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Association of the upregulation of LncRNA00673 with poor prognosis for colorectal cancer

L.-M. FENG[1], D.-W. ZHAO[1], S.-J. LI[2], J. HUANG[3]

[1]Department of Gastrointestinal Surgery, Yuhuangding Hospital, Yantai, Shandong, China
[2]Department of General Surgery, Yuhuangding Hospital, Yantai, Shandong, China
[3]Department of General Surgery, Weifang People's Hospital, Weifang, Shandong , China

Limin Feng and Dawei Zhao contributed equally to this work

Abstract. – OBJECTIVE: **To investigate the effect and clinical significance of long non-coding RNA 00673 (lncRNA00673) in the occurrence and development of colorectal cancer (CRC) through the research on the expression level, biological effect and clinical significance of lncRNA00673 in CRC.**

PATIENTS AND METHODS: **The relative expression of lncRNA00673 in 71 pairs of CRC tissues and cells was detected by quantitative Real-time polymerase chain reaction (qRT-PCR). The correlation of the relative expression of lncRNA00673 with the clinicopathological features of CRC patients was analyzed. The lncRNA00673 interference sequence was designed and synthesized, and its transfection efficiency was detected by qRT-PCR assays. 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay and clone formation experiments were performed to investigate the effect of lncRNA00673 on the proliferation ability of CRC cells.**

RESULTS: **In CRC tissues of 71 patients, there were 51 patients whose lncRNA00673 level was up-regulated compared with that of cancer-adjacent tissues. The highly expressed lncRNA00673 was positively correlated with tumor, node and metastasis (TNM) staging, regional lymph node metastasis, distant metastasis and tumor size in CRC patients. Cox proportional-hazards regression model showed that the highly expressed lncRNA00673 was an independent risk factor for the overall survival of CRC patients. Kaplan-Meier curve analysis showed that highly expressed lncRNA00673 was significantly associated with the relatively lower overall survival (OS). MTT and clone formation experiments showed that knockdown of lncRNA0673 could inhibit the proliferation of CRC cells.**

CONCLUSIONS: **The expression level of lncRNA00673 is up-regulated in CRC tissues and cells, which is related to the degree of malignancy and poor prognosis. LncRNA00673 can be used as a potential molecular marker for the prognosis of CRC.**

Key Words:
lncRNA00673, Colorectal cancer, Prognosis, Biomarkers, Proliferation.

Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal tumors. With the improvement of human living standards and changes in dietary structure, CRC incidence rate is increasing year by year, and there are 1.23 million new cases every year around the world. In developed countries, the overall mortality rate of CRC is 33%[1]. The five-year survival rate of primary CRC is about 70%, while it drops to below 50% after the local spread and distant metastasis. Even though there is no metastasis detected in the cancer peripheral lymph nodes, the recurrence rate is 25%, indicating that the traditional imaging diagnosis [computed tomography (CT), positron emission tomography (PET) and magnetic resonance imaging (MRI)] and pathological examinations are not able to detect the hidden metastatic lesions[2]. The vast majority of patients died for CRC are caused by distant metastases, but the existing tumor markers and other detection means are not able to detect the early spread CRC cells. Recent studies[3-5] have shown that long non-coding ribonucleic acid 00673 (lncRNA00673) can be used as a new molecular marker to improve the diagnostic accuracy of CRC and determine the prognosis of patients. Encyclopedia

Corresponding Author: Jie Huang, MD; e-mail: wfhuangjie@sina.com

of DNA elements (ENCODE) project revealed that less than 2% gene transcripts in the human genome can encode proteins, and the rest were non-coding RNA (ncRNA)[6]. LncRNA belongs to ncRNA with the length of more than 200 nt. According to the location where lncRNA and adjacent protein encode genes, it can be divided into intergenic lncRNA, intronic lncRNA, bidirectional lncRNA, antisense lncRNA and sense lncRNA[7]. Recent researches[8,9] have shown that the abnormal expression of lncRNA is associated with a series of biological processes that can play a role in transcriptional levels, post-transcriptional levels and epigenetic levels. It has been found that the susceptibility of some tumors is associated with lncRNA on tumor-associated gene loci at present[10-12]. Changes in the expression of lncRNA can affect the occurrence and progression of the tumor. Yue et al[13] reported that lncRNA00152 promoted the chemotherapeutic resistance of oxaliplatin to colon cancer cells by regulating AKT signaling pathway, and lncRNA homeobox transcript antisense RNA (HOTAIR) targetedly regulate cell autophagy pathway molecular marker autophagy-related protein 12 (ATG12) in chondrosarcoma so as to promote tumor cell proliferation[14]. LncRNA00673 is located in chromosome 17q25.1 region with the whole length of 2275 bp. It has been reported that lncRNA00673 acts as an "oncogene" in a variety of tumors. Huang et al[15] found that the transcription factor specificity protein 1 (SP1) can promote lncRNA00673 transcription, and the highly expressed lncRNA00673 binds Lysine-specific histone demethylase 1 (LSD1) to regulate downstream target genes, thus promoting gastric cancer cell proliferation. In non-small cell lung cancer (NSCLC) tissues and cells, lncRNA00673 regulates neurocalcin delta (NCALD) and homeobox A5 (HOXA5) genes to promote proliferation and metastasis of NSCLC[16,17]. However, the relative expression, clinical significance and biological effects of lncRNA00673 in CRC have not been reported. The research group found for the first time that lncRNA00673 was upregulated in CRC tissues and cells through experiments, so the interference with its expression could inhibit tumor cell proliferation. The expression level of lncRNA00673 can be used as a potential molecular marker for the determination of clinical diagnosis and the formulation of treatment schemes.

Patients and Methods

Tissue Specimen and Cell Culture

All clinical specimens in this investigation were collected from Yuhuangding Hospital. All the patients or their clients signed the informed consent for the applied specimens. All studies involving human specimens were approved in advance by the Ethics Committee of Yuhuangding Hospital. CRC and cancer-adjacent normal tissue specimens of 71 patients who received surgical treatment in Yuhuangding Hospital from July 2010 to December 2013 were collected. Each specimen was divided into 2 pieces, one for pathologic examination and the other for RNA extraction, which was immediately placed in liquid nitrogen. Each postoperative pathological staging was referred to the 7th edition of the tumor, node and metastasis (TNM) staging standards for CRC formulated by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC), each of which was determined by two pathologists. The survival period of each patient was calculated from the surgery date to December 2016 or the death date, and the follow-up was performed every 3 months. Patients with preoperative chemotherapy or radiotherapy, lack of clinical data or loss to follow-ups were excluded. Human CRC cell lines HT-29, LOVO, SW480, HCT-116, RKO, HCT-8, HCT-15, DLD1 and colonic normal cell line CCD841 were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) or Roswell Park Memorial Institute-1640 (RPMI-1640) (Gibco, Rockville, MD, USA) medium containing 10% fetal bovine serum (10% FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and the cell suspension was placed in a constant temperature incubator with 5% CO_2 at 37°C. The medium was replaced every 2 days, and when the cell fusion reached 80-90%, cells began passage; those in good conditions were selected for the experiment.

Detection of the Expression Level of lncRNA00673 by Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

The total RNA in CRC and the corresponding cancer-adjacent tissues was extracted by using TRIzol kits. The concentration of RNA was measured by an ultraviolet spectrophotometer. Complementary DNA (cDNA) was synthesized

according to the described procedures of the PrimeScript™ RT Master Mix (Perfect Real-time) kits. qRT-PCR system (20 µL): 10 µL SYBR qPCR Mix, 0.8 µL (10 µmol/L) upstream and 0.8 µL (10 µmol/L) downstream primers, 2 µL cDNA products and 0.4 µL 50×ROX reference dyes. Reaction conditions: after 1 min pre-change at 95°C, the reaction was lasted for 30 s at 95°C and 40 s at 60°C, and the whole process was repeated for 40 cycles. Three parallel wells were designed, and all samples were tested for three times. The relative expression of the target gene was expressed by -ΔCt and $2^{-\Delta\Delta Ct}$, respectively, using the relative quantification method.

Synthesis of lncRNAA00673 Small Interfering RNA (siRNA) and qRT-PCR Primers

The effective interference sequences of LncRNA00673: si-1# GAGAAAUAGUCUGUGUUG CCCUGAA, si-2# CAGCCGGAUACAGAGUGAAUAGUUA and si-3# UGUGCCUUUGUA CUCAGCAAUUCUU. The upstream and downstream primer sequences of qRT-PCR: lncRNA 00673 F-TACCACA CCCTTTCTTGCCC, R-GGGAGCCAAAAGGGTCA; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F-GGGAGCCAAAAGGGTCAT and R-GAGTCCTTCCA CGATACCAA. The above sequences were designed and synthesized by Invitrogen Co., Ltd. (Carlsbad, CA, USA).

Detection of Cell Proliferation Capacity by MTT Assay and Clone Formation

The siRNA was transiently transfected into CRC cells. 6 h later, the cell lines were seeded in 96-well plates at a cell density of 3×10^3/well, and 20 µL MTT solution was added to each well at 0 h, 24 h, 48 h, 72 h and 96 h (1.55 g/L), respectively. The cell lines were placed in the incubator at 37°C for 4 h. 150 µL dimethyl sulfoxide (DMSO) were added in each hole, the absorbance value was read, and the cell growth curve was drawn. The siRNA was transiently transfected into CRC cells. 48 h later, the cells were collected and counted. The cells were seeded in a six-well plate at 800 cells/well. The cell saps were replaced every three days. 12 days later, the medium was removed and fixed by formaldehyde and stained by crystal violet. The number of formed cell colonies was counted and pictures were taken.

Statistical Analysis

Statistical Product and Service Solutions 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (La Jolla, CA, USA) were used for analysis. Kaplan-Meier analysis was used to draw the survival curve, and the log-rank test was used to compare the difference in the total survival time. The linear trend of overall survival (OS) was analyzed. A Cox proportional-hazards regression model (Cox model) was used, and single-factor and multivariate analysis were conducted. Besides, the bilateral test was conducted. $p<0.05$ was statistically significant.

Results

Detection of the Expression of lncRNA00673 in CRC and its Relationship with Clinicopathologic Features

In order to detect the expression level of lncRNA00673 in CRC and cancer-adjacent tissues, we detected the relative expression of lncRNA00673 in 71 pairs of CRC and cancer-adjacent tissues by qRT-PCR. The results showed that the expression level of lncRNA00673 in 71.8% (51 of 71; fold ≥ 1.0) of CRC tissues was increased compared with that in cancer-adjacent tissues (Figure 1A). Then, we took the median of the fold change in the expression level of lncRNA00673 as a cut-off to divide CRC patients into two groups: lncRNA00673 high-expression group (n=36, fold change > 4.5) and lncRNA00673 low-expression group (n=35, fold change <4.5) (Figure 1B). The results of statistical analysis showed that the expression level of lncRNA00673 was positively correlated with TNM staging, regional lymph node metastasis, distant metastasis and tumor size, but not with age, sex, tumor origin and differentiation degree (Table I).

Correlation Between the Expression of lncRNA00673 and the Survival Rate of Patients

We examined the correlation between the expression level of lncRNA00673 and the prognosis of CRC patients by Kaplan-Meier survival analysis. The results showed that the 5-year overall survival rate in the lncRNA00673 high-expression group (13%) was significantly lower than that in the lncRNA00673 low-expression group (35.3%). The median survival time of lncRNA00673 high-expression group was 49 months while that of lncRNA00673 low-expression group was 30 months (Figure 1C).

Table I. Correlation between 00673 expression and clinicopathological characteristic of CRC patients (n = 71). *Overall $p < 0.05$.

	00673 Low no. Case (%)	00673 High no. Case (%)	p X^2-test p-value
Age (years)			
> 60	19	18	0.814
≤ 60	16	18	
Gender			
Male	20	23	0.631
Female	15	13	
Tumor location			
Rectum	24	25	1
Colon	11	11	
TNM Stage			
0+I+IIa+IIb	17	8	0.029*
IIc + IIIa+IIIb	10	14	
IIIc+IVa+IVb	8	14	
Tumor size			
≤ 5 cm	23	10	0.002*
> 5 cm	12	26	
Regional lymph node metastasis			
Negative	25	15	0.017*
Positive	10	21	
Distant metastasis			
No	30	22	0.031*
Yes	5	14	
Tumor differentiation			
Poor	19	24	0.337
Well	16	12	

Table II. Univariate and multivariate analysis of over-survival in CRC patients (n=71).

	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	p-value	HR	95% CI	p-value
Age	0.896	0.444-1.809	0.759			
Gender	1.215	0.608-2.429	0.581			
Tumor location	0.885	0.433-1.809	0.739			
Tumor differentiation	0.914	0.458-1.822	0.914			
Regional lymph node metastasis	1.456	0.742-2.855	0.275			
Tumor size	1.958	0.962-3.986	0.064			
Distant metastasis	3.030	1.504-6.105	0.002*	2.748	1..304-5.794	0.008*
TNM stage	1.720	1.142-2.589	0.009*	1.741	1.136-2.610	0.011*
LncRNA 00673 expression	3.023	1.471-6.213	0.003*	3.194	1.365-7.471	0.007*

HR, hazard ratio; 95% CI, 95% confidence interval, *Overall $p<0.05$.

Analysis of risk Factors in the whole Survival Period of CRC Patients by a Cox Proportional-hazards Regression Model

The single-factor analysis was performed on the survival data of 71 patients by a Cox proportional-hazards regression model. Results of the overall survival rate analysis showed that there was notable significance in highly expressed lncRNA00673 (p=0.003), TNM staging (p=0.009), and distant metastasis (p=0.002). Then, the multivariate analysis was further performed on the survival data of 71 patients by a Cox proportional-hazards regression model. The results showed that there was notable significance in highly-expressed lncRNA00673 (p=0.007), distant metastasis (p=0.008) and TNM staging (p=0.011). There was no notable significance in age, sex, tumor size, differentiation and other factors in single-factor or multivariate Cox proportional-hazards regression model (Table

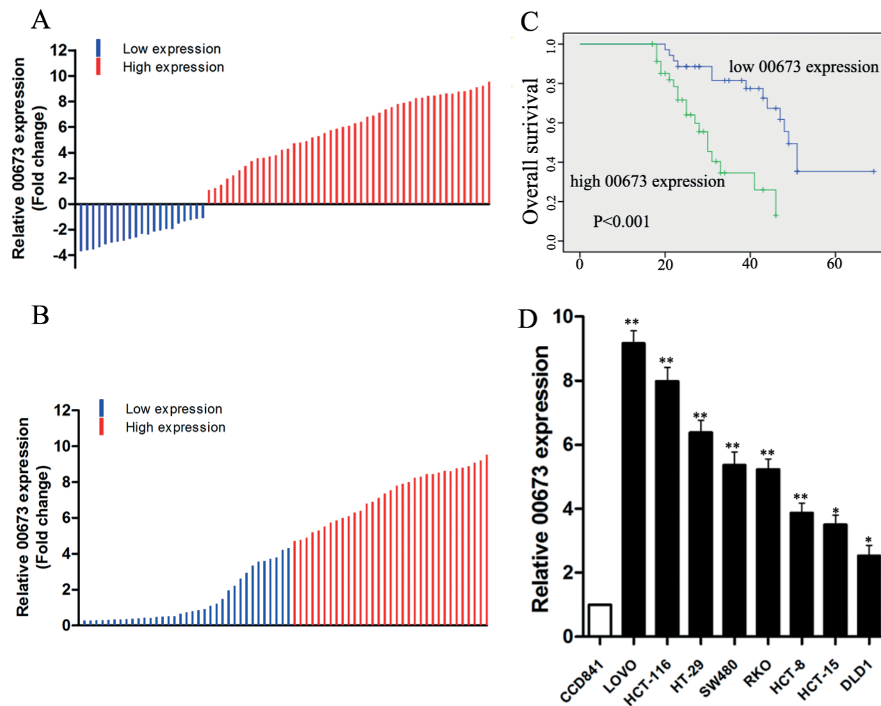

Figure 1. Relative expression and clinical significance of lncRNA00673 in CRC tissues. ***A,*** qRT-PCR assay is used to detect the relative expression of lncRNA00673 in 71 pairs of CRC and cancer-adjacent tissues, which is quantified by GAPDH. ***B,*** According to the fold change in the expression of lncRNA00673, CRC patients are divided into the lncRNA00673 high-expression group and lncRNA00673 low-expression group. ***C,*** Kaplan-Meier survival analysis is used to study the correlation of the expression level of lncRNA00673 with OS. D- qRT-PCR assay is used to detect the relative expression of lncRNA00673 in CRC cells compared with that in human normal colonic cells.

II). The results of this part showed that LncRNA00673 expression level, distant metastasis and TNM staging can be used as a separate prognostic factor for CRC patients, respectively.

Influence of lncRNA00673 on CRC Cell Proliferation

First, the relative expression levels of lncRNA00673 in human CRC cell lines HT-29, LOVO, SW480, HCT-116, RKO, HCT-8, HCT-15, DLD1 and colonic normal cell line CCD841 were detected by qRT-PCR (Figure 1D). LOVO and HCT-16 with the highest up-regulation fold change were used for further experiments. The specific interference sequence of lncRNA00673 was designed and synthesized, and then transiently transfected into model cells. Its transfection efficiency was detected by qRT-PCR. The results showed that the interference efficiency of 2# sequence was the highest (Figure 2A and B). After knocking down lncRNA00673 expression, MTT and clonal formation experiments showed that CRC cell proliferation was significantly inhibited (Figure 2C, D).

Discussion

CRC is one of the most common tumors. Global statistics in 2012 showed that the incidence rate of CRC in males and females ranked 3rd and 2nd, among all tumors, respectively. Their mortality rate ranked 4th and 3rd, respectively[18]. Epidemiological statistics of CRC between 2008 and 2013 in China also showed that the incidence and mortality rate ranked top 5 in all tumors[19]. Although the symptoms, diagnosis and treatment of CRC have been well explained, there are still large unknown areas involved in the mechanisms of proliferation, migration, invasion and metastasis of CRC to be explored. More and more evidence showed that the expression of lncRNA is significantly associated with tumor formation[20]. Early studies have shown that about 18% of non-coding protein genes of encoding lncRNA are associated with tumors whereas only 9% of genes in encoding proteins are associated with the development and progression of tumors[21]. In the past few years, the important role of lncRNA in the occurrence and development of CRC has been gradually recognized. For exam-

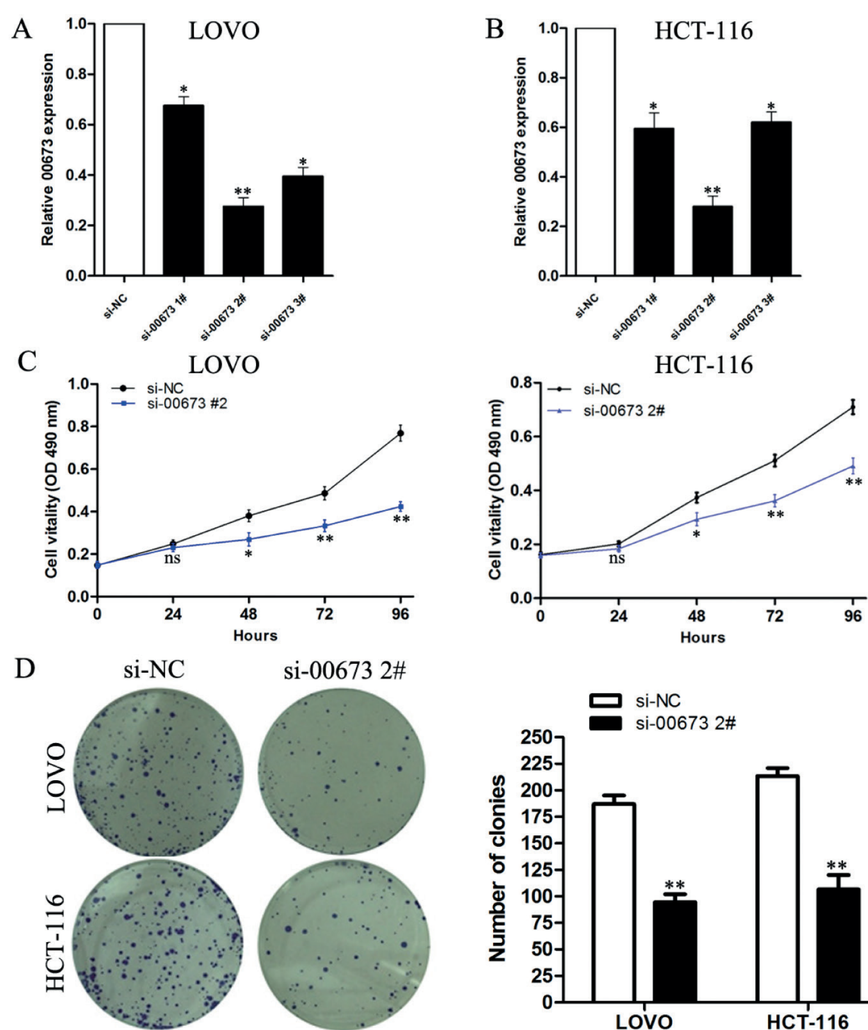

Figure 2. Influence of lncRNA00673 on CRC cell proliferation. ***A,*** and ***B,*** The interference efficiency of LOVO and HCT-116 cells transfected with siRNA. ***C,*** After the transfection with siRNA of LOVO and HCT-116 cells, MTT assay is used to detect the change in cell viability. ***D,*** Clone formation assay is used to detect the change in proliferation capacity after LOVO and HCT-116 cells are transfected with si-00673. All experiments are repeated for three times independently. $^{*}p<0.05$, $^{**}p<0.01$.

ple, lncRNA P21 associated ncRNA DNA damage activated (PANDAR), forkhead box P4 antisense RNA 1 (FOXP4-AS1), plasmacytoma variant translocation-1 (PVT-1) and sprouty RTK signaling antagonist 4-intronic transcript 1 (SPRY4-IT1) are significantly elevated in CRC and closely related to the proliferation, metastasis and drug resistance of CRC[22-25]. However, the expression levels of branched-chain amino acid transaminase 1 (BCAT1) and growth arrest-specific 5 (GAS5) in CRC are significantly reduced, while the cell cycle of CRC cells is inhibited and apoptosis of CRC cells is promoted[26,27]. However, the expression and effect of lncRNA00673 in CRC have not been reported. Various reports indicated that lncRNA00673 promotes tumor growth and metastasis in non-small cell lung cancer and gastric cancer[15-17]. It was found for the first time that the expression level of lncRNA00673 was highly expressed in CRC and closely related to TNM staging, tumor size and distant metastasis. The highly expressed lncRNA00673 suggests that the prognosis of patients is poor and lncRNA00673 can be used as an independent predicative factor for the prognosis. In addition, *in vitro* studies have shown that knocking down lncRNA00673 expression can significantly inhibit the proliferation of CRC cells.

Some transcription factors, such as C-MYC, E2F1, SP1, can induce lncRNA transcription and increase DNA copy number in the position

where lncRNA genes are located, so that lncRNA expression is up-regulated[28-31]. However, why lncRNA00673 is highly expressed in CRC tissues and cells and how to regulate the molecular mechanism of cell proliferation are the focus of our further studies.

Conclusions

The expression level of lncRNA00673 is up-regulated in CRC tissues and cells and it is related to the degree of malignancy and poor prognosis. LncRNA00673 can be used as a potential molecular marker for the prognosis of CRC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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