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High expression of LncRNA CASC15 is a risk factor for gastric cancer prognosis and promote the proliferation of gastric cancer

X.-M. YAO, J.-H. TANG, H. ZHU, Y. JING

Department of Gastroenterology, Affiliated Wujiang Hospital of Nantong University, Suzhou, China

Abstract. – OBJECTIVE: To investigate the expression of long non-coding RNA CASC15 in gastric cancer tissue and its effect on the proliferation of gastric cancer cell line MKN28.

MATERIALS AND METHODS: We found that expression of lncRNA CASC15 in gastric cancer tissue was higher than normal gastric epithelium through the TCGA and Gene Expression Omnibus (GEO) database. Then, we detect the RNA level of CASC15 from clinical samples of 42 normal gastric epithelial tissues and 60 gastric cancer tissues. In order to explore the function of CASC15 in gastric cancer, we perform gain-function and loss-function assay in gastric cancer cell lines.

RESULTS: We found that expression of lncRNA CASC15 in gastric cancer tissue was higher than normal gastric epithelium through the TCGA database and the related microarray data set was searched from Gene Expression Omnibus (GEO) database. Then, we extracted total RNA from clinical samples of 42 normal gastric epithelial tissues and 60 gastric cancer tissues. The results of quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) were consistent with those of TCGA analysis. Clinical data analysis showed that the expression of LncRNA CASC15 was correlated with the total survival, tumor size and TMN staging in clinical patients. Clinical data analysis showed that the expression level of CASC15 was correlated with tumor size and TNM stage in clinical patients. Compared with the negative control group, the proliferation and cell cloning ability of MKN28 cells overexpressing LncRNA CASC15 significantly increased ($p<0.001$), indicating that overexpression of LncRNA CASC15 promoted the proliferation of MKN28 cells.

CONCLUSIONS: The expression of LncRNA CASC15 was significantly higher in gastric cancer tissues and its expression was negatively correlated with the overall survival of clinical patients. It was positively correlated with the tumor size and TMN stage. LncRNA CASC15 could promote the proliferation of gastric cancer cells and was expected to become the molecular marker for prediction and prognosis of gastric cancer, as well as a potential therapeutic target.

Key Words:
Gastric cancer, CASC15, lncRNA, TGCA.

Introduction

In the global cancer statistics, the incidence of gastric cancer ranks 4th, and the occurrence of gastric cancer in Asia accounts for 60% worldwide[1-3]. Despite the continuous improvement of diagnostic techniques, more and more comprehensive surgical treatment-based treatments (including chemotherapy, targeted therapy, etc.), gastric cancer is still the 2nd leading cause of cancer death in the world[4]. Therefore, it is urgent to explore the molecular mechanism of gastric cancer development and find reliable biomarkers for early diagnosis and reliable therapeutic targets to improve the survival of these patients. Long non-coding RNAs (lncRNAs), which are abundantly transcribed in the genome, are potential candidates after microRNAs have been the new hot topic in tumor research. The genome sequencing project showed that the human genome comprised about 20,000 kinds of protein coding genes, accounting for only about 2% of the total genes, and over 90% of the transcripts were non-coding RNAs. The non-coding RNAs did not have an open reading frame and had no protein translation function. According to the length of RNAs, they were divided into three types: long-chain, medium-chain and short-chain noncoding RNAs. lncRNAs were transcripts with over 200 nucleotides in length and did not encode proteins[5]. More and more researches have shown that lncRNA not only participated in many biological processes but also played many roles (such as splicing, transcriptional interference, post-transcriptional regulation, genomic imprinting, chromatin modification, cell cycle regulation, epigenetic regulation, immunity surveillance, etc.)[6,7]. Now lncRNAs are known to

Corresponding Author: Yin Jing, MM; e-mail: sunny13211@sina.com

be involved in the occurrence and development of gastric cancer, such as H19, TUG1, TINCR and HOXA-AS2EZH2[8-11]. Our laboratory genetically annotated and analyzed the chip data GSE13911, GSE19826 and GSE79973 in the gene expression omnibus (GEO) database and found that the differential expression of CASC15 in gastric cancer was over 2-fold. However, the research on CASC15 in gastric cancer has not been reported. Based on the above chip results, we conducted a preliminary evaluation of CASC15 as a potential molecular marker of gastric cancer. In this study, we found that the up-regulation of CASC15 was correlated with the total survival time, tumor size and TMN stage in patients with gastric cancer, and the expression of CASC15 in gastric cancer tissues and cell lines was detected by quantitative polymerase chain reaction (PCR). RNA interference technology was utilized at the cellular level to inhibit CASC15 expression, at the same time, the impact of cell proliferation activity was also observed; the results were reported as follows.

Materials and Methods

GEO Data Analysis and TCGA Prognosis Analysis

First, we downloaded the RNA-seq data of gastric cancer and normal gastric mucosa epithelium in the TCGA database; differential expressions of them were analyzed by edger function, and prognostic analysis was performed by survival function. The expression data of GSE13911, GSE19826 and GSE79973 in GEO (Gene Expression Omnibus) database were analyzed by limma function, and it was found that the differential expression of CASC15 in gastric cancer was over 2-fold.

Cell Culture and siRNA and pcDNA Transfection

Gastric cancer cell lines (MKN453, AGS, MKN28) and normal gastric epithelial cell line (GES-1) were cultured in Roswell Park Memorial Institute-1640 (RPMI- 1640) medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA). Then, cells were seeded in a 6-well plate, and were transfected with Entranster-R4000 until cell density was about 60%. Cells were seeded in 6-well plates, which contain 1 μL of Entranster-R4000, 0.5 mg of pcDNA-CASC15, and 0.45 ml serum-free Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Rockville, MD, USA). Besides, the control group was added with the same amount Entranster-R4000 and pcDNA-NC. 6 h after transfection, the culture medium was replaced with RPM-1640 medium with 10% fetal bovine serum (FBS).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted according to the instructions of TRIzol. 50 μL reaction system was prepared according to the protocol of qRT-PCR. The reverse transcription reaction was performed under the following conditions: reverse transcription reaction at 50°C for 30 min and 92°C for 3min. The obtained cDNA was subjected to polymerase chain reaction (PCR) amplification under the following conditions: denaturation at 92°C for 10 s, annealing at 55°C for 20 s, extension at 68°C for 20 s and amplification for 40 cycles. β-actin was used as loading control, the relative expression of CASC15 was calculated by 2-ΔΔCt method. The β-actin primer sequence: upstream primer 5'-CTCCATCCTGGCCTCGCT-GT-3' and downstream primer 5'-GCTGTCACCTTCACCGT-TCC-3'.

Cell Proliferation Assay by Cell Counting kit-8 (CCK8) Method

The transfection time point was 0 h, cells in the control group and treated group were seeded in the 96-well plates, each sample had 6 same wells at a density of 5×10^3/100 μL, 5 96-well plates were prepared. 6 h later, the activity of adherent cells was measured. CCK8 assay was performed after they were cultured for 24, 48, 72 and 96 h. 20 μL of CCK8 was added to each well. After incubation at 37°C and 5% CO_2 for 2-3 h, the OD value was measured at 450 nm. Blank control was added only with CCK8 solution and medium (no cells).

Statistical Analysis

Statistic Package for Social Science (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. GraphPad Prism 5.0 was used for picture editing. Survival analysis was performed using Kaplan-Meier survival curves and statistically significant survival analysis was included in the COX regression analysis. Measurement data were compared with *t*-test and presented as mean ± standard deviation ($\bar{x}\pm s$), categorical data were compared with X^2-test. $p<0.05$ indicated significant difference; $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

Results

CASC15 is Highly Expressed in Gastric Cancer and is a Prognostic risk Factor for Gastric Cancer

By analyzing The Cancer Genome Atlas (TCGA) database, we found that CASC15 was highly expressed in gastric cancer tissues in TCGA database (Figure 1A and 1B). Next, we analyzed its relationship with prognosis and found that the higher the expression of CASC15, the worse the prognosis of patients (Figure 1C). Similarly, GSE13911, GSE19826, and GSE79973 in the GEO database also found that CASC15 was significantly overexpressed in gastric cancer (Figure 1D, 1E and 1F).

CASC15 Expression in Gastric Cancer Tissues and Cells

To investigate the expression of CASC15 in gastric cancer tissues, we first selected 42 normal gastric tissues and 60 gastric cancer tissues and detected the expression of CASC15 in the tissues by qRT-PCR. The results showed that the expression of CASC15 in gastric cancer tissues was significantly higher than that in normal tissues (p<0.001) (Figure 2A). As the TCGA showed, the

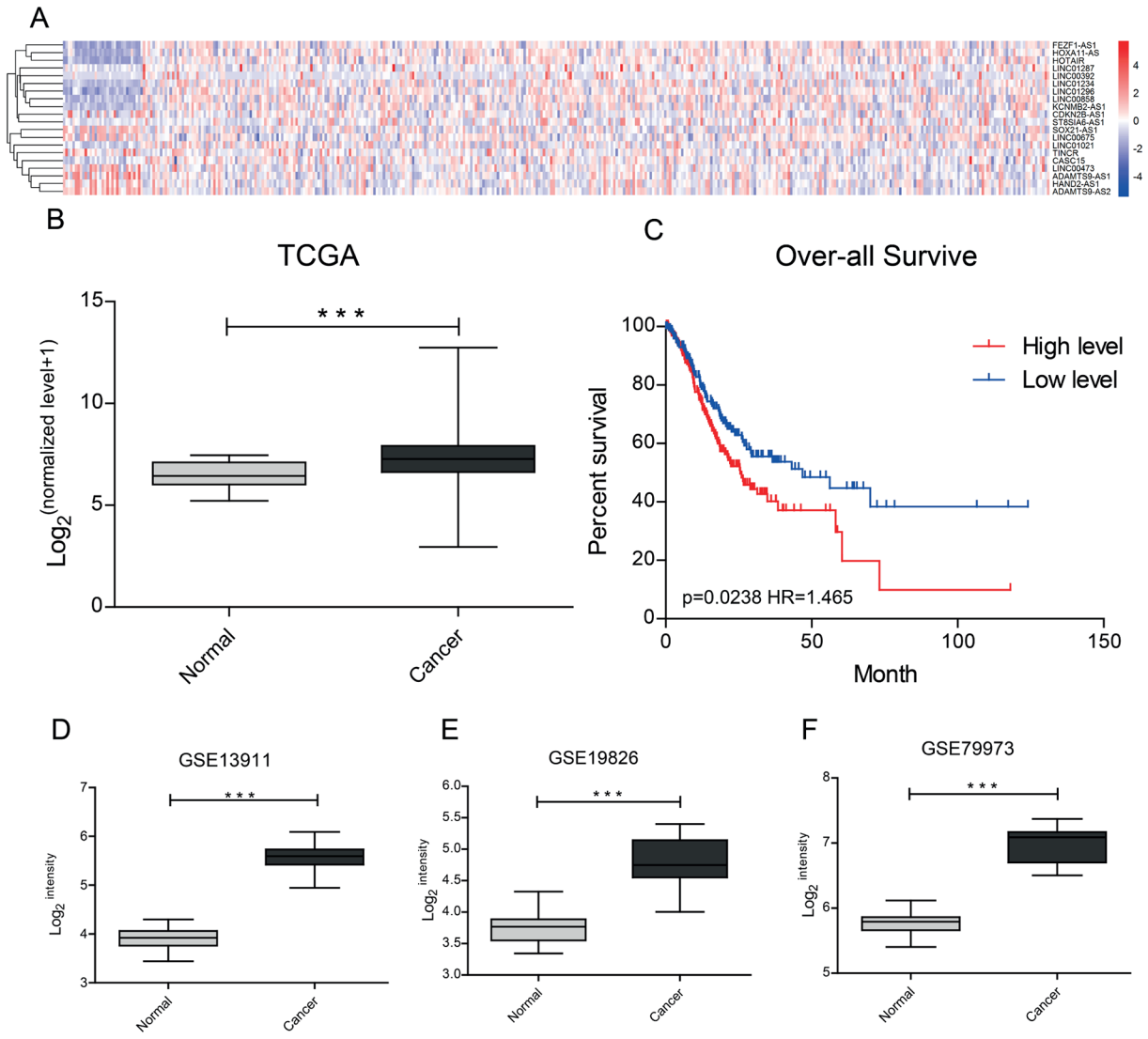

Figure 1. LncRNA CASC15 is highly expressed in gastric cancer. ***A,*** Heat map of differential expression between gastric cancer tissues and normal gastric tissues in TCGA. ***B,*** CASC15 was highly expressed in gastric cancer tissues in TCGA. C, The expression level of CASC15 is negatively correlated with over-all survive in TCGA with gastric cancer. ***D, E*** and ***F,*** CASC15 is up-regulated in GSE13911, GSE19826 and GSE779973 in GEO.

patients' outcome was negatively correlated with CACS15 level (Figure 2B).

Relationship Between the Expression of CASC15, Clinical Features and Prognosis of Gastric Cancer

Relationship study of CASC15 expression and pathological characteristics found that the expression of CASC15 was correlated with the depth of clinical stage and tumor size, but not correlated with the patient's age, sex, tumor location and regional metastasis (Table I). The expression of CASC15 in patients with T3 and T4 invasion was significantly higher than those in T1 and T2 (Figure 2C). The CASC15 expression was higher in patients with higher tumor size (Figure 2D). Therefore, it was suggested that CASC15 may be involved in the progression of gastric cancer to a certain extent.

Promotion effect of Overexpressed CASC15 on the Proliferation of Well-Differentiated Gastric Cancer cell line MKN28

In this study, we detected the expression of CASC15 in gastric cancer cell lines (MKN453, AGS, MKN28) and normal gastric epithelial cells (GES-1) at mRNA level (Figure 3A). The relative expression of CASC15 in well-differentiated gastric cancer cell line MKN28 was the lowest, while the poorly differentiated gastric cancer cell line MKN453 had the highest expression

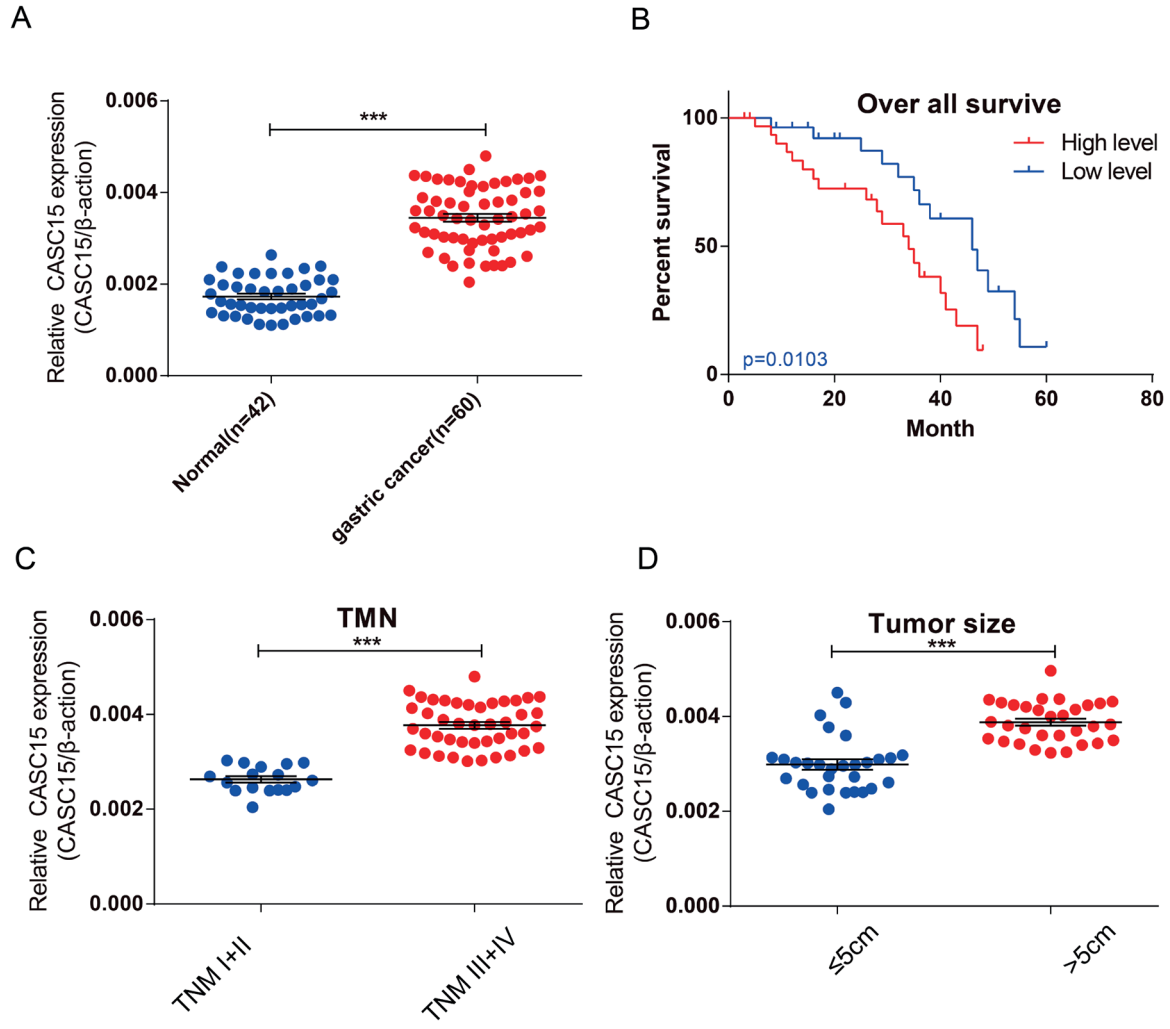

Figure 2. LncRNA CASC15 is positive related with TNM stage and tumor size. ***A,*** CASC15 in 60 patients with gastric cancer tissues was significantly higher than the expression of adjacent tissues. ***B,*** The overall survival rate of gastric cancer patients with overexpressed CASC15 was significantly lower than that of CASC15 lowly expressed group. ***C,*** CASC15 expression was positively correlated with TNM stage. ***D,*** CASC15 expression was positively correlated with tumor size.

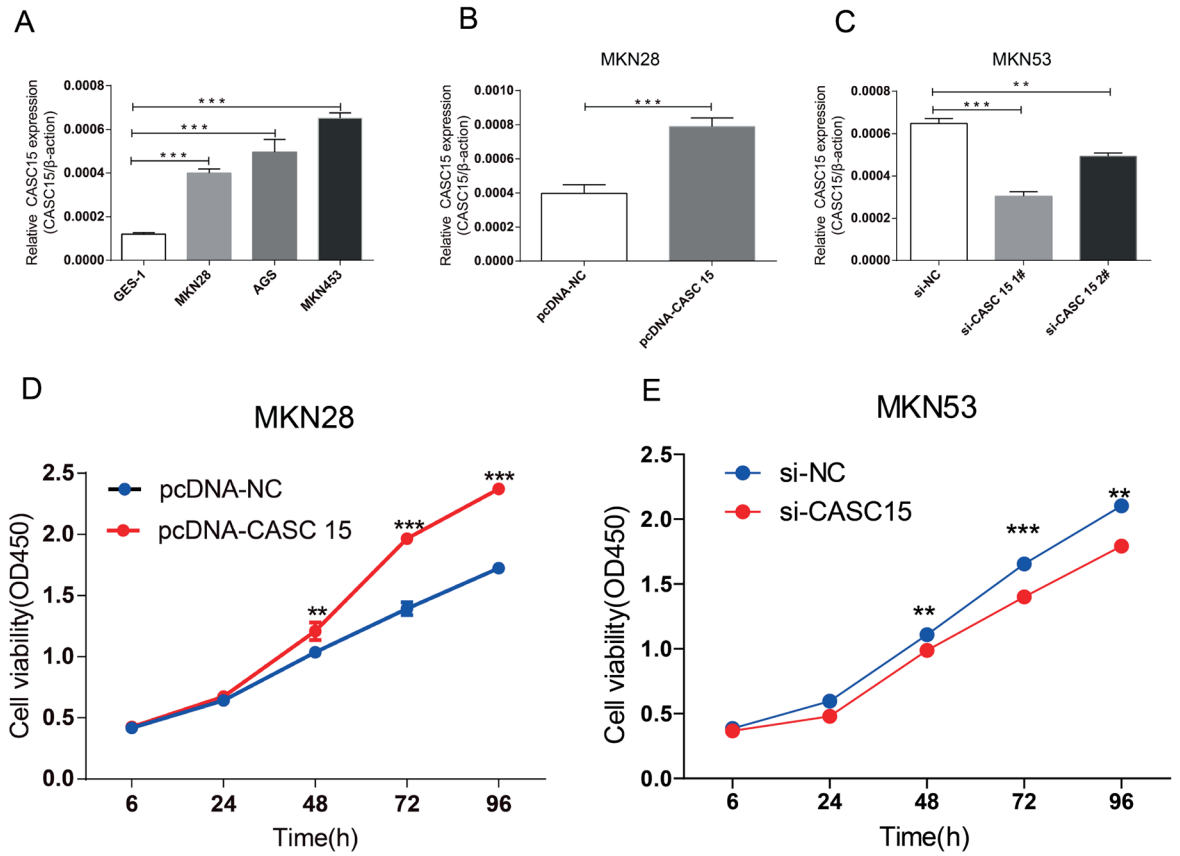

Figure 3. Effect of CASC15 on cell phenotype. ***A,*** CASC15 expression in normal gastric cell line (GES-1) and cancer cell lines (MKN28, AGS, MKN453). ***B,*** Overexpression efficiency of pcDNA-CASC15 in MKN28 cell line. ***C,*** si-RNA interference efficiency in MKN53 cells. ***D,*** CCK8 assay showed that over-expression with CASC15 simulate viability of MKN28 cells. ***E,*** CCK8 assay showed that interference with CASC15 inhibited viability of MKN53 cells.

Table I. Correlation between expression of lncRNA CASC15 and clinicopathological features in patients with gastric cancer (n = 60).

Clinicopathologic features	Number of cases	CASC15 expression		*p*-value
		Low (n=30)	High (n=30)	
Age (years)				
<50	33	15	18	0.4362
≥50	27	15	12	
Gender				
Male	32	14	18	0.3006
Female	28	16	12	
Tumor size				
≤5 cm	26	6	20	0.0003*
>5 cm	34	24	10	
TNM stage				
I-II	30	10	20	0.0098*
III-IV	30	20	10	
Histological grade				
G1-G2	33	17	16	0.7953
G3	27	13	14	
Lymph node metastasis				
Absent	38	20	18	0.5920
Present	22	10	12	

*$p<0.05$.

of CASC15 (Figure 3B), so gastric cancer cell line MKN28 was selected for the further overexpression experiments and MKN453 was selected for the further loss-function experiments (Figure 3C). After constructing the plasmid pcDNA-NC and pcDNA-CASC15 were transfected into MKN28 cell line, the degree of proliferation of gastric cells was determined by CCK8 assay. Compared with pcDNA-NC negative control, D450 value was increased after MKN28 cells transfected with pcDNA-CASC15 (Figure 3D). At the same time, the MKN453 whose CASC15 was knockdown exerted relative lower proliferative ability than control group (Figure 3E). The results showed that CASC15 overexpression promoted the proliferation of MKN28 cell line ($p<0.001$).

Discussion

LncRNA is another major research hot topic after miRNA, which is abnormally expressed in tumor tissues, resulting in the loss of biological function of tumor cells and regulation. Long non-coding RNAs played an important role in the occurrence and development of gastric cancer[12]. LncRNA CCAT2 was highly expressed in gastric cancer tissues. Patients with high expression of CCAT2 were more likely to develop local lymph nodes and distant organ metastases with shorter disease-free progression time and overall survival[13]. The expression of CCAT1 in gastric cancer was up-regulated. This lncRNA may promote the invasion and metastasis of cancer cells by binding to the C-Myc promoter region[14]. Both *in vivo* and *in vitro* studies have shown that TUSC7 can inhibit the growth of tumor cells, and TUSC7 can exert its tumor inhibitory effect by interacting with p53[15]. In gastric cancer, abnormal expression of these lncRNAs had some correlation with tumor size, macroscopic type, histological grade, tumor invasion, metastasis, etc. Therefore, these lncRNAs may have many clinical applications. For example, it may serve as a biomarker for early diagnosis and precision therapy, as well as for assessing prognosis. In this study, firstly, the expression of CASC15 in gastric cancer was found to be significantly increased by analyzing the TCGA database, and negatively correlated with the prognosis of patients. The expression of CASC15 in gastric cancer tissues was significantly higher than that in normal gastric tissues by analyzing the GSO13911, GSE19826 and GSE79973 data of GEO database.

LncRNA CASC15 is a brand new trans lncRNA, located at human chromosome 6q22.3. Systematic studies based on the lncRNA CASC15 gene and human tumors have rarely been reported. No report was found to investigate the occurrence and development of gastric cancer. There were some articles reported with liver cancer, leukemia, neuroblastoma, melanoma, but lncRNA CASC15 was not fully understood[16-19]. In this study, qRT-PCR was used to detect the expression of LncRNA CASC15 in 60 cases of gastric cancer and 42 cases of normal gastric tissues. LncRNA CASC15 was found to be for the first time highly expressed in gastric cancer. Combined with clinicopathological data, it was found that the higher expression of lncRNA CASC15, the deeper the depth of tumor invasion, the higher clinical stage and the larger the tumor volume ($p<0.001$). Invading deeper into the serosa and late stage were signs of higher-grade malignancy. As the expression of CASC15 is positively related with the depth of the tumor invasion and tumor stage, the result suggested that lncRNA CASC15 may be involved in the progress of gastric cancer. The higher the expression of LncRNA CASC15, the shorter the survival time of patients, which presented a negative correlation ($p<0.001$).

Further, it was investigated whether LncRNA CASC15 was involved in the proliferation of gastric cancer cells. In CCK8 assay, the proliferation of cancer cells was significantly increased by up-regulating the expression of lncRNA CASC15 in well-differentiated gastric cancer cell line MKN28, indicating that overexpression of lncRNA CASC15 promoted the proliferation of gastric cancer cells ($p<0.001$). In cloning assay, the ability of gastric cancer cells overexpressed lncRNA CASC15, which was enhanced compared with that of control cells, indicating that lncRNA CASC15 can promote the proliferation of gastric cancer cells. In summary, the results showed that lncRNA CASC15 in gastric cancer tissue was upregulated, which was negatively correlated with overall clinical survival, while positively correlated with tumor size and TNM stage. Overexpression of lncRNA CASC15 can promote tumor cell proliferation, suggesting that lncRNA CASC15 served as an oncogene in the development of gastric cancer. LncRNA CASC15 had the potential ability for the treatment of gastric cancer. However, its transcriptional regulation mechanism was not yet clear, further studied are still needed.

Conclusions

LncRNA CASC15 is highly expressed in gastric cancer; it promotes the proliferation of gastric cancer cells and is a risk factor for the prognosis of patients with gastric cancer.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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