Research Article

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BRAF-activated non-protein coding RNA (BANCR) advances the development of esophageal squamous cell carcinoma via cell cycle

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Abstract: Esophageal carcinoma is one of the important lethal tumors in the world, but the mechanism of esophageal cancer progression is still unclear. The long non-coding RNAs are known to regulate tumor progression. Here we report that BRAF-activated non-protein coding RNA (BANCR) is associated with the development of esophageal squamous cell carcinoma (ESCC). The expression levels of BANCR in 132 ESCC tumor tissues detected by quantitative real-time PCR were significantly higher than that in the adjacent normal tissues. The expression level of BANCR was down-regulated by transfecting small interrupting RNA against BANCR (BANCR siRNA) in Eca109 cells. Eca109 and TE-1 cells transfected with BANCR siRNA led to the delayed cell cycles compared to the cells transfected with negative control siRNA. Moreover, MTT assays demonstrated that Eca109 cells transfected with BANCR siRNA resulted in the inhibition of cell proliferation. These results suggested that BANCR advances the development and progression of ESCC cells through regulating their cell cycles.

Keywords: BANCR, Cell Cycle, ESCC

1 Introduction

Esophageal cancer is one of the main causes of death with higher incidence rates found in Eastern Asia [1]. Esophageal cancer is classified into two main types according to occurring at different parts of the body: one is squamous cell carcinoma and the other is adenocarcinoma. In the majority of cases (90%), most are squamous cell carcinomas [2, 3]. Currently, there are limited clinical approaches for the early diagnosis and treatment of ESCC, leading to a 10 % five-year survival rate for the patients [4-6].

Long non-coding RNAs (lncRNAs) usually contain more than 200 nucleotides and are involved in cancer cell proliferation and metastasis [7-9]. For example, lncRNA BCAR4 promotes breast cancer cell migration through binding SNIP1 and PNUTS [10]. Also, the up-regulation of lncRNA HNF1A-AS1 promotes tumor proliferation and metastasis in lung adenocarcinoma by regulating the expression of cyclin D1, E-cadherin and β-catenin [11].

BRAF-activated non-protein coding RNA (BANCR) was first identified by Flockhart RJ et al. via RNA sequencing in 2012. This RNA sequence is a 693bp long transcript on chromosome 9, which inhibits the progression of bladder cancer [12], and reduces the proliferation of papillary thyroid carcinoma cells [13]. Besides the tumor suppressing role, BANCR can also act as an oncogene, which promotes the proliferation of gastric cancer via regulating NF-kappaB [14, 15]. A recent study reported that the up-regulation of BANCR correlates with tumor progression and poor prognosis in ESCC cells [16].

In the present study, we analyzed the correlation between BANCR expression and ESCC progression. Flow cytometry results suggested that BANCR was involved in the regulation of cell cycles of the ESCC cells. Moreover, MTT data demonstrated that the inhibition of BANCR expression blocked the proliferation of ESCC cells. These results demonstrated that BANCR was involved in promoting ESCC cell proliferation.

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2 Materials and methods

2.1 Clinical specimen collection

A total of 132 pairs of ESCC tumor and adjacent normal tissues were obtained from patients who underwent surgical resection at the affiliated Wujin People’s Hospital of Jiangsu University and the affiliated Nanjing Drum Tower Hospital of Nanjing University. The documented clinicopathological characteristics were: age, gender, tumor size, differentiation, lymph node metastasis and TNM stage.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by Human Ethics Committee board of Jiangsu University and Nanjing University.

Informed consent: Informed consent has been obtained from all individuals included in this study prior to the research commencing with no patients receiving preoperative treatments.

2.2 Cell culture and treatment

Eca109 cell and TE-1 cell lines were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO₂ incubator. The cells were transfected with BANCR siRNA-1 (UAGCAACCACAUCAGCUUGGUUU), BANCR siRNA-2 (UAUAUCAACGUUUGCCAUUGGUC) and NC siRNA (the negative control) 100 pM respectively, according to manufacturer’s protocol.

2.3 RNA extraction and quantitative real-time PCR

Total RNA was extracted from tissues (Tumor and Adjacent normal tissues) and we cultured cells using TRIzol Reagent (Invitrogen) according to the manufacturer’s protocol. About 1 μg RNA was reverse-transcribed using Primescript RT reagent kit (Takara), qRT-PCR was performed by using SYBR green premix following the manufacturer’s instructions. BANCR expression levels were normalized against GAPDH expression in three independent experiments. The primers used for qRT-PCR include the following: BANCR forward primers: CTTCTTGTAGGGTCTGGATTGG; BANCR reversed primers: TAGGGTCAGGGGTCTTCAGT; GAPDH forward: GAAGGTGAAGGTCGGAGTC; GAPDH reverse: GAAGATGGTGATGGGATTTC.

2.4 Analysis of cell cycle by flow cytometry

Eca109 and TE-1 cells were harvested and approximately 1x10⁶ cells per treatment were re-suspended in 400 μl cold phosphate-buffer saline (PBS) in polystyrene tubes. Cells were then fixed by incubating the cell suspension with an equal volume of 75% ice cold ethanol for 1 hour on ice. Cells were then spun down and washed twice with ice cold PBS, and the cell pellets re-suspended with 500 μl PBS with added 0.1% RNase A (type I-A, Sigma Chemicals) for 30 min at 37°C. Cells were then stained with 10 μg/ml propidium iodide PI (Sigma Chemicals) for 30 min in the dark at room temperature. PI fluorescence measurements were then taken immediately using a flow cytometer as described in a previous publication [17]. Experiments were performed in triplicates.

2.5 MTT Assays

Eca109 cells were transfected with BANCR siRNA and NC siRNA for 48h and cells were then harvested and inoculated in 96 well plates. 20 μl MTT was then added to one group (5 wells per group) at different time intervals: 6h, 24h, 48h and 72h and incubated for 4 hours. Medium was then discarded and 150 μl DMSO was added and samples were then shaken for 15 min. The absorbance was tested using a Microplate reader at 490 nm absorbance [18]. The inhibition rate was calculated using the following formula: the percentage of the MTT inhibition = (NC group OD490 value – BANCR siRNA group OD490 value)/ NC group OD 490 value. Experiments were performed in triplicates.

2.6 Statistical analysis

All of the statistical analysis was performed using GraphPad Prism 5.1. The results are presented as the mean ± the standard error of the mean. All the experiments were performed at least three times. Correlation analysis was assessed by the Pearson correlation coefficient. The statistical significance of inter-group differences was calculated by the Student’s t-test.
3 Results

3.1 The expression of BANCR is positive correlated with ESCC

To investigate the interaction of BANCR expression with ESCC tumor progression, quantitative real-time PCR (qRT-PCR) was performed using 132 pairs of ESCC tumor and adjacent normal tissues. The results showed that expression levels of BANCR were significantly up-regulated in the ESCC tissues compared to adjacent normal ones (Figure 1). The results demonstrated that BANCR expression was significantly correlated with the progression of ESCC, which suggested that BANCR expression was associated with the development and progression of ESCC.

3.2 Association between BANCR and clinico-pathological characteristics

We further wondered whether the expression levels of BANCR were associated with specific clinicopathological parameters in ESCC patients. We separated equally the 132 patients into two groups according to the expression levels of BANCR. We found that BANCR expression levels were strongly associated with tumor size and an advanced TNM stage (Table 1). The analyzed clinicopathologic parameters showed that the tumor size of ESCC tissue with high BANCR expression was usually bigger than 3 centimeters. Whereas tumor size in patients with low BANCR expression showed no significant changes (Table 1). For patients with BANCR high expression, tumors

![Figure 1. BANCR expression is positively correlated with the progression of ESCC. The total RNA of fresh ESCC and adjacent normal tissues were extracted using Spin Column Animal total RNA purification Kit and performed according to the Manufacturer’s Manual, and the total RNA was then reverse-transcribed into cDNA. The mRNA levels of BANCR were detected by quantitative real-time PCR (*p<0.05, **p<0.01, ***p<0.001, Pearson’s correlation, n=132).](image)

<table>
<thead>
<tr>
<th>Clinicopathologic parameters</th>
<th>BANCR expression</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High (%)</td>
<td>Low (%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤63</td>
<td>17(45.9)</td>
<td>20(54.1)</td>
</tr>
<tr>
<td>&gt;63</td>
<td>50(52.6)</td>
<td>45(47.4)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>37(46.3)</td>
<td>43(53.7)</td>
</tr>
<tr>
<td>Female</td>
<td>30(57.7)</td>
<td>22(42.3)</td>
</tr>
<tr>
<td>Tumor Size (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3</td>
<td>11(40.7)</td>
<td>16(59.3)</td>
</tr>
<tr>
<td>≥3</td>
<td>70(66.7)</td>
<td>35(33.3)</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High/Medium</td>
<td>32(60.4)</td>
<td>21(39.6)</td>
</tr>
<tr>
<td>Low</td>
<td>48(60.8)</td>
<td>31(39.2)</td>
</tr>
<tr>
<td>Invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>20(51.3)</td>
<td>19(48.7)</td>
</tr>
<tr>
<td>T3-T4</td>
<td>43(46.2)</td>
<td>50(53.8)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
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<tr>
<td>Negative</td>
<td>22(46.8)</td>
<td>25(53.2)</td>
</tr>
<tr>
<td>Positive</td>
<td>41(48.2)</td>
<td>44(51.8)</td>
</tr>
<tr>
<td>TNM Stage</td>
<td></td>
<td></td>
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<tr>
<td>I, II</td>
<td>50(64.9)</td>
<td>27(35.1)</td>
</tr>
<tr>
<td>III, IV</td>
<td>22(40.0)</td>
<td>33(60.0)</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01, ***P<0.001, Chi-squared test P-value.
were mostly at I and II TNM stages, which is equivalent to 69.4% (50 out of 72). However, there were no statistically significant associations between BANCR expression and other clinicopathological data, including age, gender, differentiation, invasion and lymph node metastasis (All \( p > 0.05 \), Table 1).

### 3.3 BANCR expression regulates the cell cycle of ESCC cells

In order to investigate whether there was correlation between BANCR expression and the growth or apoptosis of ESCC cells, we wondered whether BANCR expression could change the cell cycle of ESCC cells. Two BANCR siRNA fragments were designed and their interrupting efficiency was tested. The quantitative real-time PCR (qRT-PCR) results showed that the expression levels of BANCR in the Eca109 cells transfected with BANCR siRNA were notably decreased compared to the ones transfected with NC siRNA (Figure 2). Next, the two BANCR siRNA fragments were used to transfect the Eca109 cells, the cell cycles were tested after the transfection for 48 hours, and the cells transfected with NC siRNA worked as the negative control group. Results of Flow Cytometry analysis indicated that more cells transfected with BANCR siRNA arrested at G0/G1 phase compared to the cells transfected with NC siRNA, and the numbers of cells transfected with BANCR siRNA decreased significantly in the S phase relative to the negative control (Figure 3). In

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**Figure 2.** The relative mRNA levels of BANCR detected by quantitative real-time PCR in Eca109 cells transfected with Negative Control siRNA (NC siRNA), BANCR siRNA-1 or BANCR siRNA-2 respectively as indicated.

**Figure 3.** BANCR regulates the cell cycles of Eca109 cells. (A-C). The cell cycles of Eca109 cells transfected with NC siRNA or BANCR siRNA were measured using a Flow Cytometer. D. The number of cells transfected with BANCR siRNA (100 nM) or NC siRNA (100 nM) at different cell cycle phages were assayed.
addition, TE-1 cells of ESCC cell lines were transfected respectively with the two BANCR siRNAs and the cell cycles were then detected and the data analyzed. The results obtained were similar to that of Eca109 cells, that is the numbers of cells transfected with BANCR siRNA reduced at the S phase (Figure 4). These results indicated that low expression levels of BANCR could significantly decrease the cell numbers arrested at S phase relative to the negative control, which suggested that BANCR expression might regulate the proliferation of ESCC cells via modulating their cell cycles.

3.4 BANCR expression affects the growth of ESCC cells

In order to further confirm the results above, which showed that BANCR expression could regulate the ESCC cell proliferation, MTT assays were performed. The results of these assays showed that the growth of cells transfected with BANCR siRNA were significantly decreased after transfection for 72 hours compared to the cells transfected with the negative control siRNA (Figure 5A). Moreover, we found that the cell proliferation ratio (BANCR siRNA OD<sub>490</sub> / NC siRNA OD<sub>490</sub>) was negatively correlated with the percentage of the MTT inhibition (Figure 5B). These results indicated that reduction of BANCR expression resulted in the decreased proliferation of ESCC cells, which suggested that BANCR could promote the development and progression of ESCC.

4 Discussion

Esophageal cancer is the main cause of cancer related deaths in China and ESCC is the main form of Esophageal cancer. Unfortunately, the average 5-year survival rate of ESCC remains lower than 10% [19, 20]. Recently, a greater number of studies have focused on the underlying mechanisms for how the development and progression of ESCC cells occurred [21-23].

Figure 4. BANCR regulates the cell cycles of TE-1 cells. TE-1 cells were transfected with BANCR siRNA-1 (100 nM), BANCR siRNA-2 (100 nM) or NC siRNA (100 nM) respectively, and the cell cycles of these cells were detected by Flow Cytometry.
Emerging evidence has reported that long non-coding RNA (lncRNA) plays an important role in the development and progression of cancer [24, 25]. BRAF-activated non-protein coding RNA (BANCR) is a 693 bp long transcript, which could promote the proliferation of gastric cancer cells via regulating NF-κB [14]. Recent studies indicate that up-regulation of BANCR correlates with tumor progression in ESCC [15, 16, 26], which is consistent with our results (Figure 1) that demonstrated that BANCR expression was positively correlated with the progression of ESCC. According to the association between BANCR and clinicopathological characteristics, BANCR expression is positively correlated with Tumor size and TNM stages (Table 1), which suggests that BANCR could be a potential biomarker for analyzing TNM stages of ESCC patients.

Recent studies have demonstrated that the proliferation of cancer is closely correlated to the altered regulation of cell cycles, which might cause uncontrolled growth and contribute to the development and progression of tumor cells [27]. In the present study, our data illustrated that fewer ESCC cells transfected with BANCR siRNA were arrested in the S phase compared to that transfected with NC siRNA (Figure 3 and Figure 4). All of this data implies that BANCR expression was associated with the progression of ESCC through its ability to regulate cell cycles.

In summary, we found that BANCR expression is positively correlated with development of ESCC, and up-regulation of BANCR expression would advance the proliferation of ESCC cells via regulating cell cycles. Therefore, our findings provide new insights into the mechanism of BANCR in the development and progression of ESCC. BANCR may function as a potential biomarker for diagnosis and treatment for ESCC patients.

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Conflict of interest: Authors state no conflict of interest.

Abbreviations

- lncRNA: long non-coding RNA
- BANCR: BRAF-activated non-protein coding RNA
- ESCC: esophageal squamous cell carcinoma
- TNM: tumor node metastasis
- siRNA: small interrupting RNA
- NC: negative control
- PI: propidium iodide

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References


