

Long non-coding RNA CARLo-5 upregulation associates with poor prognosis in patients suffering gastric cancer

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Abstract. – **OBJECTIVE:** Accumulating evidence has demonstrated that microRNAs (miRNAs) play critical roles in cancer initiation and development. The present study aims to explore the clinical significance of long noncoding RNA CARLo-5 (CARLo-5) in gastric cancer.

PATIENTS AND METHODS: CARLo-5 expression in normal gastric tissue, gastric cancer were quantified by Quantitative reverse transcription PCR. The relationship between CARLo-5 expression and the clinical features of gastric cancer was assessed. Kaplan-Meier method with log-rank test was applied to compare survival curves. The univariate and multivariate Cox regression models were employed to evaluate the prognostic value of the CARLo-5 expression in overall survival (OS) and relapse-free survival (RFS).

RESULTS: We observed that the relative expression of CARLo-5 in gastric cancer tissues was significantly higher than that of their matched adjacent normal tissues ($p < 0.001$). High CARLo-5 expression was found to be closely correlated with advanced T stage ($p = 0.003$), positive distant metastasis ($p = 0.009$), lymph node involvement ($p = 0.001$), and poor differentiation ($p = 0.001$). Furthermore, Kaplan-Meier analysis demonstrated that gastric cancer patients with high CARLo-5 expression had poorer OS ($p < 0.001$) and RFS ($p < 0.001$). Finally, univariate and multivariate Cox analysis indicated that high CARLo-5 expression were independent predictors for both OS and RFS.

CONCLUSIONS: Our findings implicated that CARLo-5 could be considered promising biomarkers for prognosis of gastric cancer.

Key Words:

Long noncoding RNA, CARLo-5, Overall survival, Relapse-free survival.

nancies and the third leading cause of cancer-related deaths worldwide². Despite advancements in the treatment of GC, the 5-year overall survival rate is still very low³. Early detection and treatment as well as blocking or slowing GC malignancy progression are necessary to improve GC prognosis.

Emerging evidence indicate that long noncoding RNAs (lncRNAs) is defined as transcripts with a minimum length of 200 nucleotides in size and limited protein-coding potential^{4,5}. lncRNAs have recently gained significant attention in delineating the complex mechanisms underlying malignant processes⁶. More and more studies report the effect of lncRNA in tumors. For instance, Zhan et al⁷ found that lncRNA PANDAR was significantly up-regulated in bladder cancer tissues and over expression of PANDAR promoted the proliferation/migration and suppressed apoptosis. Li et al⁸ reported that knockdown of lncRNA could inhibit epithelial-mesenchymal transition (EMT) phenotype in glioma cells. Zhang et al⁹ found high expression of lncRNA Sox2ot was a statistically significant risk factor affecting overall survival in gastric cancer patients. These results revealed that lncRNA may function not only as diagnostic and prognostic markers, but also as potential therapeutic targets of human tumors.

CARLo-5 was a newly identified lncRNA and its function in tumors remained largely unknown. The current study aimed to determine CARLo-5 expression in GC tissues and to explore the relationship between CARLo-5 level and clinicopathological features and patients survival.

Introduction

Cancer has become a major health problem and a large economic burden in the world¹. Gastric cancer (GC) is one of the most common malig-

Patients and Methods

Ethics Statement

The study was approved by the Institute of The Second Hospital of Shandong University, Jinan,

Shandong, People's Republic of China. All specimens were handled and identified according to ethical and legal standards. Written informed consent was obtained from every patient to participate in this study.

Patients and Tissue Samples

GC tissue samples and matched normal tissues were obtained from 240 patients at the Second Hospital of Shandong University between July 2008 and July 2011. None of the patients had received chemotherapy, radiotherapy, or endocrine therapy before surgery. The diagnosis was based on clinical examination and histopathological analysis of the tissue specimens. All tissue specimens were immediately snap-frozen in liquid nitrogen and then stored at -80°C . Clinical data of all the patients is listed in Table I.

Quantitative Real-time PCR

Total RNA was extracted from cells or tissues using RNAisoPlus (Takara, Dalian, Niaoing, China), and was reverse-transcribed into cDNA using HiFiScript cDNA Kit (Invitrogen, Carlsbad, CA, USA). Its synthesis was conducted at 37°C for 15 min, then 85°C for 5 s according to the experimental protocols. Quantitative real-time PCR was performed with SYBR Green (Takara, Dalian, Niaoing, China) and the data collection were carried out on the Applied Biosystems[®] 7500 Real-Time PCR Systems (Thermo Fisher Scientific, Franklin, MA, USA). The primer sequences were as follows: GAPDH: 5'-GTCAACGGATTTGGTCTGTATT-3' (forward), 5'-AGTCTTCT-GGGTGGCAGTGAT-3' (reverse); CARLo-5: 5'-GCCACAAATCAACAA-CAACAACAACA-3' (forward), 5'-AGAGT-GATGCCAAGGCTGTTATTGTCAA-3' (reverse).

CARLo-5 values were normalized to GAPDH. Comparative quantification was determined using the method of $2^{-\Delta\Delta\text{CT}}$.

Statistical Analysis

Data are expressed as means \pm standard errors of at least 3 independent experiments. The significance of differences was analyzed using two-tailed Student's t-test using Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). The Kaplan-Meier method was used to plot overall survival and relapse-free survival curves. The univariate and multivariate analyses were performed using log-rank tests and Cox's proportional hazard model, respectively. Differences were considered statistically significant when p was less than 0.05.

Results

CARLo-5 is Upregulated in Human GC Tissues

We assessed CARLo-5 expression by qRT-PCR in the 240 GC tissues and matched adjacent normal tissues. The relative expression of CARLo-5 in GC tissues was significantly higher than that of their matched adjacent normal tissues ($p < 0.001$, Figure 1).

Correlations Between the Levels of CARLo-5 and the Clinicopathological Factors in GC Patients

According to the set cutoff value of 0.041, this cohort of 240 GC patients was divided into two groups: low CARLo-5 expression and high CARLo-5 expression. The clinicopathological parameters of CARLo-5 were showed in Table I. We found that high CARLo-5 expression was found to be closely correlated with advanced T stage ($p = 0.003$), positive distant metastasis ($p = 0.009$), lymph node involvement ($p = 0.001$), and poor differentiation ($p = 0.001$). However, CARLo-5 expression was not significantly related to gender, age, or tumor size (all $p > 0.05$) (Table I).

Increased Expression of CARLo-5 Predicts a Poor Prognosis

Furthermore, the prognostic value of the CARLo-5 expression in malignant GC patients was evaluated. Kaplan-Meier method and log-rank test were used to evaluate the differences of OS

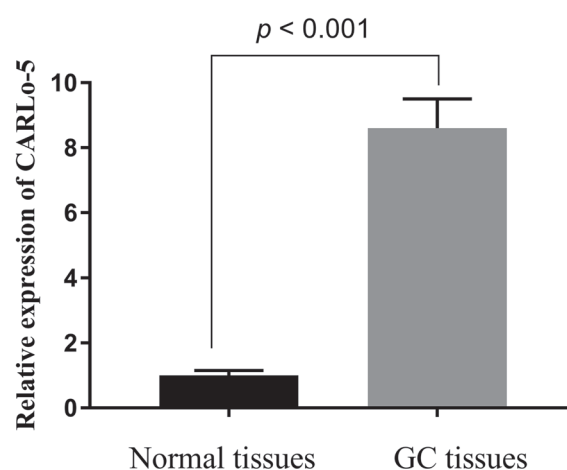


Figure 1. Expression levels of CARLo-5 in GC tissues. The expression levels of CARLo-5 were significantly upregulated in GC tissue samples, as compared with adjacent normal tissue samples ($p < 0.001$).

Table I. CARLo-5 level and clinicopathological parameters in patients with GC.

Characteristics	n	High expression (%)	Low expression (%)	p
Gender				0.445
Male	95	45 (47.4)	50 (52.6)	
Female	145	76 (52.4)	69 (47.6)	
Age				0.191
< 60	109	60 (55)	49 (45)	
≥ 60	131	61 (46.6)	70 (53.4)	
T stage				0.003
T1-T2	118	48 (40.7)	70 (59.3)	
T3-T4	122	73 (60)	49 (40)	
Lymph node metastasis				0.001
No	106	41 (38.7)	65 (61.3)	
Yes	134	80 (59.7)	54 (40.3)	
Distant metastasis				0.009
No	189	87 (46)	102 (54)	
Yes	51	34 (66.7)	17 (33.3)	
Differentiation				0.001
Well/moderate	141	58 (41.1)	83 (58.9)	
Poor	99	63 (63.6)	36 (36.4)	
Tumor size (cm)				0.194
< 5	91	41 (44.1)	50 (55.9)	
≥ 5	149	80 (53.7)	69 (46.3)	

and RFS between the low-expression group and high-expression group. The results indicated that patients with high CARLo-5 expression had shorter mean months of OS and RFS than did patients with low CARLo-5 expression ($p < 0.001$, respectively) (Figures 2, 3).

The univariate analyses showed that T stage ($p = 0.012$), Lymph node metastasis ($p = 0.007$),

distant metastasis ($p = 0.004$), differentiation ($p = 0.009$) and CARLo-5 level ($p = 0.002$) were significantly associated with OS (Table II). Multivariate analysis using Cox's regression model was performed and identified that CARLo-5 was independent prognostic factors (RR=2.41; 95% CI 1.13-5.94; $p = 0.005$, Table II). The similar results were also found in RFS (Table III). Our

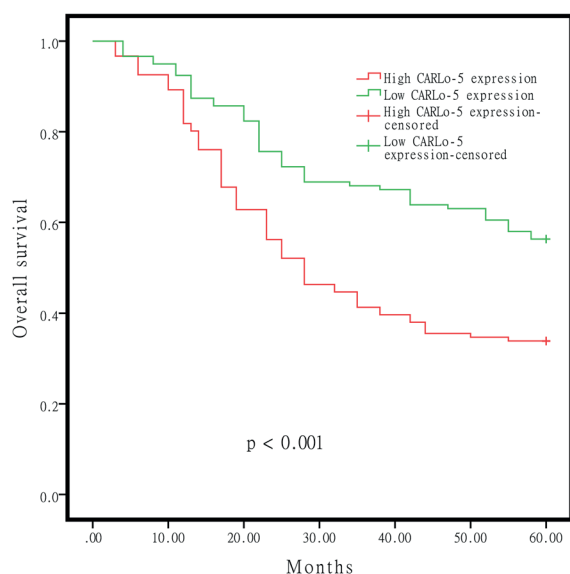


Figure 2. Overall survival rate in patients with high CARLo-5 expression was significantly lower than that in patients with low CARLo-5 expression ($p < 0.001$).

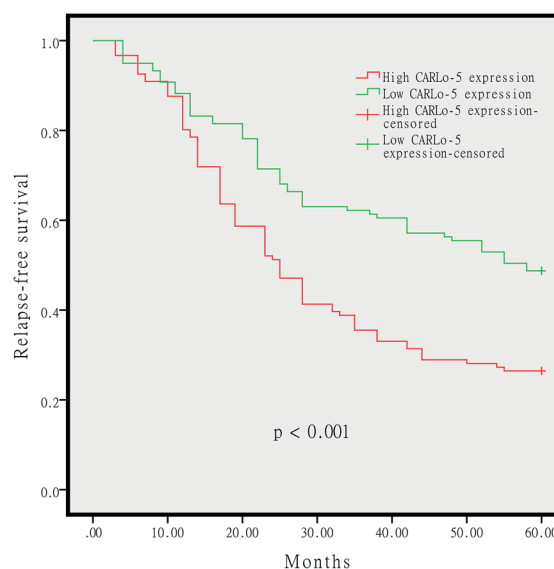


Figure 3. Relapse-free survival rate in patients with high CARLo-5 expression was significantly lower than that in patients with low CARLo-5 expression ($p < 0.001$).

Table II. Univariate and multivariable Cox regression analysis of OS.

Viable	Univariate analysis			Multivariate analysis		
	RR	95 % CI	p-value	RR	95 % CI	p-value
T stage	2.56	0.83-4.14	0.012	2.02	0.63-3.65	0.018
Lymph node metastasis	3.72	1.14-4.73	0.007	3.13	0.88-4.16	0.009
Distant metastasis	2.86	1.24-4.21	0.004	2.21	1.03-3.99	0.007
Differentiation	3.55	1.73-5.56	0.009	3.11	1.31-4.77	0.011
CARLo-5 expression	2.16	0.98-6.65	0.002	2.41	1.13-5.94	0.005

results revealed that CARLo-5 was a poor independent prognostic factor for both OS and RFS in GC patients.

Discussion

To the best of our knowledge, this is the first report of the analysis of the prognostic value of CARLo-5 in GC. We found that high CARLo-5 expression in GC tissues, providing the first evidence that CARLo-5 up-regulation is closely associated with GC. Then, we analyzed the association between CARLo-5 expression and clinicopathologic features in GC. The results showed that high CARLo-5 expression were closely correlated with advanced T stage, positive distant metastasis, lymph node involvement, and poor differentiation. Furthermore, Kaplan-Meier analysis showed that patients with high CARLo-5 expression had worse OS and RFS than patients with low CARLo-5 expression. Moreover, both univariate and multivariate analyses revealed that the CARLo-5 expression was a predictor of poor RFS and OS. These findings implicated CARLo-5 as a potentially important contributing factor in GC progression.

Mounting evidence indicates that lncRNAs are associated with a diverse range of functions in cell biology^{10,11}. The prognostic potential of

lncRNA has been demonstrated for several types of cancer, including gastric cancer^{12,13}. For example, Peng et al¹⁴ found that lncRNA SPRY4-IT1 up-regulation occurred more frequently in GC patients with multiple tumor nodes and advanced tumor stage. Moreover, SPRY4-IT1 may serve as a promising biomarker for predicting the prognosis of GC. Fei et al¹⁵ showed that lncRNA LINC00982 expression was downregulated in tumor tissues in patients with gastric cancer (GC) compared with those in the adjacent normal tissues. Furthermore, they identified lncRNA LINC00982 as a poor prognostic biomarker in GC.

Previous studies revealed that CARLo-5 served as a tumor promoter in several tumors. Luo et al¹⁶ showed that a great up-regulation of CARLo-5 was observed in cancer tissues compared to their adjacent normal tissues, and knockdown of CARLo-5 inhibits proliferation of NSCLC cells *in vitro*. Wang et al¹⁷ observed that CARLo-5 was up-regulated in hepatocellular carcinoma specimens and its high expression was associated with poor prognosis of hepatocellular carcinoma patients. Further function experiments showed that CARLo-5 overexpression significantly enhanced cell proliferation, migration, and invasion *in vitro*. More importantly, Zhang et al¹⁸ reported that CARLo-5 was observed to be up-regulated in GC, and knockdown of CARLo-5 in gastric

Table III. Univariate and multivariable Cox regression analysis of RFS.

Viable	Univariate analysis			Multivariate analysis		
	RR	95 % CI	p-value	RR	95 % CI	p-value
T stage	2.83	1.21-4.87	0.015	2.43	0.89-3.83	0.016
Lymph node metastasis	3.44	1.32-5.16	0.004	3.07	0.57-4.89	0.007
Distant metastasis	2.45	1.17-5.89	0.007	2.04	0.89-4.13	0.012
Differentiation	4.14	1.22-6.68	0.014	2.56	1.51-5.55	0.006
CARLo-5 expression	2.12	0.77-5.54	0.004	2.76	1.42-6.65	0.008

cancer cell lines significantly inhibited the cell proliferation via inducing G0/G1 cell-cycle arrest and apoptosis. Based on those results, we hypothesized that CARLo-5 may be associated with the overall survival of GC patients.

Conclusions

We observed for the first time that the up-regulation of CARLo-5 is a frequent event and predicted poor prognosis in GC patients. However, the precise molecular mechanisms by which CARLo-5 is downregulated in GC need to be further investigation.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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