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BCAR4 increase cisplatin resistance and predicted poor survival in gastric cancer patients

L. WANG[1], Q. CHUNYAN[2], Y. ZHOU[1], Q. HE[1], Y. MA[1], Y. GA[1], WANG[3]

[1]Department of Pharmacy, People's Hospital of Yuxi City, Yuxi, Yunnan Province, China
[2]The Seventh People's Hospital of Chengdu, the Tumor Hospital of Chengdu, Sichuan Province, China
[3]Physical Examination Centre, The Third Affiliated Hospital of Chongqing Medical University, Chongqing, China

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 80 cases of gastric cancer tissue and adjacent tissue, and the clinical significance was also analyzed. MTT assays and Western blot were performed to cytological determine the relationship between BCAR4 expression and cisplatin resistance, as well as investigate the potential molecular mechanism involved.

RESULTS: Compared with the adjacent tissues, we found that BCAR4 was highly expressed in gastric tissues. We also found that the expression of BCAR4 was significantly related to the size of the tumor, clinical classification and the survival time. 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 activated 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 the gastric cancer and improving the efficiency of chemotherapeutic drugs.

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

Introduction

Gastric cancer is one of the most common cancer worldwide and the emergence of new-targeted drugs has greatly improved the survival time of gastric cancer[1]. However, the survival time remains short because of drug-resistance in late-stage gastric cancer patients[2]. 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 studies[3]. 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[4,5]. 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[6-8]. The lncRNA 4 (BCAR4) was reported to be involved in anti-estrogen resistance in breast cancer[9]. Increased expression of BCAR4 is an in-

Corresponding Author: Xiang Wang, MD; e-mail: wx300@163.com

dependent indicator for poor disease-free survival after tamoxifen therapy for recurrent breast cancer disease[10]. In a recent study, Gong et al[11] found that elevated expression of lncRNA 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 ... tion. The clinical data, including age, sex, size, lymph node metastasis, histology type and pathological grade, were recorded. All gastric cancer patients received no radiotherapy and chemotherapy before surgery, and the tissue was stored at -80°C until use. All ... were informed and signed informed co... This study was approved by the Medical E... Comm... of our institution.

Cell Culture

SGC7901 and ...-resistant c... were purchased from ...ese Academy ... Sciences (Shangha... China)... stem cells, not tumor cells, ... be grown ...um-free medium, and a sph... culture can b... for the isolation an... tracterization of tumo... stem cells. Cells we... spend... a sphere serum-free medium co... Dulbecco's M...dified Eagle Medium/F12 (... 12 (1:1)... EGF (30 ng/L), fibroblast grow... tor (2... /L) and B27 supplement (... After ... s, the number of sphere in thre... rections ... iew at a low magnification was... nted. In the logarithmic growth phase, we co... s and resuspended them in pre...sly prepared sphere culture medium as a single cell suspension. All the cell lines were cultured ...7°C incubator with 5% CO_2, and 0.25 mL fresh ...mor sphere culture medium was added every day. At the 14th day, we counted and analysis the information collected from the ...

RNA Extraction and Real-tim... Quantitative PCR Assays

According to the manufacture... ...ocol, total RNA from tissue and ... was ... d by using RNAiso Plus (TaK... Otsu, Shiga... Expression of BCAR4 ... mor ti...e and ga... cancer cell lines wer... ected h... andard fluorescent quantitative P... ay ... SYBR... emix Ex Taq (TaKaR... Otsu... , Japan)... e PrimeScript™ RT... gent Kit ... ed ... etect the concentratio... RNA and syn... cDNA with gDNA Er... Ra, Otsu, Shi..., Japan).

Lentivirus Produc... nd Plasmid Tra... ion

...cloned the BCAR4 into the overexpres... vector pC... H-MSCV-mcs-EF1-GFP-T2A-... (SBI) after a... fication. The pLKO.1 vector ...used to con... the knocked-down BCAR4 s... e: ...CAG-CAGCTTGTTGCTCA-TC... and 5'-TTGCCTTGGGGACA-GTTCAC-3' (reverse). The lentiviral packaging ...asmids psPAX2 and pMD2.G were purchased ...g Yuan Biological Company (Beijing, ...).

Detection of Cell Drug Resistance

The cells were cultured under standard condi...ion 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-

Table I. Prognostic values of variables for death and disease recurrence by receiver operating characteristic analysis.

Variables	Area under curve	95% CI	p-v
Death			
Intra-tumoral BCAR	0.671	0.637-0.702	
TNM stage	0.612	0.569-0.652	.0
3-year recurrence			
Intra-tumoral BCAR	0.583	0.532-0.619	.000
TNM stage	0.644	0.626-0.692	.000

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 GraphP sm software (GraphPad Software, La Jo USA) to analyze the protein bands.

Statistical Analysis

All the experiments were inde ndently rep ated at least three times and p an ave- rage with SD. The *t*-test wa d to yze the differences between grou Overall ival of patients was analyzed b lan-M and log rank test. Ind en ostic signi- ficance of risk fact dentifi multivariate analysis was com d by the Co portional hazards model operating ch teristic (ROC) curve ysis sed to determine the predictive alue among eters. When the *p*-value<0 the result wa idered signifi- cant. G Pad P ism 6 (La Jol , CA, USA) was used al wit data.

Results

BC was H y Expressed in the Ga Cancer Tissue

e effect of BCAR4 in gastric can- we first detected the expression of BCAR4 in cases of gastric cancer tissues and adjacent t using qRT-PCR. We found that BCAR4 was hly expressed in gastric cancer tissues compared with iace es. Fu rmore, we analyzed t xpressio BC and the clinicopatho al informati e patients, founding P4 was posit y correlated with tu ize. results suggested that the expression of BCAR t be related to the de- velo of gastric ca Meanwhile, compa- r the intestinal-type gastric cancer, we also d that the ex ssion of BCAR4 was even hi- r in diffuse- gastric cancer (according to en type). Th results indicated that BCAR4 w olved in occurrence and progression of gast the mechanism was still uncle- ar (Figure 1).

ical Characteristic of BCAR4

To luate 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 II. Multivariate analysis of independent prognostic factors of gastric cancer.

Variable	Hazard	Ratio
Histological grade	2.419	0.024
Lymph node metastasis	2.627	0.041
Distant metastasis	3.159	0.019
Clinical stage	2.351	0.035
lnc RNA BCAR4 expression	2.2	0.011

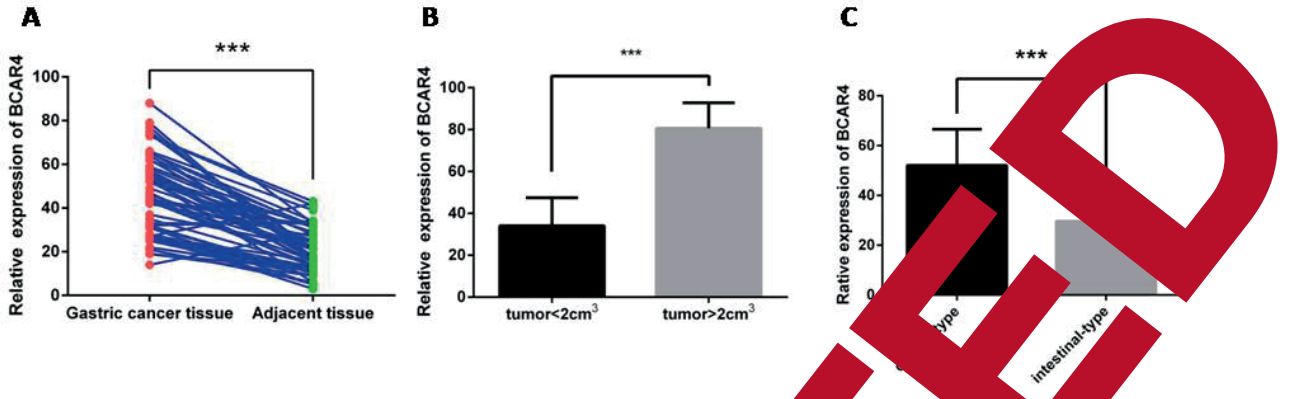
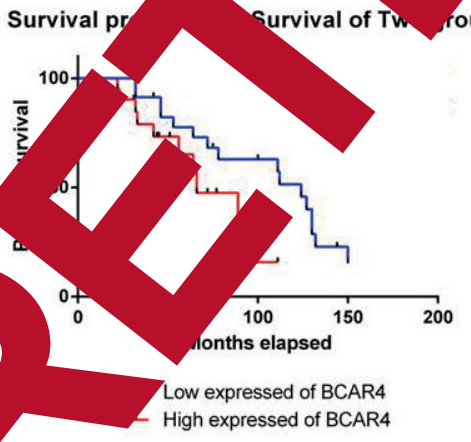


Figure 1. BCAR4 was highly expressed in the gastric cancer tissue. ***A,*** The expr of BCAR4 i cancer tissue and adjacent tissue was detected by qRT-PCR assay. $^{***}p<0.001$; ***B,*** The expres AR4 in gastric tissue was detected according to the tumour size, $^{***}p<0.001$. ***C,*** The expression of BCAR4 r tissue was analyzed according to clinical subtype, $^{***}p<0.001$.

vely correlated with the survival time of patients with gastric cancer (Figure 2).

Elevated Expression of BCAR Would Lead to the Cisplatin Resistance

Drug resistance is one of the factors resulting in poor prognosis. We detected the expr of BCAR4 in SGC7901 cell strain an splatin-resistance (SGC7901/DDP) based he mentioned results. We observed that BCAR highly expressed in SGC7901/DDP cells. T we overexpressed BCAR4 in SGC7901 cells a then knocked down the BC GC7901/ DDP cells. In the cell viabi assay found the overexpression of BC in SG 01 cells increased the cisplatin nce. decreased expressio f SGC7901



2. The clinical characteristic of BCAR4. Asso- ci tween patients' survival time (log-rank test) and expr of BCAR4.

c creased the sensitivity of cisplatin. The lts suggested the elevated expression of AR4 has a relationship with cisplatin tance (Figu .

BC es Drug Resistance of Ability of Gastric cancer Cell Line may be aused by Regulating the Expression of tem Cell Biomarker

Tu 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).

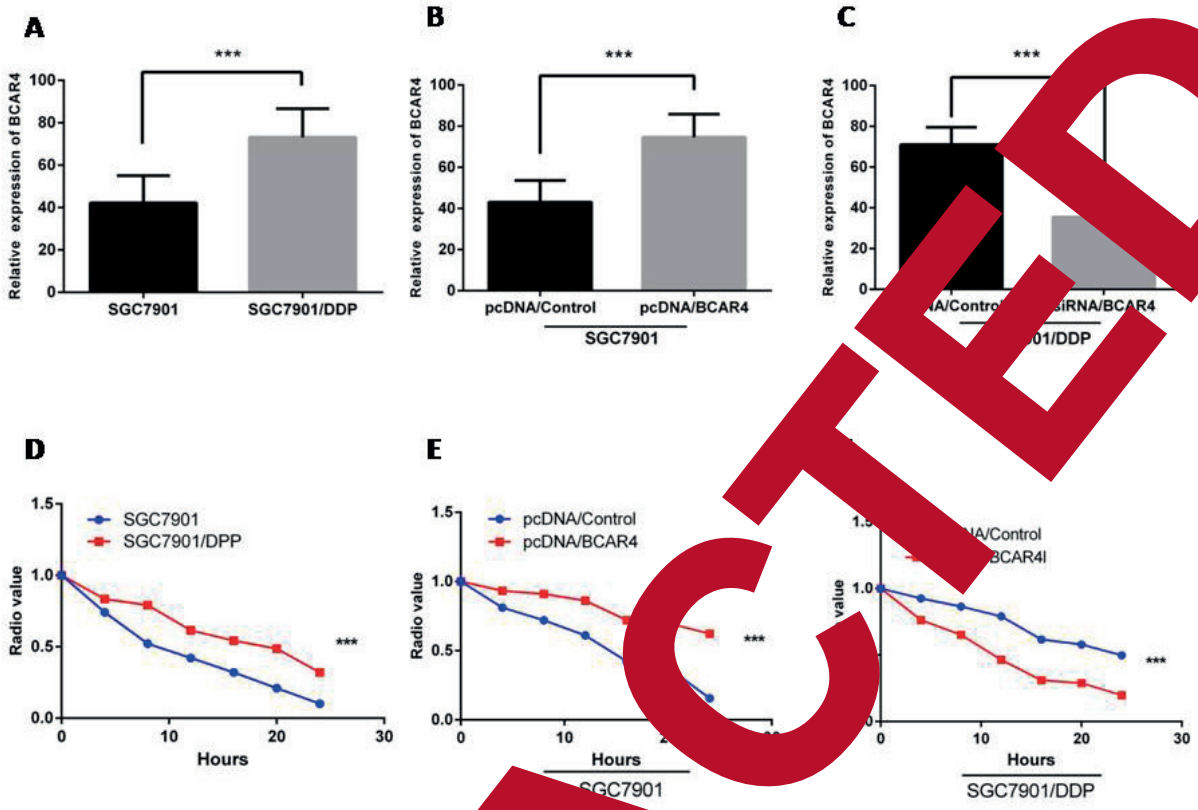


Figure 3. Improved BCAR4 would promote drug ...tance ... ...r cell lines. ***A,*** Relative BCAR4 expression in SGC7901 and SGC7901/DDP cells was detected by ...assay. ***B,*** Overexpression of BCAR4 in SGC7901 cells detected by PCR. ***$p<0.001$; ***C,*** Overexpression of BC... ...7901/DDP cells detected by PCR. ***$p<0.001$; ***D-F,*** Growth rate of each group detected by MTT. Data from each gr... detected in three separate experiments, and the results were expressed as mean ± SD. SD, standard ...ion.

Discu...n

Gastric cancer i... commo... ...ignant cancer with increasi... worldwide i... ...e. It is characterized h... ...quent recur... ...e, high drug-resistan... ...te, ...ry high mortality[12]. Cisplatin is ...first-line ch... ...rapy drug against gastric c... ...r, but accumu... ...evidence has indicat... ...at dru...-resistance b...came one of the main ...ors af...ng the prognosis of patients. In ... yea... ...ncRNA ...were reported to play impor... ...in cel... ...liferation and apoptosis, ...differ... ...on a... ...velopment, as well as in ...deve... ...d progression. For instance, t... ...xpressio... ...lncRNA LOWEG was found to b... ...reased in gastric cancer and acted as a tu... ...r by inhibiting the invasion of gastric cancers[13]. Shi et al[14] showed that downregulated lncRNA BANCR promoted the proliferation o... ...ectal cancer cells via downregulating p21 expr... ...on. Therefore, different lncRNAs 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[15] 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 tissues 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

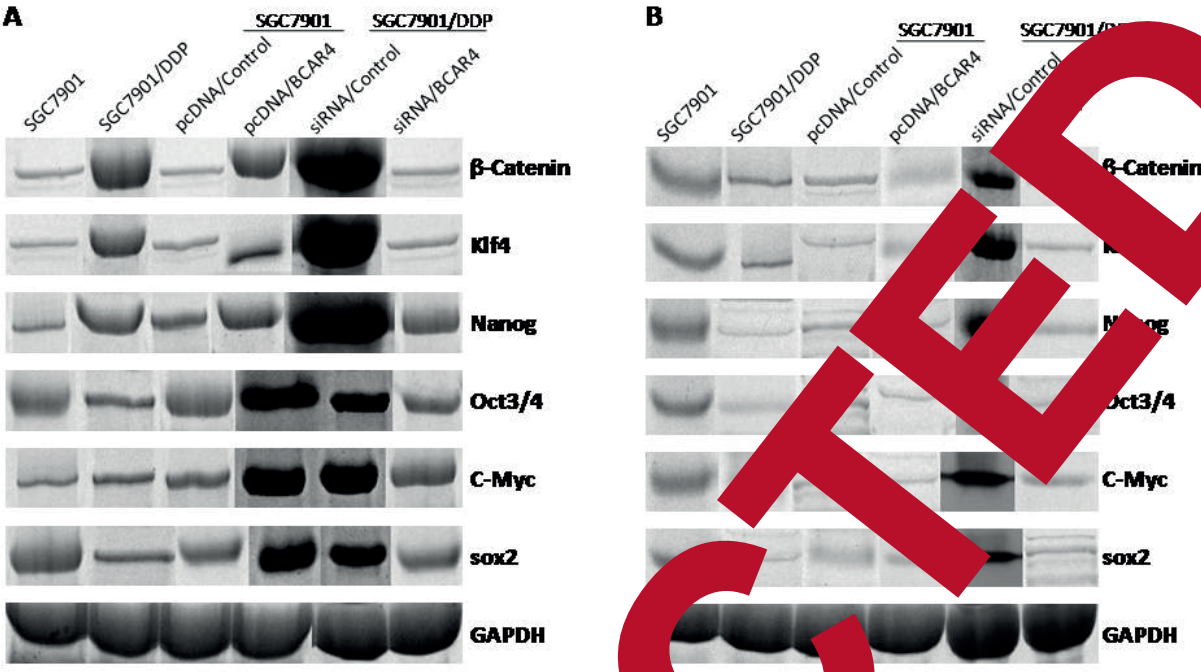


Figure 4. BCAR4 promotes drug-resistance in gastric cancer c via regulati umor stem cells biomarkers in Wnt signaling pathway. ***A,*** Elevated BCAR4 expression increased the e of β- n, Nanog, Oct3/4, Sox2, c-Myc, and Klf4. ***B,*** In the context of downregulating β-catenin, the expression o , Sox2, c-Myc, and Klf4 was no longer regulated by BCAR4.

tumor varies due to the different sensitivity , Na g, Oct3/4, Sox2, c-Myc, and Klf4, whimor cells to chemotherapy. Tumor stem cells ch were important biomarkers related to tumor less likely to be killed by chemotherapy dru stem cells[19]. We considered that the expression of than normal tumor cells. Tum lls are a β-catenin, Nanog, Oct3/4, Sox2, c-Myc, and Klf4 kind of special cells in tu ells, ch have were high in some tumors and associated with the potential of self-rene nd dif tiation, tumor invasion, metastasis, and poor prognosis. and are one of the most tant f As mentioned earlier, β-catenin is upstream of the contribute to tumor in ion, s, and drug stemness biomarkers pathway and we investigaresistance. It has be ported RNAs play ted how BCAR4 regulates the expression of these an important role e process o esistanbiomarkers[20]. In the context of increased BCAR4 ce[16-18]. Previous ave proved th cRNA expression, we knocked down the gene of β-catBCAR4 is a p tial stic factor of gastric enin and surprisingly discovered all biomarkers cancer and aimed to k hether the expres- were no longer regulated by BCAR4, indicating sion of B 4 affects the d istance in tu- that BCAR4 could influence the stemness in tumor vi luenci the stemne of tumor stem mor by regulating the expression of b-catenin in cells study wed that BCAR4 was highly Wnt signaling pathway. BCAR4 is a potential and exp d in S /901/D P cells compared with independent prognostic factor of gastric cancer SGC line. R4 overexpression in whose increased expression can promote drug-reSGC7901 prom cisplatin resistance, and sistance in gastric cancer. This research provides in res s decreased in SGC7901/ us with a novel treatment target for drug-resistanDD lls with AR4 being knocked down. ce in gastric cancer patients. The lts showed that BCAR4 could influence th ce in gastric cancer.

Conclusions

e further investigated whether BCAR4 af- BCAR4 was highly expressed in the gad stemness in tumor through Wnt signaling stric cancer tissue. BCAR4 could regulate the p thereby affecting drug resistance in tum We detected the expression of β-caten-

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.

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