Expression of BANCR promotes papillary thyroid cancer by targeting thyroid stimulating hormone receptor

HAITAO ZHENG[1*], JIE XU[1*], SHAOLONG HAO[1], XINCHENG LIU[1], JINRAO NING[1], XICHENG SONG[2], LIXIN JIANG[1] and ZONGYING LIU[3]

Departments of [1]Thyroid Surgery, and [2]Ear, Nose and Throat & Head and Neck Surgery, Yantai Yuhuangding Hospital, Yantai, Shandong 264099; [3]Department of Laboratory, People's Hospital of Pingyi County, Pingyi, Shandong 273399, P.R. China

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Abstract. Papillary thyroid carcinoma (PTC) is the most common form of non-medullary thyroid cancer, accounting for ~80% of all cases of thyroid cancer. The aim of the present study was to explore the role of BRAF-activated long noncoding RNA (BANCR) in the development of PTC. Using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), the mRNA expression levels of BANCR, thyroid-stimulating hormone receptor (TSHR) and cyclin D1 between PTC and benign control thyroid nodule tissue samples from 60 patients were determined. Using RT-qPCR and western blot analysis, the expression levels of TSHR and cyclin D1 mRNA and protein were determined in cells transfected with BANCR-small interfering (si)RNA. An MTT assay and flow cytometry were used to analyze the effect of BANCR knockdown on the proliferation and cell cycle distribution of IHH-4 PTC cells. The expression of BANCR, TSHR and cyclin D1 was increased in the PTC group compared with the control group based on the RT-qPCR data. The transfection of IHH-4 cells with BANCR-siRNA induced the inhibition of TSHR and cyclin D1 expression compared with a transfection control. In addition, the proliferation of the IHH-4 cells transfected with BANCR-siRNA was suppressed, relative to the transfection control, and cells arrested in the G0/G1 phase, potentially due to the inhibition of the expression of cyclin D1. The data suggested that the expression of BANCR may promote the development of malignant thyroid nodules via the modulation of TSHR expression and its downstream effector, cyclin D1.

Correspondence to: Dr Zongying Liu, Department of Laboratory, People's Hospital of Pingyi County, 7 Jinhua Road, Pingyi, Shandong 273399, P.R. China
E-mail: bancrtshr@yeah.net

*Contributed equally

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Introduction

Thyroid nodules are commonly identified entities in clinical practice; 50% are identified on autopsy, 33-68% by ultrasound examination and 4% by palpation (1-3). Certain sonographic, laboratory and historical characteristics are associated with an increased likelihood of malignancy, although ~95% of thyroid nodules are benign (4). Thyroid scintigraphy applies technetium-99m-(^{99m}Tc) pertechnetate or radioiodine (^{123}I, ^{131}I) to distinguish between potential diagnoses (5). The differentiation is critical, as hyperfunctioning nodules, also called 'hot', 'autonomously-functioning' or 'autonomous' nodules, may infrequently harbor malignant tumors, and diagnostic fine needle aspiration (FNA) is not conventionally applied in these cases (6). It is difficult to evaluate nodule growth as a risk factor for malignant tumors, as the majority of affected patients immediately undergo surgery for their removal. Although an elevation in nodule size is an indication for biopsy, this observation is of limited specificity for malignancy, as 9-89% of benign nodules have been demonstrated to grow over time depending on the criteria for growth that was applied (7).

Papillary thyroid carcinoma (PTC) constitutes ~80% of cases of thyroid malignancy (8). Thyroid stimulating hormone receptor (TSHR) has been reported to be highly expressed in both normal and differentiated thyroid cancer cells by previous studies, which indicated that there was no decreased expression of TSHR in differentiated thyroid cancer cells, whereas other studies identified an increased expression of TSHR in benign adenomas and thyroid carcinomas when compared with normal thyroid tissue (9,10). The binding of TSH to TSHR promotes cell development and proliferation, therefore promoting PTC progression (11).

Noncoding RNAs (ncRNA) are transcribed and functional RNA molecules that are highly expressed, but not translated into proteins. A previous transcriptome sequencing study identified a novel type of ncRNA that was relatively long, designated 'long noncoding RNA' (lncRNA) (11). lncRNA are defined as RNA oligonucleotides of an arbitrarily defined length of >200 nucleotides with low or no protein coding potential, typically of a reduced expression level compared with mRNA (12). lncRNA may also be processed at a post-transcriptional level to generate smaller RNA, including microRNA (miRNA), and

accumulating data indicates they are associated with enhancer regions (13,14).

Distinct from miRNAs, lncRNAs were previously considered as poorly conserved and biologically inactive transcriptional 'noise' (15). However, the regulatory role for lncRNAs in a variety of biological activities at the levels of post-transcriptional protein synthesis, mRNA transcription and chromosome modification has been demonstrated (16). Notably, a range of lncRNAs, including non-protein coding RNA, associated with MAP kinase pathway and growth arrest, BRAF-activated long noncoding RNA (BANCR) and papillary thyroid carcinoma susceptibility candidate 3 (non-protein coding) have been demonstrated to serve critical regulatory roles in the development and progression of thyroid cancer (17).

TSHR has been demonstrated to be associated with the development of PTC, and the differential expression of BANCR has also been identified in this malignancy (18-21). The present study aimed to study the association between BANCR and TSHR, and explored their potential role in the promotion of malignancy in thyroid nodules.

Materials and methods

Sample collection. The institution's Ethics and Research Committees of People's Hospital of Pingyi County (Pingyi, China) approved the study. A total of 60 patients with thyroid nodules were enrolled from the Department of Laboratory, People's Hospital of Pingyi County, between December 2011 and May 2014, and tissue samples were collected during surgery or FNA. A total of 18 patients were male and 42 were female, with an average age of 46±3.08 years. All samples were classified as malignant tissue (which were confirmed to be PTC, n=32) or benign tissue (control group, n=28). Patients who exhibited distant metastasis or recurrence prior to surgery were excluded. All patients signed informed consent for participation in the study, which was conducted according to the Declaration of Helsinki.

Cell culture and transfection with BANCR small interfering (si)RNA. IHH-4 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and incubated in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA), 2 mM glutamine (Sigma-Aldrich; Merck KGaA) and 15% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. When the cells reached 80% confluence, RPMI-1640 with 1% FBS was used to starve the cells. Lipofectamine 2000® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect the cells with BANCR- or control-siRNA (cat. no. 4392420; Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. The duration of transfection was 24 h.

RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR). TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract the total RNA from IHH-4 cells or tissue samples according to the manufacturer's protocol. A Turbo DNA-free kit (Ambion; Thermo Fisher Scientific, Inc.) was used to eliminate the contamination of genomic DNA according to the manufacturer's protocol. M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA) was used to perform the synthesis of cDNA with the extracted RNA and random primers (Takara Biotechnology Co., Ltd, Shiga, Japan) according to the manufacturer's protocol. The SYBR Green Real Master Mix kit (Tiangen Biotechnology, Inc., Beijing, China) was used to perform the RT-qPCR assays, following the standard protocol of the manufacturer, with the RT-qPCR Primer sets (cat no. R10033.1; RiboBio, Guangzhou, China) for BANCR, TSHR and cyclin D1. The $2^{-\Delta\Delta Cq}$ method was used to analyze the expression of the BANCR, TSHR and cyclin D1 (22). U6 was used as the internal reference gene to which the expression of BANCR was normalized. All reactions were performed three times.

Cell proliferation assay. Following growth for 1, 3 or 5 days, the medium was replaced with 100 μl of 5 mg/ml MTT (Sigma-Aldrich; Merck KGaA), and the cells were incubated for a further 3 h at 37˚C. The supernatant was removed, and 100 μl dimethyl sulfoxide was added into each well, mixed thoroughly to dissolve the formed crystal formazan and incubated for 10 min at 4˚C. A microplate reader was used to determine the viability of the IHH-4 cells based on the absorption at 570 nm. Each experiment was performed in triplicate. Cell viability rate was calculated as: (Number of total cells-Number of dead cells)/Number of total cells x100.

Western blot analysis. IHH-4 cells were harvested and washed with ice-cold PBS (Invitrogen; Thermo Fisher Scientific, Inc.). Radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA) containing 10 mM Tris/HCl (pH 7.4), 0.5% Triton X-100, 150 mM NaCl and protease inhibitors (Sigma-Aldrich; Merck KGaA) were used to lyse the cells, according to the manufacturer's protocol. The cells were then homogenized on ice. The cellular lysates were centrifuged for 15 min at 13,500 x g at 4˚C. The Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to determine the concentration of protein. A 12% SDS-PAGE gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to separate the proteins (15 μg/lane), followed by semi-dry transference to a nitrocellulose membrane (Whatman; GE Life Science, Chicago, IL, USA). Then, 5% (w/v) non-fat dried skimmed milk powder was used to block the membranes to avoid nonspecific binding at room temperature for 2.5 h, followed by washing the membranes with Tris-buffered saline with 0.05% Tween-20.

The membrane was incubated with antibodies against TSHR (dilution, 1:3,000; cat.no. 2143S), cyclin D1 (dilution, 1:1,000; cat.no. 2922S; both Cell Signaling Technology, Inc., Danvers, MA, USA) or β-actin (dilution, 1:10,000; cat. no. A5441; Sigma-Aldrich; Merck KGaA) at 4˚C for 12 h (TSHR and β-actin) or at 25˚C for 1 h (cyclin D). PBS with 0.05% Tween-20 (PBST) was used to wash the membranes three times, and then the appropriate Goat Anti-Rabbit IgG secondary antibodies conjugated to horseradish peroxidase (cat. no., 7074S; 1:10,000, Cell Signaling Technology, Inc.) were incubated with the membranes for an additional 1 h, followed by washing three times with PBST.

The Immun-Star™ HRP Chemiluminescence kit peroxide buffer and luminol/enhancer (Bio-Rad Laboratories, Inc.) were used to treat the membranes in order to detect the protein bands, according to the manufacturer's protocol. All procedures were repeated three times.

Cell cycle analysis. PBS was used to wash the cells, and Hank's Buffered Salt solution (including 137 mmol/l NaCl, 0.25 mmol/l Na_2HPO_4, 5.4 mmol/l KCl, 0.44 mmol/l KH_2PO_4, 1.0 mmol/l $MgSO_4$, 1.3 mmol/l $CaCl_2$ and 4.2 mmol/l $NaHCO_3$) with ice-cold 80% ethanol was used to fix the cells for 30 min on ice. Centrifugation at 167.7 x g, for 10 min at room temperature, was used to collect the cells and PBS was used to wash the cells twice. Propidium iodide (50 mg/ml; Sigma Aldrich; Merck KGaA) containing 0.1 mg/ml RNAse A and 0.6% NP-40 (Thermo Fisher Scientific, Inc.) was used to stain the cells in darkness for 30 min at room temperature. Centrifugation at 167.7 x g, for 10 min at 4˚C was used to collect the fixed cells, and PBS was used to wash the cells. Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and the cell-cycle profile was analyzed using FlowJo 10.0.4 (FlowJo LLC, Ashland, OR, USA). All procedures were performed three times.

Statistical analysis. All experiments were performed in triplicate and all data are presented as mean ± standard deviation. A paired two-tailed Student's t-test and one-way analysis of variance with Student-Newman-Keuls post hoc test were used to perform the statistical analysis. SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of BANCR varies between PTC and control groups. In order to identify the effect of BANCR on pathogenesis of PTC, RT-qPCR was used to examine the expression of BANCR in 32 PTC and 28 benign (control) thyroid nodule samples. As demonstrated in Fig. 1, the level of BANCR in PTC group was increased compared with the control (P<0.0001), suggesting that the upregulation of BANCR is associated with the development of PTC.

BANCR exerts an inhibitory effect on TSHR expression. The interaction between TSH and TSHR leads to the proliferation of thyroid cells. IHH-4 PTC cells were used to investigate the effect of BANCR on the expression of TSHR. IHH-4 cells were transfected with siRNA to suppress the expression of BANCR. The level of TSHR in cells transfected with BANCR- or control-siRNA was determined using RT-qPCR and western blot analysis. The knockdown of BANCR reduced the level of TSHR mRNA (Fig. 2A; P<0.05) and protein (Fig. 2B; P<0.05) expression compared with the control-siRNA group, suggesting that BANCR reduced the expression of TSHR in IHH-4 cells.

BANCR promotes IHH-4 cell proliferation and alters the IHH-4 cell cycle distribution. As demonstrated in Fig. 3A,

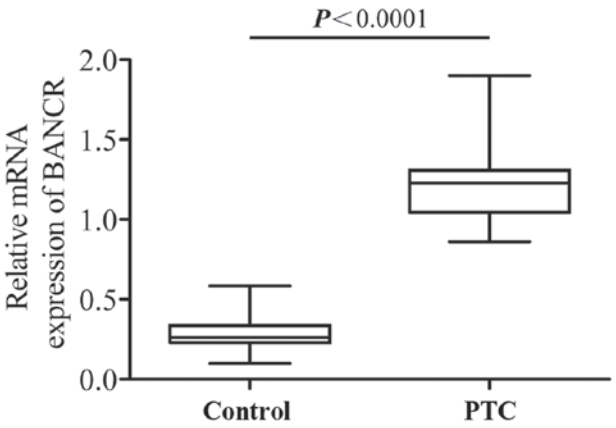

Figure 1. Expression of BANCR in the PTC group compared with the benign tissue group as detected with reverse transcription-quantitative polymerase chain reaction. BANCR, BRAF-activated long noncoding RNA; PTC, papillary thyroid cancer.

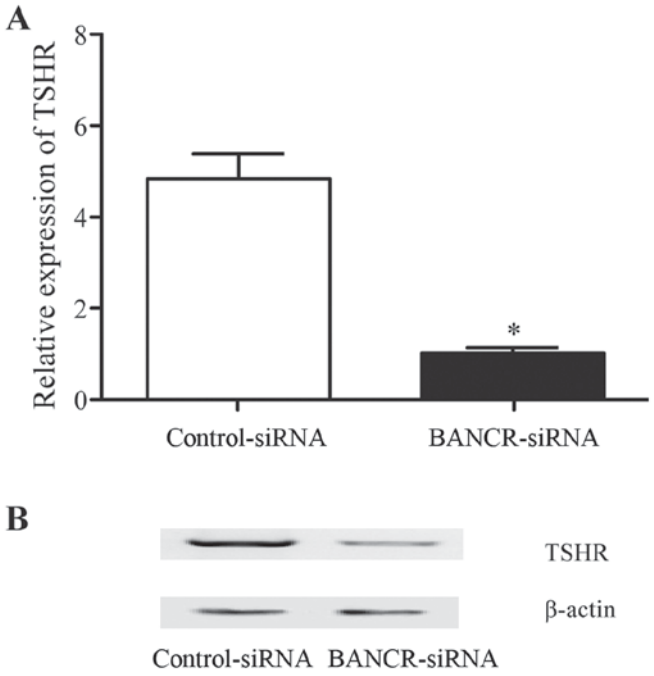

Figure 2. Effect of BANCR on TSHR expression in IHH-4 cells transfected with BANCR-siRNA or control-siRNA as determined (A) with reverse transcription-quantitative polymerase chain reaction or (B) western blotting. BANCR, BRAF-activated long noncoding RNA; TSHR, thyroid stimulating hormone receptor; siRNA, small interfering RNA. *P<0.05 vs. Control-siRNA.

the proliferation of cells transfected with BANCR-siRNA was suppressed at 3 and 5 days post-transfection compared with the control-siRNA group (P<0.05). The data indicated that BANCR knockdown exhibited a suppressive effect on the proliferation of IHH-4 cells.

Flow cytometry was used to analyze the effect of BANCR on the cell cycle distribution. As demonstrated in Fig. 3B (P<0.05), the G_0/G_1 phase proportion of cells transfected with BANCR-siRNA was significantly increased, whereas the S phase percentage was decreased, compared with the control-siRNA group, suggesting that silencing BANCR

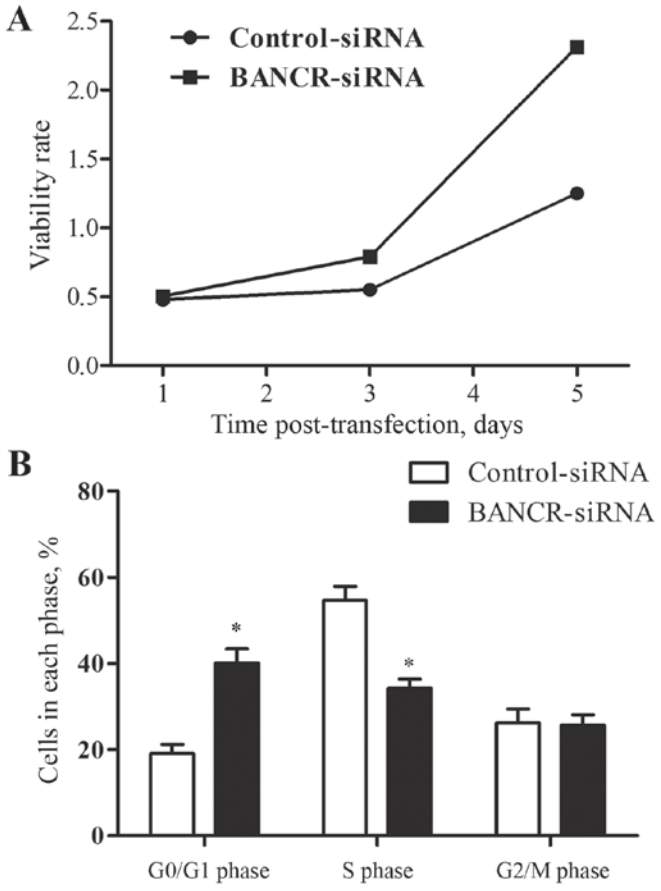

Figure 3. Effect of BANCR knockdown. (A) Effect of BANCR-siRNA or control-siRNA on the proliferation of IHH-4 cells as determined with an MTT assay. (B) Effect of BANCR-siRNA or control-siRNA on the cell cycle distribution of IHH-4 cells as determined with flow cytometry. BANCR, BRAF-activated long noncoding RNA; siRNA, small interfering RNA. *P<0.05 vs. Control-siRNA.

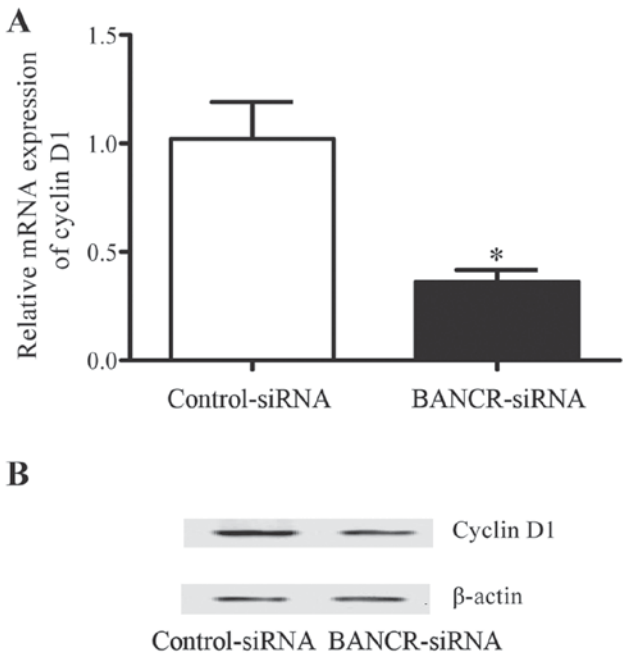

Figure 4. Effect of BANCR knockdown on cyclin D1 expression in IHH-4 cells as determined (A) with reverse transcription-quantitative polymerase chain reaction and (B) western blotting. BANCR, BRAF-activated long noncoding RNA; siRNA, small interfering RNA. *P<0.05.

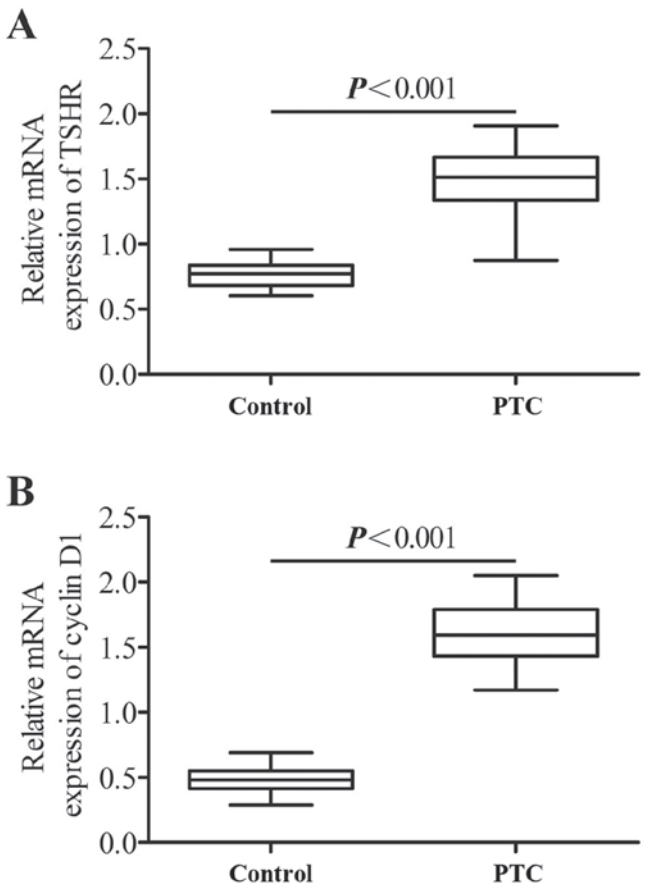

Figure 5. Expression of (A) TSHR and (B) cyclin D1 in the PTC group compared with the benign tissue group was determined using reverse transcription-quantitative polymerase chain reaction. TSHR, thyroid stimulating hormone receptor; PTC, papillary thyroid cancer; BANCR, BRAF-activated long noncoding RNA.

decreased the rate of G1 to S phase transition. Consequently, BANCR knockdown increased the rate of cell growth, and inhibited G1 to S phase transition.

BANCR affected IHH-4 cell cycle progression by targeting cyclin D1. RT-qPCR and western blotting were used to confirm that BANCR regulated the cell cycle through upregulating the expression of cyclin D1. As demonstrated in Fig. 4, IHH-4 cells transfected with BANCR-siRNA exhibited a significantly lower level of cyclin D1 mRNA (Fig. 4A, P<0.05) and protein (Fig. 4B) compared with the cells transfected with control-siRNA, suggesting that BANCR knockdown inhibited G1 to S phase transition via the downregulation of the expression of cyclin D1.

TSHR and cyclin D1 are upregulated in PTC. In order to further validate the effect of BANCR on the pathogenesis of PTC through the regulation of the expression of TSHR and cyclin D1, RT-qPCR was used to examine the level of TSHR and cyclin D1 mRNA in PTC and control thyroid nodule groups. As indicated in Fig. 5, the level of TSHR (Fig. 5A; P<0.001) and cyclin D1 (Fig. 5B; P<0.001) mRNA in the PTC group was markedly increased compared with the control group, suggesting that the upregulation of TSHR and cyclin D1 may be risk factors for PTC.

Discussion

A steadily increasing trend in the morbidity of thyroid cancer, the most frequently identified malignancy of the endocrine organs, has been observed in previous decades (23). As the major type of non-medullary thyroid cancer, PTC accounts for ~80% of all thyroid cancers (24). It has been demonstrated that lncRNAs, including BANCR, can modulate the survival or apoptosis of thyroid cancer cells (17). The function of lncRNAs in the regulation of TSHR expression has yet to be characterized, although alterations to the expression of lncRNAs have been observed in various cell types of thyroid cancer (17). In the present study, 60 participants with malignant or benign (control) diagnosed thyroid nodules were enrolled, and tissue samples were collected during surgery or FNA. In order to identify the effect of BANCR on the pathogenesis of PTC, RT-qPCR was used to examine the levels of BANCR in the PTC and control groups. As demonstrated in Fig. 1, the level of BANCR in the PTC group was increased compared with the control group, suggesting that the upregulation of BANCR was associated with the development of PTC.

Flockhart *et al* (25) originally identified BANCR as a 693-bp lncRNA in melanoma cells. Subsequently, the abnormal expression of lncRNA BANCR was demonstrated in colorectal, gastric and lung cancer, retinoblastoma and papillary thyroid carcinoma (26-29). Elevated levels of BANCR were observed in human advanced malignant melanoma tissues, and melanoma cell proliferation and metastasis were demonstrated to be inhibited by the knockdown of BANCR via the mitogen-activated protein kinase pathway (25). In stomach cancer, tumor tissues exhibited increased expression of BANCR when compared with paired adjacent normal tissues (28). Elevated BANCR levels were also associated with poor prognosis, lymph node and distant metastasis, tumor depth and clinical stage (27). In retinoblastoma, BANCR served a regulatory role in cell proliferation, metastasis and dissemination *in vitro*, and the increased expression of BANCR was correlated with optic nerve invasion, tumor size and choroidal invasion (29). In contrast to these tumor-promoting actions, Sun *et al* (28) suggested that BANCR was significantly decreased in tissue from non-small cell lung cancer tumors, and that a lower expression of BANCR was associated with shorter overall survival time, advanced stage, lymph node metastasis and a larger tumor size. Cell viability and invasion were impaired by ectopic BANCR expression, resulting in the suppression of metastasis *in vitro* and *in vivo* (29). There is data to indicate that lncRNAs, including BANCR, may have regulatory roles in the epithelial-mesenchymal transition processes in a range of cancer types (30).

TSHR is a typical G protein-coupled receptor; TSH interaction with TSHR results in heterotrimeric G protein activation (31). Subsequently, the stimulatory subunit Gsα dissociates from the G protein complex to activate adenylyl cyclases and the production of the secondary messenger cyclic adenosine monophosphate (cAMP), resulting in protein kinase A (PKA) signaling (32). Mutations in Gsα are only observed in a low proportion of thyroid cancer cases, and thyroid cancer occurs in a small number of patients with McCune-Albright syndrome (associated with increased cAMP signaling) (33). FTC spontaneously occurred in TRβPV mice, although no thyroid cancer occurred when TRβPV mice were bred with *Tshr*$^{-/-}$ mice, demonstrating that TSHR signaling was necessary for thyroid carcinogenesis in the mouse model (34). Consistent results were obtained in another mouse model, in which the development of aggressive PTC was induced by thyroid-specific knock-in of *Braf* (LSL-*Braf*^{V600E} thyroid peroxidase-Cre); thyroid cancer was not identified when these mice were bred with *Tshr*$^{-/-}$ mice (35). Notably, elevated serum levels of TSH were clinically correlated with an increased risk of malignant thyroid nodules in humans (36).

An additional two models simulating the over-activation of TSHR through constitutive Gsα activation included a mouse with expression of cholera toxin A1 subunit and a *Tg-gsp* mouse with the constitutive activation of Gsα subunit modulated by the Tg promoter. Thyroid cancer did not occur in these models despite the development of hyperthyroidism and goiters (37). A mouse model simulating the human syndrome of Carney complex, which is caused by an over-activation of PKA, was established by introducing a null allele of Protein kinase cAMP-dependent type 1 regulatory subunit α (*Prkar1a*) into *Prkar1a*-heterozygous mice. Thyroid cancers occurred in ~9% of these mice, which were identified as PTC; however, the earliest event occurred at the age of 13.5 months (38). It appears that in mice, the upregulation of PKA and/or TSHR is not adequate to trigger tumorigenesis, although it may induce a pre-disposition for PTC (30).

In the present study, RT-qPCR was performed to examine the levels of BANCR, TSHR and cyclin D1 in PTC and control tissue; it was identified that the expression of BANCR, TSHR and cyclin D1 in the PTC group was increased compared with the control group. IHH-4 cells were transfected with control- and BANCR-siRNA to confirm that the interaction between TSH and TSHR led to an alteration in the function and proliferation of thyroid cells. Western blot analysis and RT-qPCR were performed to detect the levels of TSHR and cyclin D1 in control- and BANCR-siRNA groups, and it was identified that BANCR knockdown resulted in the inhibition of TSHR and cyclin D1 mRNA and protein expression compared with control-siRNA groups. Furthermore, the effect of BANCR on cell proliferation and cell cycle was analyzed using an MTT assay and flow cytometry; it was revealed that the proliferation of cells transfected with BANCR-siRNA was suppressed at 3 and 5 days post-transfection compared with the control-siRNA group, and the proportion of cells in G_0/G_1 phase in the BANCR-siRNA group was significantly increased, whereas the S phase proportion was lower, compared with the control-siRNA group.

The data of the present study demonstrated that the expression of BANCR may promote the development of malignant thyroid nodules from benign nodules by altering the proliferation of thyroid cells, which is potentially attributed to the ability of BANCR to alter the cell cycle progression in thyroid cells by increasing the expression of TSHR and its downstream effector, cyclin D1.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

HZ planned the study, collected the literature, analyzed and interpreted the data, and prepared the manuscript; JX planned the study, collected and analyzed the data, and prepared the manuscript; SH collected the literature, collected, analyzed and interpreted the data, and prepared the manuscript; XL collected, analyzed and interpreted the data; JN collected the literature, and analyzed and interpreted the data; XS collected and analyzed the data, and prepared the manuscript; LJ collected the literature, interpreted the data; ZL analyzed and interpreted the data, and prepared the manuscript. All authors approved the final manuscript.

Ethics approval and consent to participate

The institution's Ethics and Research Committees of People's Hospital of Pingyi County (Pingyi, China) approved the study. All patients signed informed consent for participation in the study, which was conducted according to the Declaration of Helsinki.

Consent for publication

All patients signed informed consent for participation in the study, which was conducted according to the Declaration of Helsinki.

Competing interests

The authors declare that they have no competing interests.

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