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Increased Expression of Long Non-Coding RNA BCAR4 Is Predictive of Poor Prognosis in Patients with Non-Small Cell Lung Cancer

Jianhua Gong,[1] Han Zhang,[1] Li He,[1] Lixin Wang[2] and Jipeng Wang[2]

[1]Department of Respiratory Medicine, Jingzhou Central Hospital, Jingzhou, Hubei, P.R. China
[2]Department of Respiratory Medicine, Huai'an First People's Hospital, Nanjing Medical University, Huai'an, Jiangsu, P.R. China

Lung cancer is the most common human cancer, and the majority of lung cancer cases are categorized as non-small cell lung cancer (NSCLC). Long non-coding RNAs (lncRNAs) play key roles in the development and progression of human cancers. LncRNA breast cancer anti-estrogen resistance 4 (BCAR4) has been identified as an oncogenic lncRNA involved in the progression of breast cancer and osteosarcoma. However, the clinical significance of the lncRNA BCAR4 in NSCLC remains largely unclear. In the present study, real-time quantitative reverse transcriptase-polymerase chain reaction was used to examine the relative level of lncRNA BCAR4 in 68 cases of NSCLC tissues and their adjacent non-tumor tissues. Our data showed that the expression level of lncRNA BCAR4 was significantly higher in NSCLC tissues compared to their matched non-tumor tissues. Moreover, BCAR4 expression was significantly upregulated in NSCLC cell lines, when compared to the normal human bronchial epithelial cell line BEAS-2B. In addition, the BCAR4 expression was associated with the lymph node metastasis, distant metastasis and clinical stage, but not with the age, sex, tumor size, histological grade, and histological type. The increased expression of BCAR4 was significantly associated with poorer 5-year overall survival rate of NSCLC patients. Multivariate survival analysis indicated that BCAR4 was an independent prognostic factor for NSCLC patients. Taken together, our study suggests that the upregulation of lncRNA BCAR4 expression plays a promoting role in the malignant progression of NSCLC. Thus, BCAR4 is a potential biomarker for NSCLC progress and a therapeutic target for NSCLC.

Keywords: BCAR4; long noncoding RNA; metastasis; non-small cell lung cancer; prognosis
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Introduction

Lung cancer is the most common human cancer, accounting for the leading cause of cancer-related death worldwide, and the majority of lung cancer cases are categorized as non-small cell lung cancer (NSCLC) (Jemal et al. 2011; Torre et al. 2015). Squamous cell carcinoma (SCC) and adenocarcinoma (AC) are the main subtypes of NSCLC with different clinical presentations, morphologies, treatments and prognoses as well as different genetic changes (Pikor et al. 2013). Currently, epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor therapy is given to AC patients with an EGFR mutation. However, it is less effective in EGFR mutated SCC cases (Hata et al. 2014). The incidence and mortality rates of NSCLC have a high level in both the western countries and the developing countries, including China (Torre et al. 2015). Since the histological grade and tumor metastasis are important factors for the poor prognosis of NSCLC, finding novel targets associated with these two factors may help develop effective strategies for the treatment of this disease (Wu et al. 2014).

Long noncoding RNAs (lncRNAs) are novel class of molecules, with a length longer than 200 nucleotides (nt) (Lalevee and Feil 2015). In recent years, accumulating evidence has demonstrated that lncRNAs participate in the regulation of various biological processes, such as cell proliferation, differentiation, apoptosis, motility and so forth (Li and Hu 2015; Ricciuti et al. 2016). Although lncRNAs are not translated into proteins, they can regulate the expression of oncogenes or tumor suppressors to control the tumorigenesis and cancer progression (Silva et al. 2015). About 18% of the protein coding genes that produce lncRNAs are associated with cancer. For examples, lncRNA LINC00152 can promote the proliferation of hepatocellular carcinoma (HCC) cells by inhibition of EpCAM

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Correspondence: Jipeng Wang, Department of Respiratory Medicine, Huai'an First People's Hospital, Nanjing Medical University, 6 Beijing Road West, Huai'an, Jiangsu 223300, P.R. China.
e-mail: doctorwangjipeng@sina.com

expression via regulating the mTOR signaling pathway, suggesting that LINC00152 acts as an oncogene in HCC (Ji et al. 2015). On the contrary, the lncRNA LOWEG was found to be low expressed in gastric cancer and play a tumor suppressive role by inhibiting the invasion of gastric cancers (Zhao et al. 2016). Accordingly, different lncRNAs have different functions in different cancer types. Therefore, revealing of the expressions and functions of specific lncRNAs may contribute to the diagnostics and treatment of human cancers. Recently, several lncRNAs have been reported to be involved in the development and progression of NSCLC (Lin et al. 2015; Zhang et al. 2016). For instance, lncRNA RP11-397D12.4, AC007403.1, and ERICH1-AS1 were found to be upregulated in the serum of NSCLC patients, and might become potential diagnostic biomarkers for NSCLC (Tang et al. 2015).

The lncRNA breast cancer anti-estrogen resistance 4 (BCAR4) was involved in anti-estrogen resistance in breast cancer (Meijer et al. 2006). High expression BCAR4 is an independent predictive factor for poor disease-free survival after tamoxifen therapy for recurrent breast cancer disease (Godinho et al. 2010). However, the expression of BCAR4 in NSCLC has never previously been reported. In the present study, we aim to examine the expression level of BCAR4 in NSCLC tissues compared to their matched adjacent non-tumor tissues. Besides, we also analyze the association between the BCAR4 expression and the clinicpathological characteristics of NSCLC patients, including age, sex, tumor size, histological type, histological grade, lymph node metastasis, distant metastasis, clinical stage, and 5-year overall survival.

Materials and Methods

Patients and samples collection

This study was approved by the Ethical Committee of Jingzhou Central Hospital, Jingzhou, P.R. China. Informed consents were obtained from all patients and healthy controls involved in this study. The NSCLC tissues and their matched adjacent non-tumor tissues were collected from 68 cases of NSCLC patients when surgical resection at Jingzhou Central Hospital between March 2009 and January 2010, and were confirmed by histopathological evaluation (7th edition of the TNM Classification for Lung Cancer). All 68 patients with NSCLC had valid follow-up data. The overall survival (OS) was defined as the time between diagnosis and the date of death or the date last known alive. The clinicopathological characteristics are summarized in Table 1. All NSCLC patients received no preoperative radiotherapy and/or chemotherapy. The collected tissues were immediately stored at −80°C until use.

Cell lines and cell culture

Five human NSCLC cell lines L78, A549, H1229, H358, and H1650, and a normal human lung epithelial cell line BEAS-2B were purchased from Cell bank of Chinese Academy of Sciences, Shanghai, China. All cells were cultured in DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS (Life Technologies) at 37°C with 5% CO_2.

RNA extraction and Quantitative real-time reverse transcription PCR

Total RNA was isolated from NSCLC and matched adjacent tissues by using Trizol Reagent (Life Technologies), according to the manufacture's protocol. The concentration and purity of total RNA were measured on a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). The total RNA (2,000 ng) was then converted into cDNA by using a PrimeScript 1st Strand cDNA Synthesis Kit (TAKARA, Dalian, China), according to the manufacture's protocol. After that, quantitative real-time PCR was conducted using SYBR-Green RT-PCR kit (Takara), according to the manufacture's protocol. The reaction conditions was 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. GAPDH was used as an internal control. The PCR primers for GAPDH and BCAR4 were obtained from Amspring (Changsha, China). The relative expression level was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

In this study, all experiments were repeated at least three times, and all data are expressed as the mean ± standard error of the mean (SEM) SPSS 18.0 software package (SPSS, Chicago, IL, USA) was used to perform statistical analysis. Difference of lncRNA expression between two groups was compared by Independent-samples *t* test. The BCAR4 expression levels in NSCLC tissues were categorized as low expression or high expression, in relation to the mean value. Chi-square test was applied to determine the association between the BCAR4 expression and the clinicopathological parameter of NSCLC. The Kaplan-Meier method and log-rank test were used to evaluate and compare the prognosis of NSCLC Patient. Univariate and multivariate Cox proportional hazards analyses were used to analyze the independent prognostic factors for survival in NSCLC patients. The P value less than 0.05 were considered statistically significant.

Results

BCAR4 is upregulated in NSCLC tissues and cell lines

In the present study, we firstly compared the expression of lncRNA BCAR4 in 68 cases of NSCLC tissues and their matched adjacent non-tumor tissues. Total RNA was isolated from the tissues, and real-time RT-PCR was performed to examine the expression levels of lncRNA BCAR4. Our data showed that the expression of BCAR4 was markedly increased in NSCLC tissues compared to their matched non-tumor tissues (Fig. 1A, B). We further examined its expression in NSCLC cell lines (L78, A549, H1229, H358, and H1650), and the normal human lung epithelial cell line BEAS-2B was used as a control. As shown in Fig. 1B, real-time RT-PCR data indicated that the expression level of BCAR4 were also increased in NSCLC cell lines when compared with that in BEAS-2B cells. Therefore, the lncRNA BCAR4 is upregulated in NSCLC tissues and cell lines.

BCAR4 expression is associated with lymph node metastasis, distant metastasis and clinical stage of NSCLC

To further reveal the role of BCAR4 in NSCLC, we evaluated the association between its expression and the clinicopathological characteristics of NSCLC. The BCAR4

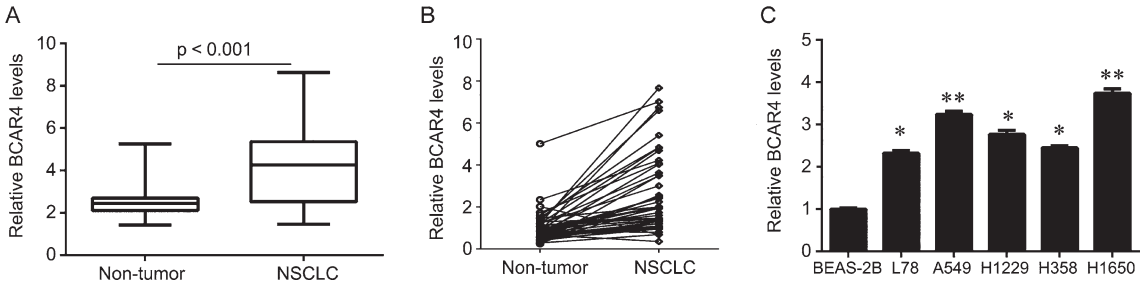

Fig. 1. Relative expression of BCAR4 in NSCLC tissues and cell lines.
(A) Expression levels of BCAR4 in a total of 68 NSCLC tissues and their matched non-tumor tissues were measured by qRT-PCR. (B) A line connects two pionts (the expression levels of BCAR4 shown in panel A) from non-tumor to NSCLC with an increased trend, suggesting that the expression level of BCAR4 was higher in NSCLC tissues than in the matched non-tumor tissues. (C) Expression levels of BCAR4 in NSCLC cell lines including (L78, A549, H1229, H358, and H1650) and the normal human lung epithelial cell line BEAS-2B was measured by qRT-PCR. *p < 0.05, **p < 0.01 vs. BEAS-2B.

Table 1. Clinical association between lncRNA BCAR4 expression and clinicopathological variables in NSCLC patients.

		lncRNA BCAR4		
Variable	Number	Low expression	High expression	χ^2 test p value
Age				
< 60	37	16	21	0.465
≥ 60	31	17	14	
Sex				
Male	40	20	20	0.81
Female	28	13	15	
Tumor size				
< 5cm	41	18	23	0.458
≥ 5cm	27	15	12	
Histological grade				
I	28	16	12	0.325
II-III	40	17	23	
Histology type				
Adenocarcinoma	48	25	23	0.431
Squamous	20	8	12	
Lymph node metastasis				
No	32	22	10	0.003
Yes	36	11	25	
Distant metastasis				
No	51	29	22	0.025
Yes	17	4	13	
Clinical stage				
I-II	30	20	10	0.014
III-IV	38	13	25	

expression levels in NSCLC tissues were categorized as low expression or high expression, in relation to the mean value. As indicated in Table 1, high expression of BCAR4 was significantly associated with the lymph node metastasis, distant metastasis and advanced clinical stage. However, we found no association between the BCAR4 expression and the age, sex, tumor size, histological grade or histological type of NSCLC patients (Table 1). These findings suggest that the increased expression of BCAR4 is involved in the malignant progression of NSCLC.

High level of BCAR4 is predictive of poor prognosis of NSCLC patients

We further analyzed the relationship between the BCAR4 expression and the survival time of NSCLC patients. Our data showed that NSCLC patients with high BCAR4 expression showed a worse prognosis when compared with those with low level of BCAR4 (Fig. 2). Univariate and multivariate Cox proportional hazards analyses were then used to analyze the independent prognostic factors for survival in NSCLC patients. Univariate analysis data indicated that the histological grade, lymph node metastasis, distant metastasis, clinical stage, and BCAR4 expression were significantly associated with the overall survival of NSCLC patients (Table 2). Moreover, in addition to histological grade, lymph node metastasis, distant metastasis and clinical stage, the BCAR4 expression was also an independent prognostic factor for the prognosis of NSCLC patients (Table 3). However, the age, sex, tumor size, and histological type were not independent prognostic factors for the overall survival of NSCLC patients (Table 3). Accordingly, our data demonstrates that high expression of BCAR4 can predicate a poor prognosis of patients with NSCLC.

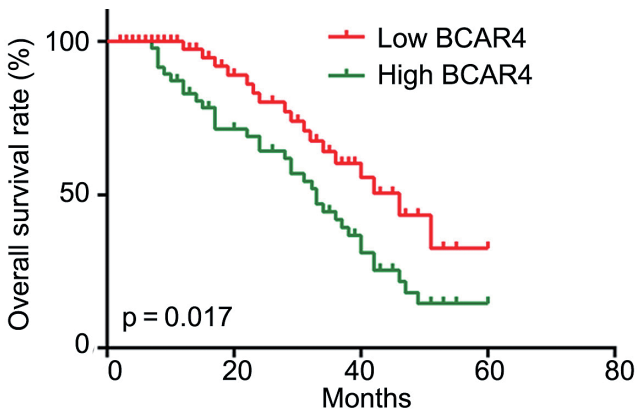

Fig. 2. Kaplan-Meier postoperative survival curve for patterns of patients with NSCLC and BCAR4 expression. NSCLC patients with High BCAR4 expression (n = 35) showed shorter suvival time than those with low BCAR4 expression (n = 33).

Discussion

Recently, accumulating evidence has suggested that lncRNAs play a critical role in the regulation of cell proliferation and apoptosis, differentiation and development, as well as cancer development and progression (Taylor et al. 2015; Ricciuti et al. 2016). For instance, lncRNA MVIH was found to be significantly upregulated in breast cancer tissues than in adjacent noncancerous tissues, and patients with high MVIH levels showed poor overall survival and disease-free survival (Lei et al. 2016). In vitro study revealed that MVIH could promote cell proliferation and cell cycle, while inhibiting apoptosis of breast cancer cells. Xue et al. (2016) reported that lncRNA urothelial cancer-associated 1 (UCA1) promoted the migration and invasion of bladder cancer cells via regulating the miR-145/zinc finger E-box binding homeobox 1 and 2 (ZEB1 and ZEB2)/fascin homologue 1 (FSCN1) pathway. Therefore, understanding the exact roles of specific lncRNAs may contribute to the development of the diagnostics and therapeutics of human cancers.

In the present study, we examined the expression of lncRNA BCAR4 in 68 tissue samples of NSCLC patients. Real-time RT-PCR data indicated that BCAR4 was remarkably upregulated in NSCLC tissues compared to their matched adjacent non-tumor tissues. Besides, its expression levels were also increased in five common NSCLC cell lines. Therefore, BCAR4 may be involved in the develop-

Table 2. Univariate analysis of prognostic factors of NSCLC.

Variable	Hazard ratio	p value
Age (≥ 60/< 60)	1.091	0.768
Sex (Male/Female)	1.215	0.513
Tumor size (≥ 5cm/< 5cm)	1.733	0.142
Histological Grade (II-III/I)	2.562	0.023
Histology type (Adenocarcinoma/Squamous)	0.966	0.858
Lymph node metastasis (Yes/No)	3.256	0.009
Distant metastasis (Yes/No)	3.811	0.001
Clinical stage (III-IV/I-II)	3.321	0.007
lncRNA BCAR4 expression (High/Low)	2.853	0.016

Table 3. Multivariate analysis of independent prognostic factors of NSCLC.

Variable	Hazard ratio	p value
Histological Grade	2.363	0.027
Lymph node metastasis	2.838	0.018
Distant metastasis	3.016	0.012
Clinical stage	2.916	0.015
lncRNA BCAR4 expression	2.643	0.021

ment of NSCLC. Further investigation indicated that high expression of BCAR4 was significantly associated with lymph node metastasis, distant metastasis and advanced clinical stage of NSCLC, but no association between the BCAR4 expression and the age, sex, tumor size, histological grade or histological type of NSCLC patients. As higher invasion and metastasis of tumor cells are generally associated with crucial poorer prognosis of patients with cancers, we speculated that BCAR4 might affect the prognosis of NSCLC patients. To clarify this speculation, we further analyzed the relationship between the BCAR4 expression and the overall survival of NSCLC patients. Our data indicated that NSCLC patients with high BCAR4 expression showed worse prognosis when compared with those with low BCAR4 expression, and the BCAR4 expression was an independent prognostic factor of NSCLC patients. Similarly, high BCAR4 levels were also found to be associated with poor prognosis of breast cancer patients treated with tamoxifen, and overexpression of BCAR4 promotes the proliferation of breast cancer cells in vitro and in vivo, suggesting that BCAR4 may play an oncogenic role in different cancer types (Godinho et al. 2011; van Agthoven et al. 2015; Xing et al. 2015). Molecular mechanism investigation revealed that in response to CC chemokine ligand 21 (CCL21) released the Smad nuclear-interacting protein 1 (SNIP1) inhibition of p300-dependent histone acetylation, BCAR4 could bind SNIP1 and Serine/threonine-protein phosphatase 1 regulatory subunit 10 (PPP1R10, also known as PNUTS), which in turn enabled the BCAR4-recruited PNUTS to bind H3K18ac and relieved inhibition of RNA Pol II via activation of the PP1 phosphatase (Xing et al. 2014). Through this mechanism, BCAR4 could activate a non-canonical Hedgehog/glioma-associated oncogene homolog 2 (GLI2) transcriptional program that promotes cell migration (Xing et al. 2014). Future studies should focus on the exact regulatory role as well as the underlying mechanism of BCAR4 in the proliferation, migration and invasion of NSCLC cells. Besides, therapeutic delivery of locked nucleic acids targeting BCAR4 significantly inhibits the metastasis of breast cancer in a mouse model, suggesting that BCAR4 may also become a therapeutic target for NSCLC (Xing et al. 2014).

In addition to BCAR4, several other lncRNAs have been identified as important biomarkers or prognostic factors for NSCLC (Yang et al. 2013). For instance, high expression of lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) predicates a poor prognosis of NSCLC patients, and overexpression of MALAT-1 induces migration and tumor growth of NSCLC cells (Schmidt et al. 2011). Our findings expand the understanding of lncRNAs in the development and progression of NSCLC.

In conclusion, our data indicate that BCAR4 expression is significantly upregulated in NSCLC tissues and cell lines, and its expression is significantly associated with the histological grade and lymph node metastasis of NSCLC patients. Moreover, increased expression of lncRNA BCAR4 is predictive of a worse prognosis in patients with NSCLC. Taken these data together, we propose that BCAR4 plays a promoting role in the malignant progression of NSCLC, and may become a prognostic biomarker for NSCLC.

Conflict of Interest

The authors declare no conflict of interest.

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