

Long non-coding RNA BANCR contributes to cervical adenocarcinoma migration by affecting epithelial-mesenchymal transition

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Summary

Purpose of Investigation: The BRAF-activated non-coding RNA (BANCR) functions are both an oncogene and a tumor suppressor; however, little is known about the role of BANCR in the development of cervical adenocarcinoma. **Materials and Methods:** The authors investigated BANCR's role in cervical adenocarcinoma by assessing BANCR levels in cervical adenocarcinoma and matched adjacent normal tissues from nine patients using qRT-PCR. They also used lentiviral vectors to establish cervical adenocarcinoma cell lines to investigate the effects of BANCR knockdown on cancer cell proliferation, apoptosis, and migration. **Results:** RT-PCR results showed that BANCR was frequently overexpressed in cancer tissues and cervical adenocarcinoma Hela cells. Knockdown of BANCR inhibited the migration of Hela cells *in vitro*. Further investigation into the mechanisms responsible for the migration effects revealed that BANCR induced the epithelial-mesenchymal transition (EMT) in Hela cells. **Conclusion:** These results revealed the important role of BANCR in the molecular etiology of cervical adenocarcinoma and implied the potential application of BANCR in cervical adenocarcinoma therapy.

Key words: Cervical adenocarcinoma; Long non-coding RNA; BANCR; Migration; EMT.

Introduction

Cervical cancer is the third leading cause of cancer-related mortality among women worldwide [1]. Although adenocarcinoma is rare, relative to squamous cell carcinoma, among uterine cervical cancers, the number of cases has increased in recent years, particularly in young women due to insufficient detection of cervical adenocarcinoma precursor lesions with the Papanicolaou smear test, and the survival of patients with cervical adenocarcinoma is significantly poorer than that for patients with squamous cell carcinoma [2]. The high invasiveness of tumor cells is the main reason for poor prognosis in cervical adenocarcinoma. Many factors affect the invasiveness of tumors [3]. A wide variety of molecular markers have been evaluated as diagnostic tools in the identification of high-risk precursors of squamous cell carcinoma and adenocarcinoma and the association with cell adhesion molecules has attracted attention recently [4]. Therefore, identification of biomarkers specific for cervical adenocarcinoma is essential for early detection and improved prognosis.

Long non-coding RNAs (lncRNAs), a member of non-coding RNAs family, are greater than 200 nucleotides in length and have no code for proteins [5], but lncRNAs can regulate gene expression in diverse cellular processes, such as in cell growth, cell cycle, apoptosis, metabolism, and

cancer migration [6-9]. Functional lncRNAs can be used for cancer diagnosis and prognosis and serve as potential therapeutic targets, which may be considered as a new cancer diagnostic and therapeutic guideline in the future [10, 11]. BRAF-activated non-coding RNA (BANCR), a 693-bp lncRNA on chromosome 9 that was firstly identified by Flockhart *et al.*, has been suggested to have the capability to control malignant melanoma cells proliferation and migration [12]. Furthermore, BANCR is often overexpressed in various types of human cancer such as gastric cancer, colorectal cancer, and papillary thyroid carcinoma [13-15]. However, the expression pattern and biological functions of BANCR in cervical adenocarcinoma remains to be elucidated.

The epithelial-mesenchymal transition (EMT) is a key step towards cancer invasion and metastasis. From a molecular point of view, EMT is characterized by the loss of epithelial markers, such as E-cadherin and cytokeratins, and the upregulation of mesenchymal markers, including N-cadherin and vimentin [16].

In the present study, the authors clarified that BANCR expression levels are upregulated in cervical adenocarcinoma, which may contribute to cervical adenocarcinoma cells migration by inducing EMT.

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Materials and Methods

Nine specimens of human cervical adenocarcinoma tissues and adjacent normal tissues were obtained between March 2015 and January 2017 from this institute, with informed consent from the Clinical Medical College of Yangzhou University. All patients gave written informed consent and specimens were anonymized and handled according to the accepted ethical and legal standards. The diagnosis of cervical adenocarcinoma was histopathologically confirmed. No patient received preoperative treatment. Resected tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. The patients were staged clinically according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO). From the nine patients, two were microinvasive adenocarcinoma (Stage Ia) and seven were invasive adenocarcinoma (six cases were Ib and one case was IIa1). Human cervical adenocarcinoma Hela cells and normal cervical epithelial cells were obtained, maintained in recommended culture conditions, and incubated at 37°C in a 5% CO_2 atmosphere.

To quantitate mRNA expression, total RNA was extracted from clinical samples with RNAiso Plus. The isolated total RNA was reverse transcribed according to manufacturer instructions. For real-time polymerase chain reaction (PCR), a final volume of 20 μL reaction was performed by using a standard protocol and the SYBR Green PCR kit on a real-time PCR system. Real-time PCR was performed in triplicate, with no template controls. The $2^{-\Delta\Delta\text{CT}}$ method was used to determine relative gene expression levels with β -actin as the endogenous control to normalize the data. The primers used in this study for BANCR were: 5'-ACAGGACTCCATGGCAAACG-3' (forward) and 5'-ATGAAGAAAGCCTGGTGCAGT-3' (reverse). The primers for E-cadherin were: 5'-GTGTCAATCCAACGGAATGC-3' (forward) and 5'-TGGCGCATTGTAGTGTTC-3' (reverse). The primers for vimentin: 5'-ATGACCGCTT CGCAACTAC-3' (forward) and 5'-CGGGCTTTGTCGTTGGTTAG-3' (reverse). The primers for β -actin: 5'-AGAAATCTGGCACCACACC-3' (forward) and 5'-TAGCACAGCCTGGATA GCAA-3' (reverse). PCR was performed by using the following cycles: 95°C for 30 seconds, 40 cycles of 95°C for 5 seconds, 60°C for 31 seconds, and the dissociation stage: 95°C for 15 seconds, 60°C for one minute, and 95°C for 15 seconds.

Recombinant lentiviruses containing short hairpin shRNA-323 (LV-BANCR-323, GGAGTGGCGACTATAGCAAAC), shRNA-540 (LV-BANCR-540, GGACTCCATGGCAAACGTTGT), and a negative control (LV-NC) were purchased. Hela cells were infected with LV-BANCR-323, LV-BANCR-540, and LV-NC (MOI = 20). The supernatant was removed after 24 hours and fresh culture medium was added to the cells. The infection efficiency was confirmed by RT-PCR at 72 hours post-infection, and the cells were treated with 2 $\mu\text{g}/\text{mL}$ puromycin for two weeks.

Cell proliferation assays were performed by using cell counting kit. The cells were plated in triplicate in 96-well plates at 2×10^3 cells per well and cultured in the growth medium. The number of cells per well was measured by the absorbance at 450 nm at the indicated time-points, according to the manufacturer's instructions.

The cells (4×10^5) were seeded in six-well plates. After 24 hours, the cells were collected and incubated with Annexin V-fluorescein isothiocyanate and 7-amino-actinomycin D for 15 minutes in the dark and apoptosis was analyzed using a flow cytometer. The cell cycle was also analyzed subsequent to propidium iodide staining for 30 minutes.

The cells (6×10^5) were seeded in six-well plates. When the cells had grown by 90% to 100%, straight wounds were generated on

them by scraping them with a 100- μL pipette tip. The wells were marked across the wounded area to ensure documentation of the same region. The medium was replaced with a serum-free medium, and the cells were treated with a medium containing 1 mM mitomycin to inhibit cell division. Phase contrast images were recorded at the time of wounding 0 h and at 48 hours after wounding. Untreated cells served as controls.

In total, 4×10^4 cells were plated in medium without serum on a non-coated membrane in the top chamber (24-well insert; 8-mm pore size). The cells were plated on a medium without serum. A medium supplemented with serum was used as a chemotactic agent in the lower chamber. The cells were incubated for 48 hours, and cells that did not migrate through the pores were removed with a cotton swab. Cells on the lower surface of the membrane were stained with Crystal violet. Cell numbers were determined by counting the penetrating cells under a microscope in random fields (five fields per chamber). Each experiment was performed in triplicate.

Proteins were extracted with RIPA and equal amounts of protein were electrophoresed on a 6%, 10%, or 12% sodium dodecyl sulphate-polyacrylamide gel and subsequently transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for two hours. The membranes were incubated with the following primary antibodies at 4°C overnight: E-cadherin (1:1000 dilution), vimentin (1:1000 dilution), and GAPDH (1:10000). The membranes were then washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:5,000) at room temperature for two hours. Following three washes with TBST, the membranes were developed using ECL Plus, and exposed to X-ray film. GAPDH was used as an internal loading control.

Data were expressed as mean \pm standard error of mean (SEM) and analysed using SPSS 19.0. The significance differences between the groups was estimated by Student's *t*-test, Pearson's χ^2 -test, or one-way ANOVA, as appropriate. Two-sided *p*-values were calculated, and a probability level of < 0.05 was used to determine statistical significance.

Results

This study was primarily aimed to investigate whether BANCR is detectable and altered in nine pairs of cervical adenocarcinoma tissues compared with adjacent normal tissues. The RT-PCR results showed that BANCR expression was significantly higher in seven out of nine of the tumor tissues compared with the adjacent normal tissues. Additionally, BANCR expression in Hela cell line was upregulated compared with the normal cervical epithelial cells ($p < 0.05$; Figure 1).

To clarify the role of BANCR in cervical adenocarcinoma cells, the authors transduced LV-BANCR-323 and LV-BANCR-540 to Hela cells. The BANCR expression was significantly downregulated in Hela cells (Figure 2A). The CCK8 assays and flow cytometric analysis showed that BANCR-knockdown had no significant effect on the proliferation and apoptosis of the Hela cells ($p > 0.05$; Figures 2B and 2C). However, both the wound healing experiment (Figure 2D) and the Transwell migration assay (Figures 2E and 2F) showed that BANCR downregulation reduce Hela

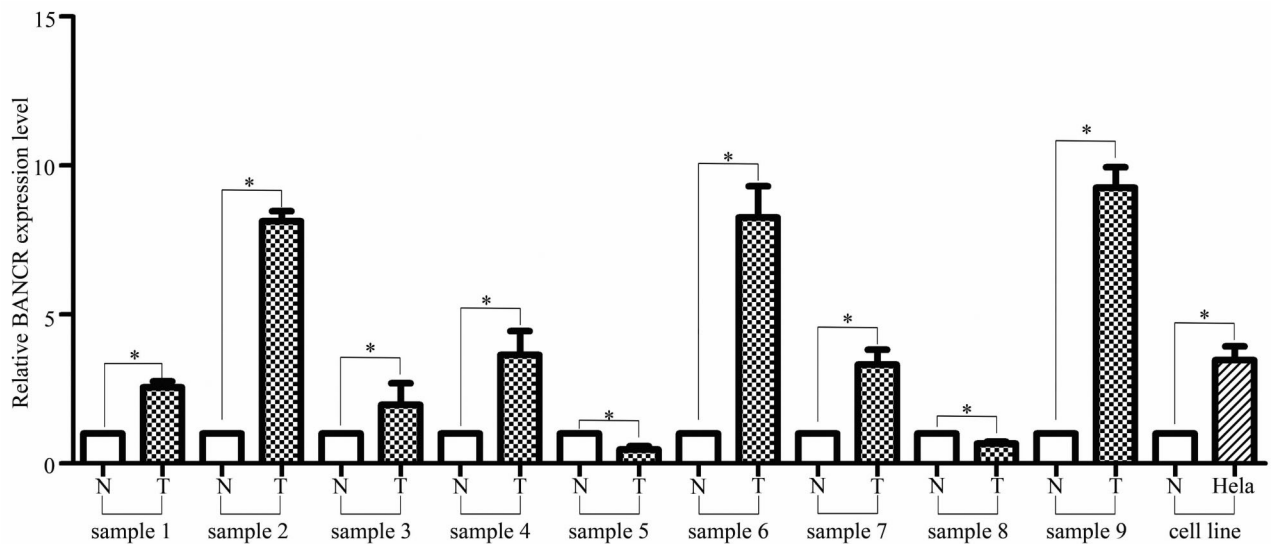


Figure 1. — BANCR levels are upregulated in cervical adenocarcinoma. RT-PCR results show that BANCR expression is significantly higher in seven out of nine of the tumor tissues compared with the adjacent normal tissues. The BANCR level in the Hela cell line is also upregulated compared with the normal cervical epithelial cells. BANCR expression levels are normalized to β -actin. Data are presented as the mean \pm standard deviation ($*p < 0.05$). BANCR: BRAF-activated long non-coding RNA; N: normal; T: tumorous tissue.

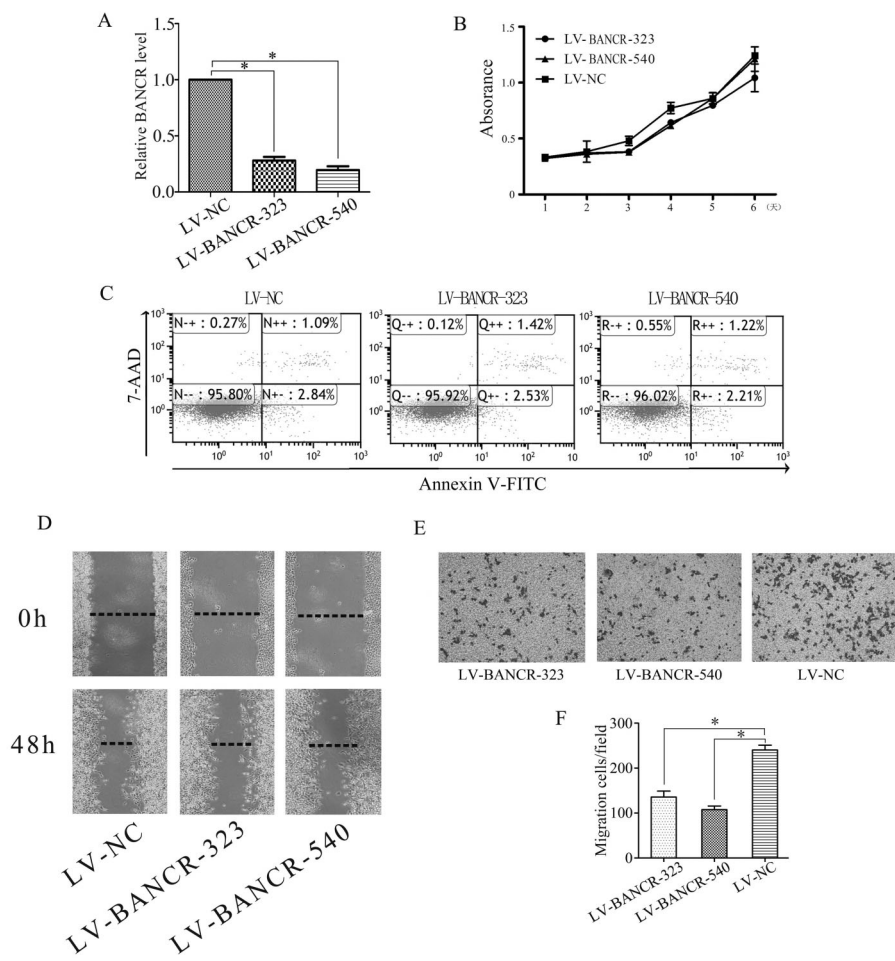


Figure 2. — Modulation of BANCR expression affects Hela cell migration. (A) Following treatment with LV-BANCR-323 and LV-BANCR-540, BANCR expression in Hela cell lines are down-regulated compared with those treated with LV-NC. (B) The Cell Counting Kit-8 assays shows that BANCR-knockdown has no significant effect on the proliferation of the Hela cells. (C) The flow cytometric analysis shows that BANCR-knockdown has no significant effect on the apoptosis of the Hela cells. (D) Wound healing experiments and (E and F) Transwell migration assays shows cell migration reduced in BANCR-downregulated cells (crystal violet stain; magnification, $\times 100$). The bar graph represents at least three independent experiments and the bars indicate the number of migrated cells per field. $*p < 0.05$. LV-BANCR-323, recombinant lentiviruses containing shRNA-323; LV-BANCR-540, recombinant lentiviruses containing shRNA-540; LV-NC, negative control; BANCR: BRAF-activated long non-coding RNA; shRNA: short hairpin RNA.

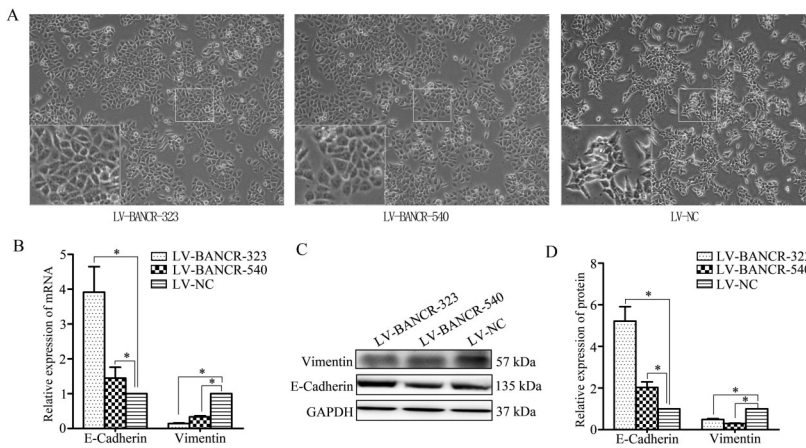


Figure 3. — BANCR induces epithelial-mesenchymal transition phenotypes. (A) Following treatment with LV-BANCR-323 and LV-BANCR-540, cells become round or oval, while LV-NC cells are spindle-shaped and similar to fibroblasts. (B) The downregulation of BANCR levels in Hela cells is associated with downregulated vimentin and upregulated E-cadherin both on the mRNA and protein levels. $*p < 0.05$. LV-BANCR-323, recombinant lentiviruses containing shRNA-323; LV-BANCR-540, recombinant lentiviruses containing shRNA-540; LV-NC, negative control; BANCR: BRAF-activated long non-coding RNA; shRNA: short hairpin RNA.

migration in Hela cells.

EMT is one of the key processes for primary tumor cells to acquire migratory capacity [16]. The authors found after the decrease of BANCR expression, the morphology of LV-BANCR-323 and LV-BANCR-540 cells became round or oval, while LV-NC cells were spindle shaped and similar to fibroblasts (Figure 3A). To define the role of BANCR in the progression of cell migration in cervical adenocarcinoma cells, the authors detected the changes in the expression of epithelial and mesenchymal makers after the BANCR expression level was modulated. The downregulation of BANCR levels in Hela cells was associated with downregulated vimentin and upregulated E-cadherin both on the mRNA and protein levels (Figure 3B).

Discussion

Identifying novel molecules that take part in cervical adenocarcinoma formation and progression may be helpful in improving the diagnosis, prevention, and treatment of this disease [17]. The relationship between lncRNAs and tumors has currently become one of the focuses of cancer studies. Abnormal expression of several lncRNAs has been reported in cervical cancer. For example, Zhang *et al.* found that lncRNA ANRIL suppressed cervical cancer cells proliferation, migration, and invasion by inactivation of PI3K/Akt signaling [18]. Cao *et al.* showed that GAS5 was downregulated in cervical cancer and associated with advanced tumor progression and poor OS [19]. Zhang *et al.* showed that the expression of lncRNA MEG3 was decreased in cervical cancer and MEG3 could suppress cervical cancer cell proliferation and induce cell apoptosis by regulating miR-21 expressions [20]. These findings suggested that lncRNAs might play important roles in cervical cancer initiation and development and have a great potential for clinical application.

In the present study, the authors observed BANCR ex-

pression levels were upregulated in seven out of nine cervical adenocarcinoma tissues compared with their adjacent normal tissues, although samples from only nine patients were used in the present study and the results may not be entirely accurate due to type I or II errors; the present data suggest a possible oncogenic role of BANCR in several human cancers. The present *in vitro* examination of the potential role of BANCR in cervical adenocarcinoma Hela cells demonstrated that the knockdown of BANCR was associated with the inhibition of cell migration. These findings revealed that BANCR might be involved in cervical adenocarcinoma progression and contribute to molecular-targeted therapy. Thus, the current results are consistent with those of previous studies, indicating that BANCR expression enhances the aggressive biological behaviour of cancer cells of various origins.

EMT processes characterised by diminished epithelial characteristics and increased mesenchymal attributes provide epithelial cells with enhanced migratory potential and are implicated in numerous physiological and pathological processes requiring cell migration. Recent studies are beginning to unravel the association of EMT with lncRNA. Ying *et al.* demonstrated that upregulated MALAT-1 contributes to bladder cancer cell migration by activating Wnt signaling and subsequently inducing EMT [21]. Luo *et al.* observed that lncRNA H19 increases bladder cancer metastasis by associating with EZH2 and inhibiting E-cadherin expression [22]. Xu *et al.* showed that the knockdown of lncRNA HOTAIR suppresses tumor invasion and reverses EMT in gastric cancer [23]. The present *in vitro* study confirmed that altered BANCR expression affects the expression of epithelial and mesenchymal markers, implying that BANCR possibly contributes to cervical adenocarcinoma migration by inducing EMT.

To the best of the present authors' knowledge, this paper is the first to report that BANCR is highly expressed in cervical adenocarcinoma and that BANCR is likely to be a

useful biomarker of cervical adenocarcinoma. Moreover, unravelling that BANCR induces cervical adenocarcinoma migration by inducing EMT adds to the understanding of the molecular mechanisms of BANCR. Most importantly, BANCR may be used as a potential molecular target to treat cervical adenocarcinoma.

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