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Long non-coding RNA ZEB1-AS1 is associated with poor prognosis in gastric cancer and promotes cancer cell metastasis

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Abstract. – OBJECTIVE: To investigate the expression of long non-coding RNA zinc-finger E-box binding homeobox 1-AS1 (lncRNA ZEB1-AS1) in gastric cancer cells and tissues, to study its effect on the gastric cancer cell metastasis capacity, and analyze its clinical significance.

PATIENTS AND METHODS: The relative expression level of lncRNA ZEB1-AS1 in gastric cancer cells was detected via quantitative reverse transcription polymerase chain reaction (qRT-PCR). Transwell assay was used to detect the effects of lncRNA ZEB1-AS1 on the invasion and metastasis capacities of gastric cancer cells. qRT-PCR was used to detect the relative expression level of lncRNA ZEB1-AS1 in 75 pairs of gastric cancer tissues, and the correlations of its expression with the pathological characteristics and prognosis of patients were statistically analyzed.

RESULTS: qRT-PCR showed that compared with that in the normal gastric epithelial cell (GES-1), the expression level of lncRNA ZEB1-AS1 was up-regulated in gastric cancer cells (MKN28, MKN45, BGC823, MGC803, KATOIII, and SGC7901). LncRNA ZEB1-AS1 interfering sequence was transfected into model cells, and Transwell assay showed that the cell invasion and migration capacities were significantly inhibited. qRT-PCR also revealed that the expression of lncRNA ZEB1-AS1 was up-regulated in 55 out of 75 cases of gastric cancer and para-carcinoma tissues (fold change > 1). Statistical analysis showed that the high expression of lncRNA ZEB1-AS1 was positively correlated with TNM staging ($p = 0.002$), lymph node metastasis ($p = 0.002$), and invasion degree ($p = 0.004$). The survival time of patients with high expression of lncRNA ZEB1-AS1 in gastric cancer tissues was shorter than that of patients with low expression ($p = 0.004$).

CONCLUSIONS: LncRNA ZEB1-AS1 is highly expressed in gastric cancer tissues and cells, and it is expected to be a new prognostic marker of gastric cancer used for the clinical diagnosis and prognostic evaluation. After intervention in lncRNA ZEB1-AS1 expression, the cell invasion and migration are inhibited, and lncRNA ZEB1-AS1 may be an important target to reverse the malignant phenotype of gastric cancer.

Key Words:
Gastric cancer, lncRNA ZEB1-AS1, Invasion and metastasis, Clinical prognosis.

Introduction

Gastric cancer is one of the malignant tumors that frequently occur in the world, whose incidence rate ranks fourth and mortality rate ranks second in all tumors. There are more than 1 million new cases every year around the world[1]. According to the pathological type, primary gastric cancer is divided into adenocarcinoma, adenosquamous carcinoma, and neuroendocrine carcinoma, etc., among which adenocarcinoma accounts for about 90%[2]. The onset of early gastric cancer is hidden, and patients often have no typical symptoms and signs, so it is clinically difficult to be found. Besides, many patients have been in the middle and advanced stage when diagnosed, often accompanied by lymph node metastasis. Invasion and metastasis of gastric cancer are the main reasons for the death of gastric cancer patients. Gastric cancer metastasis is a complex multi-factor, multi-step and multi-stage continuous development process, and its specific molecular regulation mechanism remains unclear[3,4]. Therefore, it is particularly important to deeply understand the related mechanism of gastric cancer metastasis and search the molecular target of the diagnosis and the treatment of gastric cancer.

With the development of high-throughput sequencing technique, researchers have found that the information carried by long non-coding RNA

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(lncRNA) is significantly greater than expected and very complex[5]. LncRNA is a kind of gene transcript longer than 200nt lacking the effective open reading frame without the function of encoding protein[6]. At present, it has been found that the main action mode of lncRNA is the regulation of gene expression *via* the specific dynamic interaction between it and protein, DNA or RNA[7]. Studies[8] have found that lncRNA plays an important role in the occurrence and development of a variety of diseases, especially the tumor. Wu et al[9] found that lncRNA SOX-5 can mediate the dynamic changes of micro-environment, thus promoting the development of colorectal cancer. In gastric cancer, the high expression of lncRNA DANCR can promote the tumor cell proliferation, and can be used as an important index of prognosis estimation of gastric cancer patients[10].

LncRNA ZEB1-AS1 is a total of 2232bp in length, and located in chromosome 10p11.22 region[11]. It has been reported that the expression of lncRNA ZEB1-AS1 is up-regulated in several tumors, such as bladder cancer, colorectal cancer, and osteosarcoma, playing a similar role to "oncogene" in promoting the tumor growth[12-14]. In liver cancer, the high expression of lncRNA-ZEB1-AS1 can promote the invasion and metastasis of liver cancer cells *via* regulating the epithelial-mesenchymal transition (EMT)[15]. In gastric cancer, there has been no report on the expression of lncRNA ZEB1-AS1 in gastric cancer and its biological effects.

In this study, the expression level of lncRNA ZEB1-AS1 in gastric cancer tissues and cells was detected *via* quantitative reverse transcription polymerase chain reaction (qRT-PCR) for the first time, and the correlations of its relative expression level with the clinicopathological features and prognosis of gastric cancer patients were analyzed. Besides, the effects of lncRNA ZEB1-AS1 on the invasion and migration capacities of gastric cancer cells were studied *via in-vitro* experiment. The results provided an important theoretical basis for clinically searching the therapeutic target and prognosis estimation of patients with advanced gastric cancer.

Patients and Methods

Tissues and Cells

A total of 75 patients receiving the gastric cancer operation in Department of Gastroenterology, Zhoukou Central Hospital from January 2012 to January 2015 were enrolled. They were pathologically diagnosed with gastric cancer without receiving any radiotherapy, chemotherapy, and molecular targeted therapy before the operation. After materials were obtained in the operation, the tumor site was determined according to the pathological tissue report, samples were immediately placed into the liquid nitrogen tank and stored in the refrigerator at -80°C for long-term preservation. The pathology of gastric cancer was classified, and the degree of tumor infiltration was based on the criteria of International Union against Cancer. All patients were followed-up from discharge to death. This study was agreed and signed by the patient and the authorized person, and approved by the Ethics Committee of Zhoukou Central Hospital.

Human gastric cancer cell lines (MKN28, MKN45, BGC823, MGC803, KATOIII, and SGC7901) and human normal gastric epithelial cell GES-1 were purchased from Type Culture Collection (Manassas, VA, USA) or Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) or 1640 medium (Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (Gibco BRL, Rockville, MD, USA). Penicillin (100 U/mL) and streptomycin (100 μg/mL) were added to the medium to prevent the growth of infectious microbe. The cell suspension was placed in the constant temperature incubator containing 5% CO_2 at 37°C, followed by cell digestion and passage on a regular basis.

RNA Extraction and qRT-PCR

Total RNA was extracted from gastric cancer tissues and cells using the TRIzol reagent, followed by reverse transcription into cDNA according to the instructions of reverse transcription kit (PrimeScript TM RT Master Mix, TaKaRa, Otsu, Shiga, Japan). Through the relative fluorescent quantitative PCR, lncRNA ZEB1-AS1 was detected using the Bio-Rad (Hercules, CA, USA) quantitative PCR instrument with housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control. Reaction conditions: 95°C for 5 s, 60°C for 15 s, 72°C for 15 s, and a total of 40 cycles. The computed tomography (CT) value was obtained and the gene expression level was calculated using $2^{-\Delta\Delta CT}$. Fold Change = $2^{-\Delta\Delta CT}$, which was used to present the multiple ratios of target gene expressions, and the experiment was repeated for three times.

Synthesis of siRNA and Forward and Reverse Primers

Primers were designed using Primer 5.0. For ZEB1-AS1, forward primer: 5-CCGTGGGCACTGCTGAAT-3, reverse primer: 5-CTGCTGGCAAGCGGAAC-3. For GAPDH, forward primer: 5-AGCCACATCGCTCAGACAC-3, reverse primer: 5-GCCCAATACGACCAAATCC-3. The interference sequence of ZEB1-AS1: 5-CCACAGGCCATGAATTCCTTCCTAA-3, and the sequences and primers were designed and synthesized by Invitrogen (Shanghai, China).

Transwell Assay

The interference sequence in control group was transiently transfected into the gastric cancer cells. When the cell fusion rate after transfection reached about 90%, the cells were digested with EDTA-free trypsin and were resuspended using the serum-free medium into the single-cell suspension. Cells were counted and the cell concentration was adjusted to 3.5×10^5 cells/mL. 200 μL of the above cell suspension was added into the upper chamber of Transwell chamber (the upper chamber face was coated with 50 mg/L BD Matrigel using the diluent at 1:8), while 600 μL 1640 medium or DMEM medium containing 20% fetal bovine serum (FBS) was added to the lower chamber, followed by incubation for 24/48 h under conventional conditions. The above operations were repeated for 3 samples in each group, followed by fixation *via* formaldehyde and crystal violet staining.

Statistical Analysis

Statistical Product and Service Solutions (SPSS Version X; IBM, Armonk, NY, USA) 21.0 software was used for statistical analysis. Chi-square test was used for the relationship between lncRNA ZEB1-AS1 expression and clinicopathological features of cervical cancer patients. Kaplan-Meier method was used to analyze the relationship between the lncRNA ZEB1-AS1 expression and the survival time of patients. Cox proportional hazard model was used for the univariate/multivariate analysis of prognosis of gastric cancer. $p < 0.05$ suggested that the difference was statistically significant.

Results

Effect of lncRNA ZEB1-AS1 on Cell Migration and Invasion

First, the relative expression levels of lncRNA ZEB1-AS1 in human gastric cancer cell lines (MKN28, MKN45, BGC823, MGC803, KATOIII, and SGC7901) and normal gastric epithelial cell GES-1 were detected *via* qRT-PCR. The results showed that the expression of lncRNA ZEB1-AS1 was up-regulated in gastric cancer cells (Figure 1A), the siRNA interference efficiency was detected *via* qRT-PCR (Figure 1B), and Transwell assay showed that the migration and invasion capacities of gastric cancer cells were significantly inhibited after the intervention in lncRNA ZEB1-AS1 expression (Figure 1C and 1D).

Detection of the Expression Level of lncRNA ZEB1-AS1 and Analysis of its Relationship with Clinicopathological Features

75 cases of human gastric cancer and para-carcinoma tissues were ground to extract the total RNA. The results of qRT-PCR showed that the expression of lncRNA ZEB1-AS1 was up-regulated in 55 cases (73.3%) of gastric cancer tissues (Fold change > 1) (Figure 2A). With the average expression multiple of lncRNA ZEB1-AS1 as the cutoff point, 75 patients were divided into high-expression ZEB1-AS1 group (n = 42, fold change > 4.5) and low-expression ZEB1-AS1 group (n = 33, fold change < 4.5) (Figure 2B). The results of chi-square test showed that the expression level of ZEB1-AS1 was positively correlated with TNM staging, lymph node metastasis, invasion degree of gastric cancer patients, but not correlated with the age, gender, and tumor location, etc., of patients (Table I).

Analysis of the Correlation Between the Expression of lncRNA ZEB1-AS1 and the Survival of Patients

Kaplan-Meier survival analysis showed that the survival time of patients in the high-expression ZEB1-AS1 group was shorter than that of patients in low-expression group (Figure 2C). Cox proportional hazard regression model was used for the univariate analysis of survival data of 75 patients, and the results showed that the high-expression ZEB1-AS1 ($p = 0.006$), TNM staging ($p = 0.005$) had a statistical significance. Then, Cox regression multivariate analysis showed that the clinical TNM staging and lncRNA ZEB1-AS1 expression could be used as independent prognostic factors for gastric cancer patients (Table II).

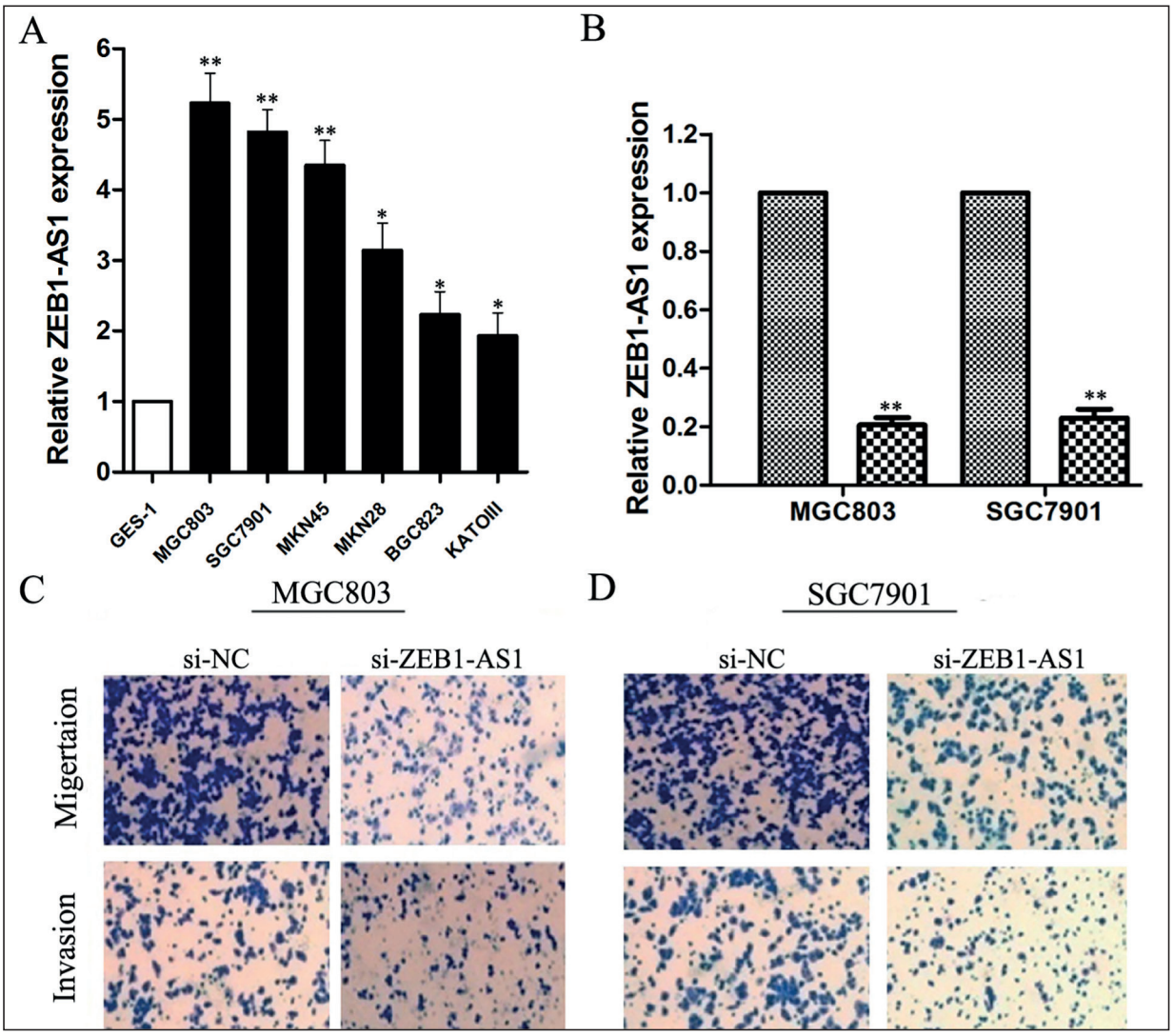

Figure 1. Effect of lncRNA ZEB1-AS1 on cell migration and invasion. ***A,*** qRT-PCR is used to detect the relative expression level of lncRNA ZEB1-AS1 in gastric cancer cells compared with that in human normal gastric epithelial cells with GAPDH as the internal control. ***B***, Specific interference sequences of lncRNA ZEB1-AS1 are designed and synthesized, transiently transfected into cells, and the interference efficiency is detected after 48h. ***C, D,*** After intervention in the expression of ZEB1-AS1, Transwell assay is used to detect its effect on cell migration and invasion capacities, and $*p < 0.05$, $**p < 0.01$.

Discussion

The occurrence and metastasis of gastric cancer is a multi-factor, multi-step, complex and continuous process, involving the changes in genes, signaling pathways, and epigenetics[16]. Current studies[17] have shown that the common molecular mechanisms of gastric cancer invasion and metastasis include extracellular matrix degradation, tumor microenvironment changes, activation or inhibition of invasion and metastasis-related genes, and EMT, etc. And lncRNA, as an important regulatory molecule, is involved in a variety of biological processes. For example, lncRNA FENDRR inhibits the invasion and migration capacities of gastric cancer by regulating the FN1, MMP2, and MMP9 expression levels[18]. Chen et al[19] demonstrated that lncRNA MALAT2 can promote cell migration and EMT by regulating MEK signaling pathway. In this study, we found for the first time that lncRNA ZEB1-AS1 was highly expressed in gastric cancer tissues and cells, and promoted the migration and invasion of tumor cells. And its potential molecular mechanism is the focus of our follow-up research.

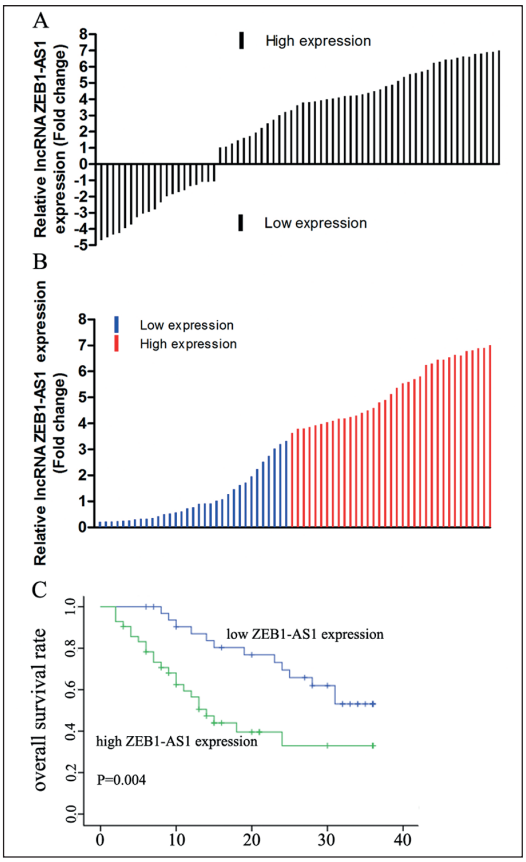

Figure 2. Expression level of lncRNA ZEB1-AS1 in gastric cancer and its clinical significance. ***A***, qRT-PCR is used to detect the relative expression levels of lncRNA ZEB1-AS1 in gastric cancer tissues and para-carcinoma tissues. ***B***, With the average expression multiple of lncRNA ZEB1-AS1 as the cutoff point, patients with gastric cancer are divided into high-expression ZEB1-AS1 group (n = 42) and low-expression ZEB1-AS1 group (n=33). ***C***, Kaplan-Meier survival analysis is used to study the correlation between the ZEB1-AS1 expression level and the survival time of patients.

Table I. Correlation between lncRNA ZEB1-AS1 expression and clinicopathological characteristics of gastric cancer patients (n = 75).

Characteristics	ZEB1-AS1 Low expression (no.)	ZEB1-AS1 High expression (no.)	*p*-value
Age (years)			
> 60	18	26	0.638
≤ 60	15	16	
Sex			
Male	13	23	0.246
Female	20	19	
Tumour size (cm)			
> 4	19	17	0.167
≤ 4	14	25	
Differentiation			
Well	6	5	0.421
Moderate	14	18	
Poor	11	26	
Undifferentiated	2	3	
TNM staging			
I+II	21	11	**0.002***
III+IV	12	31	
Lymph lode metastasis			
No	13	8	**0.002***
Yes	20	34	
Tumor location			
Gastric antrum	7	7	0.871
Corpora ventriculi	15	16	
Preventriculus	11	19	
Invasion degree			
T1	9	5	**0.004***
T2	9	4	
T3	7	16	
T4	8	17	

Table II. Univariate and multivariate analysis of overall-survival in gastric cancer patients (n = 75).

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Sex	0.756	0.386-1.480	0.414			
Age	0.854	0.443-1.645	0.637			
Tumor size (cm)	1.545	0.795-3.003	0.199			
Differentiation	0.963	0.657-1.413	0.849			
Invasion degree	0.998	0.747-1.334	0.990			
Lymph lode metastasis	1.090	0.566-2.100	0.797			
Tumor location	0.867	0.559-1.344	0.522			
TNM staging	2.798	1.356-5.775	0.005*	2.405	1.144-5.089	0.021*
LncRNA ZEB1-AS1 expression	2.675	1.327-5.392	0.006*	2.280	1.109-4.689	0.025*

HR, hazard ratio; 95% CI, 95% confidence interval, * Overall $p < 0.05$.

Early symptoms of gastric cancer often lack the specificity, and about two-third of patients have been in the middle and advanced stage or suffer from metastasis when diagnosed, and the 5-year survival rate is only 20%-30%[20]. Gastroscopy is the "golden standard" for diagnosis of gastric cancer, but many adverse reactions and poor compliance of patients in the gastroscopy limit its application in screening gastric cancer. Upper gastrointestinal barium meal fluoroscopy, CT and other imaging examinations have been widely used in the diagnosis of gastric cancer, but the imaging manifestations of early gastric cancer and precancerous lesions often lack the specificity, so imaging examinations have some limitations in the early detection of gastric cancer and small lesions[21]. Traditional tumor markers, such as CEA, CA72-4, and CA19-9, are significantly increased in the late stage of gastric cancer, and the positive diagnosis rate of gastric cancer is lower, often below 40%[22]. Therefore, searching new biological targets for gastric cancer monitoring and intervention has become an urgent problem to be solved for the gastric cancer prevention and treatment. It has been proved in many studies that the abnormal expression of lncRNA, as a new tumor marker, is closely related to the occurrence and development of tumors[23].

Conclusions

We found for the first time that lncRNA ZEB1-AS1 was highly expressed in tumor tissues compared with that in para-carcinoma tissues. The statistical analysis showed that high-expression lncRNA ZEB1-AS1 was positively correlated with TNM staging, lymph node metastasis, and invasion degree and could be used as an independent factor of prognosis prediction of gastric cancer patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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