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Overexpression of long non-coding RNA MINCR contributes to progressive clinicopathological features and poor prognosis of human hepatocellular carcinoma

X.-L. JIN[1], J.-R. LIAN[2], Y.-H. GUAN[3]

[1]Department of Infectious Disease, Luoyang Central Hospital Affiliated to Zhengzhou University, Luoyang, Henan, China
[2]Department of Pharmacy, The Xinjiang Uygur Autonomous Region Children's Hospital, Urumqi, China
[3]Department of Infection, The Second Hospital of Dalian Medical University, Dalian, Liaoning, China

Abstract. – OBJECTIVE: MYC-induced long non-coding RNA (MINCR) has been shown to be a long noncoding RNA that facilitates the progression of a number of malignancies, including hepatocellular carcinoma (HCC). However, few studies have explored the expression and role of MINCR in HCC. In this study, we aimed to investigate the clinical significance of MINCR in HCC.

PATIENTS AND METHODS: MINCR expression levels in 161 pairs of HCC tissues and pair-matched adjacent normal tissues were examined by qRT-PCR. The correlation between clinicopathological features and MINCR expression was analyzed by x^2 test. Differences in patient survival were determined using the Kaplan-Meier method and a log-rank test. The significance of survival variables was analyzed using the Cox multivariate proportional hazards model.

RESULTS: Our results showed that MINCR was significantly upregulated in HCC tissues, compared with paired adjacent nontumor tissue samples. MINCR upregulation was correlated with TNM stage ($p = 0.005$) and histological grade ($p = 0.001$). The results of Kaplan-Meier method and log-rank test indicated that the 5-years overall survival of the high MINCR group was significantly lower than that of low MINCR group ($p = 0.0035$). Univariate and multivariate analysis results indicated that MINCR was an independent prognostic factor in HCC ($p < 0.05$).

CONCLUSIONS: We firstly provided the possibility that evaluating MINCR in HCC tissues may have prognostic and predictive value in the clinical management of HCC patients.

Key Words
MYC-induced long non-coding RNA, Hepatocellular carcinoma, Prognosis.

Introduction

Hepatocellular carcinoma (HCC), one of the most common malignant tumors, is the third leading cause of cancer-related deaths worldwide[1,2]. Recently, increased morbidity and mortality are observed in many countries, including China[3]. Despite much being known about the major etiological factors, such as HBV and HCV infection, the potential mechanism of HCC remains largely unclear[4,5]. Although various aggressive surgical and non-surgical treatments have been used to improve the prognosis of HCC patients, the long-term post-treatment prognosis remains poor due to the highly metastatic potentials of HCC cells[6,7]. HCC is a complex biological process that results from the dysregulation of many cancer-related genes[8]. Therefore, it is important to investigate possible prognostic factors among the survivors in order to help management of HCC treatment and to further understand the potential mechanism. Long noncoding RNAs (lncRNAs) widely exist in the nucleus and cytoplasm of eukaryotic cells. These RNAs comprise a heterogenous group of genomic transcripts longer than 200 nucleotides that have no protein coding functions[9]. Although more than 3000 human lincRNAs have been identified, less than 1% of them have been characterized[10]. Recent evidence shows that lncRNAs have essential roles in a diverse range of cellular functions such as development, differentiation and cell fate, and they could regulate gene transcription at the transcriptional, posttranscriptional and epigenetic levels[11,12]. In addition, dysregulated lncRNAs contribute to cancer initiation

Corresponding Author: Yong-hai Guan, MD; e-mail: gupm99871@126.com

and progression by acting as proto-oncogenes or tumor suppressor genes[13,14]. Given the importance of lncRNAs in cancer progression, lncRNAs have potential to enter into cancer clinics as diagnostic and prognostic biomarkers to assess tumorigenesis, progression and response to treatment in cancer patients[15,16]. In HCC, several lncRNAs, such as lncRNA-UCA1, lncRNA PVT1 and lncRNA ZFAS1, have been identified to be important regulators[17-19]. However, most lncRNAs remain to be further analyzed. MYC induced long noncoding RNA (MINCR), also named as LOC100507316 and RP13-58209.5, was a newly identified lncRNA and correlated with MYC expression in Burkitt lymphoma[20]. Recently, it was reported that MINCR expression was dysregulated in gallbladder cancer[21] and HCC[22]. Although MINCR had been studied and was reported to be up-regulated in HCC and to serve as a tumor promoter *in vitro*, its clinical significance in HCC patients has not been reported. In this study, we detected the expression levels of MINCR in HCC and explored its prognostic value.

Patients and Methods

Patients and Clinical Samples

161 HCC and matched normal tissues samples were collected from patients, who underwent the resection of their primary HCC at the Luoyang Central Hospital Affiliated to Zhengzhou University during June 2010 to May 2014, with a median follow-up time of 32.7 months. HCC diagnosis was based on World Health Organization (WHO) criteria. The tissue samples were histologically confirmed to be tumor or non-tumor tissues, and were quickly frozen in liquid nitrogen analysis. No patients received radiotherapy or chemotherapy before the hepatectomy operation. Tumors were staged according to the Seventh Edition of the Cancer Staging Manual by the American Joint Committee on Cancer. The clinical information of all patients was shown in Table II. This study was approved by the Research Ethics Committee of Luoyang Central Hospital Affiliated to Zhengzhou University. Written informed consent was acquired from all the patients.

RNA Extraction, Reverse Transcription and Quantitative RT-PCR

Total RNA from the tissues was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA). Then, cDNA was synthesized with the Reverse Transcription System (Promega, Madison, WI, USA). PCR reaction was performed using an ABI7500 System (Applied Biosystems, Foster City, CA, USA) and SYBR Green PCR Master Mix (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was measured as an internal control for paired tumor and normal tissues. The relative expression levels of MINCR were calculated using the $2^{-\Delta Ct}$ method. The primer sequences tested in this study were shown in Table I.

Table I. PCR primer sequences.

Subject	Primer Sequences (5′-3′)
MINCR (Forward)	TGTGGCAAAACTTGAATGGA
MINCR (Reverse)	GGGGGAGGACAAGAGAAAGA
GAPDH (Forward)	GATTCCACCCATGGCAAATCC
GAPDH (Reverse)	TGGGATTTCCATTGATGACAAG

Statistical Analysis

All statistical analyses were carried out using SPSS 19.0 (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA). Paired Student's *t*-test was conducted to compare MINCR expression in paired clinical samples. The x^2-test was used to analyze the associations between MINCR expression and clinicopathological feature. Survival curves were plotted using the Kaplan-Meier method and the log-rank test. Significant variables in univariate models were further analyzed by multivariate Cox proportional hazards regression models to identify independent prognostic factors. A $p < 0.05$ was considered statistically significant.

Results

MINCR was Upregulated in HCC Tissues

Up-regulation of MINCR in HCC tissues and cell lines has been reported in previous study. However, the evidence is limited. In order to further confirm previous results, we performed RT-PCR to detect the expression levels of MINCR in HCC tissues and matched normal tissues from HCC patients at our hospital. As shown in Figure 1A, we found that the expression of MINCR in HCC tissues was significantly higher than in adjacent non-cancerous tissues ($p < 0.01$). Furthermore, we also found that the expression levels of MINCR were significantly upregulated in tumor tissues with low histologic grade compared

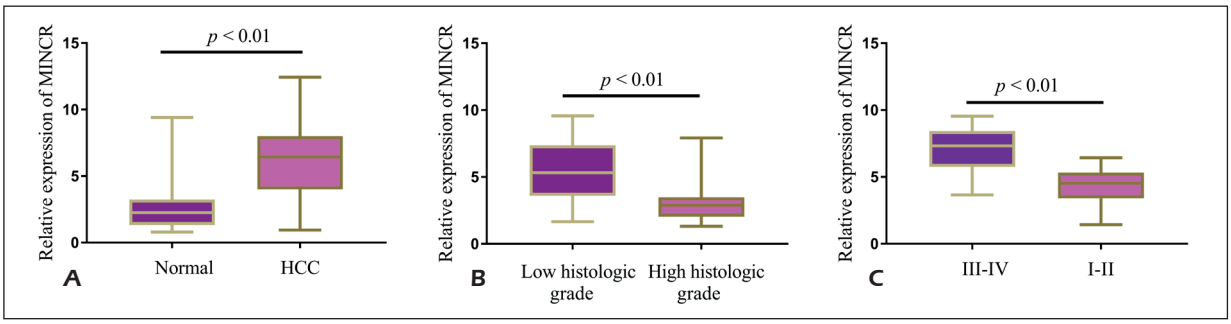

Figure 1. The relative expression of MINCR in HCC patients. **A**, The expression levels of MINCR was significantly up-regulated in HCC tissues compared to matched normal tissues (p<0.01). **B**, The expression levels of MINCR was significantly up-regulated in tumor tissues with low histologic grade compared to those with high histologic grade (p<0.01). **C**, MINCR expression was significantly higher in patients with an advanced clinical stage than in those with an early clinical stage. The experiments were repeated at least three times.

to those with high histologic grade (p < 0.01, Figure 1B). In addition, it was observed that MINCR expression was significantly higher in patients with an advanced clinical stage than in those with an early clinical stage (p <0.01, Figure 1C). Taken together, our findings were consistent with previous study and overexpression of MINCR may contribute to clinical progression of HCC.

Correlations Between MINCR Expression and Clinical Characteristics

In order to explore the clinical significance of MINCR in HCC patients, all HCC patients were subsequently divided into two groups (high expression group ≥ 4.16 and low expression group < 4.16) based on the optimal cutoff value of MINCR expression. As shown in Table II, we found that high MINCR expression was associated with lower histologic grade (p = 0.001) and advanced TNM stage (p = 0.005). However, no statistically significant association was observed between MINCR expression levels and patient's age, gender, smoking status, tumor size, tumor number, AFP and Hepatitis B (All p > 0.05). Thus, our results indicated that MINCR may act as a tumor promoter in HCC.

Correlation Between MINCR Expression and Prognosis of HCC Patients

To further investigate the correlations of MINCR expression level with survival of patients with HCC, Kaplan-Meier analyses were performed. As

Table II. Clinicopathological features and the expression of lncRNA MINCR in HCC patients.

Parameters	Group	Total	MINCR expression		p-value
			High	Low	
Gender	Male	94	50	44	NS
	Female	67	30	37	
Age (years)	< 60	75	35	40	NS
	≥ 60	86	45	41	
Tumor size (cm)	< 5	104	46	58	NS
	≥ 5	57	34	23	
Tumor number	Solitary	79	37	42	NS
	Multiple	82	43	39	
AFP	< 20	92	42	50	NS
	> 20	69	38	31	
Hepatitis B	Negative	48	25	23	NS
	Positive	113	55	58	
Histologic grade	High	94	36	58	0.001
	Low	67	44	23	
TNM stage	I-II	102	42	60	0.005
	III-IV	59	38	21	

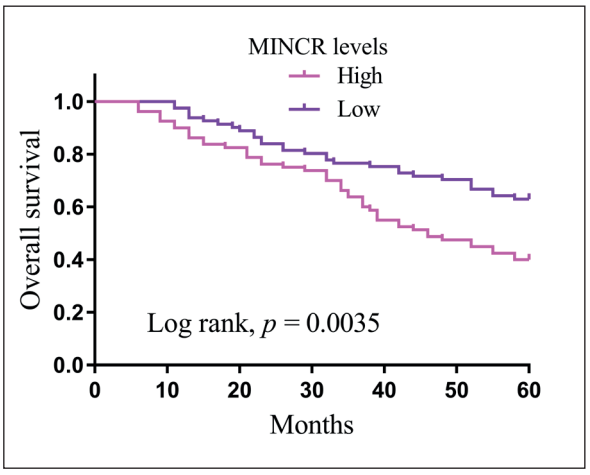

Figure 2. Kaplan-Meier analysis for the overall survival of HCC patients with the expression of MINCR. Patients with high MINCR expression had a shorter overall survival than those low expressions. Log rank test proved the difference was significant (p=0.0035).

shown in Figure 2, we found that HCC patients with higher MINCR expression level had a significantly poorer prognosis than those with lower MINCR expression level (p = 0.0035). Furthermore, Cox regression analyses were conducted to evaluate the prognostic factors in all HCC patients. Univariate analysis showed that patients with low histologic grade (p = 0.004), advanced TNM stage (p = 0.001) and high MINCR expression (p = 0.006) had markedly shorter overall survival (Table III). More importantly, multivariate analysis of the prognosis factors showed that high MINCR expression was a significant independent predictor of poor survival in HCC (HR= 3.669, 95% CI: 1.328-5.336, p = 0.006) in addition to the presence of histologic grade and TNM stage (Table III).

Discussion

Hepatocellular carcinoma (HCC) represents a unique challenge for physicians and patients[23]. There is no definitively curative treatment. Early detection of HCC is the most important factor to offer the patient the chance of cure and prediction of prognosis of HCC patients is very important for management of clinical treatment[24,25]. Up to date, several prognostic systems have been proposed. However, they have shown an unquestionable predictive value[26,27]. Therefore, it is necessary to find useful biomarkers of HCC to predict prognosis. Recently, the potential of lncRNAs as novel biomarkers attracted researcher's attention because of its dysregulation and critical biological function in tumors, including HCC[28,29]. In this study, we forced on a novel lncRNA. Recently, the expression and function of MINCR have reported in several tumors. For instance, Wang et al[21] reported that MINCR expression was significantly up-regulated in gallbladder cancer and associated with tumor volume and lymph node metastasis as well as overall survival of gallbladder cancer patients. In their *in vitro* assay, it was found that overexpression of MINCR promoted gallbladder cancer cell proliferation, invasiveness and inhibited the apoptosis by stimulating EZH2 expression, indicating MINCR served as a tumor promoter in gallbladder cancer. Cao et al[22] showed that the expression levels of MINCR were significantly up-regulated in both hepatocellular carcinoma tissues and cell lines. Loss-of-function experiments indicated that knockdown of MINCR suppressed HCC cell proliferation, migration and invasion. However, the clinical significance of MINCR remains to further be investigated. In this study, we found

Table III. Univariate and multivariate analysis of survival in HCC patients.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Gender	1.213	0.748-1.669	0.458	–	–	–
Age	1.334	0.831-1.978	0.215	–	–	–
Tumor size	1.855	0.895-2.322	0.114	–	–	–
Tumor number	1.456	0.584-2.013	0.188	–	–	–
AFP	1.579	0.744-2.328	0.116	–	–	–
Hepatitis B	1.638	0.847-2.447	0.128	–	–	–
Histologic grade	3.156	1.428-5.337	0.004	2.784	1.217-4.563	0.009
TNM stage	3.458	1.674-6.115	0.001	3.016	1.327-5.447	0.005
MINCR expression	3.669	1.328-5.336	0.006	3.126	1.248-4.478	0.011

that MINCR expression was significantly upregulated in HCC tissues compared with matched normal tissues. This result was consistent with previous study. Then, we also found that higher expression levels of MINCR were observed in HCC tissues with advanced clinical stage. Furthermore, clinical assay indicated that the level of MINCR in HCC was strongly correlated with histologic grade and TNM stage, indicating that MINCR acted as a positive regulator in progression of HCC. In addition, in order to explore the prognostic value of MINCR in HCC patients, we collected five-year follow-up data of 161 patients and performed Kaplan-Meier method, finding that patients with high levels of MINCR expression showed reduced overall survival times compared with patients with low levels of MINCR expression. More importantly, high MINCR expression could be identified as an independent poor prognostic marker for HCC patients by univariate and multivariate analysis.

Conclusions

We showed that MINCR was overexpressed in HCC tissues and correlated with poor prognosis, thereby potentially standing for a poor prognostic biomarker for HCC. Moreover, our results encourage further investigations to explore the potential mechanism via which MINCR is involved in tumor progression and to demonstrate its role as prognostic marker for clinical use.

Conflict of Interests:

The authors declare no conflicts of interest.

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