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Long non-coding RNA DILC as a potentially useful biomarker for the diagnosis and prognosis of colorectal cancer

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Abstract. – OBJECTIVE: LncRNA downregulated in liver cancer stem cells (lnc-DILC) has been implicated as a tumor suppressor in colorectal cancer (CRC). However, the clinical significance of lnc-DILC in CRC patients has not been investigated. In this study, we aimed to explore the diagnostic and prognostic value of lnc-DILC in CRC patients.

PATIENTS AND METHODS: The expression of lnc-DILC was measured in 174 paired CRC tissues and adjacent normal tissues using Real Time-Polymerase Chain Reaction (RT-PCR). The correlation of lnc-DILC expression with clinicopathological factors was statistically analyzed by the Chi-square test. Besides, overall survival analysis was carried out with the Kaplan-Meier curve with the log-rank test. Univariate and multivariate analyses were performed to explore the prognostic significance of lnc-DILC expression.

RESULTS: We found that lnc-DILC expression was downregulated in CRC tissues compared to their adjacent normal tissues ($p<0.01$). ROC analyses showed that lnc-DILC levels were reliable in distinguishing patients with CRC from normal colorectal tissues. Then, down-regulation of lnc-DILC was positively associated with aggressive clinical characteristics, including depth of invasion ($p=0.018$) and advanced TNM stage ($p=0.009$). Moreover, the Kaplan-Meier analysis demonstrated that overexpression of lnc-DILC was associated with poorer overall survival ($p=0.0205$) and disease-free survival ($p<0.001$). Finally, multivariate analyses confirmed that expression of lnc-DILC was an independent prognostic factor in CRC.

CONCLUSIONS: Our results firstly suggested that lnc-DILC could be a favorable indicator of prognosis in CRC.

Key Words
Lnc-DILC, Colorectal cancer, Prognosis.

Introduction

Colorectal cancer (CRC) is the second most common malignancy in men and a leading cause of high cancer-related deaths, with more than 600,000 deaths worldwide annually[1,2]. Most CRCs develop via a multistep process involving a series of histological genetic changes[3]. With the development of surgery, chemotherapy, radiation therapy as well as targeted therapy, CRC patients at stage I-II have a favorable prognosis, and five-year survival rate is 60-80%[4,5]. However, approximately 50-60% of patients diagnosed with CRC will develop distant metastases and, after metastasis has occurred, almost all the therapeutic tools were limited, and the therapy will become more complex[6,7]. Therefore, the identification of novel diagnostic and prognostic biomarkers is of significant importance for improving survival.

Long noncoding RNAs (lncRNAs), belonging to the noncoding RNA family with more than 200 nucleotides in length, are a type of by-products of genetic transcription without protein-coding potential[8]. Some scientific evidence[9,10] indicates that some functional lncRNAs can act through various mechanisms, thereby influencing many biological processes, such as RNA transcription, mRNA degradation, and epigenetic regulation. Furthermore, increasing studies[11,12] have indicated that several functional lncRNAs are abnormally expressed in various tumors and are involved in tumor initiation and development. For instance, lncRNA UCC, an up-regulated lncRNA, was reported to promote CRC cells proliferation and metastasis by sponging miR-143 and was associated with advanced clinical progression[13]. By contrast, lncRNA Linc00675, a down-regulated lncRNA,

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was found to suppress proliferation and invasion of CRC cells via modulating Wnt/β-catenin signaling[14]. These findings highlighted the potential of those lncRNAs as diagnostic and prognostic biomarkers for CRC patients.

LncRNA downregulated in liver cancer stem cells (lnc-DILC), a recently identified lncRNA, has been firstly reported to be down-regulated in liver cancer stem cells[15]. The studies of lnc-DILC in tumors are limited. Recent findings by Gu et al[16] reported that lnc-DILC expression was reduced in CRC tissues and cell lines, and overexpression of lnc-DILC suppressed CRC progression *in vitro*. However, for one thing, the expression pattern of lnc-DILC needed to be further confirmed; for another, the clinical significance of lnc-DILC in CRC patients remained unknown. In this study, for the first time, we provided important data indicating that lnc-DILC might be a potential biomarker for predicting the prognosis of CRC patients.

Patients and Methods

Patients and Clinical Specimens

174 CRC tissues and matched adjacent tissues were obtained by the Dongying People's Hospital. Patients' (107 males and 67 females) ages ranged from 38 to 72 years (median age 52.6 years). The diagnosis of CRC was confirmed by the clinical examination and the histopathological analysis of the samples. None of these patients received radiotherapy or chemotherapy before the surgery. All collected tissues were immediately frozen in liquid nitrogen and stored at -80°C. Follow-up data were obtained by reviewing out-patient charts or by contacting or corresponding with patients. The clinical characteristics of all these patients are shown in Table I. The use of CRC tissues for all assays was approved by all the patients and by the Ethics Committee of the Dongying People's Hospital.

RNA Extraction and Quantitative Real Time-PCR

Total RNA was isolated from tissue samples and cell lines followed by column purification using miRNeasy Mini-kit (Qiagen, Xuhui, Shanghai, China). The complementary DNA (cDNA) preparation was performed using the Reverse Transcriptase (Transgene, Xuhui, Shanghai, China). Quantitative real time-PCR was performed with the 7300-sequence detection system (Biosystems, Foster City, CA, USA) using

Table I. Correlation between lnc-DILC expression and clinicopathological features of CRC.

Characteristics	No. of cases	lnc-DILC expression		*p*-value
		High	Low	
Age (years)				0.804
≤ 60	87	41	46	
> 60	87	47	40	
Gender				0.332
Male	107	51	56	
Female	67	37	30	
Tumor size				0.175
≤ 5 cm	100	55	45	
> 5 cm	74	33	41	
Histology/differentiation				0.210
Well + Moderate	95	53	42	
Poor	79	35	44	
Depth of invasion				0.018
T1 + T2	116	66	50	
T3 + T4	58	22	36	
TNM stage				0.009
I-II	110	64	46	
III-IV	64	24	40	
Tumor site				0.863
Colon	98	49	49	
Rectum	76	39	37	

Table II. The primer sequences included in this study.

Gene name	Sequences (5'-3')
Lnc-DILC: forward	CTCTGGAGCCATACGTGACA
Lnc-DILC: reverse	TCAGGTCACTTGTGCCGTT
β-actin: forward	ACTGCTCTGGCTCCTAGCAC
β-actin: reverse	CAGCTCAGTAACAGTCCGCC

SYBR Green Master Mix (Biosystems, Foster City, CA, USA). The amplification conditions were 35 cycles of 12 s at 95°C and 1 min at 60°C. β-actin was chosen as the endogenous control in the assay. The comparative cycle threshold (CT) method was used to evaluate the relative quantification of lnc-DILC. The primer sequences are listed in Table II.

Statistical Analysis

All data were carried out using the SPSS 17.0 software package (version 17.0, SPSS Inc, Chicago, IL, USA). The statistical significance between groups was determined using a two-tailed Student's *t*-test. The Chi-Square test was applied for the examination of the relationship between lnc-DILC expression levels and clinicopathological characteristics. The receiver operating characteristic (ROC) curve analysis was performed to determine the diagnostic significance of lnc-DILC expression in CRC patients. The prognostic value of lnc-DILC was estimated through Kaplan-Meier and Multivariate analysis. A two-tailed *p*-value less than 0.05 was considered to have statistical significance.

Results

Lnc-DILC Expression is Decreased in CRC Tissues

Firstly, to explore the potential roles of lnc-DILC in CRC progression, we detected the expression levels of lnc-DILC in CRC patients. As shown in Figure 1A, the results showed that lnc-DILC expression was significantly down-regulated in CRC tissues compared to matched normal tissues ($p<0.01$). We also observed that lnc-DILC expression was significantly lower in CRC patients with advanced stages ($p<0.01$, Figure 1B). Thus, our data further proved that lnc-DILC was lowly expressed in CRC and may play an important role.

Diagnostic Accuracy of lnc-DILC for CRC

Because of frequent dysregulation of lnc-DILC expression in CRC tissues, we further explored the possibility of lnc-DILC as a diagnostic biomarker for CRC. As shown in Figure 2, the results of ROC analyses showed that lnc-DILC level could distinguish CRC tissues from normal tissues, with an AUC of 0.8264. Apart from that, the sensitivity and specificity were 0.78 (95% CI 0.723, 0.895) and 0.71 (95% CI 0.51, 0.84). Thus, detection of lnc-DILC levels may be a potential method for the diagnosis of CRC patients.

Down-Regulation of lnc-DILC Was Associated with Clinical Characteristics

In order to explore the clinical significance of lnc-DILC in CRC patients, 174 CRC patients were classified into two groups according to median

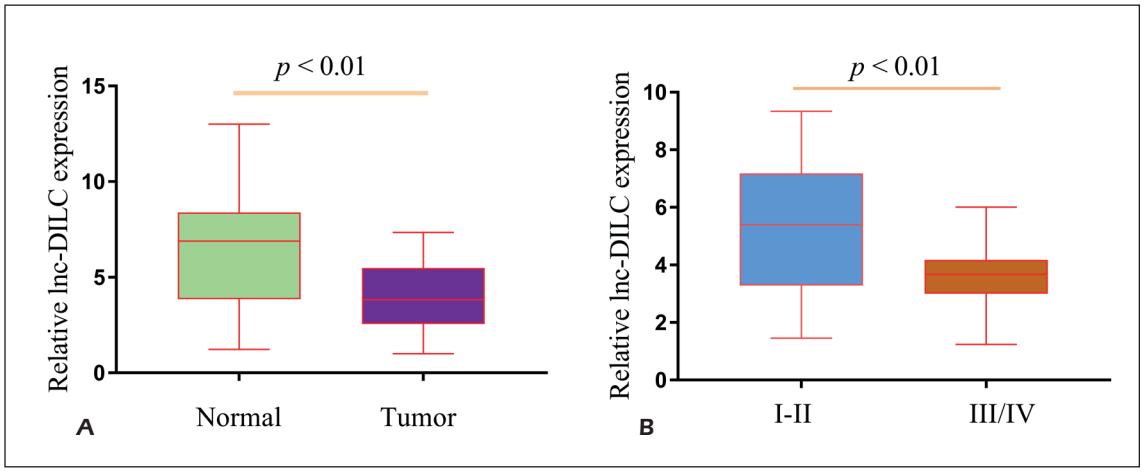

Figure 1. lnc-DILC expression is downregulated in CRC samples. **A**, The expression of lnc-DILC was measured in 174 paired CRC and adjacent non-tumor tissues by qRT-PCR. **B**, Lnc-DILC expression was significantly lower in CRC patients with advanced stages.

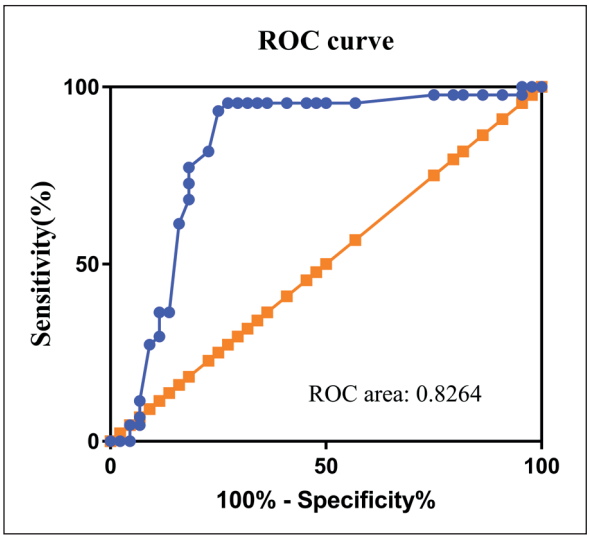

Figure 2. A receiver operating characteristic curve analysis was used to determine the diagnostic performance of lnc-DILC for CRC.

lnc-DILC levels in CRC tissues: high lnc-DILC expression group (n = 86) and low lnc-DILC expression group (n = 88). Then, a Chi-square test was performed, and the results revealed that low lnc-DILC expression was significantly associated with depth of invasion (p = 0.018) and advanced TNM stage (p = 0.009) (Table I). However, no significant difference was observed between lnc-DILC expression and patients' age, gender, tumor size, histology/differentiation, and tumor site (p>0.05).

Prognostic Values of lnc-DILC Expression in CRC Patients

Then, we explored the prognostic value of lnc-DILC in CRC patients; the survival analysis through the Kaplan-Meier method revealed that low lnc-DILC expression was correlated with a shorter overall survival (OS) (p = 0.0205, Figure 3A) and a disease-free survival (DFS) (p<0.001, Figure 3B), suggesting that the low expression of lnc-DILC had a poor prognosis in CRC patients. Subsequently, multivariate analysis of prognostic parameters for OS and DFS was performed. As shown in Table III, we found that lnc-DILC expression was an independent poor prognostic factor for both OS (HR = 3.246, 95% CI: 1.328-4.554, p = 0.007) and DFS (HR = 3.562, 95% CI: 1.499-4.982, p = 0.004) in CRC patients.

Discussion

CRC is an aggressive cancer associated with low survival rate. In recent years, the incidence of CRC has become the fifth most common among cancer-related deaths in China[17]. Growing advancements have influenced patients care, such as the development of cancers biomarkers that will undoubtedly have an important role in the management of patients with CRC[18,19]. Up to date, KRAS sequencing and determination of microsatellite instability has been used to influence treatment plans[20]. Several studies[21,22] indicated that lncRNAs could be sensitive and specific biomarkers of tumors, due to its frequent dys-

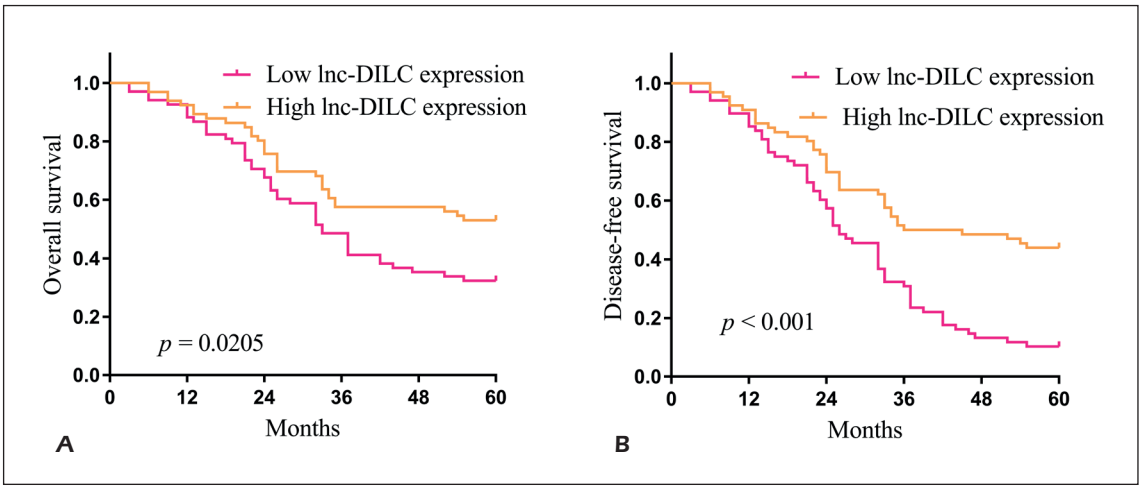

Figure 3. Kaplan-Meier curves of the overall survival and disease-free survival of 174 CRC patients. **A**, Overall survival rate in patients with low lnc-DILC expression was significantly lower than that in patients with high lnc-DILC expression (p=0.0205). **B**, Disease-free survival rate in patients with low lnc-DILC expression was significantly lower than that in patients with high lnc-DILC expression (p<0.001).

Table III. Multivariate analyses for disease-free survival and overall survival by Cox regression model.

Variable	Overall survival			Disease-free survival		
	HR	95% CI	p	HR	95% CI	p
Age	1.422	0.561-2.113	0.355	–	–	–
Gender	1.855	0.842-2.349	0.127	–	–	–
Tumor size	1.342	0.895-1.894	0.139	–	–	–
Histology/differentiation	1.642	1.123-2.342	0.131	–	–	–
Tumor site	1.543	0.643-2.166	0.423	–	–	–
Depth of invasion	3.579	1.324-4.775	0.009	3.774	1.576-5.469	0.003
TNM stage	3.985	1.492-4.884	0.005	4.213	1.583-5.377	0.001
lnc-DILC expression	3.246	1.328-4.554	0.006	3.562	1.499-4.982	0.004

regulation and critical effects in the progression of tumors. In addition, with the development of high throughput sequencing, it became possible to detect the expression levels of a large number of lncRNAs synchronously, highlighting the important clinical value of lncRNAs used as novel diagnostic and prognostic biomarkers[23,24].

Recently, the expression pattern and functional effects of lncRNAs in CRC have been reported in several studies[25]. For instance, Han et al[26] reported that lncRNA H19, a well-studied lncRNA, was highly expressed in CRC and its overexpression only predicted shorter survival of CRC patients, but also promoted the tumor growth by binding to eIF4A3. Iguchi et al[27] showed that lncRNA-ATB, a highly expressed lncRNA in CRC, was associated with poor prognosis of CRC patients. Zhang et al[28] indicated that lncRNA HNF1A-AS1 expression was significantly up-regulated in CRC and associated with advanced clinical stages and poor overall survival of CRC patients. Functional assays also confirmed its tumor-promotive roles in migration and invasion of CRC cells by modulating the Wnt/β-catenin signaling pathway. Recently, a newly identified lncRNA lnc-DILC attracted our attention because of its overexpression in liver cancer stem cells and CRC[15,16]. Gu et al[16] firstly reported that the knockdown of lnc-DILC can suppress cell proliferation and metastasis in CRC, suggesting its tumor-promotive roles. However, whether dysregulation of lnc-DILC could influence the prognosis of CRC patients has not been investigated.

In this study, consistent with previous findings, our results of RT-PCR also showed that lnc-DILC expression was significantly up-regulated in CRC tissues compared to matched normal colorectal tissues. In addition, ROC assays revealed that lnc-DILC could be served as a promising marker for early detection of CRC. Then, for the first time, we explored the clinical significance of lnc-DILC in CRC patients, finding that a low lnc-DILC expression was significantly associated with depth of invasion and advanced TNM stage, suggesting that lnc-DILC may play a negative regulatory role in clinical progression of CRC patients. Importantly, survival assays indicated that patients with low lnc-DILC expression levels had a significantly poorer prognosis. Finally, through multivariate analysis we found that lnc-DILC expression was an independent prognostic indicator for OS. However, the expression level of lnc-DILC in this study is arbitrary. The cutoff level of lnc-DILC in CRC samples should be established. On the other hand, the study populations are relatively small. Further studies are needed to confirm our findings on lnc-DILC as a novel diagnostic and prognostic biomarker for CRC patients.

Conclusions

Our findings suggested that lnc-DILC may be a novel biomarker for the detection of CRC and its down-regulation may be associated with poor prognosis of CRC patients, indicating that lnc-DILC was potential biomarkers for CRC detection and prognosis.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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