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Up-regulation of long non-coding RNA BCAR4 predicts a poor prognosis in patients with osteosarcoma, and promotes cell invasion and metastasis

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Abstract. – OBJECTIVE: The long non-coding RNA BCAR4 (BCAR4) has been reported to be associated with cancer development. The aim of our study was to investigate the expression of BCAR4 in osteosarcoma patients and its association with clinicopathologic parameters and the prognosis.

PATIENTS AND METHODS: Quantitative RT-PCR (qRT-PCR) assay was used to detect the expression of BCAR4 and its correlations with clinicopathological factors were statistically analyzed. The clinical and prognostic significance of BCAR4 expression was analyzed statistically by Kaplan-Meier estimate and Cox regression model. Furthermore, Cell proliferation, migration, and invasion were evaluated using counting assay Kit-8 (CCK-8) and transwell assay, respectively.

RESULTS: We found that BCAR4 expression was higher in osteosarcoma tissues and cell lines than that in normal controls. The BCAR4 levels were significantly correlated with clinical stage and distant metastasis. Kaplan-Meier analysis with the log-rank test indicated that high expression of BCAR4 had a decreased overall survival (OS). Univariate and multivariate analyses showed that BCAR4 expression was an independent predictor of overall survival. Furthermore, decreased expression of BCAR4 markedly inhibited osteosarcoma cell proliferation, migration, and invasion.

CONCLUSIONS: The results of the present study identified a crucial tumor promotive role of BCAR4 in the progression of osteosarcoma, and suggested that BCAR4 may be a potential therapeutic agent for the treatment of osteosarcoma.

Key Words:
Long non-coding RNAs, BCAR4, Osteosarcoma, Overall survival.

Introduction

Osteosarcoma is a highly aggressive bone tumor with a poor outcome in populations of children and young adolescents[1,2]. Both genetic and environmental factors may contribute to the development of osteosarcoma. At present, the effective treatment approaches for osteosarcoma including surgery, radiotherapy, and chemotherapy, which considerably raised the survival to 65-70%[3]. However, More than 50% of patients who are chemoresistant have an extremely poor prognosis due to lung metastasis[4]. Therefore, new therapies, clinical biomarkers and treatment targets are in demand.

Long noncoding RNAs (lncRNAs) are types of transcriptional products of the eukaryotic genome comprising > 200 nt in length[5,6]. Recently, more and more evidences have shown that lncRNAs are key regulators in several biological processes, such as embryonic growth, cell proliferation, differentiation, transcriptional, and post-transcriptional regulators of gene activity[7-9]. Undoubtedly, lncRNAs have become new players in cancer after microRNAs. Specific lncRNAs have also been documented to be involved in the pathogenesis and progression of human cancers. For instance, Hu et al[10] found that long noncoding RNA MALAT1 was markedly upregulated in ESCC tissues and significantly promoted cell migration and invasion. Zhu et al[11] found that downregulated lncRNA ANCR expression could promote osteoblast differentiation by targeting EZH2 and regulating Runx2 expression. Shi et

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al[12] showed that downregulated Long Noncoding RNA BANCR promotes the proliferation of colorectal cancer cells via downregulation of p21 expression. It was reported that lncRNA BCAR4 contributed to antiestrogen resistance and promoted breast cancer proliferation and metastasis through regulating noncanonical Hedgehog/GLI2 pathway[13]. However, to date, the clinicopathologic or prognostic value of BCAR4 expression in osteosarcoma has not been investigated.

In this work, we examined BCAR4 levels in osteosarcoma patients and investigated the correlation between BCAR4 levels and clinicopathological characteristics and patient survival. Finally, we explored the effect of BCAR4 in cell proliferation, migration, and invasion. Our results showed that the evaluation of BCAR4 levels might be a valuable prognostic marker for osteosarcoma.

Patients and Methods

Patients and Specimens

This study was approved by the Research Ethics Committee of Yishui Central Hospital. Written informed consent was obtained from all of the patients. A total of 168 samples were collected from patients with osteosarcoma and adjacent non-tumor tissues between 2007 and 2010 from patients who were undergoing surgery in Department of Orthopedics, Yishui Central Hospital. None of the patients investigated had received chemotherapy before the surgery. All patients investigated in the study were diagnosed with osteosarcoma, and the diagnosis and the pathological type were confirmed by two pathologists. The surgically removed tissues were collected and immediately placed in liquid nitrogen and then stored at -80 °C until analysis.

Cell Culture and Transfection

Cell lines: hFOB, MG-63, U2OS and SW1353 cell lines (CCTCC, Wuhan, Hebei, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA; GE Health Care, Wauwatosa, WI, USA) supplemented with 10% fetal bovine serum. All cells were cultured in a cell incubator with humidified atmosphere and 5% CO_2 at 37°C.

Antihuman siRNAs which were purchased from Life Technologies (Carlsbad, CA, USA) were used for BCAR4. For transfection, U2OS cells were cultured in a 6-well plate and transiently transfected at 70-80% confluence using the Lipo- fectamineTM 2000 reagent (Invitrogen, Carlsbad, CA USA) as per the manufacturer's instructions.

Cell Proliferation Assay

U2OS cells were seeded in 96-well culture plates at a density of 3000 cells/well. Cell proliferation was analyzed at 24, 48, 72, and 96 h after transfection using a Cell Counting Kit-8. A volume of 10 μL CCK-8 solution was added to each well, and the cells were incubated for another 1 h in a humidified incubator at 37°C. Then, optical density was measured at 450 nm using a microplate reader.

Cell Migration and Invasion Assay

For migration assay, 5× 104 transfected cells were placed in the upper chamber of each insert. For invasion assay, 5×104 transfected cells were placed in the upper chamber of each insert coated with 150 mg Matrigel. The lower chamber of transwell was then filled with DMEM medium with 20% FBS. After 36 hours of incubation, cells that had invaded the lower chamber were fixed with 4% paraformaldehyde, stained with hematoxylin, and counted using a microscope.

RNA Extraction and Real-time PCR Analysis

Total RNA was isolated from tissues or cultured cells treated with Trizol reagent (Life Technologies, Carlsbad, CA, USA). RNA was reverse transcribed into cDNAs using the Prime-Script™ one step RT-PCR kit (TAKARA, Dalian, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the SYBR Select Master Mix (Applied Biosystems, Cat: 4472908) with 0.5 μl cDNA on ABI 7300 system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. BCAR4 expression level was determined by qRT-PCR using the following primer sequences: BCAR4: 5'-ACAGCAGCTTGTTGCTCATCT-3' (forward) and 5'-TTGCCTTGGGGACAGTTCAC-3'(reverse). GAPDH was also included as an internal control with primers: 5'-GTCAACGGATTTGGTCTGTATT-3' (forward) and 5'-AGTCTTCTGGGTGGCAGTGAT-3' (reverse). The relative quantitative value was expressed by the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed in triplicates and repeated three times.

Statistical Analysis

All data were repeated at least three times and indicated that the difference was statistically significant with *t*-test, The relationships between BCAR4 expression and clinicopathological parameters were examined by chi-square test. Overall survival of patients was estimated by the Kaplan-Meier method. Cox proportional hazards regression model was used for univariate and multivariate analyses of the prognostic significance of BCAR4. All the statistical analyses were performed using SPSS13.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Elevated Expression of BCAR4 in Human Osteosarcoma Tissues and Cell Lines

To evaluate the potential role of BCAR4 in osteosarcoma, we first quantified BCAR4 in 168 pairs of osteosarcoma tissues and matched adjacent non-tumor tissues using qRT-PCR methods. We found that the BCAR4 was up-regulated in osteosarcoma tissues compared with the normal tissues ($p < 0.01$, Figure 1A). Furthermore, endogenous expression of BCAR4 was detected in a panel of osteosarcoma cell lines (MG-63, U2OS, SW1353) and normal bone cells (hFOB). We found that BCAR4 expression was significantly increased in MG-63, U2OS and SW1353 compared with hFOB (Figure 1B). This suggests that BCAR4 may be involved in the development of osteosarcoma.

Relationship Between BCAR4 Expression and Different Clinicopathological Parameters

We divided the 168 patients with osteosarcoma into a high-expression group (n = 87) and a low-expression group (n = 81), according to the median expression level of BCAR4. As shown in Table I, We found that increased BCAR4 expression in osteosarcoma was significantly associated with malignant behavior, such as clinical stage ($p = 0.002$) and distant metastasis ($p = 0.001$). However, There was no correlation between BCAR4 expression and other clinicopathological factors (gender, age, tumor size, and anatomic location ($p > 0.05$).

Prognostic value of BCAR4 Expression for the Clinical Outcome of Osteosarcoma patients

To assess the clinical significance of BCAR4 overexpression in osteosarcoma, we analyzed the relationship between the level of BCAR4 expression and patient survival. The results showed that Patients with high BCAR4 expression tended to have poorer overall survival ($p < 0.01$, Figure 2). Univariate analysis with the Cox proportional hazards model identified three prognostic factors: clinical stage, distant metastasis, and BCAR4 expression (Table II).

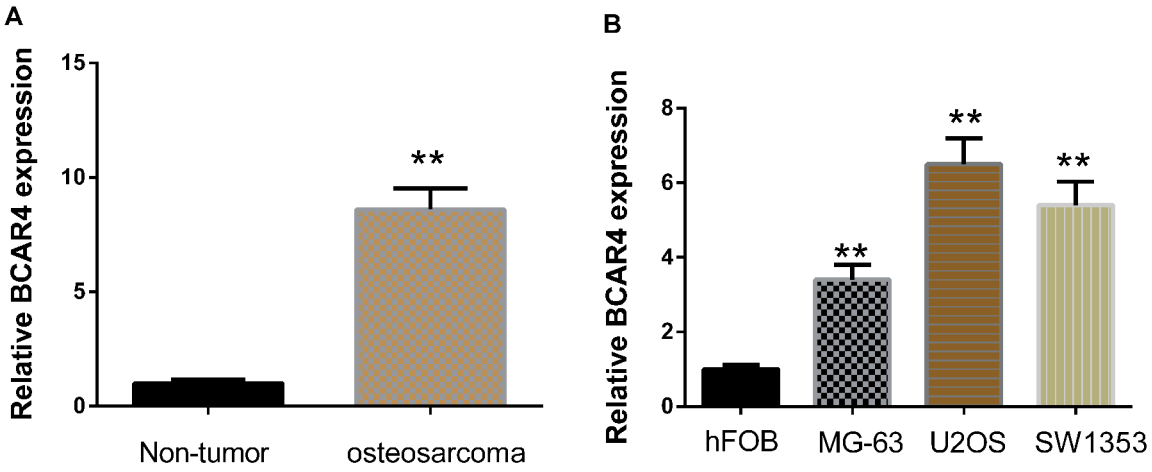

Figure 1. The BCAR4 expression is down-regulated in osteosarcoma samples and cell lines. ***(A)*** qRT-PCR analysis of BCAR4 expression in osteosarcoma tissues and normal bone tissues. ***(B)*** qRT-PCR analysis of BCAR4 expression in three human osteosarcoma cell lines (MG-63, U2OS, and SW1353) and human normal bone cell line hFOB. $^{**}p < 0.01$, $^{*}p < 0.05$.

Table I. Correlation of BCAR4 with clinicopathological features of osteosarcoma.

Clinicopathological features	Number of cases	BCAR4 Low n (%)	BCAR4 High n (%)	*p*
Age				0.494
<55 years	73	33 (45.2)	40 (54.8)	
≥55 years	95	48 (50.5)	47 (49.5)	
Gender				0.381
Male	100	51 (51)	49 (49)	
Female	68	30 (44.1)	38 (55.9)	
Tumor size				0.810
<8cm	97	46 (47.4)	51 (52.6)	
≥8cm	71	35 (49.3)	36 (50.7)	
Anatomic location				0.982
Tibia/femur	108	52 (48.1)	56 (51.9)	
Elsewhere	60	29 (48.3)	31 (51.7)	
Serum level of alkaline phosphatase				0.191
Elevated	95	50 (52.6)	45 (47.4)	
Normal	73	31 (42.5)	42 (57.5)	
Clinical stage				0.002
IIA	83	50 (60.2)	33 (39.8)	
IIB/III	85	31 (36.5)	54 (63.5)	
Distant metastasis				0.001
Absent	120	68 (56.7)	52 (43.3)	
Present	48	13 (27.1)	35 (72.9)	
Response to chemotherapy				0.841
Good	70	33 (47.1)	37 (52.9)	
Poor	98	48 (49)	50 (51)	

A multivariate analysis of the prognosis factors with a Cox proportional hazards model confirmed that high BCAR4 expression was a significant independent predictor of poor survival in CRC ($p = 0.014$) (Table II).

Knockdown of BCAR4 Attenuated Proliferation, Invasion and Migration in Osteosarcoma Cells

To investigate the roles of BCAR4 in gastric cancer, The U2OS cell line, expressing relative-

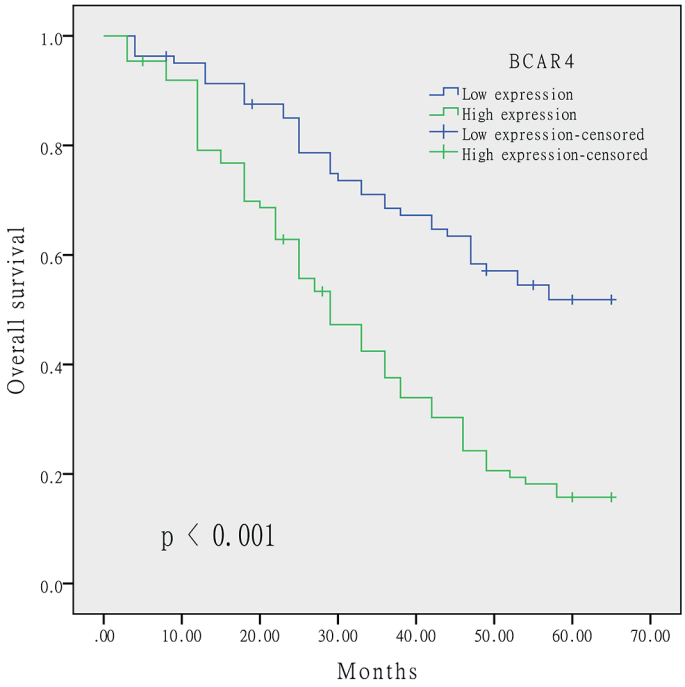

Figure 2. Survival curves of 168 osteosarcoma patients. Patients with higher BCAR4 expression in the tumor were closely correlated with poorer overall survival than that with tumor with lower BCAR4 expression ($p < 0.001$, respectively).

Table II. Univariate and multivariate analysis of factors influencing survival in 168 patients with osteosarcoma.

Variable	Univariate analysis HR (95% CI)	Multivariate analysis p-value	HR (95% CI)	p-value
Age		0.673		
<55 years	1 (Ref.)			
≥55 years	1.67 (0.86-3.23)			
Gender		0.382		
Male	1 (Ref.)			
Female	1.23 (0.77-2.18)			
Tumor size		0.177		
<8cm	1 (Ref.)			
≥8cm	3.37 (1.32-6.62)			
Anatomic location		0.215		
Tibia/femur	1 (Ref.)			
Elsewhere	2.33 (1.15-4.18)			
Serum level of alkaline phosphatase		0.438		
Elevated	1 (Ref.)			
Normal	2.77 (1.14-3.32)			
Clinical stage		0.004		0.008
IIA	1 (Ref.)		1 (Ref.)	
IIB/III	7.54 (3.36-20.82)		6.23 (2.93-18.39)	
Distant metastasis		0.007		0.012
Absent	1 (Ref.)		1 (Ref.)	
Present	4.37 (1.45-14.55)		3.88 (1.17-11.39)	
Response to chemotherapy		0.253		
Good	1 (Ref.)			
Poor	2.71 (0.33-4.29)			
Expression of BCAR4		0.008		0.014
Low	1 (Ref.)		1 (Ref.)	
High	3.83 (1.16-9.88)		3.22 (0.89-7.88)	

ly high levels of BCAR4, was transfected with si-BCAR4 or a negative si-NC. The qRT-PCR analysis confirmed that transfection with the si-BCAR4 resulted in significant decreased expression of BCAR4 (Figure 3A). After the transfection, we assayed the growth of BCAR4 cells by CKK-8 analysis and found that si-BCAR4 inhibited cell growth (Figure 3B). Then, we analyzed the effect of ectopic BCAR4 expression on cellular invasion and migration potential of U2OS cells. In the transwell invasion and migration assay, cells transfected with si-BCAR4 displayed an inhibition in invasion and migration ability when compared with the control group in U2OS cells (Figure 3C and 3D).

Discussion

The prognosis of osteosarcoma is extremely poor, therefore, understanding the mechanisms underlying osteosarcoma pathogenesis may help yield novel biomarkers for treatment. With the development of genome sequencing technologies, it is well accepted that less than 2% of the human genome encodes proteins and that the remaining 98% encodes noncoding RNAs (ncRNAs)[14]. Emerging data strongly implicate lncRNAs in the basal regulation of protein-coding genes, which are central to normal development and oncogenesis, at both the transcriptional and the post-transcriptional levels[15]. Thus, the roles of deregulated lncRNAs in osteosarcoma have also received considerable attention in the past few years.

Recently, many lncRNAs have been confirmed as tumor suppressor genes and prognosis biomarkers. For instance, expression of lncRNA MEG3 was clearly lower in osteosarcoma tissues compared with adjacent non-tumor tissues and was associated with clinical stage and distant metastasis[16]. Overexpression of MALAT1 could promote the proliferation and invasion of human osteosarcoma cell and suppressed its metastasis by activating the PI3K/Akt pathway[17]. Upregulation of TUG1 strongly correlated with poor prognosis and was an independent prognostic indicator for overall survival[18]. To our best knowledge, only few studies reported the role of BCAR4 in breast

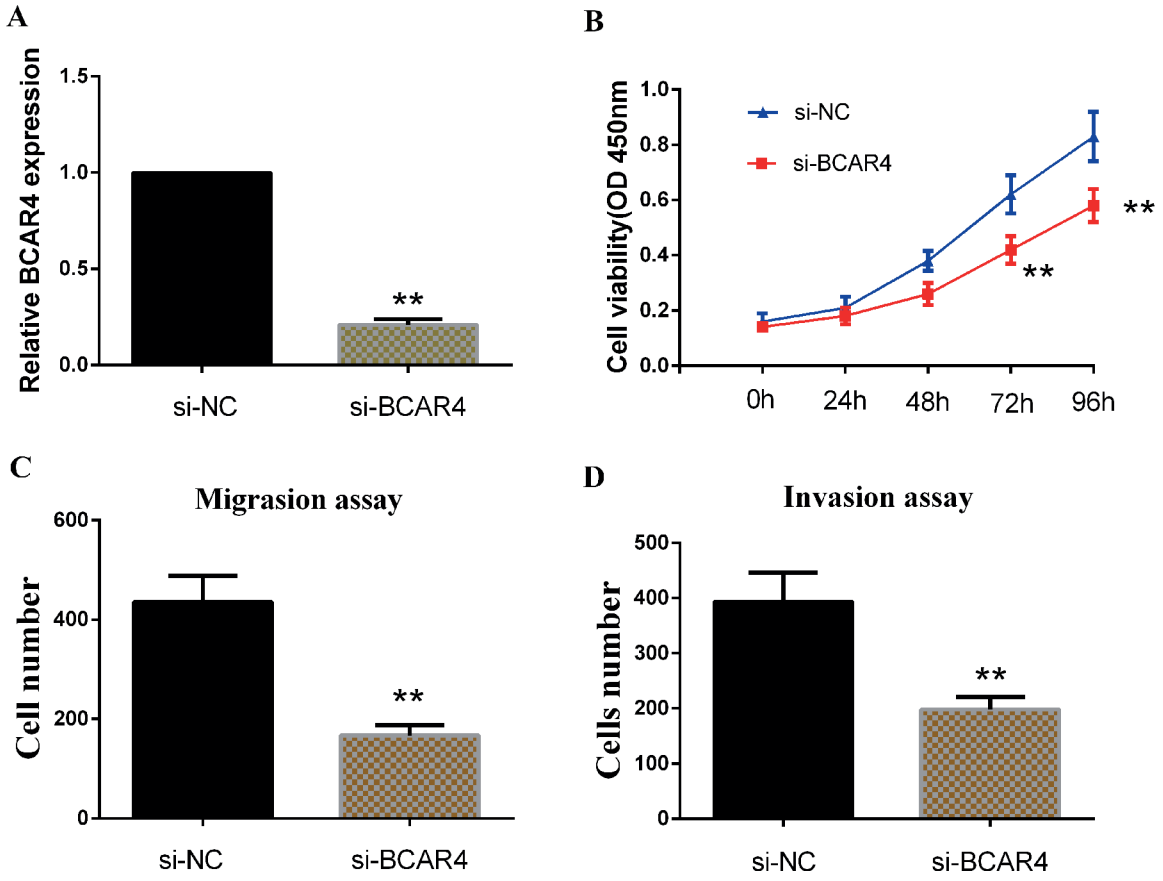

Figure 3. Down-regulated BCAR4 inhibited cell proliferation, migration, and invasion of osteosarcoma cells. ***(A)*** Expression of BCAR4 in U2OS cells transfected with NC or si-BCAR4 by qRT-PCR. *(B)* Cell viability detected by CCK8 in U2OS cells stably transfected with si-BCAR4 or NC. ***(C,D)*** Transwell migration and invasion assays were conducted using U2OS cells transfected with si-BCAR4 or NC. $^{**}p < 0.01$, $^{*}p < 0.05$.

cancer[19]. The role of BCAR4 in osteosarcoma has not been reported.

In the present work, we for the first time found that BCAR4 expression was up-regulated in osteosarcoma tissues and cell lines. The expression level of BCAR4 was strongly correlated with clinical stage and distance metastasis. Osteosarcoma patients with BCAR4 overexpression have a worse prognosis than those with low expression of BCAR4. The univariate and multivariate analyses confirmed that expression of BCAR4 was a potential prognostic biomarker for osteosarcoma. To explore the molecular mechanism modulated by BCAR4 in osteosarcoma progression. We modulated its expression in U2OS cell line. Our data suggested an inhibiting effect of BCAR4 downregulation on osteosarcoma cell growth, migration and invasion.

Conclusions

Our report reveals that the high expression profile of BCAR4 in osteosarcoma is an independent indicator of poor overall survival and served as an oncogene in osteosarcoma development. However, the detailed molecular mechanisms of BCAR4 regulating osteosarcoma and tumor progression required further exploration.

Conflicts of interest

The authors declare no conflicts of interest.

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