Introduction

Lung cancer is considered as the “leading killer” around the world. Its incidence rate ranks second in male and female patients, and the tumor-related mortality ranks first in all tumors. Among, non-small cell lung cancer (NSCLC) accounts for nearly 85% of all types of lung cancers, and includes two pathological patterns: lung adenocarcinoma (LAD) and lung squamous carcinoma. In recent years, despite of the rapid development of chemotherapy, radiotherapy and molecular targeted therapy in the comprehensive treatment of NSCLC, the 5-year survival rate (about 16.6%) is still unsatisfactory. The important reason for the low 5-year survival rate is that invasion and metastasis occur easily in NSCLC. Therefore, it is important to understand the potential molecular mechanism of NSCLC metastasis and recurrence and find effective molecular markers.

About 98% of the transcripts of the human genome have no protein translation capacity, so such kind of RNA is named non-coding RNA (ncRNA). At first, the researchers believe that ncRNA has no special biological characteristics and it is regarded as the “junk” of evolution. However, more and more research results show that ncRNA, as an important regulatory factor, is involved in cell metabolism, differentiation, stem cell induction and other biological processes.

LncRNA BCAR4 promotes proliferation, invasion and metastasis of non-small cell lung cancer cells by affecting epithelial-mesenchymal transition

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Abstract. – OBJECTIVE: Long non-coding RNAs (lncRNAs) play an important role in various cellular biological processes. It is also involved in the occurrence and development of the tumor. BCAR4 is reported to be highly expressed in breast cancer and promotes cell proliferation. However, the biological effects of BCAR4 in non-small cell lung cancer (NSCLC) remains unclear.

PATIENTS AND METHODS: qRT-PCR was performed for detecting BCAR4 expression in 76 pairs of NSCLC tissues and corresponding cancer-adjacent tissues and 6 NSCLC cell lines. BCAR4 expression was knocked down, and its effects on NSCLC cell proliferation, cycle, apoptosis, invasion and metastasis were studied via MTT, clone formation, flow cytometry, TUNEL and Transwell assay. Metastatic tumor model of nude mice was established to investigate its effects on NSCLC cell metastasis. BCAR4 downstream target gene protein expression was detected using Western blotting and immunofluorescence assay.

RESULTS: BCAR4 was higher in NSCLC tissues than that in cancer-adjacent tissues and was positively correlated with tumor size, clinical stage, and distant metastasis, suggesting that BCAR4 can be used as an independent predictor of prognosis. Results also showed that BCAR4 knockdown could inhibit tumor cell invasion, metastasis, and proliferation, induce cell cycle arrest and increase cell apoptosis. BCAR4 knockdown inhibits the metastasis and invasion of tumor cells via regulating Vimentin, N-cadherin and E-cadherin in Epithelial-Mesenchymal Transition (EMT).

CONCLUSIONS: BCAR4 promotes the invasion and metastasis of NSCLC via regulating EMT and BCAR4/EMT interaction can be used as a new target for the diagnosis and therapeutics of NSCLC.

Key Words: Carcinoma, Non-small-cell lung, Epithelial-mesenchymal transition, Neoplasm invasiveness, Neoplasm metastasis, RNA, Long noncoding.
the activation of the PI3/AKT signaling pathway. Liu et al. found that IncRNA HOTAIR (HOX transcript antisense RNA) is highly expressed in NSCLC, and the highly-expressed HOTAIR can enhance the metastasis and invasion of NSCLC cells. Though there are more and more studies on IncRNA, researchers still know little about its biological role in NSCLC.

Invasion metastasis is an important cause of NSCLC treatment failure. The molecular mechanisms of NSCLC invasion and metastasis mainly include extracellular matrix degradation, tumor microenvironment changes, activation or inhibition of invasion and metastasis-related genes. The epithelial-mesenchymal transition (EMT), as one of the important molecular mechanisms, is closely related to tumor invasion and metastasis. Its main features include decreased E-cadherin expression, a marker for cell adhesion, and increased N-cadherin expression, and the transformation from keratin-based cytoskeleton to vimentin-based cytoskeleton. However, NSCLC invasion and metastasis are the results of multi-factor network regulation, and IncRNA-mediated EMT is confirmed by more research results. It is reported in the literature that IncRNA TUG1 (taurine-up-regulated gene 1) can promote the metastasis and invasion of colon cancer, and its molecular mechanism is the regulation of EMT pathway. Li et al. found that IncRNA HULC is highly expressed in liver cancer and can promote the invasion and metastasis of hepatocellular carcinoma cells. Further mechanism research has shown that IncRNA HULC can competitively absorb miRNA-200a-3p, and the decrease of miR-200a-3p promotes the increase of ZEB1 protein expression, which induces the occurrence of EMT in hepatocellular carcinoma cells. IncRNA-mediated EMT provides a good idea for researchers to study the potential molecular mechanisms of NSCLC invasion and metastasis. IncRNA BCAR4 (breast cancer anti-estrogen resistance 4) was the first gene that was found to be related to hormone resistance in breast cancer. Godinho et al. reported that BCAR4 plays a role similar to “oncogene” in breast cancer and can promote the breast cancer metastasis and proliferation by regulating Hedgehog/GLI2 pathway, and promote its resistance to estrogen. In breast cancer patients, highly-expressed BCAR4, as a new biological marker, suggests that patients are prone to invasion and metastasis, but there has been no report on relative expression quantity of BCAR4 in NSCLC and its biological effects. In this study, the biological function of BCAR4 in NSCLC was studied by in vivo/in vitro experimental studies, and the correlation between its relative expression quantity and the clinical and pathological characteristics of NSCLC patients was analyzed. Results showed that BCAR4 was relatively highly expressed in NSCLC, which is positively correlated with tumor size, clinical stage, invasion and metastasis. Highly-expressed BCAR4 can be used as a predictive factor of prognosis of NSCLC patients. Both in vivo and in vitro studies showed that the knockdown of BCAR4 in NSCLC cells can promote cell apoptosis, inhibit proliferation and inhibit cell invasion and metastasis by affecting EMT.

**Patients and Methods**

**Tissue Collection**

Fresh and formalin-fixed, paraffin-embedded NSCLC tumor tissue samples were obtained from patients diagnosed with NSCLC. These patients received the elective surgery at the First Affiliated Hospital of Nanjing Medical University. A total of 76 pairs of fresh NSCLC and adjacent non-tumor tissues (more than 5 cm away from the tumor) were freshly frozen in liquid nitrogen and stored at −80°C until further use. The archived, formalin-fixed and paraffin-embedded NSCLC tissue samples from 76 cases, were used for clinicopathological and prognostic investigation of IncRNA BCAR4. Ethics Committee of the First Affiliated Hospital of Nanjing Medical University has approved the use of tissues for this study. Before using these clinical materials for research purposes, informed consent has been signed by all the patients. No patient received any preoperative chemotherapy or radiotherapy.

**Cell Culture and Transfection**

NSCLC cell lines (A549, SPC-A-1, PC-9, H157 and SK-MES-1) and the 16HBE cell lines were from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum was used to culture cells in 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco, Grand Island, NY, USA) at 37°C with 5% CO₂. si-NC, si-BCAR4, using Lipofectamine 2076
2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

**Western Blot Analysis**

According to the protocol described previously, Western blot was performed. The primary antibodies were used as follows: rabbit anti-E-cadherin (Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-N-cadherin (Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-vimentin (Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-GAPDH (Sigma-Aldrich, St. Louis, MO, USA).

**siRNA Knockdown Experiments**

Stealth siRNA oligonucleotides were synthesized by Invitrogen (Carlsbad, CA, USA). The siRNA sequences were used as follows for knockdown of lncRNA BCAR4: 1# 5'-GCUGCGAGGGUAGACUCUCUGUUU-3'; 2# 5’-CAGCUGGGCUUGACAUUCCUGGGAA-3'; 3# 5'-UGGACCUGCUCUGGAUGGGAA-3'. Cells were transfected with 50 pmol of siRNA and the scrambled control oligo with RNAimax Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. Quantitative PCR was performed to determine knockdown efficiency at the time of plating for assay.

**RNA Isolation and qRT-PCR**

TRIZOL reagent was used for extraction of total RNA from tissue samples or cells in accordance to the manufacturer’s instructions. Random primers were used to reversely transcribe total RNA (500 ng) in a final volume of 10 mL under standard conditions with the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). To measure PVT1 expression levels, SYBR Premix Ex Taq (TaKaRa, Dalian, China) was used according to the manufacturer’s instructions. GAPDH expression was used for normalization. GAPDH(F): 5'-GGGGAGGAGGCCAAAGGGTCTAT-3', GAPDH(R): 5'-GAGTCTCTTCCAGATACCAA-3' Real-time PCR was performed in triplicate on an ABI 7500, and comparative cycle threshold (CT) (2-ΔΔCT method) was adopted for data calculation.

**Proliferation Assay**

Cell viability was determined by cell counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Briefly, cells (2.5*10^4 cells per well) were seeded on the plate (96-well) and allowed for adhesion. CCK-8 solution (10 μL) was added into each well at different time points and then the cells were incubated for 3 h and measured at 450 nm.

**Colonies Formation Assay**

For colony formation assay, a total of 800 cells were seeded in a six-well plate to allow colony formation for 2 weeks. Methanol was used to fix colonies and Giemsa was used to stain colonies for 20 min, and the visible colonies were manually counted.

**Cell Cycle Analysis**

After being trypsinized, cells were washed with PBS and then fixed in cold ethanol (10 ml) for 2 h. After being washed with PBS, cells were incubated with 1mL RNase (0.25 mg/mL) at 37°C for 1 h. After being pelleted and resuspended in propidium iodide (PI) (50 μg/mL), cells were incubated in the dark at 4°C for 30 min. Finally, PI signal was examined using flow cytometry.

**Apoptosis Assay by Flow Cytometry**

Phosphatidylserine externalization was detected via flow cytometry with 7-aminoactinomycin (7-AAD) and Annexin V (FITC-labeled) (BD Pharmingen, San Diego, CA, USA), and taken as an endpoint indicator of apoptosis according to the manufacturer’s instructions.

**Immunofluorescence**

Cells were grown on glass coverslips, fixed with 4% formaldehyde for 10 min, and permeabilized with Triton X-100 (0.2%) for 10 min at room temperature. After being washed with PBS, cells were incubated at 4°C overnight with anti-E-cadherin (Sigma-Aldrich, St. Louis, MO, USA), anti-N-cadherin (Sigma-Aldrich, St. Louis, MO, USA), anti-vimentin (Sigma-Aldrich, St. Louis, MO, USA). The Alexa Fluor 488® IgG (Invitrogen, Carlsbad, CA, USA) was used as secondary antibody. Lamellipodia was observed via Alexa Fluor568® phalloidin (Invitrogen, Carlsbad, CA, USA). 1 mg/mL DAPI (Sigma-Aldrich, St. Louis, MO, USA) was used to stain nuclei at 37°C. Finally, coverslips and SlowFade® Gold confocal microscope (Olympus, Tokyo, Japan) were mounted.

**Results**

**BCAR4 Was Up-Regulated in NSCLC**

To explore the effects of BCAR4 on NSCLC, the qRT-PCR assay was used to detect 76 cases of NSCLC tissues and cancer-adjacent tissues. The results showed that BCAR4 was highly expressed...
for 8.62 times on average in 76 cases of NSCLC tissues compared with that in cancer-adjacent tissues (Figure 1A). Then, the chi-squared test was used for correlation analysis between the relative expression quantity of BCAR4 and the clinical and pathological characteristics of NSCLC patients. First, the median of multiple of BCAR4 differential expression was taken as the cut-off point, and patients with NSCLC were divided into two groups: BCAR4 high-expression group (n=38) and BCAR4 low-expression group (n=38). The results of the chi-squared test showed that highly-expressed BCAR4 was closely associated with tumor size ($p<0.01$), clinical stage ($p<0.001$) and lymph node metastasis ($p<0.001$), but uncorrelated with age, gender and pathological type (Table I).

**Research on Biological Function of BCAR4 in NSCLC cells via in vitro Experiment**

The results of our preliminary study showed that BCAR4 expression was relatively higher in NSCLC tissues, but its relative expression quantity in NSCLC cells and its biological function remain unknown. First, qRT-PCR was employed to detect BCAR4 expression in 5 kinds of NSCLC cell lines (A549, SPC-A-1, PC-9, H157 and SK-MES-1) and human normal bronchial epithelial cells (16HBE). Results revealed that relative
expression level of BCAR4 was higher than that of 16HBE in 4 kinds of NSCLC cell lines, but the relative expression level in H157 was not statistically significant (Figure 1B). 2 kinds of cell lines with the highest relative expression level, A549 and SPC-A-1, were selected as the model cell to design and synthesize BCAR4-specific interference sequence that was transiently transfected into A549 and SPC-A-1 cells using lip3000. After 48h, RNA was extracted from the two groups and the interference efficiency of Si-BCAR4 was detected (Figure 1C); 1# and 2# sequences were selected for subsequent experiments.

Then, MTT assay was used to study the effect of BCAR4 knockdown on the proliferation capacity of A549 and SPC-A-1 cells, and 0h, 24h, 48h, 72h and 96h were selected as the observation points. The results showed that the proliferation capacity of the experimental group was significantly decreased compared with control group (Figure 2A). And the clone formation results were consistent with MTT assay (Figure 2B). To investigate the effect of BCAR4 on the cell cycle progression and apoptosis of NSCLC cells, lip3000 was used to transiently transf ect si-BCAR4 to A549 and SPC-A-1 cell lines. After 48h, cells were collected and flow cytometry was used to detect the change in cell cycle and apoptosis. The results showed that compared with the si-NC group, the cell cycle progression of the si-BCAR4 group was blocked in G1 phase and the apoptosis rate was also increased (Figure 3A, 3B). Then, Transwell assay was used to investigate whether BCAR4 knockdown affected the invasion and metastasis of NSCLC cells. The cells of si-BCAR4 and si-NC groups were obtained by the same method. The cells were planted in transwell chamber, and after 48h, the cells crossing the chamber were counted. The results showed that the cell migration and invasion in the si-BCAR4 group were significantly decreased (Figure 3C, 3D).

### Table I. LncRNA-BCAR4 expression and clinicopathologic characteristics in non-small cell lung cancer (NSCLC).

<table>
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### Research on Influences of BCAR4 on NSCLC cell Invasion and Metastasis Via in vivo Experiment

Then the metastatic tumor model of nude mice was established, and effects of BCAR4 knockdown on metastasis and invasion capacities of NSCLC cells was studied via in vivo experiments. First, A549 cell lines with the highest transfection efficiency were screened as the model cells. Cells in the transfected sh-BCAR4 group and empty plasmid group were injected into the nude mice via caudal vein. The growth situation of nude mice was observed and they were weighed every four days. At 28d after the caudal vein injection of cells, mice were executed, and the lungs of nude mice were taken out, followed by a photograph.
As shown in Figure 4A, compared with the control group, the number of metastatic tumor in the lung of nude mice in the sh-BCAR4 group was significantly reduced. Then the metastatic tumor in the lung of nude mice was taken out and total RNA was extracted from tumor tissue. The relative expression quantity of BCAR4 in metastatic tumor tissue was detected by qRT-PCR. The results showed that compared with control group, the expression quantity of BCAR4 in the transfected sh-BCAR4-derived metastatic tumor was significantly decreased (Figure 4B).

**Figure 2.** Effects of knockdown of BCAR4 on NSCLC cell viability *in vitro*. **A,** MTT assays were conducted to determine the cell proliferation ability for si-BCAR4-transfected A549 and SPC-A1 cells. Values indicate the mean (SD from three independent experiments). **B,** Colony-forming assays were performed to determine the proliferation of si-BCAR4-transfected A549 and SPC-A1 cells.

**BCAR4 Affected the Invasion and Metastasis of NSCLC Cells by Acting on EMT Pathway**

The preliminary results showed that interference on BCAR4 expression in NSCLC cells could significantly inhibit the invasion and metastasis capacities of NSCLC cells. But there is no report on how BCAR4 regulates downstream molecular pathways and plays its role in NSCLC. Recent researches have shown that epithelial-mesenchymal transition is closely related to tumor invasion and metastasis. Howe-
ver, Sun et al. showed that lncRNA SPRY4-IT1 inhibits NSCLC cell invasion and metastasis by inhibiting EMT. Western blot assay was used to observe whether the expressions of EMT molecular markers (E-cadherin, Vimentin, and N-cadherin) change after the interference on BCAR4. The results showed that compared with control group, the expression of E-cadherin was
up-regulated and expressions of N-cadherin and Vimentin were down-regulated after the knockdown of BCAR4 expression (Figure 5A). Then immunohistochemistry was used to detect the protein expressions in E-cadherin, Vimentin, and N-cadherin in the above metastatic tumor. The results were consistent with those of Western blot (Figure 5B). It was concluded from the above in vivo/in vitro experiments that the knockdown of BCAR4 can inhibit the invasion and metastasis of NSCLC cells by acting on EMT pathway.

**Discussion**

At present, the prognosis of NSCLC patients still remains to be unsatisfactory, and the 5-year survival rate is about 16.6%. The main reason for treatment failure and death of NSCLC patients is the invasion and metastasis. The micro-environment changes around tumor cells and the interaction between tumor cells make cells lose the established biological habits, so metastasis occurs easily. Tumor cells colonize in other organs through the blood, lymph nodes and direct spreading, which leads to malignant proliferation and organ dysfunction. Therefore, the in-depth understanding of genes and proteins involved in NSCLC invasion and metastasis can provide a good idea for searching the accurate biological markers and developing effective treatment strategies.

With the rapid development of molecular biology, it has been found that lncRNA has a strong biological function in the tumor, so it attracts the attention of researchers around the world and studies on lncRNA is also becoming a forward-looking hot spot. LncRNA has a role in maintaining normal cell physiology, and its expression imbalance will
lead to many pathological and physiological phenomena in cells. For example, MALAT-1 was the first IncRNA that was confirmed to be associated with NSCLC cell invasion and metastasis\(^2^2\), and subsequent studies found that the highly-expressed MALAT-1 can also promote tumor cell invasion and metastasis in gastric cancer, ovarian cancer, and other tumors\(^2^3,2^4\). Also, Yang et al\(^2^5,2^6\) reported that IncRNA also plays an important role in the process of the cell cycle, differentiation and programmed apoptosis.

BCAR4 was first discovered by the Meijer et al\(^1^6\) in breast cancer and might have a role in the resistance to tamoxifen, a chemotherapeutic drug in breast cancer. And Chen et al\(^2^7\) found that BCAR4 can promote the occurrence and development of osteosarcoma by promoting the transcription of target gene GLI2 (GLI family zinc finger 2). Here, we found for the first time that IncRNA BCAR4 is highly expressed in NSCLC tissues and cells and can be used as a predictive factor of prognosis of NSCLC patients. In vivo/

**Figure 5.** BCAR4 inhibiton suppresses NSCLC cell invasion and metastasis by affecting EMT. **A**, Analysis of E-cadherin, N-cadherin, Vimentin expression in A549 and SPC-A1 cells treated with si-BCAR4 by Western blot. **B**, Analysis of E-cadherin, N-cadherin, Vimentin expression in lungs from mice by immunohistochemical.
*in vitro* experiments have proved that the knockdown of BCAR4 expression can inhibit the cell proliferation, invasion, and metastasis, and promote the cell apoptosis. The above experiments showed that BCAR4 plays a role similar to “oncogene”, which is closely related to the occurrence and development of NSCLC. Moreover, the study on BCAR4-specific interference sequence also provides a theoretical basis for clinical treatment of NSCLC patients.

Although BCAR4 has been proved to play a role similar to “oncogene” in a variety of tumors, the underlying molecular mechanisms that affect the metastasis and invasion of tumor cells are still unclear, and there has been no report about it so far. Epithelial-mesenchymal transition plays an important role in epithelial-derived tumors (such as esophageal cancer, non-small cell lung cancer)\(^{28-30}\). Epithelial cells lose cell polarity and lose connection to the basement membrane, but obtain higher migration and invasion via EMT\(^{31}\). In recent years, several studies have shown that lncRNA-mediated EMT is confirmed in a variety of tumors\(^{13,14}\). Western blot and immunofluorescence assay showed that knockdown of BCAR4 expression in NSCLC cells can increase the expression of E-cadherin (EMT molecular marker) and inhibit the expressions of N-cadherin and vimentin.

Methylation of gene promoter region includes DNA methylation and histone methylation\(^{32}\). DNMT1 (DNA (cytosine-5)-methyltransferase 1) is an important protein for maintaining high methylation of CPG island in gene promoter region\(^{33,34}\), and enhancer of zeste homolog 2 (EZH2) is an important enzyme mediating the histone H3K27-site methylation. If there is methylation in gene promoter region, the expression of this gene will be silent. And such a phenomenon exists in E-cadherin gene promoter region\(^{35}\). Wu et al\(^{36}\) confirmed through RIP and CHIP experiments that lncRNA HNF1A-AS1 and lncRNA 01133 can bind DNMT1 and EZH2 to the E-cadherin promoter region, and inhibit their expressions at the epigenetic level\(^{37}\). And there is no report on the relation of direct regulation and control between lncRNA and N-cadherin and Vimentin, and maybe the expressions of them are regulated by other mechanisms at the transcription or post-transcription levels. However, this paper did not further investigate whether there is a direct relation between BCAR4 and E-cadherin, N-cadherin and Vimentin, which is also the research direction in the future.

**Conclusions**

We observed for the first time that the knockdown of BCAR4 expression in NSCLC cells can inhibit the proliferation, invasion, and metastasis and promote apoptosis of tumor cells. And *in vitro* and *in vivo* experiments, our study further proved that BCAR4 promotes cell invasion and metastasis by influencing the EMT process. BCAR4 can be used as a predictive factor of prognosis of NSCLC patients. And blocking the interaction between BCAR4 and EMT can also provide an important theoretical basis for clinical reversal of NSCLC recurrence and metastasis.

**Conflict of interest**

The authors declare no conflicts of interest.

**References**


