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Original Article

Clinical significance of long non-coding RNA ZEB2-AS1 in locally advanced colorectal cancer

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Abstract: Objective: The aim of this study was to investigate the clinical significance of differential expression of long non-coding RNA (lncRNA) ZEB2-AS1 in patients with colorectal cancer (CRC). Methods: mRNA expression of lncRNA ZEB2-AS1 was evaluated by real-time quantitative PCR on eighty-seven cancerous tissues and adjacent normal mucosal tissues from patients with CRC tissue. Correlation between the lncRNA ZEB2-AS1 expression and clinicopathological characteristics of the colorectal cancer patients was evaluated, and five-year overall survival (OS) was also analyzed according to the lncRNA ZEB2-AS1 expression of the CRC patients. Moreover, Cox Regression Analysis was performed in screening prognosis factors. Results: A significantly upregulated lncRNA ZEB2-AS1 expression, with a fold change of 18.75, was found in CRC tissue compared to the normal tissue. lncRNA ZEB2-AS1 expression in CRC was correlated with death (P<0.001). The five-year OS was 43.2% and 76.7%, respectively, in patients with higher and lower lncRNA ZEB2-AS1 expression. Cox regression analysis showed that location (P=0.020), N1 staging (P=0.021) and lncRNA ZEB2-AS1 lower expression (P<0.001) were independent prognosis factors associated with a better OS. Conclusion: Expression of lncRNA ZEB2-AS1 was significantly upregulated in stage III CRC patients and affects the prognosis.

Keywords: Colorectal cancer, lncRNA ZEB2-AS1, prognosis

Introduction

Colorectal cancer is the fourth most common cancer and the fifth most common cause of cancer-related deaths in China, with an estimated 331,300 newly diagnosed patients and 159,300 deaths in 2012 [1]. Surgical resection, followed by adjuvant chemotherapy, is the most commonly used strategy for colorectal cancer management. Despite the overall five-year survival rate of colorectal cancer improving to 65%, only a 15% five-year survival rate could be found in patients presented with distant metastasis [2], which reflects poor treatment response in some of the patients with colorectal cancer. Therefore, it is necessary to search for effective biomarkers in patients with colorectal cancer to improve the therapeutic benefits of current agents and to develop individualized therapies.

Long noncoding RNAs (lncRNAs) with a length >200 nucleotides are a recently discovered novel class of genes with regulatory function but lacking in protein-coding ability. Multiple studies have proven the critical role of lncRNA in a wide range of cellular processes, including X chromosome inactivation, splicing, imprinting, epigenetic control and gene transcription regulation [3-5]. Furthermore, studies show that dysregulated expression of lncRNAs also exists in various human diseases, especially in cancers, including breast cancer, lung cancer, gastric cancer and colorectal cancer (CRC) [6-8]. According to the most recent evidence, lncRNAs were verified to be involved in the development and progression of human CRC and may serve as novel therapeutic targets [9-11]. However, the role of lncRNAs in CRC remains largely unknown.

Here, we investigated the clinical significance of differential expression of lncRNA ZEB2-AS1 in CRC patients and performed association analyses between the expression levels and the clinicopathological characteristics, thereby

Table 1. Primers for real-time PCR

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
LncRNA ZEB2-AS1	ATGAAGAAGCCGCGAAGTGT	CACACCCTA ATACACATG CCCT
GAPDH	TCGACAGTCAGCCGCATCTT	GGCGCCCAATACGACCAAAT

Table 2. Relationship between lncRNA ZEB2-AS1 expression and clinical characteristics in patients with colorectal cancer

Characteristics	No.	LncRNA ZEB2-AS1		χ^2	*P*
		Low	High		
Gender					
Male	41	23	18	1.381	0.24
Female	46	20	26		
Age					
≤60	49	27	22	1.446	0.229
>60	38	16	22		
Location					
Colon	42	21	21	0.011	0.918
Rectum	45	22	23		
Histology					
High	5	3	2	1.527	0.466
Moderate	61	32	29		
Low	21	8	13		
T staging					
I	1	1	0	5.746	0.125
II	8	1	7		
III	39	21	18		
IV	39	20	19		
N staging					
N1	56	27	29	0.092	0.761
N2	31	16	15		
Death					
Yes	35	10	25	10.88	0.001
No	52	33	19		

providing information for CRC diagnosis and prognosis.

Materials and methods

Patients

A total of eighty-seven cases of surgically resected CRC specimens with complete clinical data were collected from the Second Affiliated Hospital of Soochow University between January 2009 and June 2012. The inclusion criteria of the patients were: (1) diagnosis of primary CRC, (2) no history of chemotherapy and radiotherapy, and (3) histologically confirmed adenocarcinoma. All of the patients were diagnosed and classified by two experienced pathologists according to The Union for International Cancer Control (UICC). Moreover, eighty-seven cases of non-cancerous mucous tissue were obtained as controls. The study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University. Written informed consent was provided by each patient that was included.

Most of the patients were followed up by the Outpatient Department. The survival period was recorded from the date of surgery to the date of death, or until the last follow up time. The last follow up was done in June 2017. The minimum follow up period was six months and the maximum period was eighty-eight months for surviving patients.

Real-time quantitative PCR for lncRNA ZEB2-AS1

Cancer tissues and normal control tissues were collected and grinded into powder before total tissue RNA extraction with TRIzol Reagent (Invitrogen). RT-PCR was carried out using a One Step SYBR® PrimeScript™ RT-PCR kit (Takara, Dalian, China) and an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was assayed simultaneously with samples as an internal control. Relative gene expression was determined by the 2-ΔΔCT method [14]. Oligonucleotide primers specific for LncRNA ZEB2-AS1 and GAPDH are listed in **Table 1**.

Statistical analysis

Descriptive statistics for the patient group were reported as mean ± standard deviation (SD) or median and range. The category data was presented as number and percentages. The correlation analysis between relative expression of lncRNA ZEB2-AS1 and clinical data of the patients was performed using a Chi-square Test. The Kaplan-Meier method was used to determine five-year overall survivals. Multivariate analysis was performed using the Cox proportional hazards model. Statistical significance was accepted at the $P<0.05$ level. Statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL).

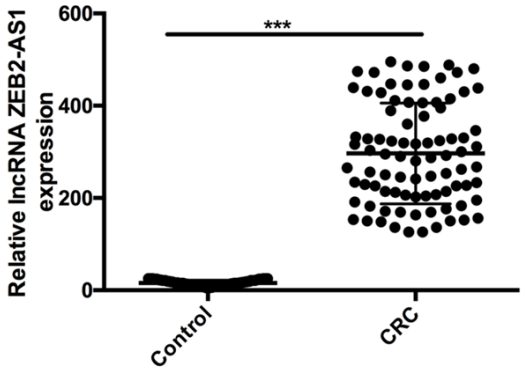

Figure 1. Increased expression of lncRNA ZEB2-AS1 in colorectal cancer tissue. The expression level of lncRNA ZEB2-AS1 was determined by real-time quantitative PCR. A fold of 18.75 was found on colorectal cancer tissues compared to the control tissue.

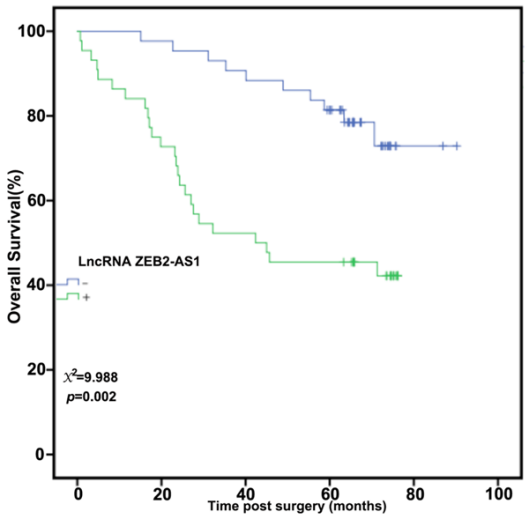

Figure 2. Kaplan-Meier curves for overall survival in patients with colorectal cancer according to the lncRNA ZEB2-AS1 expression.

Results

Patient demographic data

A total of eighty-seven CRC patients were included in the present study. The median age of the CRC patients was 60 (30-82) years. Of these CRC patients, 41 were male and 46 were female. The mean follow up time of these CRC patients was 55.4±22.3 months, ranging from 6-88 months. The demographic data of these patients are listed in **Table 2**.

Increased lncRNA ZEB2-AS1 expression in CRC tissues

To determine the lncRNA ZEB2-AS1 expression in CRC, we performed quantitative PCR analysis with the tissue samples collected from the above-mentioned patients. As shown in **Figure 1**, a significantly increased level of lncRNA ZEB2-AS1, with a fold change of 18.74, was found on CRC tissues compared to the control tissue (P<0.001).

Correlation analysis between lncRNA ZEB2-AS1 expression and clinicopathological characteristics of CRC

We also performed correlation analysis between lncRNA ZEB2-AS1 expression and clinicopathological characteristics of CRC. As shown in **Table 2**, no correlation was found between the lncRNA ZEB2-AS1 expression level and gender (P=0.24), or age (P=0.229), or differentiation (P=0.466), or T stage (P=0.125), or N stage (P=0.761), while significance was found between lncRNA ZEB2-AS1 expression and death (P= 0.001) (**Table 2**).

Survival analysis

The median OS of the patients with high and low lncRNA ZEB2-AS1 expression was, respectively, 23.2±4.8 months and 40.1±10.8 months (**Figure 2**). The accumulated survival ratio in patients with high and low lncRNA ZEB2-AS1 expression was, respectively, 43.2% and 76.7%. Significantly prolonged OS was found in patients with low lncRNA ZEB2-AS1 expression compared to those with high lncRNA ZEB2-AS1 expression. Then, we used the Cox proportional hazards model to assess the clinicopathological characteristics and we found that location (P=0.020), N staging (P=0.021), and differential expression levels of lncRNA ZEB2-AS1 (P< 0.001) were the independent risk factors of five-year OS (**Table 3**).

Discussion

Searching for effective biomarkers in patients with colorectal cancer could be of great importance in improving the therapeutic benefits of current agents and in the development of individualized therapies [12].

In this present study, we performed real-time quantitative PCR to evaluate the expression level of lncRNA ZEB2-AS1 on CRC tissues to explore its clinical significance in CRC tissues. Our results demonstrated that significantly upregulated lncRNA ZEB2-AS1 expression, with a fold change of 18.75, was found in CRC tissue compared to normal tissue. Moreover, the cor-

Table 3. Cox regression analysis of the prognosis in the patients with stage III colorectal cancer

Characteristics	RR	95.0% CI		*P* value
		Upper	Lower	
Gender	1.423	0.666	0.363	3.044
Age	0.851	0.375	0.701	1.932
Location	0.390	0.176	0.020	0.865
T staging	1.117	0.659	0.682	1.894
N staging	2.347	1.135	0.021	4.850
Differentiation	1.649	0.864	0.129	3.145
LncRNA ZEB2-AS1 level	4.209	1.948	0.000	9.093

RR: risk ratio; CI: confidence interval.

relation analysis revealed that lncRNA ZEB2-AS1 expression in CRC was correlated with death ($P<0.001$), and the five-year OS was, respectively, 43.2% and 76.7% in patients with higher and lower lncRNA ZEB2-AS1 expression. In addition, Cox regression analysis showed that location ($P=0.020$), N1 staging ($P=0.021$) and lncRNA ZEB2-AS1 lower expression ($P<0.001$) were independent prognosis factors associated with a better OS. To the best of our knowledge, this is the first study concerning the role of lncRNA ZEB2-AS1 in CRC.

As a class of newly discovered genes, lncRNAs, with gene regulatory function but without protein coding ability, are suggested to play a critical role in physiological function regulation. According to previous studies, approximately 18% of lncRNAs are associated with human tumors and have been shown to act as major contributors in the development and progression of human cancers [13]. Multiple mechanisms have been suggested about the regulatory role of lncRNAs in physiological functions, including trans-regulatory and cis-regulatory mechanisms [14-17], and the representative lncRNAs including PTENP1 [18], H19 [19], and CCAT1 [20].

ZEB, as a transcriptional factor, has been identified to play an important role in the process of EMT, which is closely associated with carcinogenesis. Beltran et al. first discovered that a non-coding antisense transcript located from the promoters of ZEB2 (ZEB2-AS1) had the ability to activate ZEB2 expression [21]. Recently, Li et al. reported that the ZEB1-AS1 gene was involved in the tumorigenesis of hepatocellular carcinoma (HCC) and functioned as a non-coding oncogene [22]. They found upregulated lncRNA ZEB1-AS1 expression level in HCC tissues compared with the adjacent normal tissues. Moreover, Li et al. also described the effects of lncRNA ZEB1-AS1 on cell proliferation, migration and invasion, and cell cycle regulation. In addition, ZEB1-AS1 may promote tumor growth and metastasis in HCC patients through regulating expression levels of ZEB1. However, Lan et al. recently found that down-regulation of lncRNA ZEB2-AS1 was associated with reduced tumor growth and metastasis in HCC tissues [23]. However, no study has focused on the role of lncRNA ZEB2-AS1 on CRCs. Here, we report an upregulated pattern of lncRNA ZEB2-AS1 on CRC tissue which was consistent with its pattern in HCC. Moreover, we also found that lncRNA ZEB2-AS1 was correlated with overall survival, which may serve as prognosis marker.

There are also some limitations in our study. First, this is a single-center small study and insufficient sample size could affect the final conclusion. Second, the diagnosis value of lncRNA ZEB2-AS1 was also implicated by the results obtained here, however, the accurate cut-off value of lncRNA ZEB2-AS1 expression quantification should be calculated by an ROC curve in future studies to maximize the diagnostic value of lncRNA ZEB2-AS1. Third, according to previous studies, elevated lncRNA ZEB2-AS1 was associated with early death and high incidence of death. Due to the lack of detailed data of the cause of death, we could not analyze the associations of these features with lncRNA ZEB2-AS1 level. Fourth, the detailed mechanisms that resulted in the upregulation of lncRNA ZEB2-AS should be elucidated for the exact role of lncRNA ZEB2-AS1 in CRC. Taken together, further investigation is of great importance to identify the biomarker role of lncRNA ZEB2-AS1 by a multi-center clinical large sample size study with prognosis results.

In conclusion, our findings suggest that expression of lncRNA ZEB2-AS1 is significantly upregulated in the stage III CRC patients, and thus may be employed as a biomarker for cancer prognosis and malignant diagnosis.

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Disclosure of conflict of interest

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