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Long non-coding RNA XLOC_010235 correlates with poor prognosis and promotes tumorigenesis of hepatocellular carcinoma

F. YANG, Y. JIANG, L.-Z. LV

Department of Hepatobiliary Surgery, Fuzhou PLA General Hospital, Fuzhou, China

Abstract. – OBJECTIVE: Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies worldwide. Long non-coding RNAs (lncRNAs) are novel proposed non-coding RNAs, and play critical roles in tumorigenesis. However, the clinical significance, biological role and molecular mechanism of ncRNAs in HCC still remain largely elusive. The aim of this study was to uncover the clinical value and biological role of XLOC_010235 (XLOC), which has been demonstrated as an oncogene in gastric cancer, in HCC.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (QRT-PCR) was conducted to measure the level of XLOC in HCC tissues and cell lines. The relationship between XLOC expression and clinicopathological features of HCC patients was analyzed. Then loss-of-function assays were conducted to determine the biological effect of XLOC in HCC cells.

RESULTS: Our investigations revealed that XLOC was increased in HCC tissues and cell lines, and high level of XLOC was significantly correlated with poor prognosis. Additionally, silenced XLOC significantly inhibited cell proliferation, induced cell apoptosis and facilitated cell migration of HCC *in vitro*.

CONCLUSIONS: Our findings advance our understanding of the role of XLOC as an oncogene in HCC, which may help in the development of new therapeutics.

Key Words:
Hepatocellular carcinoma, LncRNA, XLOC_010235, Proliferation, Migration.

Introduction

Hepatocellular carcinoma (HCC), a typical hypervascular tumor, is one of the most prevalent malignancies worldwide, and both the mortality and morbidity have been escalating in the past decades[1]. Frequent intrahepatic and extrahepatic metastasis and limited therapeutic approaches result in a high mortality rate. Therefore, it is essential to understand the molecular mechanisms underlying metastasis and angiogenesis of HCC which is favor to exploit suitable therapeutic targets for HCC patients.

Long non-coding RNAs (lncRNAs), longer than 200 nucleotides with limited or no protein coding ability, have been attracted more and more attention worldwide[2-5]. Currently, lncRNAs, characterized by the complexity and diversity of their sequences and mechanisms of action, have been identified to be dysregulated in multiple biological processes through regulating gene expression at transcriptional, post-transcriptional and epigenetic levels. For instance, Zhao et al[6] reported that lncRNA SNHG5/miR-32 axis regulates gastric cancer cell proliferation and migration by targeting KLF4. Liu et al[7] demonstrated that lincRNA FEZF1-AS1 represses p21 expression to promote gastric cancer proliferation through LSD1-Mediated H3K4me2 demethylation. Thus, lncRNAs play important roles in carcinogenesis, and present a broad application prospects. However, the biological role of lncRNAs in the prognosis of HCC remains largely elusive.

In the present study, we focus on lncRNA XLOC_010235 (XLOC), a 302-bp lncRNA on chromosome 12. Recently, Liu et al[8] showed that up-regulation of XLOC regulates epithelial-to-mesenchymal transition to promote metastasis by associating with Snail1 in gastric cancer. Song et al[9] revealed that XLOC was over-expressed in gastric cancer cells and closely related to tumor metastasis and patient prognosis. However, the biological function and underlying mechanism of XLOC in HCC is hardly clarified.

In current study, we revealed that XLOC was upregulated in HCC tissues and cell lines, and high level of XLOC was associated with poor prognosis of patients with HCC. Moreover, we also explored the biological effect of XLOC

Corresponding Author: Lizhi Lv, MD; e-mail: lizhilv3647@sina.com

expression on HCC cell phenotypes and uncovered that ectopic expression of XLOC significantly suppressed the proliferation ability of HCC cells, caused cell apoptosis, repressed cell migration and reversed epithelial mesenchymal transition (EMT) phenotype to MET. All these investigations proposed for the first time that XLOC contributed to the progression of HCC.

Patients and Methods

Patients

A total of 68 HCC tissues and matched tumor-adjacent tissues were obtained from the Department of Hepatobiliary Surgery, Fuzhou PLA General Hospital. Tissue specimens were conserved in liquid nitrogen for RNA isolation or 10% formalin for immunostaining until use. No patients received immunotherapy, radiotherapy or chemotherapy before surgery. All samples were used after obtaining informed consent. This study was approved by the Ethics Committee of Fuzhou PLA General Hospital.

Cell Culture

For routine culture, HepG2, Hep3B, SMMC-7721, Bel-7402 HCC cell lines and a normal liver epithelium cell line L02 purchased from the Shanghai Cell Collection (Shanghai, China) were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA, USA) medium with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in humidified air at 37°C with 5% CO_2.

Cell Viability Assay

2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfothenyl)-2H-tetrazolium salt (CCK-8, Dojindo, Kumamoto, Japan) assay was conducted to evaluate the rate of cell proliferation, according to manufacturer's instructions. Briefly, log-phase cells were trypsinized into a single-cell suspension and plated into 96-well plates at a density of 2×10^3 per well. CCK-8 solution was added to each well. After 1 hour, the absorbance of each well was recorded at 450 nm and read on a microplate reader victor (Enspire 2300 Multilabel Reader, PerkinElmer, Singapore).

Colony Formation Assay

Cells (500 cells/wells) were transfected with indicated vectors, which were seeded in six-well plates and cultured at 37°C for two weeks. Then the cells were stained with 0.1% crystal violet after fixed. Visible colonies number was counted manually.

Flow Cytometric Analysis of Apoptosis

Cells transfected with indicated plasmid or negative control, were reaped after 48 hours. Apoptosis were performed using flow cytometric analyses with Annexin V: FITC Apoptosis Detection Kits (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's instructions. All samples were assayed in triplicate.

Quantitative Real-time PCR

RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacture's protocols. The first strand cDNA was compounded using a Tianscript RT kit (Tiangen Biotech., Beijing, China). PCR amplifications for XLOC mRNA were performed with the Kit of TaqMan Human MiRNA Assay (Genecopoeia, Guangzhou, Guangdong, China) and the SYBR Premix Ex Taq™ Kit (TaKaRa, Otsu, Shiga, Japan) in ABI 7300 system (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as reference genes to normalize the expression XLOC.

Wound Healing Assays

Cell migration capacity was calculated by wound healing assay. 2×10^5 cells with or without transfection were plated into 12-well plates and incubated in DMEM with 10% FBS at 37°C. After reaching 100% confluence, cells were wounded by scraping with a 200 μL tip; next, they were washed 3 times in serum-free medium and incubated in regular medium. Wounds were observed at 0 and 48 h. The cell migration distance was calculated by subtracting the wound width at each time point from the wound width at the 0 h time point. Three independent assays were assayed.

Transwell Assay

Cell migration experiments were performed using Transwell chamber to measure cells (8 um pore size, Corning, NY, USA). Cells were placed into the upper chamber in serum-free medium 48 hours after transfection. The lower chamber was supplemented with media containing 10% bovine calf serum (BCS). After 48 hours culture, the upper membrane cells were removed and the transitional cells were fixed in methanol, stained

with 0.1% crystal violet and counted under a microscope. Each experiment was performed three times independently.

Western Blotting

Total proteins were collected with Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and protein concentrations were measured following the bicinchoninic acid (BCA) method (Beyotime, Nanjing, Jiangsu, China), and 40 ug protein were subjected to 4-20% SDS gel electrophoresis (Sigma-Aldrich, St. Louis, MO, USA) and were then transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Indianapolis, IN, USA). Then, 5% milk blocked membranes were incubated with E-cadherin (Abcam, Cambridge, MA, USA), N-cadherin (Abcam, Cambridge, MA, USA), α-SMA (Abcam, Cambridge, MA, USA) or vimentin (Abcam, Cambridge, MA, USA) primary antibody and subsequently incubated with matched secondary antibodies (Cell Signaling Technology, Beverly, MA, USA). GAPDH (US Biological, Swampscott, MA, USA) was used as a loading control.

Statistical Analysis

SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data was presented as means ± standard deviation (SD). The Pearson χ^2-test was used to evaluate the links between XLOC expression and clinicopathological factors. Differences between two groups were analyzed by Student's *t*-test. One-way ANOVA was performed when multiple comparisons were made, and Least Significant Difference served as its post-hoc test. Survival analysis was performed using the Kaplan-Meier method, and the log-rank test was used to compare the differences between patient groups. Through multivariate survival analysis of HCC, Cox proportional hazard regression model was established to identify factors related with overall survival. $p<0.05$ indicated statistical significance.

Results

XLOC was Over-Expressed in HCC Tissues and Cell Lines

To determine the biological role of XLOC in HCC, we detected the level of XLOC in 68 HCC tissues by RT-qPCR. As demonstrated in Figure 1A, the level of XLOC was significantly overexpressed in HCC tissues, compared with corresponding normal tissues ($p<0.001$). Then, we measured the endogenous level of XLOC in a serious of HCC cell lines (SMMC7721, Hep3B, HepG2, Bel-7402) and a human normal liver epithelial cell line (L02). As shown in Figure 1B, we found that XLOC were notably up-regulated in HCC cells, in comparison to the human normal liver epithelial cell line (L02). Collectively, these findings indicated that XLOC might act as an oncogene in HCC progression.

High Level of XLOC was Correlated with Poor Prognosis of HCC Patients

To evaluate the clinical value, we analyzed the association between XLOC and clinical pa-

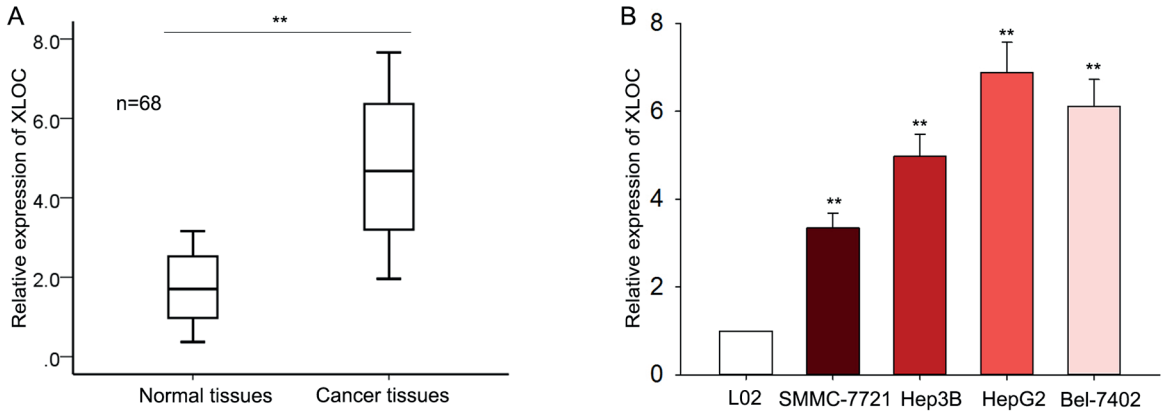

Figure 1. XLOC was over-expressed in HCC tissues and cell lines. ***A-B,*** The expression level of XLOC in HCC tissues and cell lines by qRT-PCR. Error bars represented the mean±SD of at least three independent experiments. $^{*}p<0.05$ and $^{**}p< 0.01$ *vs.* control group.

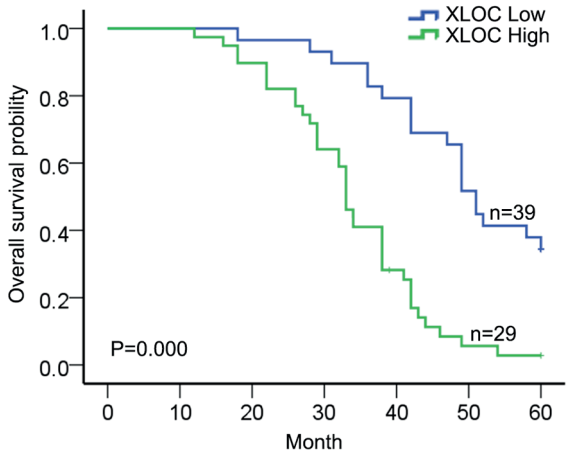

Figure 2. High expression of XLOC predicted the poor prognosis for HCC patients according to Kaplan-Meier method.

thological parameters of HCC. 68 HCC patients were divided into XLOC high expression group (n=39) and XLOC low expression group (n=29) based on the cutoff value, according to the mean level of XLOC. As illustrated in Table I, high level of XLOC was significantly correlated with vascular invasion (p=0.003), tumor size (p=0.006) and Edmindson grade (p=0.014), but was no significant correlation with age, gender, cirrhosis HbsAg, alanine aminotransferase (ALT) and alpha fetoprotein (AFP) stage (p>0.5). Moreover, results from Kaplan-Meier method analysis (log-rank test) showed that high level of XLOC was associated with poor prognosis (p=0.000, log-rank test; Figure 2). Additionally, proportional hazards method analysis revealed that high level of XLOC could

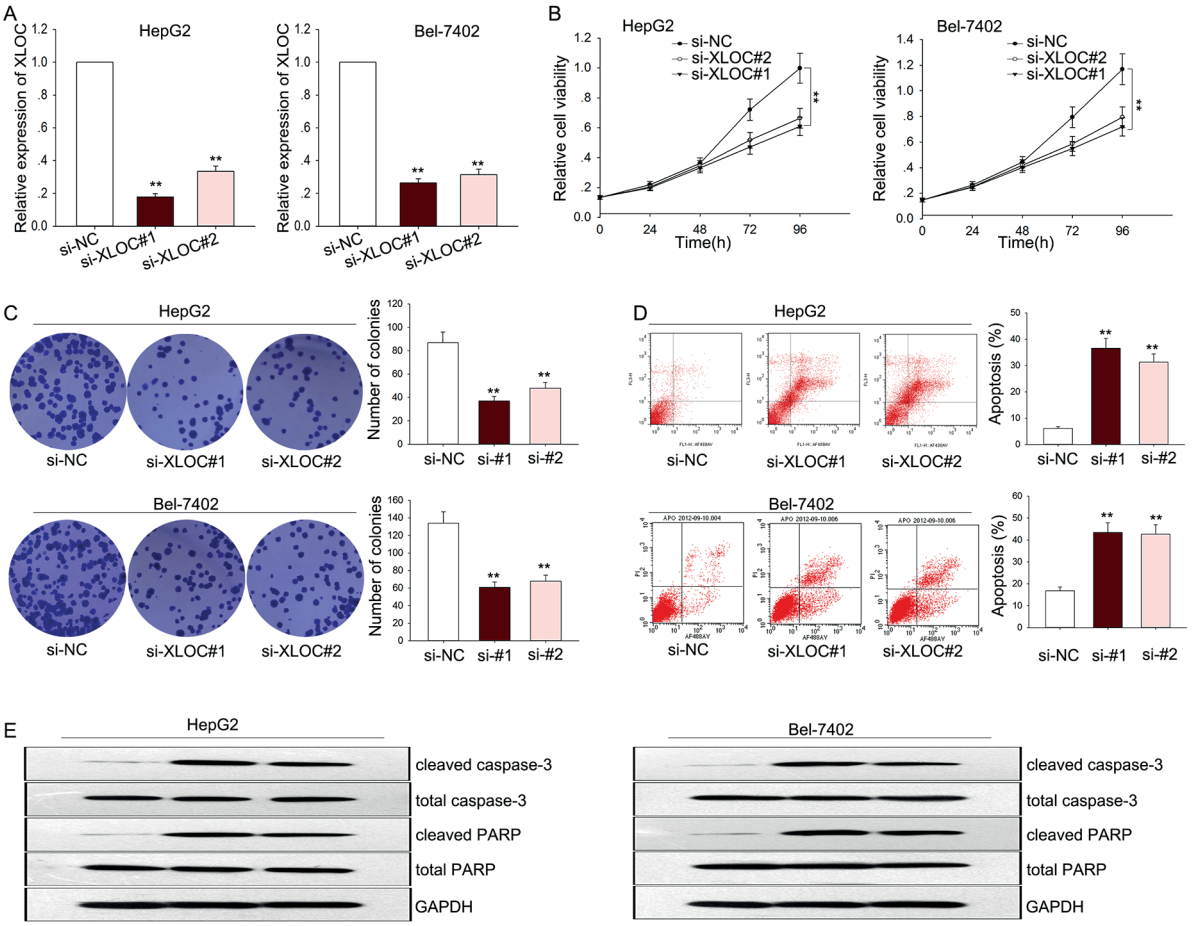

Figure 3. Silencing XLOC impaired HCC cell proliferation ability and induced apoptosis. ***A,*** The transfection efficiency in cells transfected with si-XLOC by qRT-PCR. ***B-C,*** The effect of silenced XLOC on the cell proliferation based on CCK8 and colony formation assays. ***D-E,*** The effect of silenced XLOC on apoptosis according to flow cytometry and Western blot assays. Error bars represented the mean±SD of at least three independent experiments. $^{*}p$<0.05 and $^{**}p$<0.01 *vs.* control group.

be proposed as an independent prognostic factor (p=0.001) (Table II). These findings suggest that XLOC might be a promising prognostic marker for HCC patients

Silencing XLOC Impaired HCC Cell Proliferation Ability and Induced Apoptosis

To better understand the effect of XLOC on cervical cancer cell, loss-of-function assays were performed. XLOC specific siRNA was used to silence the endogenous level of XLOC, and si-RNA was used as negative control. QRT-PCR showed that XLOC expression was significantly down-regulated both in HepG2 and Bel-7402 cells compared with si-RNA group (Figure 3A). CCK8 assay revealed that decreased level of XLOC significantly suppressed cell proliferation both in HepG2 and Bel-7402 cells (Figure 3B). Likewise, results from colony formation also revealed that silenced XLOC could reduce the colony formation ability of HCC cells (Figure 3C). Additionally, result from flow cytometric analysis of apoptosis revealed that silenced XLOC increased the apoptosis rate of HepG2 and Bel-7402 cells (Figure 3D). Moreover, apoptosis-related proteins cleaved caspase-3 and cleaved PARP was also increased when XLOC was silenced (Figure 3E). Taken together, these results suggested that XLOC could act as a tumor promoter in HCC proliferation.

Knockdown XLOC Suppressed HCC Cells Migration Through Regulating EMT Formation

To evaluate the effect of XLOC knockdown on HCC cells migration, wound healing and transwell assays were performed. As illustrated in Figure 4A-B, results from wound healing and transwell assays showed that XLOC knockdown inhibited cell migration capacity in HepG2 and Bel-7402 cells. To understand the underlying mechanism, we focus on the MET process, which plays critical role in cancer cells metastasis. As shown in Figure 4C, Western blot assays revealed that silencing XLOC could increase the level of epithelial protein markers (E-cadherin), but reduce the mesenchymal markers (N-cadherin, α-SMA and vimentin) of HCC cells. These re-

Table I. Correlation between XLOC expression and clinical features (n=68).

Variable	XLOC Expression		p-value
	low	high	
Age			
<60	12	20	0.468
≥60	17	19	
Gender			
Male	17	17	0.327
Female	12	22	
HbsAg			
Negative	15	17	0.130
Positive	14	22	
ALT			
≤45	13	19	0.809
>45	16	20	
AFP			
≤13.6	12	22	0.327
>13.6	17	17	
Vascular invasion			
Absent	21	13	0.003**
Present	8	26	
Cirrhosis			
Absent	16	14	0.142
Present	13	25	
Tumor size			
≤5	22	16	0.006**
>5	7	23	
Edmindson grade			
I-II	21	16	0.014**
III-IV	8	23	

Low/high by the sample median. Pearson χ^2 test. p<0.05 was considered statistically significant.

Table II. Multivariate analysis of prognostic parameters in patients with XLOC by Cox regression analysis.

Variable	Category	p-value
Age	<60	0.682
	≥60	
Gender	Male	0.209
	Female	
HbsAg	Negative	0.955
	Positive	
ALT	≤45	0.712
	>45	
AFP	≤13.6	0.083
	>13.6	
Vascular invasion	Absent	0.042*
	Present	
Cirrhosis	Absent	0.121
	Present	
Tumor size	≤5	0.099
	>5	
Edmindson grade	I-II	0.028*
	III-IV	
XLOC	Low	0.001*
	High	

Proportional hazards method analysis showed a positive, independent prognostic importance of XLOC expression (p = 0.001). *p < 0.05 was considered statistically significant.

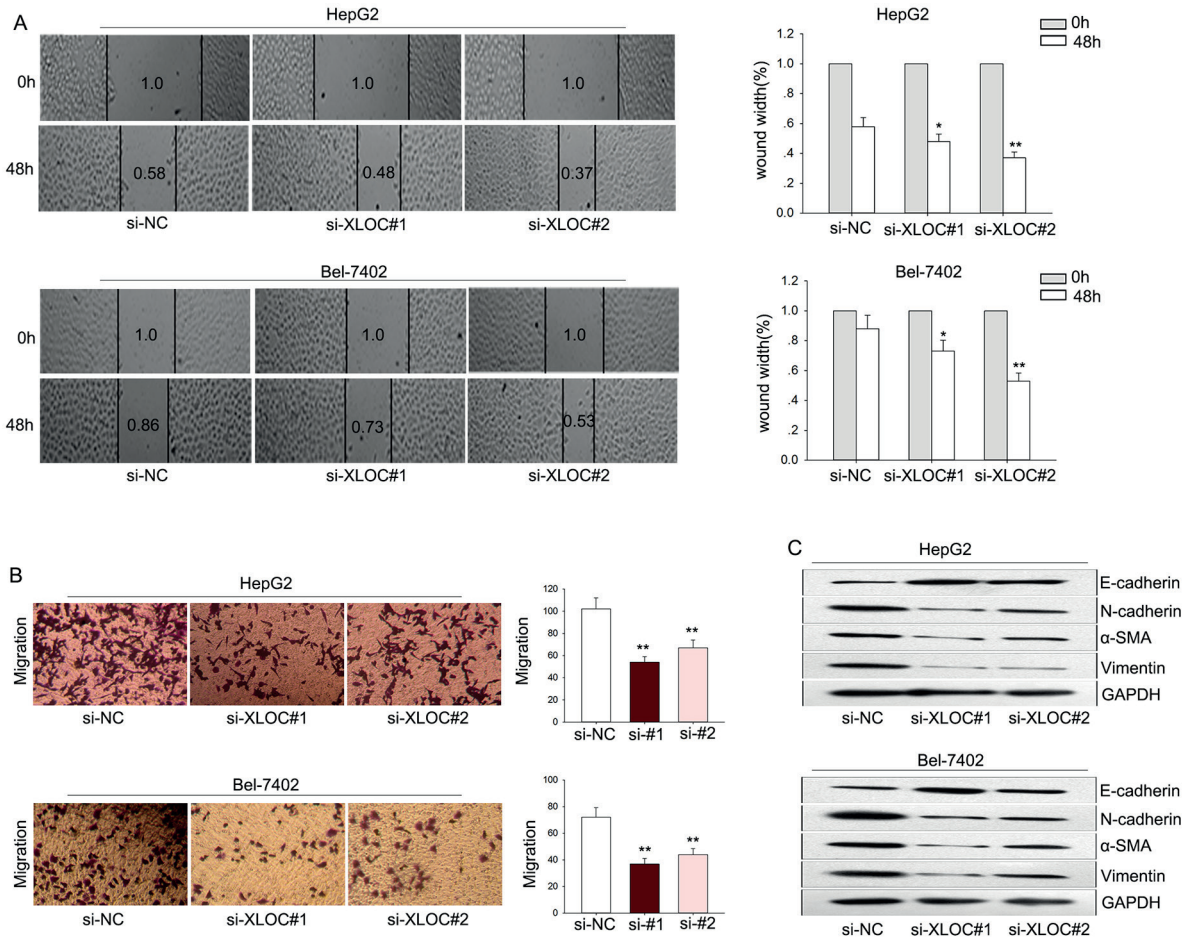

Figure 4. Knockdown XLOC suppressed HCC cells migration through regulating EMT formation. ***A-B,*** Wound healing and transwell assays defined the effect of silenced XLOC on the cell migration. ***C,*** Western blot was used to detect the effect of down-regulated XLOC on the markers of EMT process. Error bars represented the mean±SD of at least three independent experiments. $^{*}p<0.05$ and $^{**}p<0.01$ *vs*. control group.

sults suggested that XLOC could facilitate HCC cells migration, at least partially, through regulating EMT process.

Discussion

HCC is one of the commonest cancers worldwide with a poor prognosis. The exploration of early biomarkers and therapeutic targets for HCC is of great importance. It has been uncovered that more than 98% of the total mammalian genome is non-coding portion[10]. Based on the length, the no coding RNAs (ncRNAs) can be divided into two subtypes including short ncRNAs with the length <200 nt, and long ncRNAs with the length longer than 200 nt. Currently, the expression pattern and biological role of lncRNAs have attracted more and more attention from researchers, suggesting that lncRNAs can act as major contributors to carcinogenesis and cancer progression. The roles of dysregulated lncRNAs in the proliferation, invasion and migration of many kinds of cancers have garnered increased scientific interest in recent years[11-14]. For example, Liu et al[12] had reported the role of lncRNA PCA3 in epithelial ovarian carcinoma; He et al[15] related the oncogenic role of lncRNA FEZF-AS1 in non-small cell lung cancer through inhibiting E-cadherin and regulating WNT pathway; Wu et al[16] discussed that lncRNA CASC9 promoted esophageal squamous cell carcinoma through modulating PDCD4 by EZH2. Furthermore, there is a range of papers discussing about lncRNA XLOC series such as lncRNA XLOC_010235 in gastric cancer[8], lncRNA XLOC_006390 in cervical cancer[17],

lncRNA XLOC_008466 in human non-small cell lung cancer[3], lncRNA XLOC_010588 in cervical cancer[18]. However, the articles about lncRNA XLOC_010235 were few.

As for this paper, we firstly measured the expression level of XLOC in HCC tissues and cell lines, and found that it was up-regulated both in tissues and cells. Its high expression was associated with the terrible prognosis for HCC patients. Then, in order to examine its biological function in HCC, we decreased its expression in HCC cells. It was discovered that the knockdown of XLOC impaired cell proliferation but induced more apoptosis. Moreover, we also unmasked that the silence of XLOC blocked the migration *via* reversely regulating EMT to MET process.

Conclusions

All these findings suggested that XLOC functioned as an oncogene in the development of HCC. Such a paper discussing about lncRNA XLOC not only added new insight to the researches on lncRNA XLOC but also to that on hepatocellular carcinoma.

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Conflict of interest

The authors declare no conflicts of interest.

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