal violet and observed under the microscope.

Hoechst 33342 staining
The various NSCLC cells with a density of $5 \times 10^5$ cells/mL were plated in 12-well plates prior to transfection.

Annexin V-FITC/PI analysis
The A549, SPC-A1, H1299, H1650, H1975, and PC-9 cells were harvested at 48 hrs post transfection. Annexin V-FITC/PI apoptosis detection kit was utilized to detect cell apoptosis according to the manufacturer’s instructions, and the percentage of apoptotic cells was calculated using a Beckman Coulter FACS flow cytometer (Beckman Coulter).

Caspase-3/7 activity detection
Caspase-3/7 activity was measured using a synthetic rhodamine labeled caspase-3/7 substrate performed immediately after the detection of viability in the same wells.

Western blot
Cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, pH 8.0) supplemented with a protease inhibitor cocktail. The protein concentration was measured using a BCA Protein Quantitation Kit. Protein samples were separated by 10% SDS-PAGE and electroblotted onto 0.45 µm Immobilon polyvinylidene difluoride membranes. The membranes were blocked with 5% BSA in PBST for 1 hr at room temperature and incubated overnight at 4°C with the respective antibodies: rabbit anti-BRAF (1:2,500), rabbit anti-Bcl-2 (1:2,000), rabbit anti-Bax (1:1,000), or mouse
anti-GAPDH antibody (1:5,000). Membranes were incubated for 1 hr at room temperature with Amersham ECL peroxidase-linked secondary antibodies: goat anti-mouse IgG (1:10,000) or goat anti-rabbit IgG (1:10,000). WB immune-reactivity was detected using a Super Signal West Femto Maximum Sensitivity Substrate Kit (Thermo) with a C-DiGit Blot Scanner.

RNA extraction and real-time PCR
Total RNA was extracted from NSCLC cells after different treatments or lung tissues using Trizol reagent according to manufacturer’s instruction. The levels of BANCR were determined using SYBR Green incorporation on Roche LightCycler 480 Real-Time PCR system (Roche, Germany), with GAPDH as an internal control for BANCR, Bcl-2, and Bax. The sequences of primers used were listed as follows:

BANCR F: 5′-ACAGGACTCCATGGCAACG-3′,
BANCR R: 5′-ATGAAGAAAGCCTGTGCAAT-3′;
Bax F: 5′-CCCCGAGAGTTTTCGGAG-3′,
Bax R: 5′-CCAGCCCATATGTTCTCAGT-3′;
Bcl-2 F: 5′-CTTTGAGTTCGGTGGAAT-3′,
Bcl-2 R: 5′-GGGCCGTACAGTTCCACAAA-3′;
GAPDH F: 5′-GGAAAGCTGTGGCGTGAT-3′,
GAPDHR: 5′-AAGGTTGGAAGAATGGGAGTT-3′.

Quantitative real-time PCR was performed in 20 μL volumes with SYBR Green PCR Master Mix at 95°C for 10 mins and 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s, using Light Cycler 480. The amount of target (2−ΔΔCT) was obtained by normalizing to endogenous reference and relative to a calibrator (average of the control samples).

Statistical analysis
Results are presented as mean ± SD. Comparisons between groups were made by one-way ANOVA or two-tailed Student’s t test. Differences were considered statistically significant at P<0.05.

Result
BANCR expression in human and mouse NSCLC cells
To identify whether BANCR are critical regulators for NSCLC development, BANCR expression was examined in six different NSCLC cell lines (A549, H1975, H1299, H1650, SPC-A1, and PC-9), NSCLC mouse model and human NSCLC lung tissues. Real-time PCR and western blot (WB) for BANCR expression determination revealed that BANCR was significantly reduced in these six NSCLC cells (Figure 1A). To confirm the findings of these NSCLC cells, expression of BANCR was measured in the pneumonocytes of normal mouse and lung cancer cells from NSCLC mouse. We found that BANCR was also nearly undetectable in NSCLC tumors of mouse model, compared to these normal pneumonocytes in NC group (Figure 1B). In addition, the expression levels of BANCR were also evaluated from 30 human NSCLC lung tumor tissues and 15 adjacent normal tissues, BANCR expression deficiency was observed in human lung tissue during the study (Figure 1C). Generally, our data suggested that NSCLC cells or tissues displayed a decreased expressing of BANCR, comparing with normal cells or lung tissues.

Effect of BANCR overexpression on NSCLC cell viability
BANCR and NC were transfected into A549, H1975, H1299, H1650, SPC-A1, and PC-9 cells, respectively, to assess the capacity of BANCR on the growth and viability of NSCLC cells. The expression of BANCR was confirmed up-regulated in the above six cells after transfection with BANCR in comparison to cells transfected with NC by real-time PCR (Figure 2A). CCK-8 assay was performed to assess the proliferation of six cell lines. Compared with the NC group, BANCR caused a decreased cell growth rate with a concentration of 100 nM in the cells at 0, 24, 48, and 72 hrs after transfection (Figure 2B). BrdU immunofluorescence assay was also performed to measure the cell viability at 48 hrs after transfection. In contrast, BANCR transfection inhibited the NSCLC cells DNA synthesis by approximately 30%, 50%, 40%, 50%, and 30%, respectively, in comparison to NC group (Figure 2C). Totally, the BANCR overexpression reflected an inhibitory effect in a time-dependent manner in these cells.

BANCR mediates invasion of NSCLC cells
Compelling studies reported invasion of NSCLC cells was major issue for mortality during the NSCLC development and progression. To determine whether BANCR influenced the invasion of NSCLC cells, Transwell migration was carried out after transfection with the BANCR and NC plasmids transfection in A549, H1975, H1299, H1650, SPC-A1, and PC-9 cells. The results showed that abnormal overexpression of BANCR resulted in an obvious suppression of cell invasion in NSCLC cells as shown in the
Transwell migration assay. In Transwell migration assays, ectopic expressing of BANCR in cells presented an inhibitory ability on invasion of these six cell lines, especially for H1299, H1975, H1650, and SPC-A1 cells (Figure 3A, B). The results suggest that BANCR overexpression suppressed the invasive property of NSCLC cells in vitro.

**Ectopic overexpression of BANCR up-regulates apoptosis level of NSCLCs**

Reduced apoptotic activity in NSCLC cell lines was a crucial property of NSCLC. In the study, an evaluation whether BANCR played a vital role in NSCLC cellular apoptosis was performed. Hoechst 33342 staining and Annexin V-FITC/PI flow cytometry were performed in A549, H1975, H1299, H1650, SPC-A1, and PC-9 cells transfected with BANCR and other plasmids. The phenomenon of BANCR expressing cells revealed that a higher amounts of positive Hoechst 33342-stained cells at 48 hrs post-transfection, compared with control groups (Figure 4A, B). Meanwhile, overexpression of BANCR caused an increased apoptotic activity in NSCLC cell lines has been detected via flow cytometry examination (Figure 4C, D).

Because BANCR suppressed cell viability and promoted NSCLC cells apoptosis, its role in regulating the expression of apoptosis-related proteins has been further studied. Caspase-3 and caspase-7 are typical apoptotic markers; therefore, we tested the caspase-3/7 activity in these NSCLC cell lines to evaluate the effect of BANCR on apoptosis. The result showed that BANCR is able to induce the apoptosis and caspase activity at 72 hrs post-transfection (Figure 5A). Bcl-2 and Bax, typical anti- and pro-apoptotic proteins, were also examined by WB and real-time PCR. As shown in Figure 5B and C, BANCR transfection led to an
Figure 2 Effect of BANCR overexpression on NSCLC cell viability. (A) The expression of BANCR in each NSCLC cells transfected with BANCR expressing vector and empty plasmid was measured by real-time PCR. (B) The cell viability of six NSCLC cells was measured by CCK-8 assay at 0, 24, 48, and 72 hrs post transfection. (C) At 48 hrs post transfection, the cell proliferation of NSCLC cells expressing BANCR or not was determined by BrdU incorporation assays. Data in NC group was setting as 100%. Data represent mean ± SD.* \( P < 0.05 \), ** \( P < 0.01 \).

Abbreviations: BANCR, BRAF activated non-coding RNA; NC, negative control; NSCLC, non-small-cell lung cancer.

Figure 3 BANCR overexpression suppressed NSCLC cells invasion. After BANCR plasmids transfection, invasive capacity of six NSCLC cell lines was measured by Transwell migration assay (A and B). Representative data from three independent experiments represent mean ± SD. * \( P < 0.05 \), ** \( P < 0.01 \).

Abbreviations: BANCR, BRAF activated non-coding RNA; NC, negative control; NSCLC, non-small-cell lung cancer.
expression decline on Bcl-2, in contrast, it increased Bax expression when compared with NC group in both protein and mRNA level (Figure 5D, E). These findings also revealed a mechanism of how BANCR regulated the apoptosis and were coincided with previous findings.

**BANCR displays the strong therapeutic effect on NSCLC in mice**

To determine the effect of BANCR on xenograft tumor formation, BALB/c mice were subcutaneously injected with SPC-A1 cells constitutively expressing BANCR or normal SPC-A1 cells, and monitored daily for tumor
growth. At 28 days after the injection, these mice were sacrificed and the lung tissues were excised and weighed (Figure 6A). The data unraveled that the average volumes of BANCR-expressing tumors grew at a comparatively slower rate than the tumors in the control group, and the mean tumor weight was significantly less than those in control groups (Figure 6B and C).

**Discussion**

The development of tumor was a synergetic process of many oncogenes activation as well as tumor suppressor genes stimulation, for instance, epigenetic changes in the tumor pathogenesis. Although NSCLC has been extensively investigated in the previous studies, the underlying mechanisms of NSCLC were still poorly understood. During the study, we found a specific lncRNA, **BANCR**, which was down-regulated with its expression in various NSCLC cell lines. It showed the capacity of the inhibition in key properties of NSCLC cells. Overexpression of **BANCR** in six NSCLC cell lines (A549, H1975, H1299, H1650, SPC-A1, and PC-9 cells) inhibited cell viability, invasion and simultaneously promoted their apoptosis. In vivo experiment revealed that lung tissue of NSCLC mouse model injected with BANCR-expressing cells exhibited the robust therapeutic ability on NSCLC tumor growth. Our findings suggested that **BANCR** suppressed NSCLC development via regulation of cell viability, invasion, and apoptosis, in which provided evidence that **BANCR** can be served as therapeutic agent on NSCLC treatment.

MiRNAs and lncRNAs were regarded as two new categories of RNAs and formed an essential component among the repertoire of ncRNAs. Recently, accumulating studies have demonstrated the crucial roles of lncRNAs and miRNAs shed an insight into the carcinogenesis and cancer-associated molecular biology. Several lncRNAs enhanced the importance in lung cancer, such as HOTAIR, NEAT1, and PVT1. HOTAIR supported invasion and migration of lung cancer cell via interaction between chromatin remodeling factor LSH and HOTAIR to affect the ratio of FOXA1 to FOXA2. NSCLC development and progression was also proved to be promoted by NEAT1 via acting as a competing endogenous RNA for the hsa-miR-377-3p. lncRNA LINC00152 mediated NSCLC cell proliferation through inhibition of IL24 expression, which was regulated by binding with EZH2. Down-regulation of another lncRNA SNHG20 caused a significant inhibition of NSCLC cell proliferation and migration in vitro and suppression of NSCLC tumor growth in vivo. Among these oncogenic lncRNAs and tumor suppressor, lncRNA **BANCR** has been drawn sufficient attention. BANCR is frequently overexpressed or

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**Figure 5** Overexpression of **BANCR** regulated expression level of apoptosis-associated proteins. (A) **BANCR** overexpression increased Caspase-3/7 activity in six NSCLC cell lines. (B and D) Western blot and (C and E) real-time PCR were performed to assess the Bcl-2 and Bax expression in protein and mRNA level, which was regulated by **BANCR** overexpression. Data represent mean ± SD.*P<0.05, **P<0.01 versus NC group. **Abbreviations:** **BANCR**, BRAF activated non-coding RNA; **NC**, negative control; **NSCLC**, non-small-cell lung cancer.
down-regulated in the tumor tissues, and played bidirectional roles in the progression of cancers, such as endometrial cancer,20 esophageal squamous cell carcinoma,21 hepatocellular carcinoma,22 gastric cancer,23 retinoblastoma,24 colorectal cancer,25 melanoma.26 Down-regulation of BANCR was detected in 30 tissues of NSCLC patients in comparison with 12 adjacent normal lung tissues. The data concealed that the cell viability, metastasis, and apoptosis of A549, H1975, H1299, SPC-A1, and PC-9 cells were significantly inhibited after transfection with BANCR. Sun et al also found that BANCR expression was significantly decreased in 113 NSCLC tumor tissues compared with normal tissues. Their work demonstrated that BANCR expression was highly associated with larger tumor size, advanced pathological stage, and metastasis. Ectopic expression of BANCR impaired cell viability and invasion, leading to the inhibition of metastasis in vitro and in vivo.27 In our study, it mainly focused on the relation between BANCR expression and NSCLC cell apoptosis. BANCR Overexpression was shown to play a pivotal role in reinforcing the apoptotic level in different NSCLC cell lines and NSCLC tissue in vivo via the regulation of Bel-2 and BAX expression.

Conclusion

Taken together, our study revealed that a multi-functional anti-tumor effect of IncRNA BANCR in major malignant properties of three NSCLC cell lines and NSCLC progressing in a mouse model. Furthermore, the endogenous factors which BANCR targeted to and how it contributed to the NSCLC pathogenesis still remain unknown. Hence that, to screen and identify the targets for biotin-labeled BANCR in NSCLC cell lines, a modified HITS-CLIP method combined by RNA-Trap might be applied in our further investigation. This method would provide us a precise and comprehensive interpretation for the role of BANCR in NSCLC development.

Disclosure

The authors report no conflicts of interest in this work.

References


Figure 6. BANCR suppresses xenograft tumor formation. (A) SPC-A1 cells with stable expression of BANCR or not were injected subcutaneously into nude mice (n=8 per group). Representative photographs of nude mice at day 30 post-inoculation were displayed. Mice were sacrificed and tumor block were weighted at day 28 post inoculation. (B) Tumor growth curve during 28 days post inoculation was displayed. The volume of the largest tumor detected in the study was 0.82 cm^3 (C) Tumors were weighted after removal from each group.

Abbreviations: BANCR, BRAF activated non-coding RNA; NC, negative control.

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