

Long non-coding RNA CRNDE is a novel tumor promoter by modulating PI3K/AKT signal pathways in human gastric cancer

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Abstract. – **OBJECTIVE:** Long non-coding RNA CRNDE (CRNDE) recently emerged as a carcinogenic promoter in various cancers including gastric cancer (GC). However, the functions and molecular mechanisms of CRNDE to GC are still largely unclear. The aim of this study was to investigate the clinical significance and functional mechanisms of CRNDE expression in GC.

PATIENTS AND METHODS: The expression of CRNDE was detected by quantitative Real-time PCR (qRT-PCR) in GC specimens and cell lines. The correlation between the CRNDE expression and clinicopathological parameters was investigated. Survival rate was determined with Kaplan-Meier and statistically analyzed with the log-rank method between groups. Subsequently, the significance of survival variables was analyzed using the Cox multivariate proportional hazards model. Then, MTT and Transwell assays were used to assess cell proliferation, migration and invasion capacity. Finally, Western blot analysis was performed to explore the effects of CRNDE knockdown on the PI3K/Akt pathway.

RESULTS: We observed that expression of CRNDE was higher in GC tissues and cells compared with the normal gastric tissue and normal gastric cell lines. High expression of CRNDE was correlated with invasion depth ($p = 0.006$), TNM stage ($p = 0.010$) and lymph node metastasis ($p = 0.005$). Furthermore, high CRNDE expression was associated with shorter overall survival ($p = 0.0066$) of GC patients. Multivariate analysis confirmed that high CRNDE expression was a significant independent predictor of poor survival in GC. In vitro assay indicated that knockdown of CRNDE inhibited cell proliferation, migration and invasion of GC. Finally, the data of Western blot showed that CRNDE exerted its oncogenic role by affecting PI3K/AKT signaling pathways.

CONCLUSIONS: Our findings indicate that CRNDE plays an important role in promoting GC progression and may represent a novel prognostic biomarker in GC.

Key Words:

Long non-coding RNA, CRNDE, PI3K/Akt, Prognosis.

Introduction

Gastric cancer (GC) is one of the most common types of malignancies and the second leading cause of cancer-related death around the world, with particularly high incidence in China^{1,2}. Although the recent advances in diagnosis and treatment provide excellent survival for patients with early gastric cancer, those patients who were diagnosed at moderate and advanced stage have a poor overall survival rate³. In addition, lack of effective early diagnostic methods and obvious clinical symptoms make GC usually be diagnosed at advanced stages⁴. Therefore, it is urgent to understand the genetic alterations underlying the development and progression of GC.

Long non-coding RNAs (lncRNAs) are defined as transcribed RNA molecules greater than 200 nt in length which are not translated into proteins⁵. Multiple studies have suggested that lncRNAs participate in a large range of biological processes, including cell differentiation, proliferation, and apoptosis⁶. Recently, growing evidence indicates that lncRNAs play critical and extensive roles in the development and progression of cancer. For instance, Lv et al⁷ found that lncRNA Uni-gene56159 promoted cell migration, invasion and EMT by acting as a ceRNA of miR-140-5p in hepatocellular carcinoma cells. Zhang et al⁸ reported that lncRNA NEAT1 served as a tumor promoter and was associated with poor prognosis of breast cancer patients. Other findings by Li et al⁹ suggested that knockdown of lncRNA AB073614 inhibited cell migration, and invasion in glioma

cell lines via affecting EMT. Those findings highlight the role of lncRNAs in progression of cancer. However, the effect of most lncRNAs remains unclear.

Colorectal neoplasia differentially expressed (CRNDE) located on chromosome 16, has been reported to be aberrantly expressed in several tumors, such as colorectal cancer¹⁰, glioma¹¹, medulloblastoma¹² and GC¹³. However, the functional role and underlying mechanism of CRNDE in GC remain unclear. In the present study, we firstly determined the prognostic value of CRNDE in GC, and explored the possible mechanism by which CRNDE regulated GC progression.

Patients and Methods

Patients and Specimens

GC tissues and adjacent non-tumor tissues from patients who had undergone curative resection were collected between 2008 and 2011 from the Beijing Shijitan Hospital of Capital Medical University. All patients had complete 5-year follow-up, and informed written consents were obtained from all cases. None of the patients had received chemotherapy or radiotherapy before surgery. The histological grade of cancers was assessed according to criteria set by the World He-

alth Organization. The clinical and pathological data for the patients are reported in Table I. This study was approved by the Human Research Ethics Committee of Beijing Shijitan Hospital.

Cell Culture and Transfection

Four GC cell lines (MGC-803, MNK-45, SGC-7901, HGC-27) and a normal gastric mucosa cell line (GES) were obtained from Shanghai Institute Chinese Academy of Science (Shanghai, China), maintained in Roswell Park Memorial Institute 1640 (RPMI-1640) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) and streptomycin in a humidified atmosphere. According to the manufacturer's instructions, we transfected the GC cells CRNDE siRNA (si-CRNDE) or negative control siRNA (si-NC) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) to decrease their expression of CRNDE. si-CRNDE was purchased from GenePharma Company (Pudong, Shanghai, China). The transfected cells were harvested 48 h after transfection.

RNA Extraction and qRT-PCR

Total RNA of GC tissues and cell lines were isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For cDNA synthesis, 1 µg of total RNA was retro-transcribed with the PrimeScript RT Master Mix

Table I. Association between CRNDE expression and different clinicopathological features of human GC.

Viable	Cases	CRNDE expression		p-value
		Low	High	
Age (years)				0.813
< 60	51	24	27	
≥ 60	67	33	34	
Gender				0.153
Male	48	27	21	
Female	70	30	40	
Tumor size				0.379
≥ 5 cm	42	18	24	
< 5 cm	76	39	37	
Histological grade				0.070
Well-moderate	52	30	22	
Poor	66	27	39	
Invasion depth				0.006
T1-T2	55	34	21	
T3-T4	63	23	40	
TNM stage				0.010
I-II	60	36	24	
III-IV	58	21	37	
Lymph node metastasis				0.005
Present	53	18	35	
Absent	65	39	26	

(Perfect Real-time) (TaKaRa, Otsu, Shiga, Japan). qPCR-PCR was performed using SYBR Premix EX Taq™ II (TaKaRa, Otsu, Shiga, Japan) on 7900HT Fast Real-time System (Applied Biosystems, Foster City, CA, USA). The transcription levels were normalized to GAPDH expression. The relative amount of CRNDE was calculated using the equation $2^{-\Delta\Delta C_t}$. The assay was performed in triplicate for each case. The primer sequences used for the studies were purchased from Invitrogen (Carlsbad, CA, USA).

Analysis of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium Bromide (MTT)

Cell viability was measured using MTT method. In brief, cells were seeded into 96-well plates and transfected. Then, 100 μ L of MTT solution (0.5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) were added to each well, and further cultured for another 4 h. Subsequently, the medium was replaced by 100 μ L of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Carlsbad, CA, USA) and plates were shaken at room temperature for 10 min. The optical density was measured at 590 nm wavelengths with a microplate reader.

Cell Migration and Invasion Assays

A total of 1×10^4 cells were transfected with si-CRNDE or si-NC for 48 h. Then, cells were planted in the top chamber with a non-coated membrane. The cells were seeded in a serum-free medium, and a medium with 10% serum was used as a chemoattractant in the lower chamber. After incubation at 37°C under 5% CO₂ for 24 h, the non-filtered cells were gently removed with a cotton swab, and cells on the lower membrane were fixed with 95% ethanol and stained with crystal violet. The migrated cells were counted and photographed under an optical microscope. The cell invasion experiment was performed in a similar method, except that the transwell chambers were coated with Matrigel (Sigma-Aldrich, St. Louis, MO, USA).

Western Blot Analysis

Western blot analysis was performed by previous study¹⁴. The primary and second antibody for Western blot was purchased from Calbiochem (San Diego, CA, USA).

Statistical Analysis

All data were analyzed SPSS 17.0 using the software package (SPSS Inc., Chicago, IL, USA).

Data were analyzed using independent two-tailed *t*-test. The χ^2 test was used to explore the relationship between CRNDE expression level and clinicopathological characteristics. Overall survival rates were calculated actuarially according to the Kaplan-Meier method. The survival data were evaluated by univariate and multivariate Cox regression analyses. $p < 0.05$ was considered statistically significant.

Results

Increased Expression of CRNDE in GC Tissues and Cell Lines

First, we measured the CRNDE expression levels by RT-qPCR in GC tissues and matched normal gastric tissues. As shown in Figure 1A, we found that CRNDE expression was remarkably increased in GC tissues compared with adjacent normal tissues ($p < 0.01$). Then, we have also examined the CRNDE expression in GC cell lines and normal gastric cell line. As shown in Figure 1B, the results showed that CRNDE was highly expressed in all the examined GC cell lines than GES-1 cells (all $p < 0.01$).

Association Between Clinicopathological Features and CRNDE Expression Levels in GC Patient Tissues

We next analyzed the correlation between CRNDE expression and the clinicopathological characteristics of GC. The median value of serum CRNDE level in 118 GC patients (4.38) was used as the cutoff point to divide these patients into CRNDE-low ($n = 57$) and CRNDE-high ($n = 61$) groups. As shown in Table I, by statistical analyses, we found that high expression of CRNDE was correlated with invasion depth ($p = 0.006$), TNM stage ($p = 0.010$) and lymph node metastasis ($p = 0.005$). However, no significant difference was observed between CRNDE expression and patients' age, gender, tumor size and histological grade (all $p > 0.05$).

High CRNDE Expression Predicts Poor Prognosis in Patients With GC

To further explore whether increased CRNDE expression in GC patients correlates with a worse prognosis, we performed Kaplan-Meier survival curves. As shown in Figure 2, the results indicated that high CRNDE expression was associated with shorter overall survival ($p = 0.0066$) of GC patients. Then, univariate and multivariate

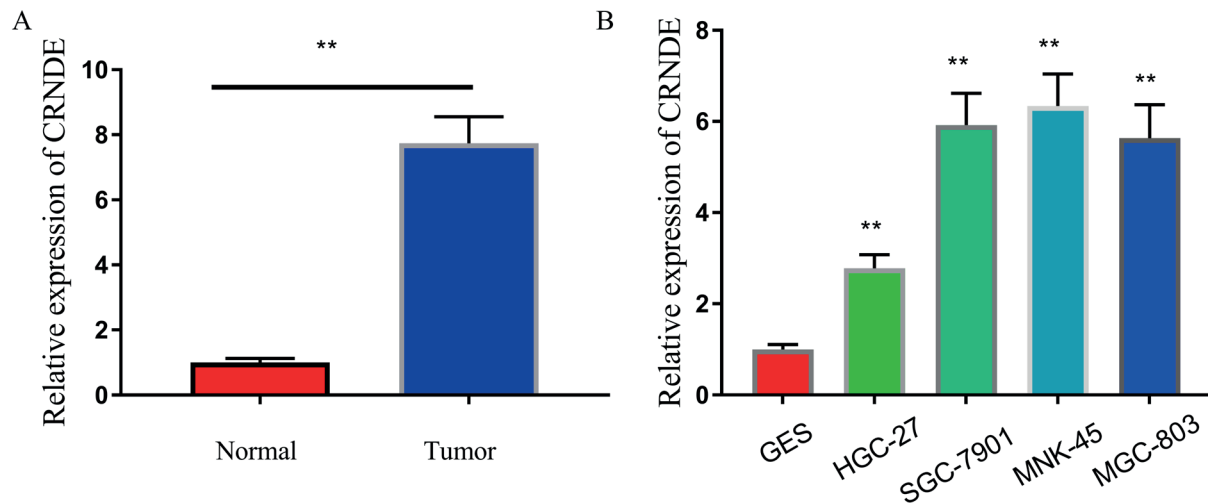


Figure 1. Expression of CRNDE in GC tissues and cell lines. **A**, qRT-PCR analysis of the expression of CRNDE in GC tissues and adjacent normal tissues. **B**, qRT-PCR analysis of the expression of CRNDE in GC cell lines (MGC-803, MNK-45, SGC-7901 and HGC-27) and normal gastric mucosa cell line (GES). The data represented the mean values of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

analyses were used to check the effect of CRNDE expression on GC prognosis. The results showed that CRNDE expression was an independent predictor for overall survival of GC patients (Table II, $p = 0.001$).

Downregulation of CRNDE Suppressed the Proliferation, Invasion and Migration of GC Cells

In order to explore the effects of CRNDE on GC cells, the MGC-803 and MNK-45 cells were transfected with si-CRNDE or si-NC. Decreased expression of CRNDE was found in cells transfected with si-CRNDE (Figure 3A, $p < 0.01$). MTT assay showed that knockdown of CRNDE significantly inhibited MGC-803 and MNK-45 cells proliferation (Figure 3B and 3C). Subsequently, we performed transwell assays in MGC-

803 and MNK-45 cells. Knockdown of CRNDE markedly decreased migration and invasion of MGC-803 and MNK-45 cells compared with the negative control (Figure 3D and 3E). Taken together, our data suggested that CRNDE could serve as an oncogene in GC progression.

CRNDE Regulated PI3K/Akt Pathway in GC Cells

To further explore the molecular mechanism by which CRNDE exerts its biological function, we performed Western blot to analyze the role of CRNDE knockdown on the PI3K/Akt pathway. As shown in Figure 4, knockdown of CRNDE significantly decreased the expression levels of p-PI3K, and p-Akt in GC. Our results indicated that CRNDE can promote the migration and invasion of GC cells by regulating PI3K/Akt pathway.

Table II. Univariate and multivariate Cox regression analyses of parameters correlated with overall survival of all GC patients.

Parameters	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Age	1.452 (0.773-2.218)	0.233	—	—
Gender	1.744 (0.621-2.449)	0.177	—	—
Tumor size	1.631 (0.599-3.322)	0.113	—	—
Histological grade	2.213 (0.521-3.421)	0.089	—	—
Invasion depth	3.782 (1.432-5.566)	0.004	3.236 (1.211-4.774)	0.008
TNM stage	3.231 (1.774-6.652)	0.006	2.842 (1.421-4.587)	0.011
Lymph node metastasis	4.213 (1.334-7.742)	0.001	3.872 (1.219-6.649)	0.002
CRNDE expression	2.663 (1.458-5.532)	0.000	2.453 (1.321-4.777)	0.001

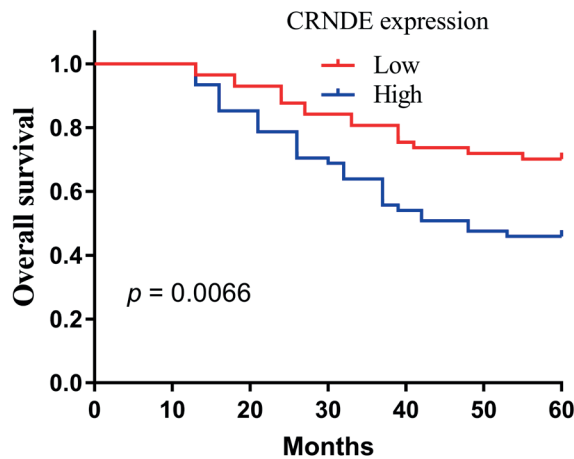


Figure 2. Relationship between CRNDE expression and survival time in GC.

Discussion

Identification of cancer-specific lncRNAs and their targets are important for understanding their role in tumorigenesis. Recently, CRNDE attracted

our interest due to its important role in various tumors. For example, Huan et al¹⁵ reported that CRNDE promotes the migration, invasion and mobility of breast cancer cells by activates Wnt/ β -catenin signaling through inhibiting miR-136. Furthermore, they demonstrated that high CRNDE expression was associated with poor prognosis of breast cancer patients. Zheng et al¹⁶ showed that CRNDE played an oncogenic role of glioma stem cells through the negative regulation of miR-186. Hu et al¹³ indicated that CRNDE promotes proliferation of gastric cancer cells by targeting miR-145. Those results indicated that CRNDE played a positive role in progression of tumors including GC. However, to our best knowledge, the clinical significance of CRNDE has not been reported.

In the present study, we investigated CRNDE expression by Real-time PCR in GC tissues and cell lines. Our results showed that the expression levels of CRNDE were up-regulated in both GC tissues and cell lines. High CRNDE expression level was significantly associated with invasion depth, TNM stage and lymph node metastasis. Moreover, Kaplan-Meier analysis revealed that

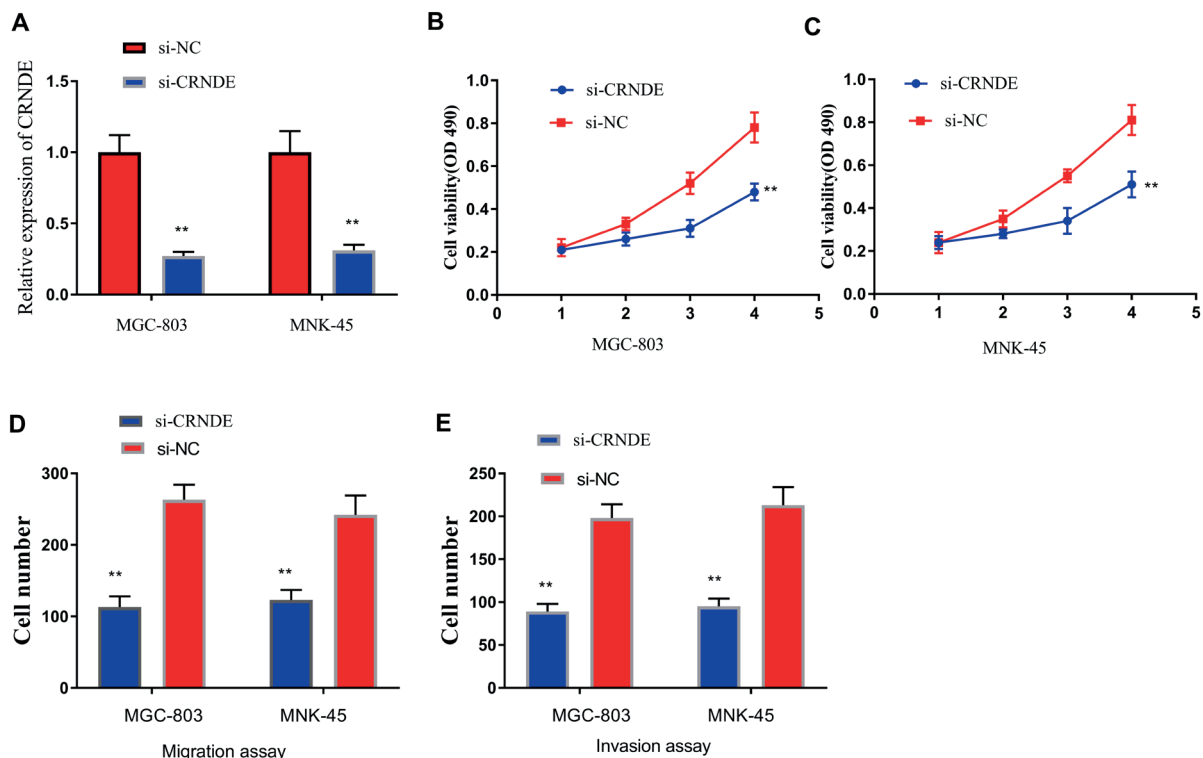


Figure 3. CRNDE knockdown inhibits GC cells proliferation, migration and invasion. **A**, CRNDE expression levels were evaluated using qRT-PCR in si-CRNDE transfected MGC-803 and MNK-45 cells. **B**, **C**, Determination of MGC-803 (**B**) and MNK-45 (**C**) cell proliferation with an MTT assay after transfection with si-CRNDE or si-NC. **D**, Cell migration was determined in MGC-803 and MNK-45 cells transfected with si-CRNDE or si-NC by transwell assay. (**E**) Cell invasion was determined in MGC-803 and MNK-45 cells transfected with si-CRNDE or si-NC by transwell assay. * $p < 0.05$, ** $p < 0.01$.

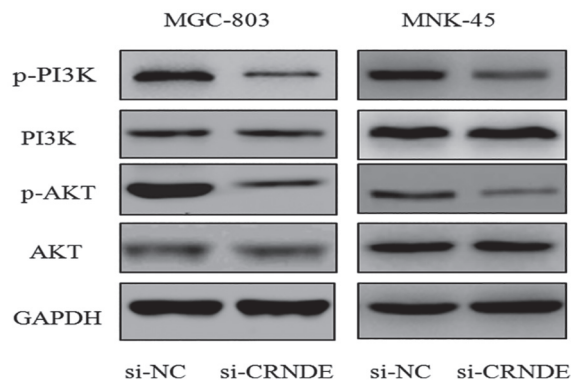


Figure 4. CRNDE regulates PI3K/Akt signaling. Western blotting explored the expression of PI3K/Akt pathway-related proteins after CRNDE knockdown. GAPDH served as the loading control. * $p < 0.05$.

GC patients with high CRNDE expression had poorer overall survival. Subsequently, Cox regression analyses showed that high CRNDE expression might be an independent prognostic parameter to predict poor prognosis. In addition, *in vitro* assay indicated that knockdown of CRNDE suppressed GC cell proliferation, migration and invasion. These results were in line with previous study¹³.

PI3K/Akt signaling pathway is abnormal in many different tumors and has been demonstrated to be involved in cancer cell proliferation, migration and invasion^{17,18}. Previous reports revealed that lncRNAs participated in the regulation of PI3K/Akt signaling pathway. For instance, Lu et al found¹⁹ that lncRNA HULC promoted cell proliferation by regulating PI3K/AKT signaling pathway in chronic myeloid leukemia. Dong et al²⁰ reported that lncRNA MALAT1 promoted the proliferation, migration and invasion in osteosarcoma by activating the PI3K/Akt pathway. More importantly, Shen et al²¹ found that CRNDE served as a tumor promoter in gallbladder carcinoma by activating PI3K-AKT pathway. Thus, we wonder whether CRNDE has the similar effect by GC progression. In the present study, we observed that knockdown of CRNDE significantly decreased the expression levels of p-PI3K, and p-Akt in GC, suggesting CRNDE was involved in the regulation of PI3K-AKT pathway.

Conclusions

We showed that CRNDE served as a novel prognostic biomarker for GC. Further experiment

showed that knockdown of CRNDE inhibited cell proliferation, migration and invasion by modulating PI3K/Akt pathway. Thus, our findings are expected to present new therapy for the diagnosis and treatment of GC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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