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High-expression of lncRNA CEBPA-AS1 promotes liver cancer progression

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Abstract. – OBJECTIVE: Long non-coding RNAs (lncRNAs) have been confirmed to play important roles in the progression of different cancers. The aim of this study was to detect the expression level of lncRNA CEBPA-AS1 in liver cancer and to study its influence on cell proliferation, invasion and prognosis.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, transwell assay, Western blot, Kaplan-Meier survival curve and Cox regression were used to evaluate lncRNA CEBPA-AS1 expression, cell proliferation, invasion, epithelial-mesenchymal transition (EMT)-related molecules expression and prognosis, respectively.

RESULTS: The expression of lncRNA CEBPA-AS1 increased significantly in liver cancer tissues ($p<0.05$). Meanwhile, CEBPA-AS1 expression was associated with tumor size, portal vein tumor thrombus and invasion and metastasis ($p<0.05$). *In vitro* experiments indicated that downregulation of lncRNA CEBPA-AS1 could effectively reduce cell proliferation, invasion and EMT process.

CONCLUSIONS: LncRNA CEBPA-AS1 acts as an oncogene in liver cancer, which may be a novel biomarker in liver cancer progression.

Key Words:
LncRNAs, CEBPA-AS1, Liver cancer, EMT, Invasion.

Introduction

In the world, the incidence of primary liver cancer ranks fifth among all malignant tumors. Meanwhile, its incidence has increased year by year, resulting in the third death, second only to lung cancer and stomach cancer1. Among them, primary liver cancer accounts for 85-90% of liver cell liver cancer[2]. The main cause of liver cancer in China is a viral hepatitis infection, mainly type B and type C[3]. Internal and external factors, including the contamination of *Aspergillus flavus* in the fifteenth and the pollution of drinking water in the countryside, fundamentally lead to changes in the genetic characteristics of cells. Previous studies have indicated that these changes include the inactivation of tumor suppressor genes, activation of oncogenes, chromosomal aberrations, as well as abnormalities in growth factors and their receptors. In recent years, with the enhancement of diagnostic methods, surgical techniques have been significantly improved. However, the prognosis of liver cancer has not been greatly improved, with a five-year recurrence rate of 50%-70%. Therefore, the long-term prognosis of liver cancer is still far from satisfactory[4,5]. Currently, few studies have elucidated the molecular mechanisms of hepatocellular carcinoma. Therefore, it is of great importance to search for reliable biomarkers to help predict the prognosis of patients with hepatocellular carcinoma.

CEBPA-AS1, also known as LOC80054, is located at 19q13.11. It was first found enriched in human gastric cancer tissues and plasma of patients in March 2017. A combination of CEBPA-AS1 with circulating long non-coding RNA (lncRNA) AK001058, INHBA-AS1, MIR4435-2HG in plasma has been found to be used as diagnostic or prognostic markers for gastric cancer[6]. LncRNA CEBPA-AS1 is correlated with poor differentiation, lymph node metastasis and high clinical stage in oral squamous cell carcinoma. Meanwhile, it is correlated with poor prognosis

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and promotes tumorigenesis of oral squamous cell carcinoma *via* CEBPA/Bcl27. However, the specific role of CEBPA-AS1 in hepatocellular carcinoma remains unclear.

In this work, quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the expression level of lncRNA CEBPA-AS1 in fresh liver cancer tissues. Combined with clinicopathological and follow-up data of liver cancer patients, we found that lncRNA CEBPA-AS1 expression correlated with tumor size, portal vein tumor thrombus and invasion and metastasis. LncRNA CEBPA-AS1 was silenced in HepG2 cells to observe the changes in biological behaviors of liver cancer cells. The above results indicated that lncRNA CEBPA-AS1 might play a carcinogenic in the development of hepatocellular carcinoma. Meanwhile, it as closely related to long-term prognosis of liver cancer patients. LncRNA CEBPA-AS1 was an independent factor for predicting the prognosis of liver cancer patients, and might become a new target for hepatocellular carcinoma targeted therapy. Therefore, it is necessary to further explore its specific carcinogenic effects and related mechanisms in liver cancer.

Patients and Methods

Tissue Samples

Liver cancer tissues and corresponding adjacent tissues were removed from patients who received treatment in our hospital. No patient received radiotherapy or chemotherapy before the operation. Collected liver cancer tissues were diagnosed as hepatocellular carcinoma by pathology. Adjacent normal tissues were derived from un-invasive tissues more than 3 cm from the edge of the tumor. Collected tissue samples were immediately stored in liquid nitrogen for subsequent use. Informed consent was obtained from each subject before the study. This study was approved by the Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University.

Cell Culture and SiRNA Interference Test

HepG2 liver cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 100 mL/L of fetal calf serum (FCS), penicillin and streptomycin in an incubator at 37°C with 5% CO_2. HepG2 cells were first seeded into 12-well plates 24 h before transfection. According to the relevant instructions, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and lncRNA CEBPA-AS1 siRNA were mixed, followed by incubation in a Petri dish for 20 min at room temperature for transfection. Transfection efficiency of siRNA was verified by qRT-PCR. Control siRNA was used as a control group.

Quantitative Real Time-Polymerase Chain Reaction Detection

QRT-PCR was used to detect the expression of lncRNA CEBPA-AS1 in hepatocellular carcinoma tissues and transfected cells. Total RNA in tissues or cells was first extracted according to the instructions of the TRIzol kit (Invitrogen, Carlsbad, CA, USA). The purity and concentration of extracted RNA were determined by an ultraviolet spectrophotometer. RNA sample with a ratio of 1.8 to 2.0 was subjected to reverse transcription. Subsequently, extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) according to TaKaRa (Otsu, Shiga, Japan) kit instructions. The specific reaction procedure was: 42°C for 15 min, 85°C for 5 s, and 4°C for use. Each experiment was repeated 3 times. Primer sequences used in this study were as follows: CEBPA-AS1, F: 5'-GGCACCAATTATTCCGGCTC-3', R: 5'-CGGTATTCGATGCCTCCGATAAGA-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide)

Cells were seeded into 96-well plates at a density of 1×10^3 cells/well. 20 μl MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for 4 h, followed by addition of 150 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). Finally, the absorbance wavelength was measured.

Transwell Assay

Matrix gel and serum-free medium were first diluted at 1:5, which was then added to each chamber (100 μL). The gel was coagulated after 30 min. Transfected cells were resuspended in a serum-free medium and added to the upper chamber of transwell inserts at a density of 3×10^5 cells per well. Meanwhile, 600 μL of the culture medium containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) was added to the lower chamber and cultured for 48 h. After washing, the cells were fixed with 90% ethanol for 30 min and stained with 0.1% crystal violet.

Unmigrated cells were gently wiped with a cotton swab. 5 fields of view were randomly selected for each sample. The number of migrated cells were counted under a microscope at 400 times.

Western Blot Detection

The total protein in tissues and cells was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking with 5% skim milk powder solution for 2 h, the membranes were incubated with primary antibodies of E-cadherin (Cell Signaling Technology, Danvers, MA, USA), N-cadherin (Abcam, Cambridge, MA, USA) and Vimentin (Cell Signaling Technology, Danvers, MA, USA) (diluted at 1:1000). After washing with Tris-Buffered Saline and Tween 20 (TBST; Sigma-Aldrich, St. Louis, MO, USA), the membranes were incubated with the corresponding secondary antibody (dilution concentration: 1:5000, Southern Biotech, Birmingham, AL, USA) for 2 h. Then, the membranes were washed with TBST again. The gray value of the target protein was measured by an image analyzer.

Statistical Analysis

Statistical processing was performed using GraphPad Prism 5.0 (La Jolla, CA, USA) and Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA), consistent with the normal distribution of measurement data. The relationship between the expression level of lncRNA CEBPA-AS1 and clinicopathological features of patients was evaluated by X^2 test. $p<0.05$ was considered statistically significant.

Results

Correlation Between the Expression of LncRNA CEBPA-AS1 and Clinico-Pathological Features of Patients with Hepatocellular Carcinoma

The expression of lncRNA CEBPA-AS1 in liver cancer tissues was significantly higher than that of the adjacent normal tissues (Figure 1). High expression of lncRNA CEBPA-AS1 was correlated with tumor size, portal vein tumor thrombus and invasion and metastasis ($p<0.05$). However, CEBPA-AS1 expression was not associated with age and gender ($p>0.05$, Table I). To further analyze the relationship between the expression level of lncRNA CEBPA-AS1 and the prognosis of patients with hepatocellular carcinoma, the Kaplan-Meier survival analysis was performed. The results showed that patients with a higher expression of lncRNA CEBPA-AS1 showed significantly worse prognosis than that of patients with lower expression ($p<0.001$; Figure 2). Cox multivariate regression analysis showed that tumor size (HR=2.972, $p=0.006$), portal vein tumor thrombus and invasion (HR=3.206, $p=0.018$), metastasis (HR=4.124, $p=0.022$) and lncRNA CEBPA-AS1 (HR=2.741, $p=0.009$; Table II). These results suggested that increased expression of lncRNA CEBPA-AS1 might be an independent indicator for the prognosis of hepatocellular carcinoma patients.

Downregulation of LncRNA CEBPA-AS1 Repressed Cell Proliferation

Firstly, we downregulated lncRNA CEBPA-AS1 expression in HepG2 cells by transfection of lncRNA CEBPA-AS1-siRNA. QRT-PCR results showed that the mRNA expression of lncRNA CEBPA-AS1 was significantly down-regulated in HepG2 cells transfected with lncRNA CEBPA-AS1-siRNA ($p<0.05$), suggesting successful transfection (Figure 3A). Then,

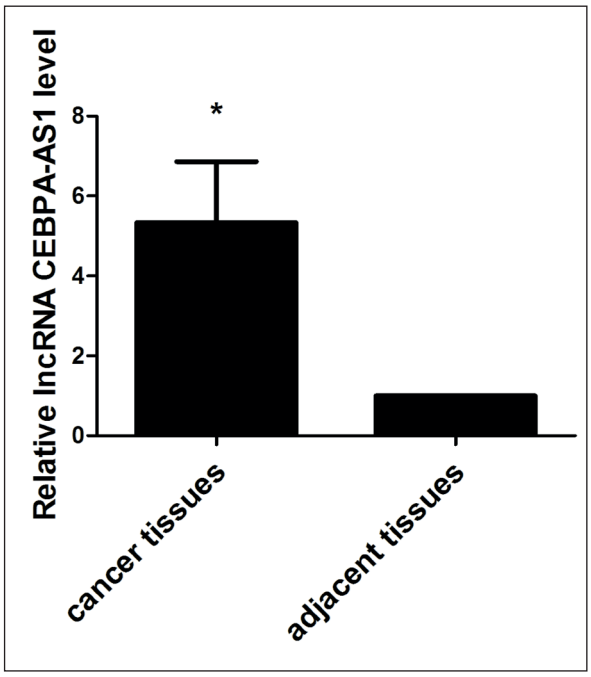

Figure 1. The expression of lncRNA CEBPA-AS1 in liver cancer tissues and adjacent tissues was detected by qRT-PCR. *$p<0.05$.

Table I. Correlation between the expression of lncRNA CEBPA-AS1 and clinicopathological features of liver cancer.

Parameters	Total (No.)	CEBPA-AS1		*p*-value
		Low (No.)	High (No.)	
Age (years)				
<50	44	20	24	
≥50	47	21	26	0.941
Gender				
Male	47	23	24	
Female	44	18	26	0.442
Tumor size (cm)				
<5	42	28	14	
≥5	49	13	36	<0.001*
Portal vein tumor thrombus and invasion				
No	49	27	22	
Yes	42	14	28	0.037*
Metastasis				
No	44	27	17	
Yes	47	14	33	0.002*

*$p<0.05$

Table II. Cox multivariate regression analysis.

Variable	HR	95%CI	p-value
Tumor size	2.972	1.375-6.426	0.006*
Portal vein tumor thrombus and invasion	3.206	1.221-8.415	0.018*
Metastasis	4.124	1.222-13.920	0.022*
LncRNA CEBPA-AS1	2.741	1.284-5.850	0.009*

*$p<0.05$

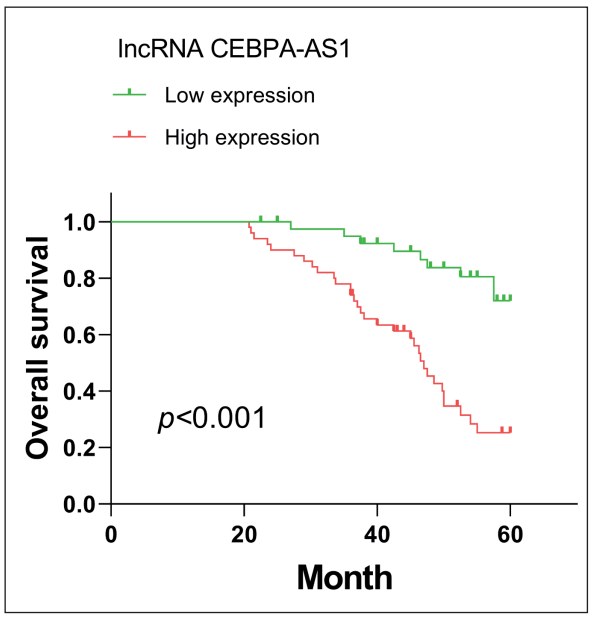

Figure 2. Kaplan-Meier survival analysis was performed.

MTT assay was performed to detect the effect of lncRNA CEBPA-AS1 on cell proliferation. The results indicated that the proliferative rate of cells transfected with lncRNA CEBPA-AS1-siRNA was significantly lower than that of the control group (Figure 3B). The above findings revealed that downregulation of lncRNA CEBPA-AS1 repressed cell proliferation.

Knockdown LncRNA CEBPA-AS1 Inhibited Cell Invasion

Based on the relationship between high expression of lncRNA CEBPA-AS1 and metastasis in liver cancer, transwell assay was then performed to assess the potential regulatory function of lncRNA CEBPA-AS1 on cell invasion *in vitro*. Interestingly, compared with the control group, the number of invading cells transfected with lncRNA CEBPA-AS1-siRNA was significantly reduced (Figure 3C), suggesting that knockdown lncRNA CEBPA-AS1 inhibited cell invasion.

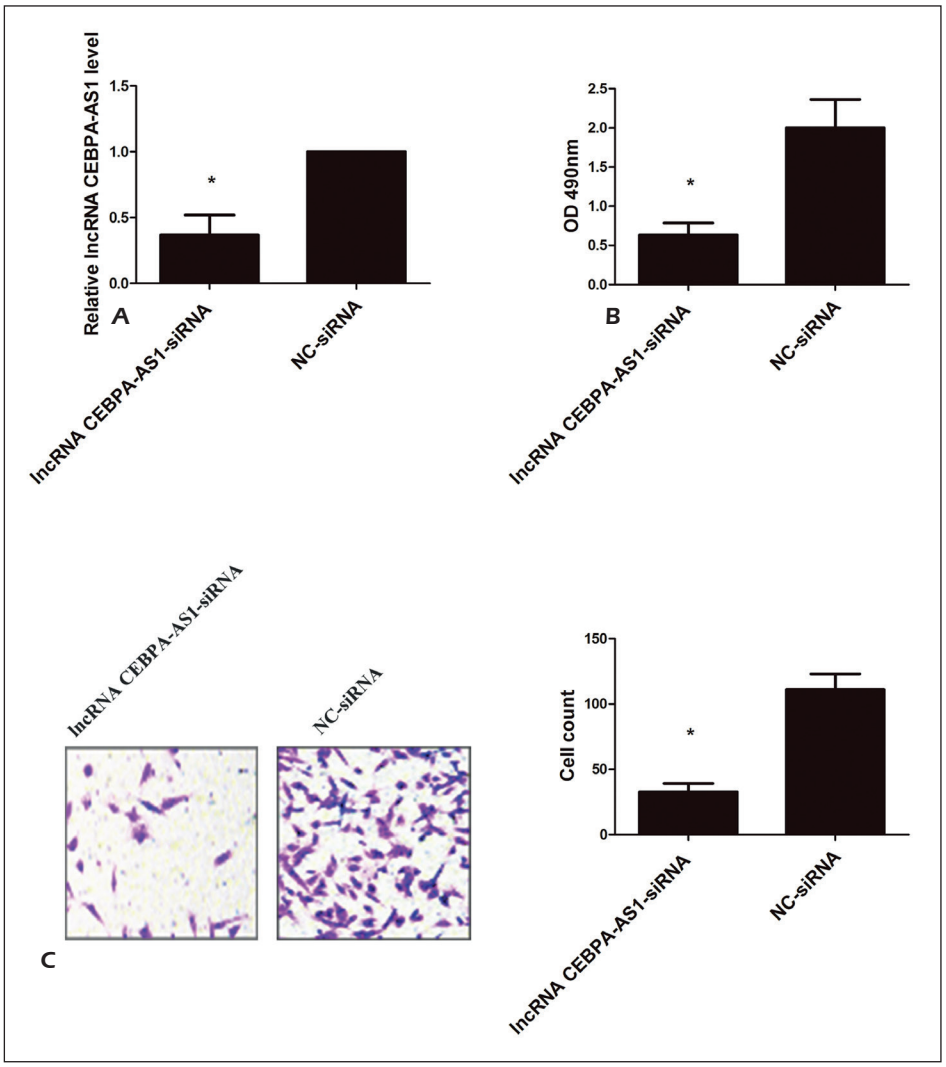

Figure 3. Downregulation of lncRNA CEBPA-AS1 repressed cell proliferation and invasion. **A**, The mRNA expression of lncRNA CEBPA-AS1 was down-regulated in HepG2 cells transfected with lncRNA CEBPA-AS1-siRNA. **B**, The effect of lncRNA CEBPA-AS1 on cell proliferation was detected by MTT assay. **C**, The function of lncRNA CEBPA-AS1 on cell invasion was detected by transwell assay (magnification × 400). *$p<0.05$.

Downregulation of LncRNA CEBPA-AS1 Impacted EMT Process

To evaluate whether lncRNA CEBPA-AS1 could influence EMT in liver cancer cells, we conducted qRT-PCR and Western blot assays. Interestingly, the results demonstrated that compared with the NC-siRNA group, both the mRNA and protein expressions of E-cadherin increased significantly in the lncRNA CEBPA-AS1-siRNA group. However, the mRNA and protein expression levels of N-cadherin and Vimentin were markedly down-regulated in the lncRNA CEBPA-AS1-siRNA group (Figure 4A, 4B). These findings demonstrated that downregulation of lncRNA CEBPA-AS1 impacted the EMT process.

Discussion

Non-coding RNAs (ncRNAs) are a kind of RNA molecules that are widely present in living organisms. The previous studies[8-11] showed that ncRNAs play important roles in regulating life activities. According to different sizes, structures and functions, ncRNAs can be classified into miRNA, Piwi interaction RNA (piRNA) and lncRNA12. In recent years, the role of lncRNA and miRNA in tumorigenesis and development is a hot topic. LncRNA is a non-coding RNA with over than 200 bp in length. It can regulate gene expression through genetic regulation, transcriptional regulation and post-transcriptional regulation[13-16]. The results of this research disclosed that the expression of ln-

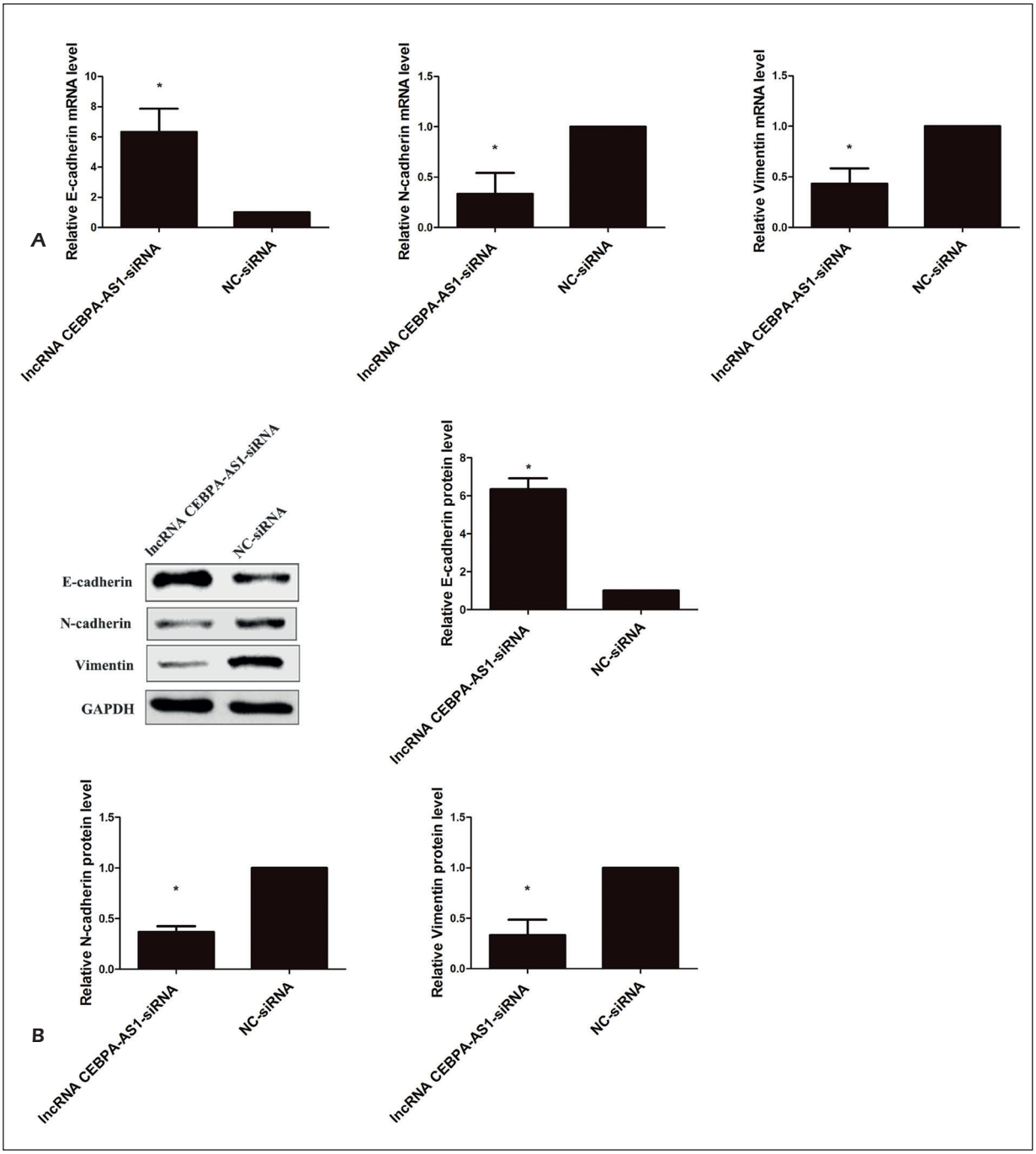

Figure 4. Downregulation of lncRNA CEBPA-AS1 impacted EMT process. To evaluate whether lncRNA CEBPA-AS1 influenced EMT in liver cancer cells, we conducted qRT-PCR **A**, and Western blot **B**, assays. *$p<0.05$.

cRNA CEBPA-AS1 increased significantly in liver cancer tissues, acting as an oncogene.

High expression of lncRNA CEBPA-AS1 was found significantly correlated with tumor size, portal vein tumor thrombus and invasion and metastasis. Patients with a higher expression of lncRNA CEBPA-AS1 showed remarkably worse prognosis than those with lower expression. Cox multivariate regression analysis showed that tumor size, portal vein tumor thrombus and invasion, metastasis and lncRNA CEBPA-AS1. All the results demonstrated that lncRNA CEBPA-AS1 was related to liver cancer development. Meanwhile, these findings prompted us to investigate the exact role and function of lncRNA

CEBPA-AS1 in liver cancer *in vitro*. MTT assay results indicated that the proliferative rate of cells transfected with lncRNA CEBPA-AS1-siRNA was significantly lower than that of the control group. This revealed that downregulation of lncRNA CEBPA-AS1 repressed cell proliferation. The transwell assay demonstrated that the number of invading cells transfected with lncRNA CEBPA-AS1-siRNA was remarkably reduced, suggesting that knockdown lncRNA CEBPA-AS1 inhibited cell invasion. Downregulated lncRNA CEBPA-AS1 led to an inhibitory effect on cell proliferation and invasion. However, the potential mechanism of this inhibition regulation still needs to be explored in the future.

EMT is a biological process in which epithelial cells lose cell adhesion and obtain mesenchymal cell invasion characteristics. Loss of E-cadherin, and upregulated expressions of mesenchymal markers (Vimentin and N-cadherin) are important changes in the EMT process. These findings suggest that the mechanism of EMT is complex[17,18]. Increasing studies have reported that EMT is closely implicated in cell invasion and metastasis of various malignancies, such as breast cancer[19,20], colon cancer[21,22], and lung cancer[23,24]. However, its specific mechanism needs further verification. Current studies have demonstrated that lncRNAs play important roles in the EMT process. For example, lncRNA CPS1-IT1 suppresses EMT and metastasis *via* repressing hypoxia-induced autophagy by inactivation of HIF-1α in colorectal cancer[25]. LncRNA H19 promotes EMT by targeting miR-484 in human lung cancer cells[26]. LncRNA ATB promotes EMT during silica-induced pulmonary fibrosis by competitively binding miR-200c[27]. LncRNA FEZF1-AS1 enhances EMT by suppressing E-cadherin and regulating the Wnt pathway in non-small cell lung cancer (NSCLC)[28]. In the present study, qRT-PCR and Western blot results showed that compared with the si-NC group, the mRNA and protein expressions of E-cadherin increased significantly in the lncRNA CEBPA-AS1-siRNA group, whereas N-cadherin and Vimentin were markedly down-regulated. All these findings illustrated that downregulation of lncRNA CEBPA-AS1 impacted EMT process.

Conclusions

Our study demonstrated for the first time that lncRNA CEBPA-AS1 increased significantly in liver cancer. High expression of lncRNA CEBPA-AS1 was related to tumor size, portal vein tumor thrombus and invasion, metastasis and poor prognosis. Downregulation of lncRNA CEBPA-AS1 markedly inhibited the proliferation and invasion of liver cancer cells, and repressed EMT process. All the findings might provide strong evidence that lncRNA CEBPA-AS1 acted as an oncogene in liver cancer, and could be a new biomarker in liver cancer progression.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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