Abstract. – OBJECTIVE: Gastric cancer is a common malignancy with increasing worldwide incidence, and chemotherapeutic drugs for gastric cancer are not effective. Long non-coding RNA (lncRNA) has been proved to be important in different cancer progression. In this research, we investigated whether lncRNAs have relations with drug resistance in gastric cancer to find new potential targets for therapy, which can increase the survival time of the drug-resistant gastric patient.

PATIENTS AND METHODS: qRT-PCR was used to detect the expression of BCAR4 in 113 cases of gastric cancer tissue and adjacent tissue, and the clinical significance was also analyzed. MTT assays and Western blot were performed to cytologically determine the relationship between BCAR4 expression and cisplatin resistance, as well as to investigate the potential molecular mechanism involved.

RESULTS: Compared with the adjacent tissues, we found that BCAR4 was highly expressed in gastric cancer tissues. We also found that the expression of BCAR4 was significantly related to the size of the tumor, clinical classification and the survival time. In cytological experiments, we found the expression of BCAR4 was enhanced in cisplatin-resistant cell strains (SGC7901/DDP). What’s more, overexpression of BCAR4 in SGC7901 cells increased resistance to cisplatin while reduced BCAR4 expression increased the sensitivity of SGC7901/DDP cells to cisplatin. Western blot experiments indicated that elevated expression of BCAR4 upregulated tumor stem cell-related biomarkers via regulating Wnt signaling pathway.

CONCLUSIONS: We showed that BCAR4 was closely related with the cisplatin-resistant gastric cancer. It might be a promising target for treating gastric cancer and improving the efficiency of chemotherapeutic drugs.

Key Words: Gastric cancer, BCAR4, Cisplatin-resistance, Wnt signaling pathway.

Introduction

Gastric cancer is one of the most common cancers worldwide and the emergence of new-targeted drugs has greatly improved the survival time of gastric cancer1. However, the survival time remains short because of drug-resistance in late-stage gastric cancer patients2. A lot of efforts have been done to clarify the tumorigenesis and progression of gastric cancer, but there is still a long way to go. How to prolong the survival time of the gastric cancer patients, especially late-stage and drug-resistant patients, is a hot spot in oncology studies3. Long non-coding RNA (lncRNA) has been reported to be involved in a lot of bioactivities, especially in cancer. lncRNAs are a diverse class of transcribed RNA molecules, with a length longer than 200 nucleotides. They play important roles in protein regulation by regulating the chromatin state to influence the expression of neighboring genes. It has been reported that lncRNA could sponge microRNA, indirectly regulated gene expression, indicating that lncRNA could act as competing endogenous RNA (ceRNA) in the cell. Although lncRNAs are not translated into proteins, they can regulate the expression of oncogenes or tumor suppressor genes to control the occurrence and progression of tumors. The lncRNA 4 (BCAR4) was reported to be involved in anti-estrogen resistance in breast cancer. Increased expression of BCAR4 is an in-
dependent indicator for poor disease-free survival after tamoxifen therapy for recurrent breast cancer disease\(^1\). In a recent study, Gong et al.\(^{11}\) found that elevated expression of IncRNA BCAR4 could be used as an indicator of poor prognosis in non-small cell lung cancer patients. However, the role of BCAR4 in gastric cancer has never been discussed. The goal of our study was to detect the expression level of BCAR4 in patients with gastric cancer and analyze the relationship between BCAR4 and drug resistance in the cancer cells. Meanwhile, we would like to discuss the mechanism of drug resistance to discover a novel therapeutic target.

**Patients and Methods**

**Patients Specimens and Clinical Assessment**

The data were collected from 113 patients with gastric cancer in the hospital from January 2015 to June 2016 (Yunan Province, China). All the specimens were divided into equal size and then treated with liquid nitrogen after the operation. The clinical data, including age, sex, tumor size, lymph node metastasis, histology type, and pathological grade, were recorded. All gastric cancer patients received no radiotherapy and/or chemotherapy before surgery, and the tissue was stored at -80°C until use. All patients were informed and signed informed consent. This study was approved by the Medical Ethics Committee of our institution.

**Cell Culture**

SGC7901 and drug-resistant cell lines were purchased from the Chinese Academy of Sciences (Shanghai, China). Tumor stem cells, not tumor cells, can be grown in serum-free medium, and a spheroid culture can be used for the isolation and characterization of tumor stem cells. Cells were suspended in a sphere serum-free medium containing Dulbecco’s Modified Eagle Medium/F12 (DMEM)/F12 (1:1) with EGF (30 ng/L), fibroblast growth factor (30 ng/L) and B27 supplement (1:50). After three days, the number of sphere in three directions of view at a low magnification was counted. In the logarithmic growth phase, we collected the cells and resuspended them in previously prepared sphere culture medium as a single-cell suspension. All the cell lines were cultured at 37°C incubator with 5% CO\(_2\), and 0.25 mL fresh tumor sphere culture medium was added every day. At the 14\(^{th}\) day, we counted and analysis the information collected from the spheres.

**RNA Extraction and Real-time Quantitative PCR Assays**

According to the manufacturer’s protocol, total RNA from tissue and cell was extracted by using RNAiso Plus (TaKaRa, Otsu, Shiga, Japan). Expression of BCAR4 in tumor tissue and gastric cancer cell lines were detected by standard fluorescent quantitative PCR assay with SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan). The PrimeScript\(^{TM}\) RT reagent Kit was used to detect the concentration of RNA and synthesize cDNA with gDNA Eraser (TaKaRa, Otsu, Shiga, Japan).

**Lentivirus Production and Plasmid Transfection**

We cloned the BCAR4 into the overexpression vector pCDH-MSCV-mcs-EF1-GFP-T2A-Pu (SBI) after amplification. The pLKO.1 vector was used to connect the knocked-down BCAR4 sequence: 5’-ACAG-CAGCTTGTTGCTCA-TCT-3’(forward) and 5’-TTGCCTTGGGGACAGTTCTAC-3’(reverse). The lentiviral packaging plasmids psPAX2 and pMD2.G were purchased from Zhong Yuan Biological Company (Beijing, China).

**Detection of Cell Drug Resistance**

The cells were cultured under standard condition for 48 h with 25 µL of previously prepared Thiazolyl blue solution (MTT, Sigma-Aldrich, St. Louis, MO, USA) in the absence of light. Cells were incubated for 4 h, after which the culture medium was discarded and 150 µL of dimethyl sulfoxide (DMSO) were added to each well; the plate was gently stirred for 15 min at room temperature. Optical density (OD) was measured with an absorbance at 490 nm using a microplate reader. The formula for calculating cell viability was: cell survival rate = (OD value of drug-treated group – OD value of empty control group)/(OD value of normal cell control group − OD value of empty control group)×100%.

**Western Blot Assays**

Whole cell lysates were prepared via lysis buffer (1% Triton-X100, 150 mM NaCl, 50 mM Tris-HCl, 1 mM each CaCl\(_2\), MnCl\(_2\), and MgCl\(_2\), 10 mM sodium fluoride and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and were tran-
sferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). 100 ug of samples were added to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% denaturing gel. The protein was transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) after electrophoresis, which was blocked in the 5% non-fat milk for 45 min at room temperature. Then, phosphate buffered saline (PBS) was used to wash the membranes. We used the respective secondary antibody to incubate the membrane. The immunoblots were tested by electrochemiluminescent (ECL) detection system. Finally, we used GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) to analyze the protein bands.

Statistical Analysis
All the experiments were independently repeated at least three times and presented as an average with SD. The t-test was used to analyze the differences between groups. Overall survival of patients was analyzed by Kaplan-Meyer method and log rank test. Independent prognostic significance of risk factors identified by multivariate analysis was computed by the Cox proportional hazards model. Receiver operating characteristic (ROC) curve analysis was used to determine the predictive value among parameters. When the p-value<0.05, the result was considered significant. GraphPad Prism 6 (La Jolla, CA, USA) was used to deal with all data.

Results

BCAR4 was Highly Expressed in the Gastric Cancer Tissue
To evaluate the effect of BCAR4 in gastric cancer, we first detected the expression of BCAR4 in 113 cases of gastric cancer tissues and adjacent tissues using qRT-PCR. We found that BCAR4 was highly expressed in gastric cancer tissues compared with adjacent tissues. Furthermore, we analyzed the expression of BCAR4 and the clinicopathological information of the patients, founding that BCAR4 was positively correlated with tumor size. These results suggested that the expression of BCAR4 might be related to the development of gastric cancer. Meanwhile, compared with the intestinal-type gastric cancer, we also found that the expression of BCAR4 was even higher in diffuse-type gastric cancer (according to Lauren type). These results indicated that BCAR4 was involved in the occurrence and progression of gastric cancer, but the mechanism was still unclear (Figure 1).

The Clinical Characteristic of BCAR4
To evaluate the clinical significance of BCAR4 in gastric cancer, we wanted to know which clinical characteristics were related with BCAR4. The relationship between expression of BCAR4 and the survival time of gastric cancer patients were analyzed. It was found that the gastric cancer patients with low BCAR4 expression showed a better prognosis compared with those with high level of BCAR4. The predictive values of BCAR4 were determined by ROC analysis (Table I). 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 patients (Table II). Also, the expression of BCAR4 was negati-

<table>
<thead>
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<th>Variables</th>
<th>Area under curve</th>
<th>95% CI</th>
<th>p-value</th>
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<tr>
<td>Death</td>
<td>0.671</td>
<td>0.637-0.702</td>
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<tr>
<td>Intra-tumoral BCAR</td>
<td>0.612</td>
<td>0.569-0.652</td>
<td>.000</td>
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<td>TNM stage</td>
<td>0.583</td>
<td>0.532-0.619</td>
<td>.000</td>
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<tr>
<td>3-year recurrence</td>
<td>0.644</td>
<td>0.626-0.692</td>
<td>.000</td>
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Table II. Multivariate analysis of independent prognostic factors of gastric cancer.

<table>
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<th>Variable</th>
<th>Hazard Ratio</th>
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<tr>
<td>Histological grade</td>
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<tr>
<td>Lymph node metastasis</td>
<td>2.627</td>
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<td>Distant metastasis</td>
<td>3.159</td>
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<td>Clinical stage</td>
<td>2.351</td>
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<td>Inc RNA BCAR4 expression</td>
<td>2.2</td>
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BCAR4 increase cisplatin resistance and predicted poor survival in gastric cancer patients

Elevated Expression of BCAR Would Lead to the Cisplatin Resistance

Drug resistance is one of the factors resulting in poor prognosis. We detected the expression of BCAR4 in SGC7901 cell strain and its cisplatin-resistance (SGC7901/DDP) based on the mentioned results. We observed that BCAR4 was highly expressed in SGC7901/DDP cells. Then, we overexpressed BCAR4 in SGC7901 cells and then knocked down the BCAR4 in SGC7901/DDP cells. In the cell viability assay, we found the overexpression of BCAR4 in SGC7901 cells increased the cisplatin resistance. However, the decreased expression of BCAR4 in SGC7901 cells increased the sensitivity of cisplatin. The results suggested the elevated expression of BCAR4 has a close relationship with cisplatin resistance (Figure 3).

BCAR4 Promotes Drug Resistance of Ability of Gastric cancer Cell Line may be Caused by Regulating the Expression of Tumor Stem Cell Biomarker

Tumor stem cell is one of the main factors on occurrence and development of drug resistance. We aimed to know whether the change of BCAR4 expression could influence tumor stem cell biomarkers thus affecting drug-resistance. We performed tumor sphere formation experiments with SGC7901 cells, SGC7901 cells overexpressing BCAR4, normal SGC7901/DDP cell and SGC7901/DDP cells with BCAR4 being knocked-down. Western blot experiments showed that elevated BCAR4 expression increased the expression of tumor stem cell biomarkers such as β-catenin, Nanog, Oct3/4, Sox2, c-Myc, and Klf4. Downregulation of BCAR4 expression resulted in decreased expression of biomarkers mentioned above. In the signaling pathway, the gene encoding β-catenin is upstream of those encoding gene in Wnt signaling pathway. Surprisingly, we found that the expression of Nanog, Oct3/4, Sox2, c-Myc, and Klf4 was no longer increased by the overexpression of BCAR4, when the expression of β-catenin was controlled in a steadily low level. These results led us to believe that BCAR4 changed the expression of those stemness factors by regulating the expression of β-catenin. It might be the molecular mechanism of drug resistance in gastric cancer (Figure 4).

Figure 1. BCAR4 was highly expressed in the gastric cancer tissue. A, The expression of BCAR4 in the gastric cancer tissue and adjacent tissue was detected by qRT-PCR assay. **p<0.001; B, The expression of BCAR4 in gastric cancer tissue was detected according to the tumour size, ***p<0.001. C, The expression of BCAR4 in gastric cancer tissue was analyzed according to clinical subtype, ***p<0.001.

Figure 2. The clinical characteristic of BCAR4. Association between patients’ survival time (log-rank test) and expression of BCAR4.
Discussion

Gastric cancer is a common malignant cancer with increasing worldwide incidence. It is characterized by its frequent recurrence, high drug-resistance rate, and very high mortality. Cisplatin is a first-line chemotherapy drug against gastric cancer, but accumulating evidence has indicated that drug-resistance became one of the main factors affecting the prognosis of patients. In recent years, IncRNAs were reported to play important roles in cell proliferation and apoptosis, differentiation and development, as well as in cancer development and progression. For instance, the expression of IncRNA LOWEG was found to be decreased in gastric cancer and acted as a tumor suppressor by inhibiting the invasion of gastric cancers. Shi et al. showed that downregulated IncRNA BANCR promoted the proliferation of colorectal cancer cells via downregulating p21 expression. Therefore, different IncRNAs have different functions and expression level in different types of cancer. Highly expressed BCAR4 is an independent predictive factor for poor disease-free survival after tamoxifen therapy for recurrent breast cancer disease. Ju et al. showed that the increased expression profile of BCAR4 in osteosarcoma is an independent indicator of poor overall survival and served as an oncogene in osteosarcoma development. However, the role of BCAR4 in gastric cancer has never been reported. In this research, we found that BCAR4 expression was up-regulated in gastric cancer cell lines and cell lines. The expression of BCAR4 was closely related with tumor size and type. Our work further showed that gastric cancer patients with overexpression of BCAR4 had worse prognosis compared with those with those with low expression of BCAR4, which indicated that expression level of BCAR4 was a potential and independent prognostic factor of gastric cancer patients. We know that the mechanism of drug resistance in
tumor varies due to the different sensitivity of tumor cells to chemotherapy. Tumor stem cells are less likely to be killed by chemotherapy drugs than normal tumor cells. Tumor stem cells are a kind of special cells in tumor cells, which have the potential of self-renewal and differentiation, and are one of the most important factors, which contribute to tumor invasion, metastasis, and drug resistance. It has been reported that lncRNAs play an important role in the process of drug resistance. Previous studies have proved that lncRNA BCAR4 is a potential prognostic factor of gastric cancer and we aimed to know whether the expression of BCAR4 affects the drug resistance in tumor via influencing the stemness of tumor stem cells. The study showed that BCAR4 was highly expressed in SGC7901/DDP cells compared with SGC7901 cells line. BCAR4 overexpression in SGC7901 cells promoted cisplatin resistance, and cisplatin resistance was decreased in SGC7901/DDP cells with BCAR4 being knocked down. The results showed that BCAR4 could influence the drug resistance in gastric cancer.

We further investigated whether BCAR4 affected stemness in tumor through Wnt signaling pathway, thereby affecting drug resistance in tumor. We detected the expression of β-catenin, Nanog, Oct3/4, Sox2, c-Myc, and Klf4, which were important biomarkers related to tumor stem cells. We considered that the expression of β-catenin, Nanog, Oct3/4, Sox2, c-Myc, and Klf4 were high in some tumors and associated with tumor invasion, metastasis, and poor prognosis. As mentioned earlier, β-catenin is upstream of the stemness biomarkers pathway and we investigated how BCAR4 regulates the expression of these biomarkers. In the context of increased BCAR4 expression, we knocked down the gene of β-catenin and surprisingly discovered all biomarkers were no longer regulated by BCAR4, indicating that BCAR4 could influence the stemness in tumor by regulating the expression of β-catenin in Wnt signaling pathway. BCAR4 is a potential and independent prognostic factor of gastric cancer whose increased expression can promote drug-resistance in gastric cancer. This research provides us with a novel treatment target for drug-resistance in gastric cancer patients.

**Conclusions**

BCAR4 was highly expressed in the gastric cancer tissue. BCAR4 could regulate the
expression of β-catenin by Wnt signaling pathway to promote the drug-resistance of gastric cancer. In the future, BCAR4 may be a potential treatment target for drug-resistance in gastric cancer.

Conflict of interest
The authors declare no conflicts of interest.

References