

Research Paper

# Downregulation of *BANCR* Promotes Aggressiveness in Papillary Thyroid Cancer via the MAPK and PI3K Pathways

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## Abstract

Recent evidence indicates that long non-coding RNAs play important roles in tumorigenesis and cancer progression. *BRAF*-activated non-protein coding RNA (*BANCR*) is a novel and potential regulator of cancer cell proliferation and migration. However, little is known regarding the role of *BANCR* in papillary thyroid cancer (PTC). The current study used quantitative PCR to demonstrate that *BANCR* was significantly downregulated in 60 paired PTC tissues compared with normal tissues. In addition, *BANCR* was significantly correlated with lymph node metastasis ( $p = 0.02$ ). Furthermore, Cell Counting Kits and Transwell assays were used to demonstrate that knocking down *BANCR* with short hairpin RNA (shRNA) transfection significantly promoted the proliferation and invasion of PTC cell lines. The flow cytometric analysis of apoptosis and the cell cycle revealed that the overexpression of *BANCR* inhibited cancer cell proliferation and invasion, which was associated with the induction of cell-cycle G2/M phase arrest and increased apoptosis. Moreover, western blotting was used to show that the MAPK and PI3K-Akt pathways were aberrantly activated during *BANCR*-mediated PTC cell proliferation and migration. These findings revealed that *BANCR* functions as a tumor suppressor during thyroid carcinogenesis.

Key words: papillary thyroid cancer; *BRAF*-activated non-protein coding RNA; MAPK signaling pathway; PI3K-Akt signaling pathway; cell cycle; apoptosis

## Introduction

Thyroid cancer is a common endocrine malignancy that has rapidly increased in global incidence in recent decades [1-3]. In China, the incidence of thyroid cancer increased by 20.1% between 2003 and 2011, which was the highest increase among all cancers [4]. Follicular thyroid cell-derived tumors include papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), poorly differentiated thyroid cancer (PDTC), and anaplastic thyroid cancer (ATC) [5]. PTC is the primary component of the rapid rise in the incidence of

thyroid cancer; it accounts for 80-90% of all thyroid cancers [6]. Although PTC is a highly curable and indolent cancer that has excellent prognosis, 10% of patients with aggressive tumors will develop progressive disease [7, 8]. Therefore, it is important to understand molecular mechanisms involved in the development and progression of thyroid cancer. Although several molecules contributing to tumor invasion and migration have been identified [5,9,10], the precise mechanisms underlying tumor pathogenesis remain to be fully elucidated.

Recently, thousands of long non-coding RNAs (lncRNAs) were uncovered by deep sequencing; they have a large variety of roles in both gene expression and remodeling of the eukaryotic genome [11]. lncRNAs are transcripts that are longer than 200 nucleotides, devoid of evident open reading frames, and often polyadenylated. Emerging evidence suggests that lncRNAs play a crucial role in gene regulation, which is strongly associated with cell fate determination and human disease, especially tumorigenesis [12-14]. For example, *HOTAIR* is highly expressed in ~10% of breast cancer patients [15, 16], *MALAT1* is significantly associated with non-small cell lung cancer [17], and the aberrant expression of *GAS5* is observed in prostate cancer [18]. Together, these findings suggest that lncRNAs are important for understanding the molecular biology of cancer progression, including thyroid cancer.

*BRAF*-activated non-protein coding RNA (*BANCR*) is a 693-base pair (bp) lncRNA originally identified using massively parallel cDNA sequencing in melanoma cells. It is highly expressed in melanoma and contributes to cell migration [19, 20]. Previous studies revealed that *BANCR* functions as both an oncogene and a tumor suppressor. Wang et al. demonstrated that *BANCR* expression was upregulated in six pairs of PTC and matching adjacent normal tissues and that *BANCR* increased PTC cell proliferation by activating autophagy [21]. Interestingly, Tian et al. reported that *BANCR* expression was downregulated in 68.5% (63/92) of PTC tissues compared with normal tissues [22]. However, the significance of *BANCR* in thyroid cancer is still unclear. In the present study, we assessed the expression of *BANCR* and performed *in vitro* studies to elucidate its role in PTC.

## Materials and methods

### Tissue samples

Tissue was obtained from patients (18 male and 42 female) who underwent surgery at the Department of Thyroid and Breast Surgery, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology in 2016. The study was carried out in accordance with the institutional ethical guidelines, and the use of human tissues was approved by the Medical Ethics Committee of Tongji Hospital (Institution Review Board Approval: TJ-C20141222). Each patient involved in the study was asked to sign a written informed consent form and the specimens were anonymized and handled according to accepted ethical and legal standards. The diagnosis of papillary thyroid cancer was confirmed histopathologically by an experienced pathologist. All

samples were snap-frozen in RNAlater solution and stored at  $-80^{\circ}\text{C}$  until use. Detailed patient descriptions are provided in Supplementary Table 1.

### Cell line and culture conditions

Three human PTC cell lines NPA, BCPAP, TPC1 and normal thyroid tissue cell lines Nthy-ori3-1 were provided by The Institute of Interdisciplinary Research (Berkeley, CA, USA). The cell lines were authenticated at Shanghai Biowing Applied Biotechnology Co. Ltd using short tandem repeat (STR) DNA profiling (ABI 3730XL Genetic Analyzer, Life Technologies, Waltham, MA, USA). The cells were used in experiments within 25 passages. TPC1 and BCPAP cells were maintained in RPMI-1640 medium (Boster, Wuhan, China). NPA and Nthy-ori3-1 cells were maintained in DMEM medium (Boster), both supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), in a  $37^{\circ}\text{C}$  incubator with a humidified atmosphere of 5%  $\text{CO}_2$ .

### RNA interference using shRNA

*BANCR* was knocked down by transfecting cells with *BANCR* short hairpin RNA (shRNA, Genepharma, Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The target sequences were as follows:

LV3-*BANCR* -323 (shRNA1#), 5'-GGAGTGGCG ACTATAGCAAAC-3'

LV3-*BANCR* -540 (shRNA2#), 5'-GGACTCCAT GGCAAACGTTGT-3'

*BANCR* shRNA stable transfectants were selected in medium containing 2  $\mu\text{g}/\text{mL}$  puromycin (Goodbio, Wuhan, China). LV3-NC empty vector was used as the control. The specific silencing of *BANCR* expression was assessed using qRT-PCR.

### Plasmid DNA transfection

The ectopic expression of *BANCR* was achieved by transfecting cells with pEX-2-*BANCR* (Genepharma, Shanghai, China) using Lipofectamine 3000. *BANCR*-overexpressing stable transfectants were selected in medium containing G418 (Goodbio, Wuhan, China; 800  $\mu\text{g}/\text{mL}$  for NPA cells and 1100  $\mu\text{g}/\text{mL}$  for TPC1 cells). pEX-2-NC empty vector was used as the control. The expression of *BANCR* was assessed using qRT-PCR.

### RNA extraction and quantitative real-time PCR

Total RNA was extracted from tissues or cells using Trizol reagent (Invitrogen). *BANCR* expression was then measured in triplicate using SYBR Green qPCR Mix (Toyobo, Shanghai, China). Primer sequences were 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 the 2- $\Delta\Delta$  CT method and normalized to *GAPDH* expression.

### Cell viability assays

Cell viability was monitored using Cell Counting Kit-8 (CCK-8, Dojindo, Kyushu, Japan) according to the manufacturer's instructions. NPA and TPC1 cells were seeded into six-well plates (Corning, New York, NY, USA) and incubated overnight. They were then transfected with *BANCR* shRNA1# and 2#, pEX-2-*BANCR*, or the respective negative control. The cells were maintained for 48 h and then seeded into 96-well plates (3000 cells per well). The cells were maintained for 4 h, and CCK-8 was then added to each well and incubated at 37°C for 3 h. The optical densities (ODs) were measured at a wavelength of 450 nm using a microplate reader. The results are represented as the mean of five different wells; wells were assessed in triplicate for each treatment group.

### Transwell migration and invasion assays

For Transwell invasion assays,  $3 \times 10^4$  NPA and TPC1 cells in serum-free medium were plated in the upper chamber that was coated with Matrigel (24-well insert, 8-mm pore size; Corning, NY, USA). For the Transwell migration assays the upper chamber was not coated with Matrigel. The media, which were supplemented with serum, served as a chemotactic agent in the lower chamber. The cells were incubated for 72 h and 48 h for invasion and migration assays, respectively. The cells that did not migrate through the pores were removed with a cotton swab, and the cells on the lower surface of the membrane were stained with crystal violet (Goodbio, Wuhan, China). The number of cells that penetrated the membrane was then counted in five random fields per chamber under a microscope (Olympus Corp. Tokyo, Japan). Each experiment was performed in triplicate.

### Protein extraction and western blotting

Western blotting was performed as described previously [23]. The blots were probed with primary antibodies, including those against p-MEK, ERK1/2, p-ERK1/2, EGFR, p-EGFR, p-P13K, AKT, p-AKT, p-FAK, p-STAT1, and p-STAT3 (all from Cell Signaling Technology, Danvers, MA, USA), followed by HRP-conjugated secondary antibodies. Signals were visualized using ECL reagent (Goodbio, Wuhan, China). The intensity of the bands was quantified using densitometry (Quantity One software; Bio-Rad,

Berkeley, CA, USA); *GAPDH* was used as a control.

### Flow-cytometric analysis of the cell cycle and apoptosis

pEX-2-*BANCR*, *BANCR* shRNA1# and 2#, and empty vector-transfected NPA and TPC1 cells were cultured in six-well plates for 48 h, washed with ice-cold phosphate-buffered saline (PBS), and fixed with 75% ethanol overnight. The cells were then collected by centrifugation (1000 rpm for 5 min) and resuspended in 1 ml of propidium iodide (PI) solution (50 mg/mL in PBS) containing 0.25 mg/mL of RNaseA. After incubation for 30 min in the dark at 4°C, the cells were analyzed by flow cytometry (FACScan; BD Biosciences, Franklin Lakes, NJ, USA) using an instrument equipped with Cell Quest software (BD Biosciences). The percentage of cells in G0-G1, S, and G2-M phase was counted and compared.

For apoptosis analysis, pEX-2-*BANCR*, *BANCR* shRNA1# and 2#, and empty vector-transfected NPA and TPC1 cells were harvested 48 h after transfection. Floating and adherent cells were collected using 0.1% trypsin, washed twice with cold PBS, and suspended in 0.5 mL binding buffer (10 mmol/L HEPES buffer pH 7.4, 140 mmol/L NaCl, and 2.5 mmol/L CaCl<sub>2</sub>). The cells were then treated with an Annexin V-APC/7-AAD Apoptosis Detection Kit (Abnova, Atlanta, GA, USA) according to the manufacturer's instructions. Flow cytometry was carried out using FACScan (BD Biosciences). The percentage of early and late apoptotic cells were compared among groups in each experiment. This assay was repeated three times.

### Fluorescence in situ hybridization (FISH)

Cells were plated onto coverslips, washed with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 10 min, and permeabilized with 0.25% Triton-100 for 5 min. Cy3-labeled lncRNA *BANCR* probes were obtained from RiboBio (Guangzhou, China). Slides of tissues were hybridized with probes overnight, washed as the above description. RNA FISH was performed using a FISH kit (Ribobio Co., Guangzhou, China) following the manufacturer's instructions. Then, the slides were counterstained with 4, 6-diamidino-2-phenylindole (DAPI) to mark the nuclei, U6 and 18S were used as cytoplasmic and nuclear positive control. Fluorescently labeled cells were visualized and imaged under a confocal laser scanning microscope (Olympus Corp. Tokyo, Japan).

### Statistical analysis

Statistical analyses were performed using SPSS software version 21 (SPSS, Inc., Chicago, IL, USA) and

GraphPad prism 5.0 for Windows (GraphPad Software, La Jolla, CA, USA). The Wilcoxon signed rank tests were applied to assess the expression of *BANCR* in cancer tissues compared with paired normal tissues. Chi-square tests or Fisher's exact test were used to examine the relationship between *BANCR* expression and clinicopathologic characteristics. The differences among groups in proliferation assays, western blotting, and PCR were estimated using Student's *t*-tests or one-way ANOVA. The results are reported as means  $\pm$  SDs. Statistical significance was assigned as  $p < 0.05$  (\*) or  $p < 0.01$  (\*\*).

**Table 1.** Correlation between *BANCR* expression and clinical pathological characteristics in papillary thyroid carcinoma

Characteristics	n	High expression (%)	Low expression (%)	P
<b>Gender</b>	60			
Female	42	11(26.2)	31(73.8)	0.755
Male	18	6(33.3)	12(66.7)	
<b>Age (y)</b>				
<45	35	11(31.4)	24(68.6)	0.575
$\geq$ 45	25	6(24.0)	19(76.0)	
<b>Clinical stage</b>				
I-II	49	16(32.7)	33(67.3)	0.155
III-IV	11	1(9.1)	10(90.9)	
<b>Tumor depth</b>				
T1-T2	55	17(30.9)	38(69.1)	0.309
T3-T4	5	0(0.0)	5(100)	
<b>Tumor size</b>				
<1cm	16	2(12.5)	14(87.5)	0.122
1-4cm	27	11(40.7)	16(59.3)	
>4cm	17	4(23.5)	13(76.5)	
<b>Lymph node metastasis</b>				
Y	36	6(16.7)	30(83.3)	0.020*
N	24	11(45.8)	13(54.2)	
NIA	20	4(20.0)	16(80.0)	0.043*
NIB	16	2(12.5)	14(87.5)	

Notes: *BANCR*, *BRAF*-activated non-protein coding RNA

$P < 0.05$  on the parallelism test, indicating that the clinical and pathological characteristics are significantly correlated with *BANCR* expression.

## Results

### Expression of *BANCR* in PTC tissues and PTC cell lines

qRT-PCR was used to examine *BANCR* levels in 60 PTC tissues and matched normal thyroid tissues. The expression of *BANCR* was significantly downregulated ( $p < 0.01$ ) in 71.7% (43/60) of cancerous tissues compared with normal tissues (Figure 1A). Furthermore, the correlation between *BANCR* expression and the patients' clinicopathological parameters (including gender, age, and TNM stage) was assessed to determine its clinical significance. *BANCR* expression in PTC was significantly correlated with lymph node metastasis ( $p = 0.02$ ). *BANCR* expression was lower in 83.3% (30/36) of the patients with lymph node metastasis (Table 1). Two patients with advanced disease, one

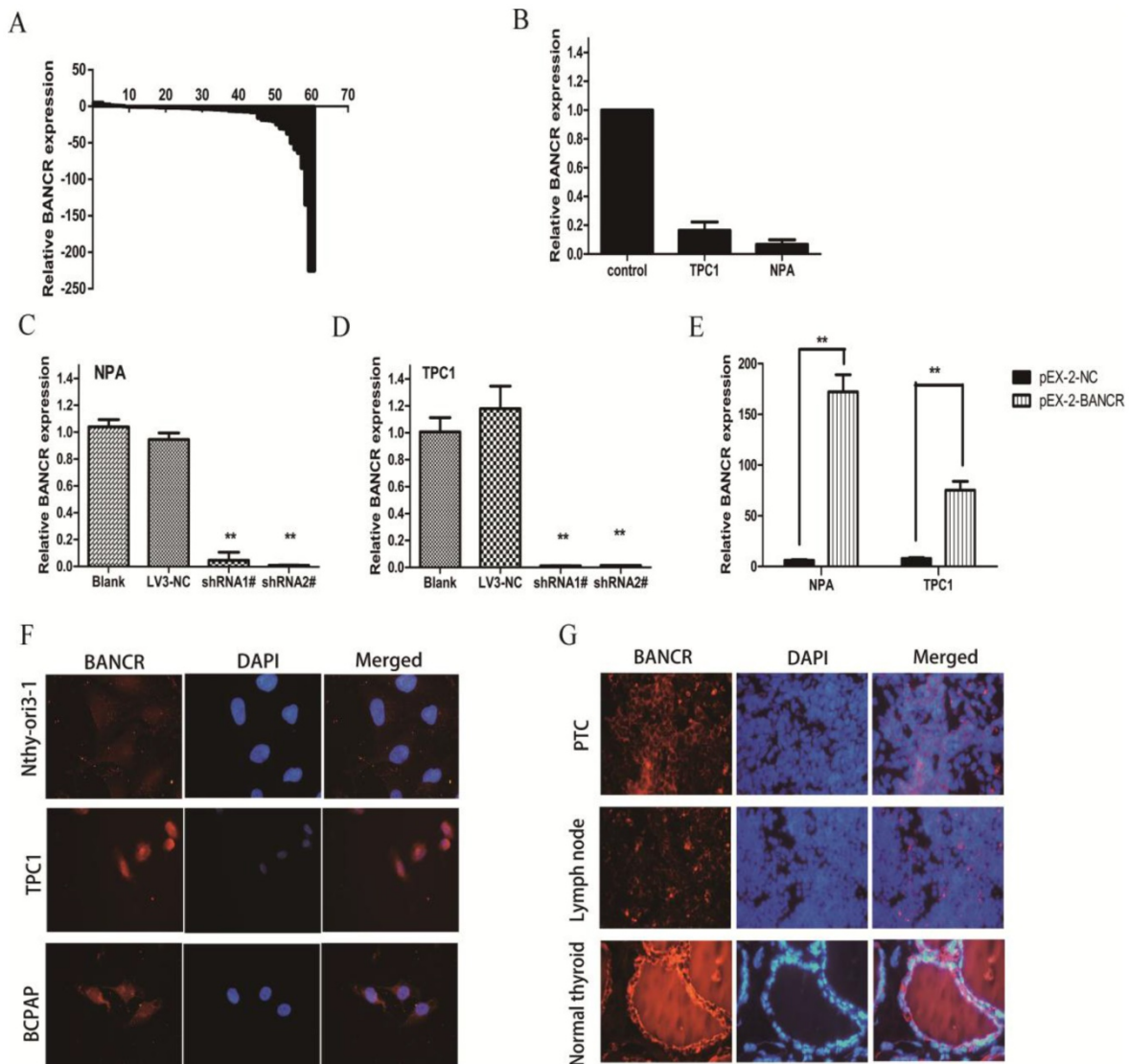
with invasion into subcutaneous soft tissues and the other with invasion into the larynx (Supplementary Table 1), both had lower *BANCR* expression. However, *BANCR* expression was not associated with other PTC parameters such as gender ( $p = 0.755$ ), tumor stage ( $p = 0.155$ ), and patient age ( $p = 0.575$ ) (Table 1). The clinicopathological parameters for all patients were summarized in Supplementary Table 1. These analyses demonstrate that *BANCR* may be a good diagnostic biomarker for PTC.

*BANCR* expression was also detected in two human PTC cell lines (NPA and TPC1) using qRT-PCR. The two cell lines exhibited a notable downregulation in *BANCR* compared with control thyroid tissues (Figure 1B,  $p = 0.024$ ). In the FISH experiment, which involved PTC cell lines (BCPAP and TPC1) and PTC patient specimens, we also confirmed the decreased *BANCR* expression, which further suggested that lncRNA *BANCR* was mainly localized in the cytoplasm of PTC cells and tissues (Figure 1F and 1G).

### Modulating *BANCR* expression affects cell proliferation and motility

To clarify the role of *BANCR* in PTC cells, CCK-8 was used to assess the impact of *BANCR* overexpression or knockdown on NPA and TPC1 cells. Our clinical data indicated that *BANCR* expression was inversely correlated with tumor size and lymph node metastasis. Thus, the shRNAs LV3-*BANCR*-323 (shRNA1#) and LV3-*BANCR*-540 (shRNA2#) were used to downregulate endogenous *BANCR* expression in NPA and TPC1 cells. CCK-8 assays were then performed to monitor the effects of *BANCR* shRNA on cell proliferation. The results showed that the proliferation of *BANCR* shRNA-transfected was higher than control cells transfected with the empty vector at 48 h ( $p < 0.01$ ; Figure 2A and 2B). Transwell migration and invasion assays showed that downregulating *BANCR* significantly promoted cell motility ( $p < 0.01$ ; Figure 2C and 2D).

Consistent with these observations, the growth of NPA and TPC1 cells transfected with pEX-2-*BANCR* was impaired compared with control cells ( $p < 0.01$ ; Figure 2A and 2B). To further examine the effects of *BANCR* on the motility of NPA and TPC1 cells, transwell migration and invasion assays were performed. *BANCR* overexpression impaired the migration and invasion of PTC cells ( $p < 0.01$ ; Figure 2E and 2F), suggesting that *BANCR* is at least partially necessary for PTC cell motility *in vitro*. These observations suggest that *BANCR* contributes to PTC progression by enhancing cell proliferation and motility.



**Figure 1. Relative expression of *BANCR* in papillary thyroid cancer tissues and cell lines.** (A) Relative expression of *BANCR* in papillary thyroid cancer tissues ( $n = 60$ ) compared with corresponding normal tissues ( $n = 60$ ). *BANCR* expression was examined by qPCR and normalized to *GAPDH* expression. The results are presented as the fold-change in tumor tissues relative to normal tissues. (B) *BANCR* expression in NPA and TPC1 papillary thyroid cancer cell lines compared with control thyroid tissues pooled from three controls. (C and D) qPCR analysis of *BANCR* expression following the treatment of NPA and TPC1 cells with *BANCR*shRNA1#, shRNA2#, and empty vector. (E) qPCR analysis of *BANCR* expression following the treatment of NPA and TPC1 cells with pEX-2-*BANCR* and empty vector. (F) Fluorescence in situ hybridization in Nthy-ori3-1, TPC1 and BCPAP cells (G) Fluorescence in situ hybridization in papillary thyroid cancer, metastatic lymph node and normal thyroid tissues. \*\*,  $p < 0.01$ . shRNA1#, shRNA2#, and LV3-NC, plasmid containing shRNA1# and shRNA2# for *BANCR* and negative control shRNA, respectively; pEX-2-*BANCR* and pEX-2-NC, pEX-2 plasmid containing human full-length *BANCR* cDNA and negative control, respectively; *BANCR*, *BRAF*-activated non-protein coding RNA; shRNA, short hairpin RNA; DAPI, 4',6-diamidino-2-phenylindole.

### ***BANCR* promotes G2/M arrest and causes apoptosis**

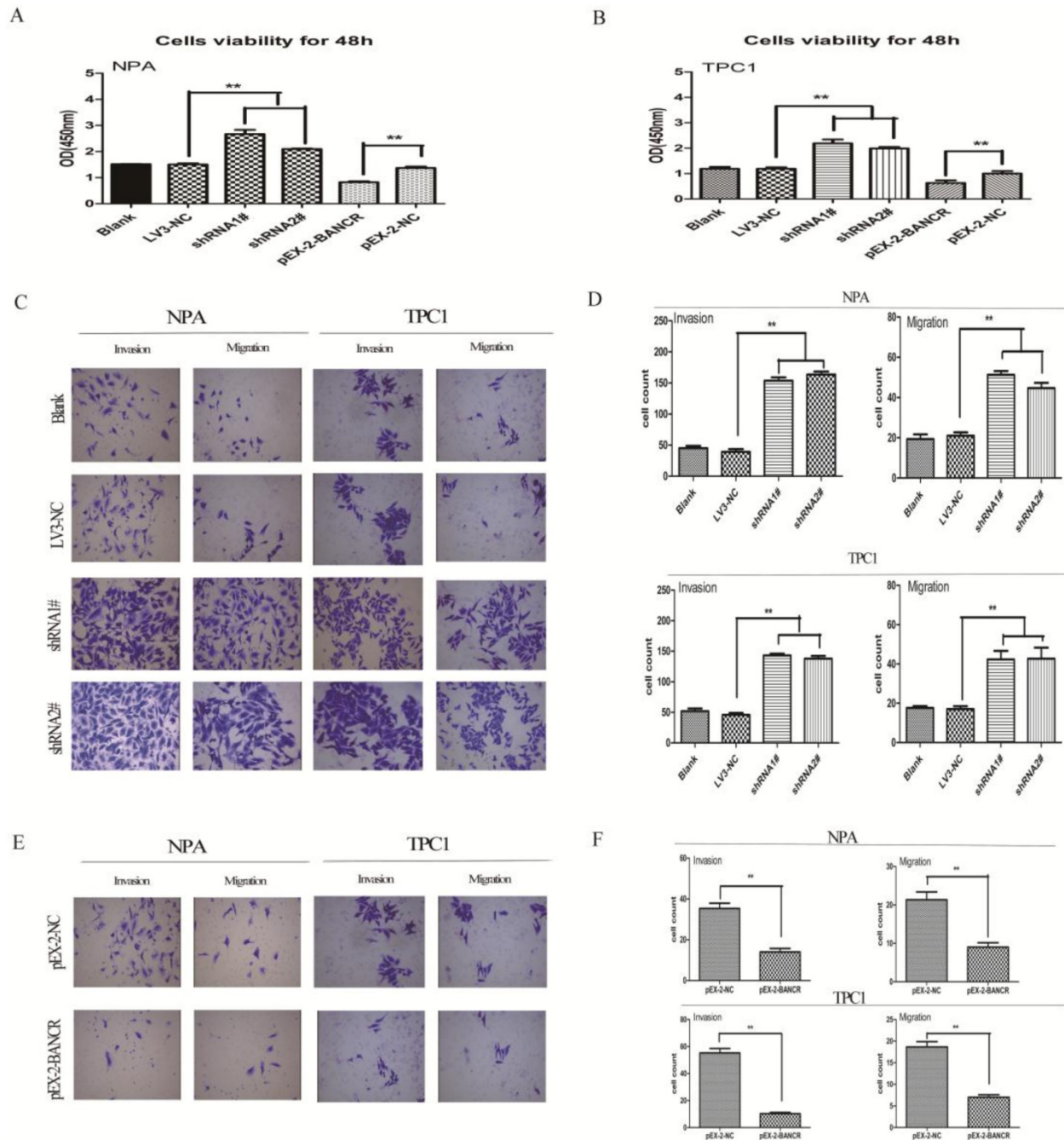
To determine whether the effects of *BANCR* on the proliferation and motility of PTC cells were mediated by inhibiting cell cycle progression, flow cytometry was used to assess the cell cycle in NPA cells (Figure 3A and 3B). *BANCR* overexpression led to the significant accumulation of cells in G2/M-phase and a significant decrease in cells in S-phase compared with control (both  $p < 0.05$ ). Conversely, knocking down *BANCR* led to a significant decrease in the percent of cells in G1/G0- and G2/M-phases and a significant increase in S-phase compared with

control (both  $p < 0.01$ ). Next, the effects of *BANCR* on apoptosis were assessed in NPA cells (Figure 3C and 3D). The percentage of cells undergoing late apoptosis and necrosis was significantly increased in the *BANCR* overexpression group compared with control ( $p < 0.01$ ). The percentage of early apoptotic cells was significantly decreased in the *BANCR* shRNA group compared with control ( $p < 0.01$ ). Taken together, these data suggest that *BANCR* overexpression could induce G2/M phase arrest and enhance apoptosis in PTC cells, whereas *BANCR* downregulation promotes the entry of PTC cells into S phase and protects from early apoptosis.

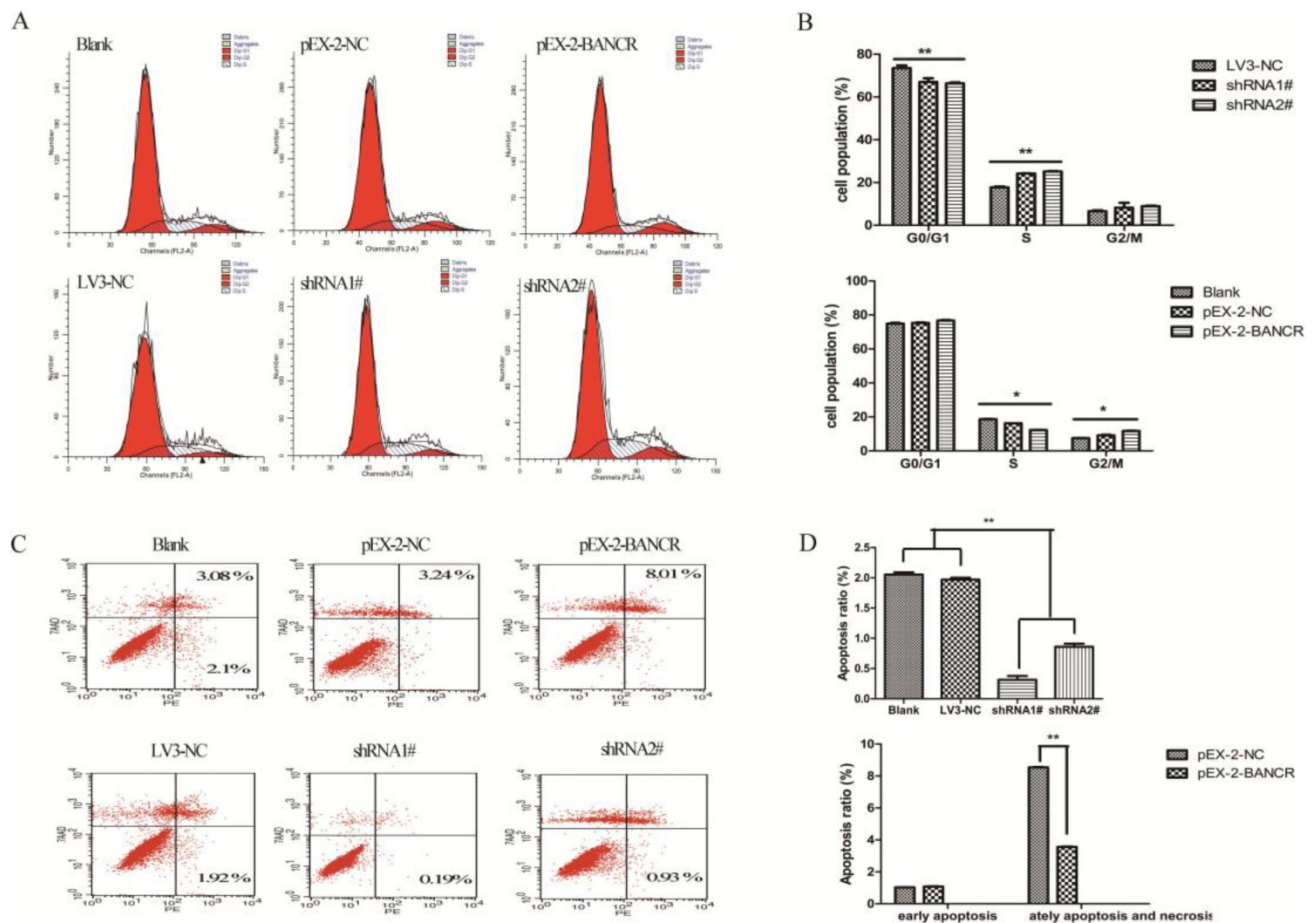
## BANCR regulates PTC proliferation and migration by inactivating the MAPK and PI3K-Akt pathways

Next, the activation of the MAPK and PI3K-Akt pathways was assessed using western blotting to explore the potential mechanism underlying the role of *BANCR* in cell proliferation and migration. *BANCR* downregulation activated the MAPK (Figure 4) and PI3K-Akt (Figure 5) pathways in NPA and TPC1 cells.

Interestingly, although the MAPK pathway was activated by *BANCR* knockdown, there was no effect on upstream EGFR or the classical activator MEK1/2; only ERK1/2 phosphorylation was upregulated (Figure 4A and 4C). There was also no difference in the phosphorylation of STAT-1 or STAT-3 (Figure 4E). When *BANCR* was upregulated, no significant differences were observed in MAPK pathway through either cell line (Fig 4B, 4D and 4F).



**Figure 2. Effects of *BANCR* on papillary thyroid cancer cell proliferation and motility.** (A and B) CCK-8 assays were performed to measure NPA and TPC1 cell proliferation. The data represent the means  $\pm$  SDs of three independent experiments. (C and E) Transwell migration and invasion assays showed that knocking down *BANCR* promoted NPA and TPC1 cell motility. However, cell motility was reduced in *BANCR*-overexpressing NPA and TPC1 cells (crystal violet stain; magnification,  $\times 200$ ). (D and F) The bar graph represents at least three independent experiments and the bars indicate the number of migrated cells per field. \*\* $p < 0.01$ . shRNA1#, shRNA2#, LV3-NC respective plasmid containing shRNA for *BANCR* and negative control; pEX-2-BANCR and pEX-2-NC, pEX-2-plasmid containing human full-length *BANCR* cDNA and negative control, respectively; *BANCR*, *BRAF*-activated non-protein coding RNA; shRNA, short hairpin RNA.



**Figure 3. The effects of *BANCR* on the cell cycle and apoptosis in papillary thyroid cancer cells *in vitro*.** (A and B) The bar chart represents the percentage of cells in G0/G1, S, or G2/M phase. (C and D) The percentage of apoptotic cells was determined by flow cytometry. The data represent the means  $\pm$  SDs of three independent experiments. \*  $p < 0.05$ ; \*\*  $p < 0.01$ . shRNA1#, shRNA2#, LV3-NC respective plasmid containing shRNA for *BANCR* and negative control; pEX-2 *BANCR*, pEX-2-NC respective plasmid containing human full length *BANCR* cDNA and negative control; *BANCR*, *BRAF*-activated non-protein coding RNA; shRNA, short hairpin RNA.

The PI3K-Akt pathway was activated by *BANCR* knockdown, as revealed by increased PI3K and Akt phosphorylation, but there was no effect on FAK (Figure 5A and 5C). In contrast, the PI3K-Akt pathway was inactivated when *BANCR* was upregulated in NPA cells. Although there were no significant differences in TPC1 cells, while was a similar a trend (Figure 5B and 5D).

## Discussion

Genomic studies have demonstrated that <2% of the total human genome can be transcribed, and that noncoding RNAs likely account for the greater complexity transcriptome in eukaryotes [24, 25]. lncRNAs were once neglected and considered to be noise in cells for a long time. However, accumulating evidence has assigned new functions to lncRNAs, including roles in the transcriptional, epigenetic, and post-transcriptional regulation of gene expression [26-28]. It was reported that lncRNAs affect many cellular processes in tumorigenesis such as survival, proliferation, apoptosis, the cell cycle, and migration

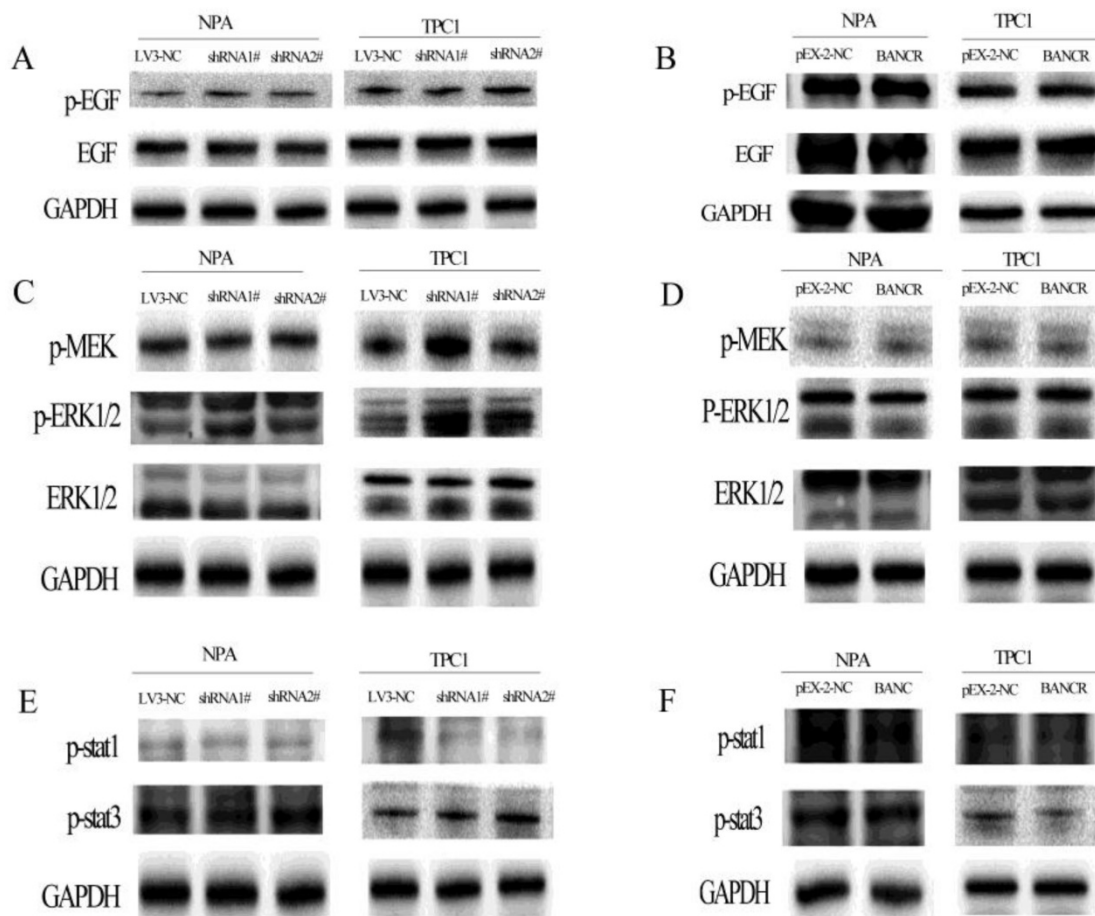
[29, 30]. Unlike microRNAs, lncRNAs are not currently well understood. *BANCR*, a recently found lncRNA, can regulate proliferation and migration in melanoma, colorectal cancer, and lung cancer [19,31,32]. In a recent meta-analysis [20], high *BANCR* expression was correlated with lymph node metastasis, tumor stage, and poor prognosis in gastrointestinal cancer patients, but not in other cancers. However, little is known about the role of *BANCR* in the development of papillary thyroid carcinoma; the mechanism underlying tumorigenesis and its clinical significance is still unclear. According to previous studies, the function of *BANCR* in PTC is controversial. Liao et al. demonstrated that *BANCR* expression was downregulated in 92 pairs of PTC tissues, as well as in the cell lines TPC-1, K1, and BCPAP. In other studies, Zheng et al. and Wang et al. demonstrated that *BANCR* is upregulated in human PTC tissues and IHH4 cells and that this promotes cell growth and proliferation [33]. In the current study, we investigated the clinical significance of *BANCR* and its function and molecular mechanism in PTC. Our

results revealed that *BANCR* levels were significantly reduced in 60 pairs of PTC tissues from patients and PTC cell lines (TPC1 and NPA). The discrepancy with our results may stem from differences in the numbers of tissue samples tested (60 in the current study, 92 in Liao et al., 40 in Zheng et al., and six in Wang et al.), tumor heterogeneity (especially in Zheng et al., who had only 21 cases of classical PTC among 40 samples) and in the *BANCR* expression patterns in different cell lines (Zheng et al. and Wang et al. used IHH4 cells). In the current study, low *BANCR* expression levels were significantly correlated with lymph node metastasis, whereas Liao et al. reported that downregulated *BANCR* expression was correlated with tumor size, multifocal lesions, and an advanced pathological stage; this difference may stem from tumor heterogeneity. Overall, both studies confirm that low *BANCR* levels are associated with poor outcomes in PTC patients.

Liao et al. reported that the overexpression of *BANCR* promotes apoptosis in TPC1 and K1 cell lines. However, the current study reported that *BANCR* overexpression could induce G2/M phase arrest and

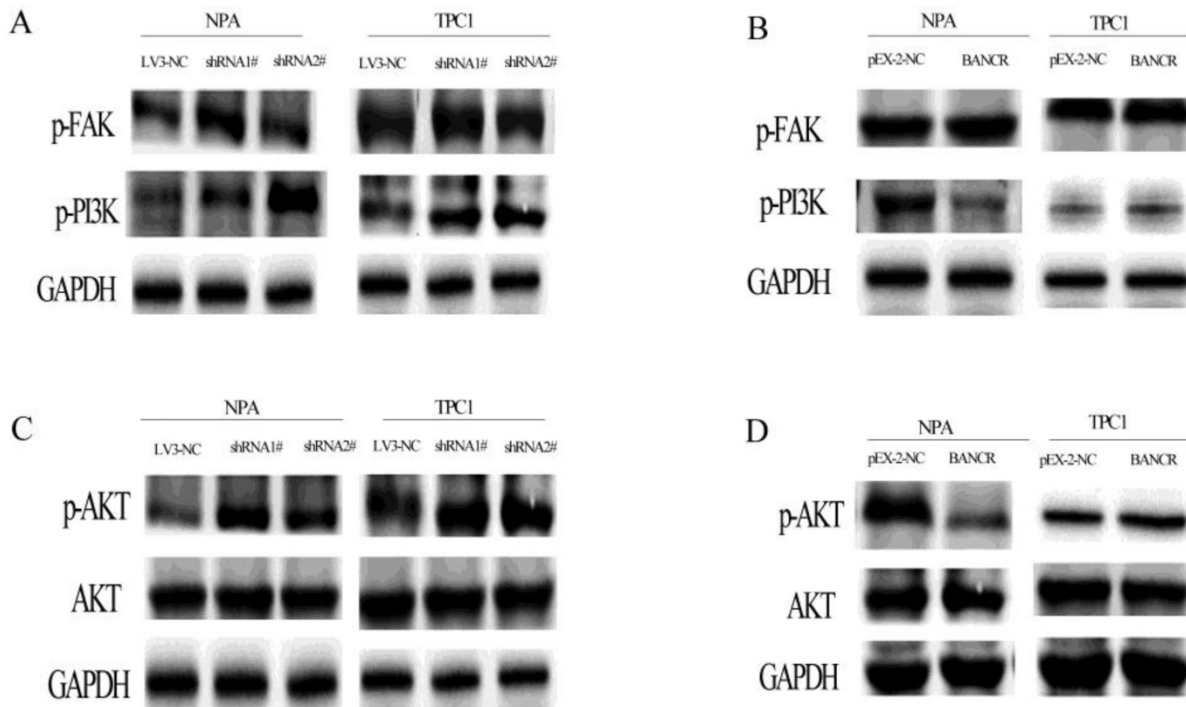
enhance apoptosis in PTC cells whereas *BANCR* downregulation promoted the entry of PTC cells into S phase and protected from early apoptosis.

The MAPK and PI3K-Akt signaling pathways play important roles in complex cellular programs including proliferation, survival, and migration [34, 35]. The MAPK and PI3K-Akt pathways are involved in differentiated PTC, and the simultaneous activation of both pathways becomes more frequent as the grade of thyroid tumors increases [36-38]. Activation of the MAPK and PI3K-Akt pathways is an important mechanism that drives the progression of thyroid cancer. The current study investigated the activation of the MAPK and PI3K-Akt pathways in PTC cells by measuring the phosphorylation of proteins in the MAPK and PI3K-Akt pathways, including EGFR, MEK, ERK1/2, STAT-1, STAT-3, FAK, AKT, PI3K, in *BANCR* overexpressing and knockdown PTC cells. MAPK and PI3K-Akt were activated in *BANCR* knockdown PTC cells, but the phosphorylation of EGFR, MEK1/2, STAT-1, STAT-3, and FAK was not affected. The overexpression of *BANCR* by transfection with pEX-2-*BANCR* inhibited the activation of the



**Figure 4. The phosphorylation of EGFR, MEK, ERK1/2, STAT-1, and STAT-3 in *BANCR*-silenced or -overexpressing NPA and TPC1 cells was detected by western blotting.** The loss of *BANCR* induced the phosphorylation of ERK1/2 (C). However, there was no activation of upstream molecule including EGFR (A), MEK (C), MEK, STAT-1, and STAT-3 (E). *BANCR* overexpression had no effect on the phosphorylation of EGFR, MEK, ERK1/2, STAT-1, and STAT-3 in either cell line (B, D and F). GAPDH expression was used to normalize protein loading.





**Figure 5. The phosphorylation of FAK, PI3K, and Akt in *BANCR*-silenced or overexpressing NPA and TPC1 cells was detected by western blotting.** The loss of *BANCR* induced the phosphorylation of PI3K (A) and Akt (C). However, there was no activation of the upstream molecule FAK (A). In NPA cells, *BANCR* overexpression induced the inactivation of PI3K (B) and Akt (D), but there was no activation of the upstream molecule FAK. However, in TPC1 cells *BANCR* overexpression had no effect on the phosphorylation of FAK, PI3K, and Akt. (B, D) GAPDH expression was used to normalize protein loading.

PI3K-AKT pathway in NPA cells (which contain *BRAF*<sup>V600E</sup>), whereas there was no significant difference in the MAPK pathway. Liao et al., demonstrated *BANCR* overexpression inactivates ERK1/2 and p38 in K1 cells (which contain *BRAF*<sup>V600E</sup> and PI3K [E542K]). In contrast, the current study found that *BANCR* knock down increased p44 ERK1 and p42 ERK2 MAPK levels but did not affect the upstream molecules. In addition, the PI3K-Akt pathway was activated by *BANCR* knockdown and inactivated when *BANCR* was upregulated in NPA cells. The discrepancy between these observations and the current results may stem from the different cell lines used. Mammalian cells contain four well-characterized and widely studied MAPKs.

The terminal serine/threonine kinases (MAPKs) include ERK1/2, c-Jun amino-terminal kinases (JNK-12/3; also called SAPKs), p38 kinases (p38a/b/g/d), and ERK5. In the current study, we were most concerned with the EGFR-Ras-Raf-MEK-ERK MAPK signaling pathway because previous studies revealed that *BRAF* mutation activate MAPK signaling pathway in thyroid cancer. When *BANCR* was knocked down with shRNA, only p44 ERK1 and p42 ERK2 MAPKs were increased; the upstream molecules were not affected. When *BANCR* was overexpressed, the MAPK signaling pathway was not inactivated, as previous studies show that *BANCR* can recruit zeste homolog 2 (EZH2) to enhance thyroid

stimulating hormone receptor which can activate MAPK pathway, we speculate may be this affect the final activation of this pathway. The PI3K-Akt pathway was activated by *BANCR* knockdown, as represented by increased PI3K and Akt phosphorylation. In contrast, the PI3K-Akt pathway was inactivated when *BANCR* was upregulated in NPA but not TPC1 cells. TPC1 cells are a thyroid cancer cell line that contain a RET/PTC rearrangement that can activate the PI3K-Akt pathway; therefore, these complex observations may stem from the different gene expression profiles in the two cancer cell lines. Therefore, we concluded that the effects of *BANCR* on the proliferation and migration of PTC cells were exerted via the ERK MAPK and PI3K-Akt activations pathways.

Clinical studies performed in *BRAF*-mutant metastatic melanoma patients treated with selective *BRAF*<sup>V600E</sup> inhibitors treatment revealed that some patients eventually relapse and succumb to acquire chemoresistance via mechanisms involving the reactivation of MAPK signaling [39-41]. Because *BANCR* was upregulated by the *BRAF*<sup>V600E</sup> mutation, the effects of *BANCR* on the MAPK pathway provide a novel mechanism behind the regulation of *BRAF*<sup>V600E</sup> mutations in PTC. These findings will help us fully understand the oncogenesis of PTC.

In summary, the current study demonstrated that *BANCR* is downregulated in PTC tissues. *BANCR*

expression in PTC was significantly correlated lymph node metastasis. The effects of *BANCR* on cell proliferation and migration were mediated by inducing cell cycle arrest in G2/M phase and inducing apoptosis. Finally, *BANCR* regulated cell proliferation by activating the ERK-MAPK and PI3K-Akt pathways. Taken together, these findings suggest that *BANCR* plays a role in the oncogenic process; it may be both a new potential target and prognostic factor for PTC. Further investigations are required to investigate this in more detail.

## Abbreviations

*BANCR*, *BRAF*-activated non-protein coding RNA; PTC, papillary thyroid cancer, lncRNAs, long non-coding RNAs; shRNA, short hairpin RNA; FTC, follicular thyroid cancer; PDTC, poorly differentiated thyroid cancer; ATC, anaplastic thyroid cancer; *HOTAIR*, *HOX* transcript antisense RNA; *MALAT1*, metastasis associated lung adenocarcinoma transcript 1; *GAS5*, growth arrest specific 5; *STR*, short tandem repeat; MAPK, mitogen activated protein kinase; PI3K, phosphatidylinositol 3'-kinase.

## Supplementary Material

Supplementary figure and table.

<http://www.jcancer.org/v09p1318s1.pdf>

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## Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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