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Increased expression of the lncRNA BANCR and its prognostic significance in human osteosarcoma

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ABSTRACT. Long noncoding RNAs (lncRNAs) play important roles in the formation and progression of many types of human malignancies. The aim of our study was to investigate the expression and biological functions of the lncRNA BRAF-activated noncoding RNA (BANCR) in human osteosarcoma. BANCR expression was quantified by real-time PCR in human osteosarcoma cell lines and tissues. We analyzed the association between BANCR levels and clinicopathological factors and patient prognosis. MTT, flow cytometric, and transwell invasion assays were performed to observe the effects of BANCR on MG-63 cell biological behaviors. BANCR overexpression was observed in osteosarcoma cell lines and clinical specimens. Increased BANCR expression was significantly associated with large tumor size, positive distant metastasis, and advanced clinical stage. High BANCR expression in osteosarcoma was an independent predictor of poor survival. Downregulation of BANCR inhibited MG-63 cell proliferation and invasion and promoted cell apoptosis

in vitro. These findings suggested that BANCR may act as a tumor promoter in osteosarcoma and could serve as a potential therapeutic target for this disease.

Key words: Long noncoding RNA; BANCR; Osteosarcoma; Prognosis; Invasion

INTRODUCTION

Osteosarcoma is the most common primary bone tumor that occurs predominantly in adolescents and young adults (Geller and Gorlick, 2010). Despite recent advancements in multimodal treatments (e.g., wide tumor excision, adjuvant chemotherapy, and radiotherapy), survival expectancies have not improved much over the past 25 years (Kager et al., 2003), especially for patients with metastasis and recurrent osteosarcoma. Previous studies have provided some clues to the molecular pathogenesis of osteosarcoma (Bilbao-Aldaiturriaga et al., 2015; Chen et al., 2015; Sun et al., 2015a; Yuan et al., 2015; Zhao et al., 2015). However, the exact mechanisms underlying its formation and progression are still obscure and further identification of new candidate molecules participating in these processes is important for improving the diagnosis, prevention, and treatment of this disease.

Long noncoding RNAs (lncRNAs), members of the noncoding RNA family, are longer than 200 nucleotides and encode proteins. lncRNAs can regulate gene expression at the transcriptional or posttranscriptional level (Guttman and Rinn, 2012; Cheetham et al., 2013). lncRNAs have been implicated in a large number of cellular processes, such as proliferation, cell cycle progression, growth, and apoptosis (Mercer et al., 2009). In the last 10 years, an increasing number of studies have reported that lncRNA expression is obviously dysregulated in various cancers and that these lncRNAs play critical roles in tumor development, progression, and metastasis (Gibb et al., 2011; Zhang et al., 2013a). In human osteosarcoma, Sun et al. (2015b) demonstrated that the lncRNA HULC was significantly upregulated in osteosarcoma tissues and that the overexpression of HULC was correlated with advanced clinical stage and distant metastasis. Moreover, higher HULC expression was associated with shorter overall survival (OS) (Sun et al., 2015b). Furthermore, decreased expression of HULC markedly suppressed osteosarcoma cell proliferation, migration, and invasion. Zhang et al. (2013b) showed that downregulation of the lncRNA TUG1 inhibited osteosarcoma cell proliferation and promoted cell apoptosis.

BRAF-activated noncoding RNA (BANCR), a 693-bp lncRNA, was originally identified in melanoma cells by Flockhart et al. (2012). Subsequently, aberrant BANCR expression was confirmed in papillary thyroid carcinoma (Wang et al., 2014), retinoblastoma (Su et al., 2015), lung cancer (Sun et al., 2014, Jiang et al., 2015), gastric cancer (Li et al., 2015), and colorectal cancer (Guo et al., 2014). BANCR regulated cell proliferation, migration, and invasion in these tumors and thus may serve as a potential oncogene or candidate tumor suppressor. However, the significance of BANCR in osteosarcoma is still unclear. In the present study, we examined BANCR expression in osteosarcoma samples and cell lines using real-time (RT) PCR. The association of BANCR levels with clinicopathologic features and prognosis was also analyzed. Furthermore, we investigated the biological functions of BANCR in osteosarcoma cells.

MATERIAL AND METHODS

Patients and clinical specimens

Paired osteosarcoma tissues and adjacent nontumor tissues were obtained from 84 pathological diagnosed osteosarcoma patients at The First Affiliated Hospital of Guangxi Medical University (China) between January 2008 and December 2010. All samples were frozen immediately in liquid nitrogen and stored at -80°C until analysis. None of the patients had previously received radiotherapy, chemotherapy, or immunotherapy. Patients characteristics are described in Table 1. Tissue sample use was approved by the Ethics Committees of our hospital and written informed consent was obtained from all study participants.

Table 1. Correlation between BANCR expression and different clinicopathological features in 84 osteosarcoma patients.

Clinicopathological features	Number of cases	BANCR expression		P
		Low [N (%)]	High [N (%)]	
Age				
<20 years	50	27 (54.0%)	23 (46.0%)	0.505
≥20 years	34	15 (44.1%)	19 (55.9%)	
Gender				
Female	36	20 (55.6%)	16 (44.4%)	0.509
Male	48	22 (45.8%)	26 (54.2%)	
Tumor size				
≤8 cm	43	28(65.1%)	15(34.9%)	0.008
>8 cm	41	14(34.1%)	27(65.9%)	
Anatomic location				
Tibia/femur	58	30 (51.7%)	28 (48.3%)	0.814
Elsewhere	26	12 (46.2%)	14 (53.8%)	
Serum level of alkaline phosphatase				
Elevated	53	24 (45.3%)	29 (54.7%)	0.366
Normal	31	18 (58.1%)	13 (41.9%)	
Clinical stage				
IIA	38	26 (68.4%)	12 (31.6%)	0.004
IIB/III	46	16 (34.8%)	30 (65.2%)	
Distant metastasis				
Absent	54	32 (59.3%)	22 (40.6%)	0.02
Present	30	10 (33.3%)	20 (66.7%)	

Cell culture and transfection

Human osteosarcoma cell lines (HOS, Saos-2, U2OS, and MG-63) and human normal bone cell line hFOB were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate. Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C.

BANCR small interfering RNA (si-BANCR) and a nontargeting siRNA (si-NC) were purchased from RiboBio (China) and transfected into osteosarcoma cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer instructions. The cells were harvested for cell proliferation and invasion assays 48 h after transfection.

RNA extraction, reverse transcription, and quantitative RT-PCR

Total RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer

instructions. RNA was reverse transcribed into cDNA using the Prime-Script One Step RT-PCR kit (Takara, Dalian, China). BANCR expression levels were measured by qRT-PCR using an ABI7500 system and SYBR Green PCR Master Mix (Takara). GAPDH was used as an internal control. The primer sequences for BANCR were 5'-ACAGGACTCCATGGCAAACG-3' (forward) and 5'-ATGAAGAAAGCCTGGTGCAGT-3' (reverse). Each assay was performed in triplicate and relative BANCR expression was normalized to GAPDH using the $2^{-\Delta Ct}$ method.

Cell proliferation assay

Cell proliferation was analyzed using the MTT assay. Briefly, approximately 3 x 10^3 cells/well were plated onto 96-well plates and routinely cultured. At the indicated time point, 20 µL MTT solution (5 mg/mL) was added into each well and incubated 4 h at room temperature. The reaction was terminated by adding 150 µL DMSO (Sigma, USA) and the absorbance at 490 nm was measured on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Detection of apoptosis by flow cytometry

Apoptosis was detected by flow cytometric analysis. After transfection, the cells were washed twice with cold PBS and resuspended in 1X binding buffer at a concentration of 1 x 10^6 cells/mL. The cells were stained with annexin V and propidium iodide, using the annexin V apoptosis detection kit. After incubation at room temperature in the dark for 15 min, cell apoptosis was analyzed on an FACSC-LSR (Becton, Dickinson and Company, USA).

Cell invasion assay

A transwell chamber system (8-µm pore size; Corning, USA) was used to investigate the effects of BANCR on cellular invasion. The upper chambers were coated with Matrigel (Sigma). Twenty-four hours after transfection, 1 x 10^5 osteosarcoma cells in serum free media were seeded into the upper chambers. DMEM containing 20% FBS was added to the lower chamber. Following a 24-h incubation, cells on the upper surface of the membrane were removed with cotton wool. Invasive cells located on the lower surface of the chamber were stained with Giemsa stain (Sigma) and counted using a microscope (Olympus Corp., Tokyo, Japan).

Statistical analysis

All statistical analyses were performed using the SPSS 17.0 software package (SPSS, Chicago, IL, USA). Differences between groups were analyzed by the Student *t*-test or the chi-square test. Survival curves were constructed with the Kaplan-Meier method and compared by log-rank tests. A Cox proportional hazard regression analysis was used for univariate and multivariate analyses of prognostic values. $P < 0.05$ was considered significant.

RESULTS

Increased BANCR expression in osteosarcoma tissues and cell lines

We performed qRT-PCR analysis to measure BANCR expression levels in osteosarcoma

tissues and cell lines. Our results showed that BANCR expression in human osteosarcoma tissues was significantly higher than in paired nontumor tissues (P < 0.05; Figure 1A). BANCR expression was also significantly higher in four osteosarcoma cell lines than in the hFOB cell line (P < 0.05; Figure 1B). The MG-63 cell line, which exhibited the highest BANCR expression among all tested cell lines, was chosen for the subsequent *in vitro* experiments.

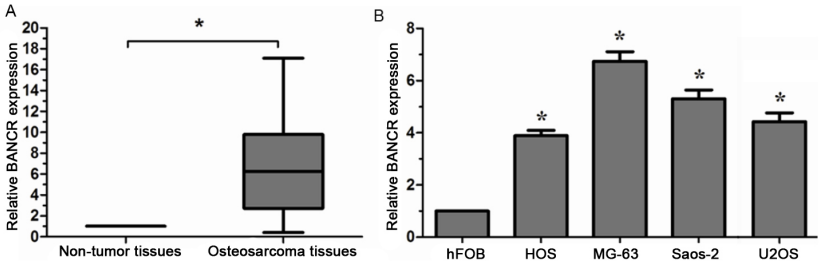

Figure 1. BANCR expression levels in osteosarcoma tissues and cell lines. **A.** BANCR expression was significantly higher in osteosarcoma tissues than in the corresponding noncancerous bone tissues. **B.** BANCR expression was upregulated in osteosarcoma cell lines HOS, Saos-2, U2OS, and MG-63, relative to the human normal bone cell line hFOB. *P < 0.05.

Correlation between BANCR expression and clinical features and prognosis of osteosarcoma patients

To evaluate the correlation between BANCR expression and clinicopathological characteristics, the 84 osteosarcoma patients were classified into either the low BANCR expression group (N = 42) or the high BANCR expression group (N = 42) according to the median BANCR level. We found that high BANCR levels significantly correlated with larger tumor sizes (P = 0.008), positive distant metastasis (P = 0.02), and advanced clinical stage (P = 0.004). No significant difference was observed between BANCR expression and patients' age, gender, anatomic location, and serum alkaline phosphatase level.

Kaplan-Meier survival analysis showed that patients in the low BANCR expression group had better OS than those in the high BANCR expression group (P = 0.001; Figure 2).

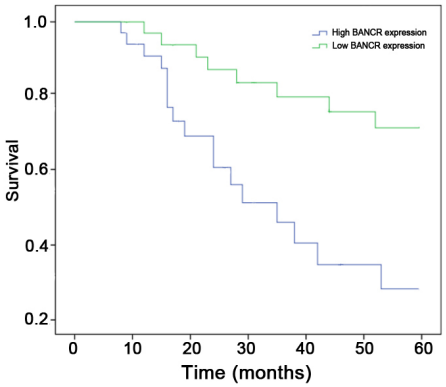

Figure 2. Kaplan-Meier survival curves of patients with osteosarcoma based on BANCR expression status. Patients in the high BANCR expression group had significantly poorer prognoses than those in the low BANCR expression group (P = 0.001, log-rank test).

Aside from BANCR expression, univariate Cox proportional hazard regression analysis revealed that tumor size, metastasis status, and clinical stage were also predictive factors for prognosis. Multivariate Cox proportional hazard regression analysis confirmed high BANCR expression (P = 0.028, HR = 2.934) as an unfavorable prognostic factor independent of other clinicopathological factors, including advanced tumor stage (P = 0.001, HR = 5.395) and distant metastasis (P = 0.005, HR = 4.878; Table 2).

Table 2. Univariate and multivariate analysis of overall survival in 84 patients with osteosarcoma.

Variable	Univariate analysis		Multivariate analysis	
	HR	P	HR	P
Age (years)	1.643	0.217	-	-
Gender	1.145	0.762	-	-
Anatomic location	1.715	0.176	-	-
Tumor size	2.338	0.024	2.015	0.082
Serum AKP	1.405	0.273	-	-
Clinical stage	3.266	0.018	5.395	0.001
Distant metastasis	5.406	0.006	4.878	0.005
BANCR level	5.738	0.001	2.934	0.028

BANCR: BRAF-activated noncoding RNA; AKP: alkaline phosphatase level; HR: hazard regression.

Effects of BANCR downregulation on the biological behaviors of MG-63 cells

We explored the role of BANCR in the development of osteosarcoma. BANCR expression in MG-63 cells was evidently inhibited by si-BANCR transfection (Figure 3A). As shown in Figure 3B and C, BANCR downregulation impaired MG-63 proliferation and promoted apoptosis relative to the si-NC group. In addition, the invasion ability of MG-63 cells was significantly reduced after si-BANCR transfection (Figure 3D).

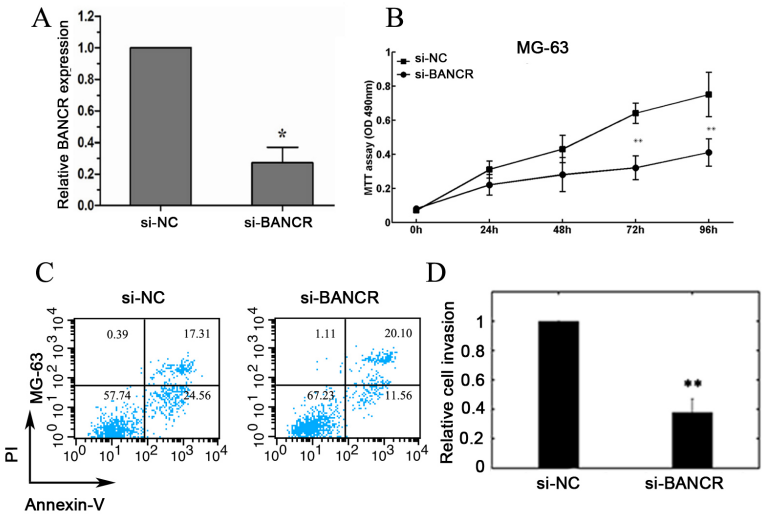

Figure 3. Effects of BANCR on the biological behaviors of MG-63 cells. **A.** Expression of BANCR was significantly silenced after si-BANCR transfection. **B.** Cell proliferation was measured by MTT assays in MG-63 cells transfected with si-BANCR or si-NC. **C.** Flow cytometric analysis showed induced cell apoptosis after si-BANCR transfection. **D.** The transwell invasion assay showed that the number of invaded cells was significantly lower in the si-BANCR-transfected group than in the si-NC-transfected group. *P < 0.05; **P < 0.01.

DISCUSSION

lncRNAs, a newly discovered class of noncoding genes, have gained attention because of their crucial roles in gene-regulatory processes. Functional lncRNAs may be applied for cancer diagnosis and prognosis and also may act as potential novel therapeutic targets (Luo et al., 2013; Liu et al., 2014). In the present study, we confirmed increased BANCR expression in osteosarcoma samples and its correlation with larger tumor size, incidence of distant metastasis, advanced tumor stage, and shorter OS. Multivariate Cox hazard regression analysis identified high BANCR expression as an indicator of unfavorable prognosis independent of other clinicpathological factors. Furthermore, *in vitro* experiments demonstrated that BANCR suppression impeded MG-63 cell proliferation and invasion and induced apoptosis. To our knowledge, this is the first report investigating aberrant BANCR expression and its biological functions in osteosarcoma.

BANCR was firstly identified by Flockhart et al. (2012). They found that BANCR knockdown reduced melanoma cell migration. Li et al. (2014) further reported that BANCR was overexpressed in human malignant melanoma cell lines and tissues and increased expression with tumor stage (Li et al., 2014). Knockdown of BANCR suppressed melanoma cell proliferation via regulation of the MAPK pathway. Similarly, Guo et al. (2014) showed that increased BANCR expression in human colorectal cancer tissues correlated with clinical stage and lymph node metastasis. Furthermore, BANCR contributes to colorectal cancer migration by inducing epithelial-mesenchymal transitions (Guo et al., 2014). In gastric cancer, high BANCR levels were positively associated with clinical stage, tumor depth, lymph node and distant metastasis, and poor prognosis (Li et al., 2015). In addition, increased BANCR expression was a poor independent prognostic factor for retinoblastoma patients and downregulation of BANCR significantly suppressed retinoblastoma cell proliferation, migration, and invasion *in vitro* (Su et al., 2015).

In contrast to the tumor-promoting properties mentioned above, a few studies indicated that BANCR might serve as a potential tumor suppressor gene. Both Jiang et al. (2015) and Sun et al. (2014) showed decreased BANCR expression in lung cancer tissues and cells lines. Reduced BANCR expression was associated with lymph node metastasis, advanced TNM stage, and shorter OS in patients with non-small cell lung cancer (Sun, et al., 2014). Downregulation of BANCR obviously promoted growth, migration, and invasion and upregulation of BANCR significantly inhibited growth, migration, and invasion in lung cancer cell lines (Sun et al., 2014; Jiang et al., 2015). Taken together, the role of BANCR in human malignancies may be multifaceted, depending on the tissue involved.

In summary, our study revealed that the expression level BANCR was significantly increased in human osteosarcoma and significantly associated with tumor development. High BANCR expression may imply a poor prognosis. Knockdown of BANCR significantly suppressed osteosarcoma cell proliferation and invasion *in vitro*. These findings suggested that BANCR might act not only as a novel diagnostic and prognostic marker, but also as a potential therapeutic target for osteosarcoma.

Conflicts of interest

The authors declare no conflict of interest.

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