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# MicroRNA-876-5p inhibits epithelial-mesenchymal transition and metastasis of hepatocellular carcinoma by targeting BCL6 corepressor like 1

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ABSTRACT

Our previous study has reported that BCL6 corepressor like 1 (BCORL1) plays an oncogenic role in hepatocellular carcinoma (HCC) via promoting epithelial-mesenchymal transition (EMT) and tumor metastasis. However, the regulation of BCORL1 mediated by microRNAs (miRNAs) remains poorly known. The analysis of our clinical samples indicated that BCORL1 expression was markedly higher in HCC tissues than that in tumor-adjacent normal tissues. The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) datasets revealed that high BCORL1 expression associated with high tumor grade, advanced tumor stage and poor survival of HCC patients. miR-875-5p expression was down-regulated and negatively correlated with BCORL1 mRNA expression in HCC tissues. Furthermore, miR-876-5p inversely regulated BCORL1 abundance in HCC cells by directly targeting the 3′-untranslated region (3′-UTR) of BCORL1. Ectopic expression of miR-876-5p suppressed cell migration and invasion in both HCCLM3 and MHCC97H cells. In accordance, miR-876-5p knockdown promoted the metastatic behaviors of Hep3B cells. Mechanistically, miR-876-5p suppressed the EMT progression of HCC cells. HCC tissues with high miR-876-5p level showed a higher E-cadherin staining compared to cases with low miR-876-5p level. Moreover, the repression of cell metastasis mediated by miR-876-5p was rescued by BCORL1 restoration in HCCLM3 cells. Notably, low miR-876-5p expression associated with venous infiltration, high tumor grade and advanced tumor stage. HCC patients with low miR-876-5p expression had a significant poorer overall survival and disease-free survival. To conclude, miR-876-5p inhibits EMT progression, migration and invasion of HCC cells by targeting BCORL1. Therefore, miR-876-5p/BCORL1 axis may represent as a novel therapeutic target for HCC treatment.

## 1. Introduction

Hepatocellular carcinoma (HCC) ranks the 3rd leading cause of cancer-related deaths worldwide, and approximately 750,000 new cases of HCC are diagnosed each year [1]. Over the past decades, the better understanding of epidemiologic risk factors, developments of imaging diagnosis, improvement of surgical techniques and discovery of targeted drugs have obviously improved the prognosis of HCC patients. But the long-term survival of patients with advanced HCC is still unsatisfactory due to the high recurrence and metastasis rate [2]. Therefore, it is necessary to disclose the molecular mechanism of tumor metastasis and develop new treatment strategies for HCC.

BCL6 corepressor like 1 (BCORL1) is primarily reported as a corepressor, which represses E-cadherin expression by interacting with C-terminal Binding Protein (CtBP) corepressor [3]. Somatic mutation analysis suggest BCORL1 as a novel candidate tumor suppressor gene in

*Abbreviations:* BCORL1, BCL6 corepressor like 1; HCC, hepatocellular carcinoma; EMT, epithelial-mesenchymal transition; miRNAs, microRNAs; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; 3′-UTR, 3′-untranslated region; CtBP, C-terminal Binding Protein; CREBBP, CREB binding protein; OFMT, ossifying fibromyxoid tumor; NSCLC, non-small cell lung cancer; ELF74, E74 like ETS transcription factor 4; qRT-PCR, quantitative real-time PCR; IHC, immunohistochemistry; EV71, enterovirus 71; cHL, classical Hodgkin lymphoma; BMP-4, bone morphogenetic protein 4

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adult acute myelogenous leukemia [4]. CREB binding protein (CREBBP)-BCORL1 fusion may be implicated in the pathogenesis of ossifying fibromyxoid tumor (OFMT) via epigenetic deregulation [5]. BCORL1 is highly expressed in human non-small cell lung cancer (NSCLC) and promotes migration and invasion of cancer cells probably by repressing E-cadherin [6]. BCORL1- E74 like ETS transcription factor 4 (ELF4) fusion transcript is identified and overexpressed in HCC compared to noncancerous liver tissues [7]. Our previous study reveals that increased expression of BCORL1 is associated with metastatic features of HCC and indicates poor prognosis of patients [8]. Functionally, BCORL1 facilitates epithelial-mesenchymal transition (EMT) and HCC cell metastasis via repressing E-cadherin [8]. However, the mechanism underlying the upregulation of BCORL1in HCC remains unclear.

MicroRNAs (miRNAs) are a class of endogenously expressed small non-coding RNAs with a length of approximately 22 nucleotides [9]. MiRNAs post-transcriptionally modulates gene expression by incompletely base-pairing with the 3′-untranslated region (3′-UTR) of target mRNAs [10]. Our previous studies demonstrate that miRNAs, such as miR-542-3p, miR-1296, miR-187-3p, miR-519a, and miR-1468, regulate growth, EMT and metastasis of HCC by suppressing different target genes [11–15]. However, the specific miRNAs involved in the regulation of BCORL1 in HCC has not been identified.

In this study, we further confirmed the expression level of BCORL1 and disclosed its clinical significance in HCC. We also recognized a novel miRNA involved in the regulation of BCORL1, as well as EMT and metastasis in HCC cells. BCORL1 overexpression and miR-876-5p underexpression were detected in HCC tissues. miR-876-5p suppressed EMT, migration and invasion of HCC cells via directly targeting BCORL1.

## 2. Materials and methods

### 2.1. Patients and tissue samples

Eighty HCC and tumor-adjacent tissues that were surgically resected in the Department of Hepatobiliary Surgery, 1st Affiliated Hospital of Xi'an Jiaotong University were enrolled in this study. All samples were histologically confirmed by two professional pathologists. Patients, who received radiotherapy, chemotherapy and radiofrequency ablation before surgery, were excluded. The study was approved by Ethic Committee of 1st Affiliated Hospital of Xi'an Jiaotong University in accordance with the guidelines outlined in the Declaration of Helsinki and all participants signed informed consent statements.

### 2.2. Cell culture and transfection

HCC cell lines including Hep3B, HepG2, HCCLM3, MHCC97H and normal human hepatic cell line LO2 were obtained from Shanghai Cell Bank of Chinese Academy of Science (Shanghai, China) and maintained in a standard culture condition [8]. To modulate miR-876-5p level, miR-876-5p mimics (HMI0929; Sigma-Aldrich, St. Louis, MO, USA) and miR-876-5p inhibitors (HSTUD0929, Sigma-Aldrich) were respectively transfected into HCC cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). For the BCORL1 restoration experiment, HCCLM3 cells engineered to stably express BCORL1 cDNA by transduction with a lentiviral vector [8].

### 2.3. Quantitative real-time PCR (qRT-PCR)

The total RNA from HCC tissues and cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara, Dalian, China). The mRNA expression levels of BCORL1 and miR-876-5p were measured by qPCR with a SYBR® Premix Ex Taq™ II (Perfect Real-Time) kit (Takara) in the Applied Biosystems 7500 Sequence Detection system. GAPDH and U6 were used as internal controls. All primers were designed and synthesized by Sangon Biotech (Shanghai, China).

### 2.4. Western blotting

All Western blotting analyses were performed as described previously [8]. In brief, 48–72 h after transfection, HCC cells were lysed with RIPA buffer and the absorption spectrophotometry (BIO-RAD, Hercules, CA) was used to measure protein concentration. Then, 5–10 μg protein was separated by SDS-PAGE and transferred to PVDF membranes. The primary antibodies were BCORL1 (PA5-24333, Thermo Fisher Scientific Pierce, Waltham, MA, USA), E-cadherin (ab1416, Abcam, Cambridge, MA, USA), Vimentin (ab92547, Abcam), N-cadherin (ab18203, Abcam) and GAPDH (sc-47724, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibodies were sheep-anti-mouse or donkey-anti-rabbit horseradish peroxidase(HRP)-conjugated antibody (NXA931-1ML and NA934-1ML, GE Healthcare Life Sciences, Beijing, China). The blots were detected with Chemiluminescence Reagents (Sigma, St-Louis, MO, USA) using Amersham™ Imager 680 from GE Healthcare Life Sciences.

### 2.5. Luciferase reporter assay

The 3′-UTR of BCORL1 containing predicted miR-876-5p binding sites (both wild type and mutant) were cloned into the pGL3 luciferase reporter vectors (Promega, Madison, WI, USA). For the luciferase reporter assay, Hep3B cells were transfected with different combinations of miR-876-5p mimics or inhibitors and pGL3-BCROL1 3′-UTR wild type or mutant. The relative luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and normalized to Renilla activity.

### 2.6. Cell proliferation, migration and invasion assay

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's recommendations [15]. Cell migration and invasion was determined using matrigel-uncoated and -coated transwell insert chambers (8 μm pore size; Millipore, Billerica, MA, USA). HCC cells ($2 \times 10^4$) that were transfected with corresponding were harvested and added to the upper chamber in 150 μL serum-free medium. Then 700 μL of chemoattractants (medium supplemented with 20% FBS) was added to the basal chambers. After incubating for 24 h, migrated and invaded cells were fixed with 20% methanol and stained with 0.1% crystal violet. Cells beneath the upper chambers were counted under microscope.

### 2.7. Immunohistochemistry (IHC)

IHC analysis of E-cadherin was performed according to the protocol in our previous study [16]. Briefly, sections were deparaffinized and rehydrated in xylene and graded ethanol, respectively. Then, 0.01 M citrate buffer (pH = 6.0) and 3% hydrogen peroxide were used for antigen retrieval and blocking of endogenous peroxidase activity. Subsequently, sections were incubated with primary antibody against E-cadherin (Abcam) overnight at 4 °C. The following day, sections were incubated with HRP-conjugated secondary antibody (ZSGB-BIO, Beijing, China).

### 2.8. Statistical analysis

All data are presented as the mean ± SD and subjected to GraphPad Prism software version 5.0 (San Diego, CA, USA) for statistical analysis. Data were compared using two-tailed Student's t-test and ANOVA. The overall survival (OS) and disease-free survival (DFS) between two groups was analyzed using Kaplan-Meier method and Log-rank test. The correlation of miR-876-5p expression with