

Long Noncoding RNA *EBLN3P* Promotes the Progression of Liver Cancer via Alteration of microRNA-144-3p/*DOCK4* Signal

This article was published in the following Dove Press journal:
Cancer Management and Research

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Background: Therapy for patients with liver cancer in the advanced stage remains a great challenge, and there are very few approved treatments. Although accumulated evidence demonstrates the importance of lncRNAs in liver cancer, data on the functional roles and molecular mechanisms of endogenous bornavirus-like nucleoprotein (*EBLN3P*) have been rarely reported.

Materials and Methods: The bioinformatics prediction software ENCORI was used to predict the putative binding sites of *EBLN3P*. The regulatory roles of *EBLN3P* and *miR-144-3p* in cell proliferation, migration and invasion ability were verified by the Cell Counting Kit-8, wound healing and Transwell assays, respectively. The interactions among *EBLN3P*, *miR-144-3p* and *DOCK4* were explored by a luciferase assay and Western blotting. The expression of *EBLN3P* and microRNA (miR)-144-3p in liver cancer tissues was quantified by reverse transcription-quantitative PCR, and the expression of dedicator of cytokinesis 4 (*DOCK4*) was quantified by immunohistochemical analysis.

Results: The present results revealed that overexpression of *EBLN3P* or knockdown of *miR-144-3p* promoted liver cancer cell proliferation, migration and invasion. Bioinformatics analysis and a luciferase assay demonstrated that *EBLN3P* directly interacts with *miR-144-3p* to attenuate *miR-144-3p* binding to the 3'-untranslated region of *DOCK4*. Furthermore, the mechanistic investigations showing that the miR-144-3p/*DOCK4* regulatory loop was activated by knockdown of miR-144-3p or overexpression of *DOCK4* validate the roles of *EBLN3P* in promoting liver cancer cell proliferation, migration and invasion in vitro. Elevated levels of *EBLN3P* and *DOCK4* and decreased *miR-144-3p* expression were observed in both liver cancer tissues and cell lines.

Conclusion: The present study is the first to demonstrate that *EBLN3P* may act as a ceRNA to modulate *DOCK4* expression by competitively sponging *miR-144-3p*, leading to the regulation of liver cancer progression, which provides new insights for liver cancer diagnosis and treatment.

Keywords: competing endogenous RNAs, dedicator of cytokinesis 4, long noncoding RNAs, microRNAs, liver cancer, endogenous bornavirus-like nucleoprotein

Introduction

Liver cancer is one of the most common types of cancer in the world.¹ Although its incidence and mortality rates have declined significantly in western countries, liver cancer is still a significant health threat in certain regions of the world, such as China and Japan.^{2,3} Surgery with radical resection is suitable for only a limited percentage of patients, whereas many patients are often diagnosed at later stages or

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experience postsurgical diseases such as relapse or metastasis, which worsens their prognoses.⁴ Comprehensive treatment of advanced liver cancer remains unsatisfactory, and thus, it is of vital importance to elucidate the detailed mechanisms of liver cancer progression.⁵

Long noncoding RNAs (lncRNAs), defined as genome transcripts consisting of >200 nucleotides that are not translated into proteins, are associated with various biological developmental processes, such as tumor proliferation and metastasis.⁶ Tumor gene expression can be positively or negatively modulated by lncRNAs, either through epigenetic transcriptional regulation or posttranscriptional regulation.⁷ Epigenetically, lncRNAs may interact with the transcription preinitiation complex at the promoter region or directly form base pairs with RNA and DNA.⁸ Posttranscriptionally, lncRNAs act as precursors of microRNAs (miRNAs/miRs) and competing endogenous RNAs (ceRNAs) to control cell fate.⁹ Among those regulatory RNAs, ceRNAs, which regulate gene expression via miRNA mediation, have been widely studied and recognized.¹⁰ It has been reported that the interaction of human epidermal growth factor receptor 2 (HER2) with miR-331-3p may underlie the oncogenic roles of the lncRNA Hox transcript antisense intergenic RNA (HOTAIR) in gastric cancer.^{11,12} Similarly, another study illustrated that lncRNA H19 has oncogenic functions, which can be attributed to its ceRNA activity through which it sequesters miR-138 and miR-200a in colorectal cancer (CRC).¹³ A previous study revealed that *miR-144-3p* is associated with abnormal expression in various tumors. It has been reported that *miR-144-3p* facilitates the progression of nasopharyngeal carcinoma via directly targeting phosphatase and tensin homolog (PTEN).¹⁴ However, the roles of lncRNA *EBLN3P* and *miR-144-3p* in liver cancer remain unclear.

In mammals, the Rho GTPase family plays critical roles in cell proliferation, cell motility, tumor cell malignant transformation, and cancer metastasis and invasion.¹⁵ Guanine nucleotide exchange factors (GEFs) “turn on” Rho GTPases by inducing the exchange of GDP for GTP.¹⁶ Rho GEFs include Db1-related classical GEFs and atypical Dock family Rho GEFs.¹⁷ *DOCK4* is a member of the Dock180 family of proteins, which mediates the outgrowth of patient-derived glioblastoma cells through activation of Rac.¹⁸ However, the expression profile and regulatory mechanism of *DOCK4* in liver cancer remains unknown. In a previous study, the ceRNA network that may regulate *DOCK4* expression was discovered by

bioinformatics analysis and verified by molecular biology techniques. The data revealed that the pseudogene-derived lncRNA *EBLN3P* can act as a ceRNA that bind with *miR-144-3p* and further upregulates the protein expression of its target *DOCK4*. *EBLN3P* is a pseudogene of endogenous bornavirus-like nucleoprotein 3 (EBLN3) on chromosome 9. However, its expression and its effects in human disease have not been reported. The aim of the present study was to explore the biological effects of *DOCK4* in liver cancer progression and its potential regulatory mechanisms. It was demonstrated that *DOCK4* is highly expressed in liver cancer tissues and that the *EBLN3P*/miR-144-3p/*DOCK4* regulatory loop axis may be a potential therapeutic target for liver cancer treatment.

Materials and Methods

RNA Extraction and Reverse Transcription-Quantitative PCR

Nuclear RNA and cytoplasmic RNA were separately isolated from cell lines using the Cytoplasmic & Nuclear RNA Purification Kit (Norgen Biotek Corp) according to the manufacturer’s protocol. Total RNA was extracted from tissues and cell lines using an RNA extraction kit (Takara Biotechnology Co., Ltd.). Reverse transcription into cDNA was conducted with Prime Script RT Master Mix (Takara Biotechnology Co., Ltd.) according to the manufacturer’s protocol. qPCR was performed via a SYBR green-based fluorescence method (SYBR Premix Ex Taq Kit; Takara Biotechnology Co., Ltd.) and the MX3000P[®] qRT-PCR system (Agilent Technology, Inc.) according to the following parameters: 95°C for 10 min followed by 40 cycles of 95°C for 10 sec and 60°C for 50 sec. The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method. GAPDH and U6 were used as the internal references for mRNA and miRNA expression, respectively. The primer sequences are listed in Table 1.

Western Blot Assay

The Western blot procedure was carried out as previously described.²⁰ Cells were plated in each well of a 6-well plate and cultured in DMEM supplemented with 10% FBS. Then, the cells were washed with ice-cold phosphate-buffered saline (PBS) three times, and cell lysates were prepared with RIPA lysis buffer (Beyotime Institute of Biotechnology). Proteins were extracted from liver tissues using T-PER Tissue Protein Extraction Reagent (cat. no. 78,510; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. Subsequently,

Table 1 Primer Sequences for Quantitative Real-Time PCR

Gene	Forward Sequence	Reverse Sequence
<i>EBLN3P</i>	5'-CAGACTAAAGGATCAAGCGAGA-3'	5'-ATCAATTGCCACAGGTTGAAGA-3'
<i>hsa-miR-144-3p</i>	5'-GCCCTACAGTATAGATGATGTA-3'	5'-GGATGCAGGTGCTGGAGGT-3'
<i>DOCK4</i>	5'-GGATACCTACGGAGCACGAG-3'	5'-AGCCATCACACTTCTCCAGG-3'
<i>U6</i>	5'-CTCGCTTCGGCAGCAC-3'	5'-AACGCTTCACGAATTTGCGT-3'
<i>GAPDH</i>	5'-CCAGTGGTCTCCTCTGA-3'	5'-GCTGTAGCCAAATCGTTGT-3'

the protein concentration was determined using a BCA assay kit (Bio-Rad Laboratories, Inc.). The proteins were loaded and electrophoresed on a 10% SDS-PAGE gel. Subsequently, the proteins were transferred onto nitrocellulose membranes (EMD Millipore). The membranes were blocked with 5% skimmed milk for 1 h and coincubated with an anti-*DOCK4* primary antibody (1:10,000; cat. no. ab85723; Abcam) at 4°C overnight. The membranes were washed three times with TBST containing 0.1% Tween 20. Following coincubation with HRP-labeled goat anti-mouse/rabbit IgG (1:5000; Sigma-Aldrich; Merck KGaA) for 2 h at room temperature, the bands were analyzed by an ECL detection kit (Pierce; Thermo Fisher Scientific, Inc.).

Immunohistochemical (IHC) Analysis

Routine hematoxylin and eosin staining was performed prior to immunohistochemical analysis. Briefly, paraffin-embedded samples were cut into 3- μ m sections and then dewaxed with xylene and rehydrated in graded ethanol. Antigen retrieval was conducted with citrate buffer, pH6.0 (1:300; cat. no. ZLI-9065; OriGene Technologies, Inc.) For antigen retrieval, the sections were heated at 97°C for 20 min. Following a brief proteolytic digestion and peroxidase blocking, the sections were incubated with a *DOCK4* polyclonal antibody (1:500; cat. no. ab85723; Abcam) overnight at 4°C, and HRP/Fab polymer conjugate (1:1000; cat. no. PV-6000-D; OriGene Technologies, Inc.) was applied as the secondary antibody. Finally, the sections were stained with diaminobenzidine substrate and counterstained with hematoxylin. Two independent investigators semiquantitatively evaluated *DOCK4*-positive staining without prior knowledge of the clinicopathologic data. The final immunoreactivity scores (IRSs) were assessed based on the percentage of positively stained cells (0 points, 0–5% positive cells; 1 point, 6–25% positive cells; 2 points, 26–50% positive cells; 3 points, 51–75% positive cells; and 4 points, 76–100% positive cells) as well as staining intensity scores (0 points, no staining; 1 point, weak staining; 2 points, moderate staining; and 3

points, strong staining). A final IRS of >4 indicated strong positivity, while all other scores indicated weak positivity.

Cell Culture

The hepatocellular carcinoma cell lines (PLHC1, Hep3B, and SNU398), a hepatoblastoma cell line (HepG2) and a human hepatocyte cell line (THLE3) were obtained from the American Type Culture Collection and had been authenticated by short tandem repeat profiling. Dulbecco's modified Eagle medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) was used to culture all cells at 37°C in 5% CO₂.

Cell Transfection and Plasmid Construction

NC (negative control) mimics, *miR-144-3p* mimics, NC inhibitor and *miR-144-3p* inhibitor were acquired from Guangzhou RiboBio Co., Ltd. The *EBLN3P* overexpression plasmid pcDNA-EBLN3P, the *EBLN3P* knockdown siRNA plasmid si-EBLN3P and a corresponding negative control siRNA (si-NC) were synthesized by Shanghai GenePharma Co., Ltd. Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transfection according to the manufacturer's instructions. Cells were collected for further use after 48 h of transfection.

Cell Counting Kit-8 (CCK-8) Assay

The CCK-8 assay (Dojindo Molecular Technologies, Inc.) was used to evaluate cell proliferation capacity as previously described.¹⁹ Briefly, 2x10⁴ cells were seeded in a 96-well plate in triplicate for each condition. At different time points (12, 24, 36, 48, 60 and 72 h), 10 μ L of CCK-8 solution was added to each well for an additional 4 h of incubation at 37°C. The absorbance was recorded at a wavelength of 490 nm by a microplate reader (Thermo Fisher Scientific, Inc.).

Cell Colony Formation Assay

For the colony formation assay, cells were seeded in 6-well plates at a density of 1×10^2 cells/well and then cultured for 15 days under standard conditions. Thereafter, the cell colonies were washed with PBS twice, subsequently fixed in 70% methanol for 10 min and stained with 0.5% crystal violet for 5 min. Finally, images of the cell colonies were captured, and the colonies were counted.

Wound Healing Assay

The cells were seeded in 6-well plates and incubated until they reached 90% confluence in serum-free medium. A sterile pipette tip was used to create ~1-mm-wide wounds. The detached cells were gently washed off twice, and the medium was replaced with complete medium containing 1% FBS. Images of the wound were captured at 0, 24 and 48 h using a light microscope.

Transwell Assay

Cells were seeded on Matrigel-coated upper chambers for the invasion assay (BD Biosciences). Culture medium without FBS and culture medium containing 10% FBS was added to the upper and lower wells, respectively, and the cells were incubated for another 24 h. After wiping off the noninvaded cells, the filters were fixed in 90% ethanol and stained with crystal violet. Cells were counted in random fields perform each chamber by using an inverted microscope (Olympus Corporation). The procedure was carried out as described previously.²¹

Dual-Luciferase Reporter Assays

The assay was carried out as described previously.²² Wild-type and mutant *EBLN3P* and *DOCK4* reporter plasmids (*EBLN3P*-WT-luc, *EBLN3P*-MUT-luc, *DOCK4*-WT-luc, *DOCK4*-MUT-luc) containing wild-type or mutant *miR-144-3p* mimics or NC binding site mimics, were synthesized by Shanghai GenePharma Co., Ltd. The synthesized reporter plasmids were cotransfected with *miR-144-3p* mimics or NC mimics with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) when the cells reached 70% confluence. Luciferase activity was analyzed via the dual-luciferase reporter assay system (Promega Corporation) after 48 h.

Clinical Tissue Sample Collection

Tumor specimens were collected from 74 patients with hepatocellular carcinoma (HCC), and 72 nonneoplastic

hepatic tissues were collected from patients who underwent surgery at The First Hospital of Jilin University. The patients were selected based on the following criteria: pathological diagnosis of HCC; no history of anticancer therapy before tumor resection; and no diagnosis of any additional malignancies. Pathological staging was based on the UICC/AJCC TNM Classification (8th edition of 2016). Collection of clinical specimens was approved by the Ethics Committee of the First Hospital of Jilin University according to the Declaration of Helsinki (approval no. JLU01698). Informed consent was collected from all participants. The status of each HCC patient was validated by an outpatient or telephone interview in order to construct a 5-year Kaplan-Meier survival curve. Patients who did not survive due to diseases other than HCC were excluded from the present study.

Statistical Analysis

All experimental data are presented as the mean \pm SD, and each experiment was conducted in triplicate. Statistical analyses were performed and graphs were generated with SPSS V17.0 (SPSS, Inc.) and GraphPad Prism V5.02 (GraphPad Software, Inc.) software. Comparisons between two groups were performed with the Student's *t*-test, and ANOVA with Dunnett's multiple comparisons test was used for multiple group comparisons. $P < 0.05$ was considered to indicate a statistically significant difference. The association between the survival rate of patients with liver cancer and the *DOCK4*, *miR-144-3p* or *EBLN3P* expression level was investigated using Kaplan-Meier survival curves and the Log rank test. Additionally, the χ^2 test was applied for association analysis of clinical case indicators.

Results

EBLN3P Knockdown Suppressed the Proliferation, Migration and Invasion of Liver Cancer Cells in vitro

Bioinformatics analysis demonstrated that *EBLN3P* can directly interact with *miR-144-3p* and that *miR-144-3p* can directly interact with *DOCK4* (Figure 1A). Furthermore, dual-luciferase reporter assays were performed to verify this hypothesis. *miR-144-3p* was overexpressed by transfecting hepatoblastoma HepG2 cells with *miR-144-3p* mimics. The luciferase assay results demonstrated that transfection of *miR-144-3p* mimics significantly weakened the luciferase signal of reporter plasmids containing *EBLN3P*-WT but had no influence on the activity of

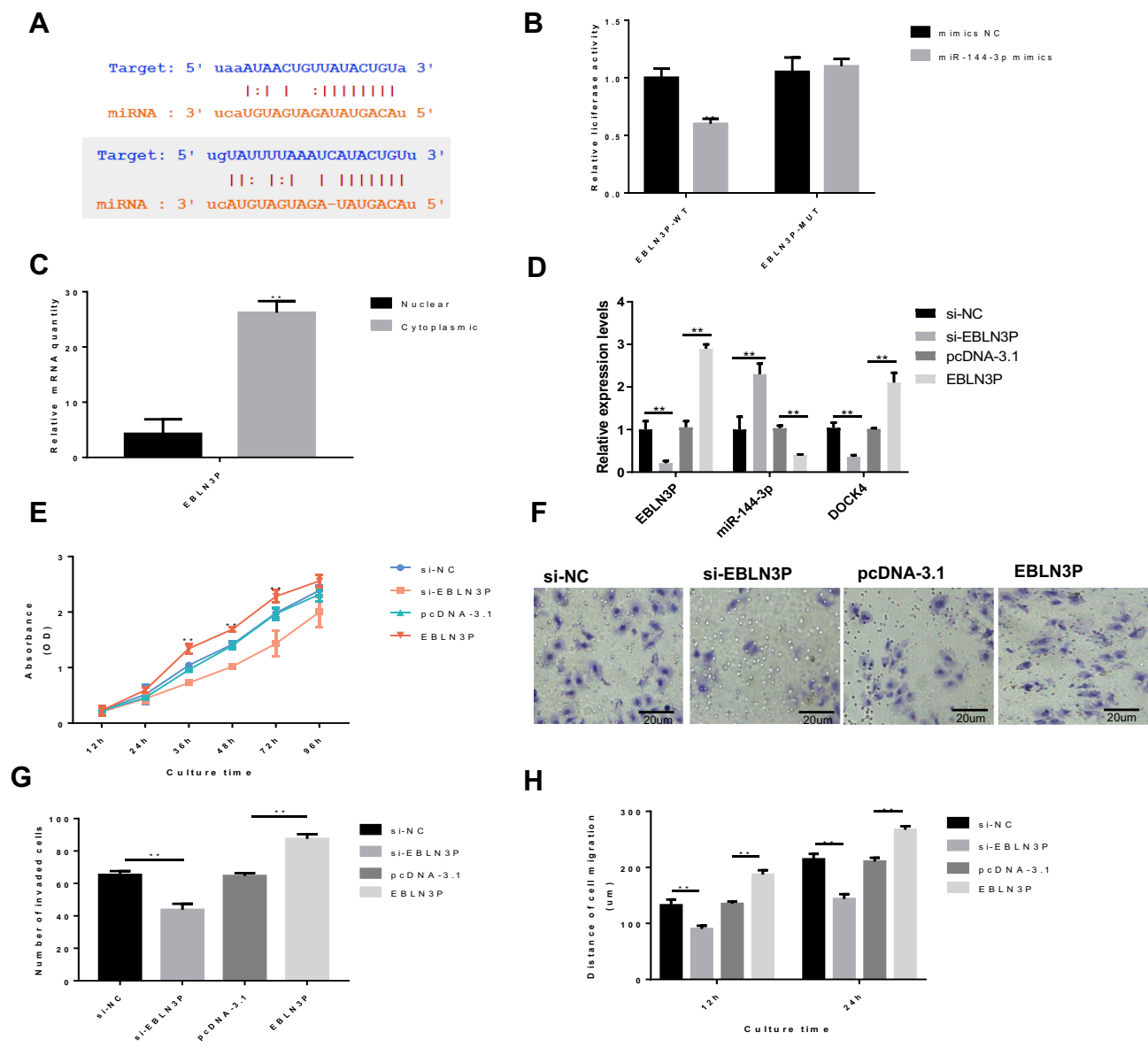


Figure 1 EBLN3P knockdown inhibits the proliferation, migration and invasion of liver cancer cells in vitro. HepG2 cells were transfected with *EBLN3P*-targeted siRNA (si-*EBLN3P*), negative control siRNA (si-NC), pcDNA3.1-*EBLN3P* overexpression vector (pcDNA-*EBLN3P*) or blank vector plasmid (pcDNA-3.1). **(A)** Bioinformatics analysis demonstrated that *EBLN3P* can directly interact with *miR-144-3p* (up) and that *miR-144-3p* can directly bind to the 3'-UTR regions of *DOCK4* (down). **(B)** Dual-luciferase reporter assays were performed to verify the impact of *miR-144-3p* on the luciferase signal of reporter plasmids containing *EBLN3P*. **(C)** The nuclear and cytoplasmic expression levels of *EBLN3P* in HepG2 cells. **(D)** The expression levels of *EBLN3P* and *miR-144-3p* were explored via reverse transcription-quantitative PCR. **(E)** Cell proliferation was tested by the Cell Counting Kit-8 assay. **(F)** The number of invaded cells. **(G)** Cell invasion was detected by the Transwell assay. **(H)** Cell migration was detected by the wound healing assay. That data is shown as the mean \pm SD. ** $P < 0.01$.

Abbreviations: siRNA, small interfering RNA; miR, microRNA

reporter plasmids containing *EBLN3P*-MUT (Figure 1B). Next, hepatoblastoma HepG2 cells were transfected with *EBLN3P* siRNA (si-*EBLN3P*) or the pcDNA3.1-*EBLN3P* overexpression vector (*EBLN3P*) to further investigate the possible impact of *EBLN3P* on the cellular behaviors of liver cancer cells. Negative control siRNA (si-NC) and the blank vector plasmid (pcDNA-3.1) were used as knockdown and overexpression controls, respectively. First, the nuclear and cytoplasmic expression levels of *EBLN3P* in

HepG2 cells were explored via RT-qPCR, and the data revealed that the *EBLN3P* was mainly expressed in the cell cytoplasm in HepG2 cells (Figure 1C). Moreover, the expression levels of *EBLN3P*, *miR-144-3p* and *DOCK4* were explored via RT-qPCR (Figure 1D). The RT-qPCR results indicated a negative association between *EBLN3P* and *miR-144-3p* expression and that the expression levels of *DOCK4* were positively correlated with the expression levels of *EBLN3P* (Figure 1D). Together, these results

demonstrated that *EBLN3P* serves as a ceRNA for *miR-144-3p* to inhibit *miR-144-3p* expression. Moreover, knock-down of *EBLN3P* notably decreased the proliferation, invasion and migration of HepG2 cells, which was enhanced by *EBLN3P* overexpression, as shown by the CCK-8 assay, transwell assay and wound healing assay, respectively (Figure 1EH). In contrast, overexpression of *EBLN3P* accelerated the proliferation, invasion and migration of HepG2 cells (Figure 1EH). Thus, these results implied that *EBLN3P* has the oncogenic potential to induce liver cancer cell proliferation, migration and invasion.

***miR-144-3p* Inhibits the Proliferation, Migration and Invasion of Liver Cancer Cells by Downregulating *DOCK4* in vitro**

DOCK4 has been demonstrated to promote tumor progression in lung adenocarcinoma (ADC) metastasis. However, its impact on liver cancer and interaction with *miR-144-3p* have not been studied yet. The potential target genes mediated by *miR-144-3p* in liver cancer cells were evaluated, and dedicator of cytokinesis protein 4 (*DOCK4*) was identified as one of the target genes of *miR-144-3p* through the bioinformatics software ENCORI. Next, a dual-luciferase reporter gene assay illustrated that the luciferase signal of cells cotransfected with *miR-144-3p* mimics and the wild-type *DOCK4* vector was notably decreased compared with that of cells cotransfected with *miR-144-3p* mimics and the mutant *DOCK4* vector (Figure 2A). This implied that *miR-144-3p* probably binds to the 3'-UTR of *DOCK4*. Furthermore, RT-qPCR and Western blotting confirmed that overexpression of *miR-144-3p* significantly decreased *DOCK4* expression, whereas knock-down of *miR-144-3p* significantly enhanced the expression of *DOCK4* at both the mRNA and protein levels in HepG2 cells (Figure 2BD). Taken together, these results implied that *miR-144-3p* acts as an upstream regulator of *DOCK4* expression. The CCK-8 assay revealed a notable decrease in the proliferation rate in the *miR-144-3p* mimics group compared to the NC mimics group, while the proliferation rate was significantly increased in the *miR-144-3p* inhibitor group compared with the control group (Figure 2E). Analysis of migration and invasion further verified this pattern. The migration and invasion capacities of liver cancer cells transfected with *miR-144-3p* mimics were decreased, but those of cells transfected with the inhibitor were increased (Figure 2FH). Collectively, these data indicate that *miR-144-3p* acts as a tumor suppressor in liver

cancer cells that can restrain multiple malignant behaviors of HepG2 cells, including cell proliferation, migration and invasion.

***EBLN3P* Regulates Liver Cancer Cells via the *miR-144-3p/DOCK4* Pathway**

Rescue experiments were carried out to assess the effects of the *EBLN3P-miR-144-3p-DOCK4* pathway on HepG2 cell activities. Overexpression of *DOCK4* was achieved by transfecting cells with pcDNA3.1-*DOCK4* or *miR-144-3p* inhibitor (Figure 3A and B). The results demonstrated that knockdown of *EBLN3P* markedly decreased cell proliferation, migration and invasion. However, compared to transfected with si-*EBLN3P* alone, cotransfection with si-*EBLN3P* and *miR-144-3p* inhibitor or *DOCK4* significantly increased cell proliferation, migration and invasion (Figure 3CF). Accordingly, the data suggests that *EBLN3P* exerts its regulatory role through the *miR-144-3p/DOCK4* axis to promote the development of liver cancer cells.

***EBLN3P* and *DOCK4* are Upregulated While *miR-144-3p* is Downregulated in Liver Cancer Tissues and Cell Lines**

To investigate the regulatory role of *EBLN3P* in liver cancer, whether *EBLN3P* and *miR-144-3p* are dysregulated in liver cancer was investigated. The RTq-PCR and Western blotting results showed that *EBLN3P* and *DOCK4* were expressed at higher levels in HCC cell lines (PLHC1, Hep3B, and SNU398) and the hepatoblastoma cell line (HepG2) than in the normal human hepatic astrocyte cell line (THLE3), while *miR-144-3p* was expressed in the reverse manner (Figure 4A and B). These data demonstrated that *EBLN3P*, *miR-144-3p* and *DOCK4* may play an important role in regulating the development of liver cancer, including HCC and hepatoblastoma. To further explore the expressions of *EBLN3P*, *miR-144-3p* and *DOCK4* in HCC, *EBLN3P*, *miR-144-3p* and *DOCK4* expression levels were similarly assessed in HCC tissues and nonneoplastic hepatic tissues. As expected, *EBLN3P* and *DOCK4* were expressed at higher levels and *miR-144-3p* was expressed at a lower level in HCC tissues than in hepatic tissues (Figure 4C and D). Moreover, the associations between *EBLN3P* and *miR-144-3p* expressions and the prognosis of patients with HCC were explored via Kaplan-Meier survival curves and the Log rank test. As shown in Figure 4E, F and H, patients with HCC and positive *miR-144-3p* expression had longer overall survival

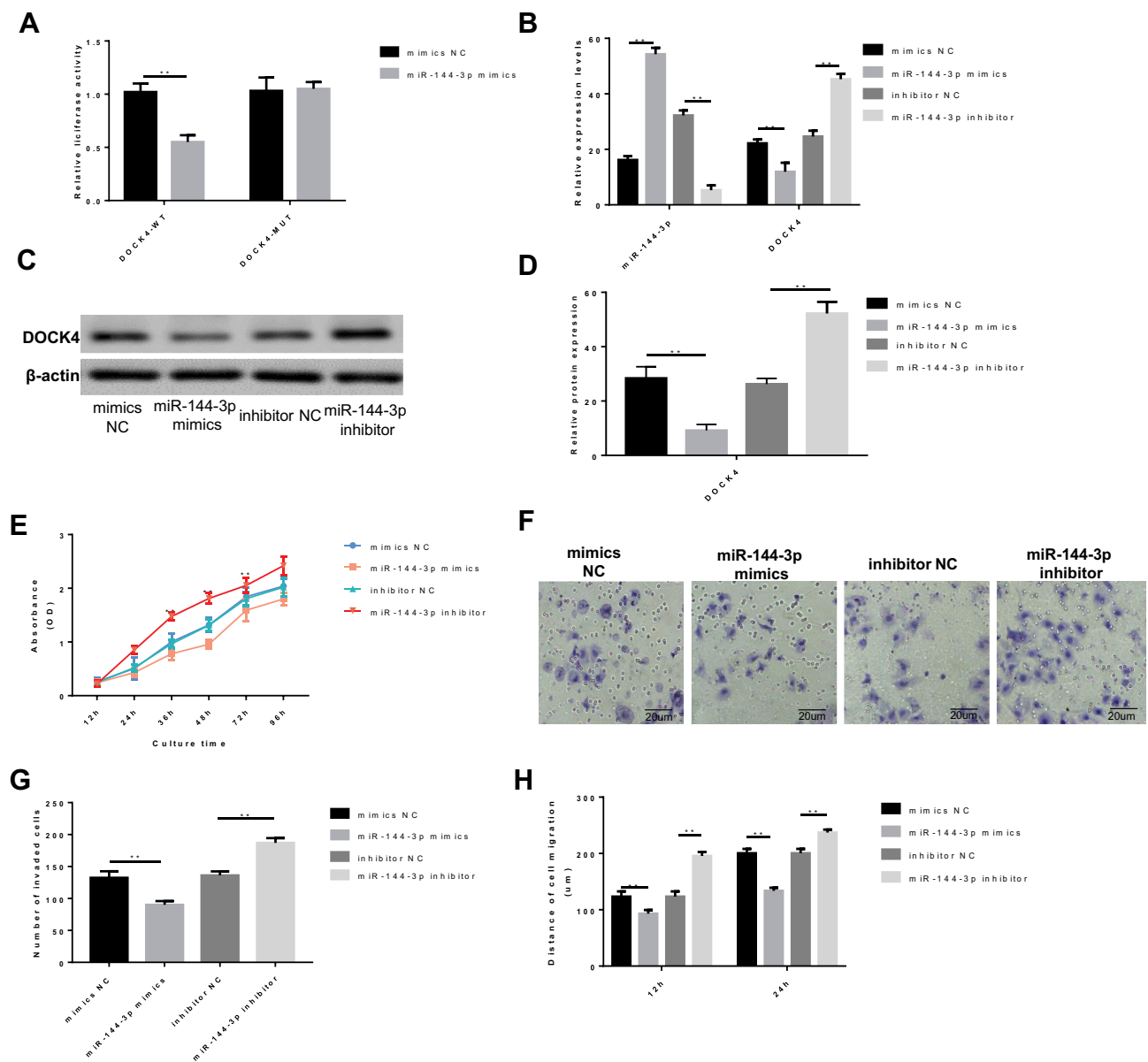


Figure 2 miR-144-3p inhibits the proliferation, migration and invasion of liver cancer cells by downregulating *DOCK4*. (A) The *miR-144-3p* mimics and luciferase reporter plasmids containing wild-type or mutant *DOCK4* 3'-UTR were cotransfected into HepG2 cells. The dual luciferase reporter gene assay was performed to verify the direct binding association between *miR-144-3p* and *DOCK4*. (B) HepG2 cells were transfected with NC mimics, *miR-144-3p* mimics, NC inhibitor, or *miR-144-3p* inhibitor, and then, reverse transcription-quantitative PCR was conducted to evaluate the relative expression levels of *miR-144-3p* and *DOCK4*. (C) Western blotting validated that the overexpression of *miR-144-3p* significantly decreased *DOCK4* expression. (D) Corresponding analysis of *DOCK4* expression. (E) Cell proliferation was tested by the Cell Counting Kit-8 assay. (F) Cell invasion was detected by the Transwell assay. (G) The number of invaded cells. (H) Cell migration was detected by the wound healing assay. The data are shown as the mean \pm SD. ** $P < 0.01$.

Abbreviations: miR, microRNA; UTR, untranslated region.

times than patients with negative *miR-144-3p* expression ($P = 0.004$). Furthermore, patients with HCC and positive *EBLN3P* or *DOCK4* expression had shorter overall survival times than patients with negative *EBLN3P* or *DOCK4* expression ($P = 0.012$; $P = 0.019$). Furthermore, *DOCK4* expression in HCC tissues was investigated via IHC analysis, as shown in Figure 4G and Table 2, and it was found that *DOCK4* was expressed at a higher level in HCC tissues (54/

74) than in hepatic tissues (14/72). Moreover, *DOCK4* was revealed to be associated with distant metastasis ($P < 0.01$) and tumor-node-metastasis stages (AJCC) ($P < 0.01$).

Discussion

The role of lncRNAs in carcinogenesis was initially identified due to the differential expression of these lncRNAs in cancer tissues compared with normal tissues.^{23–27} Thus far,

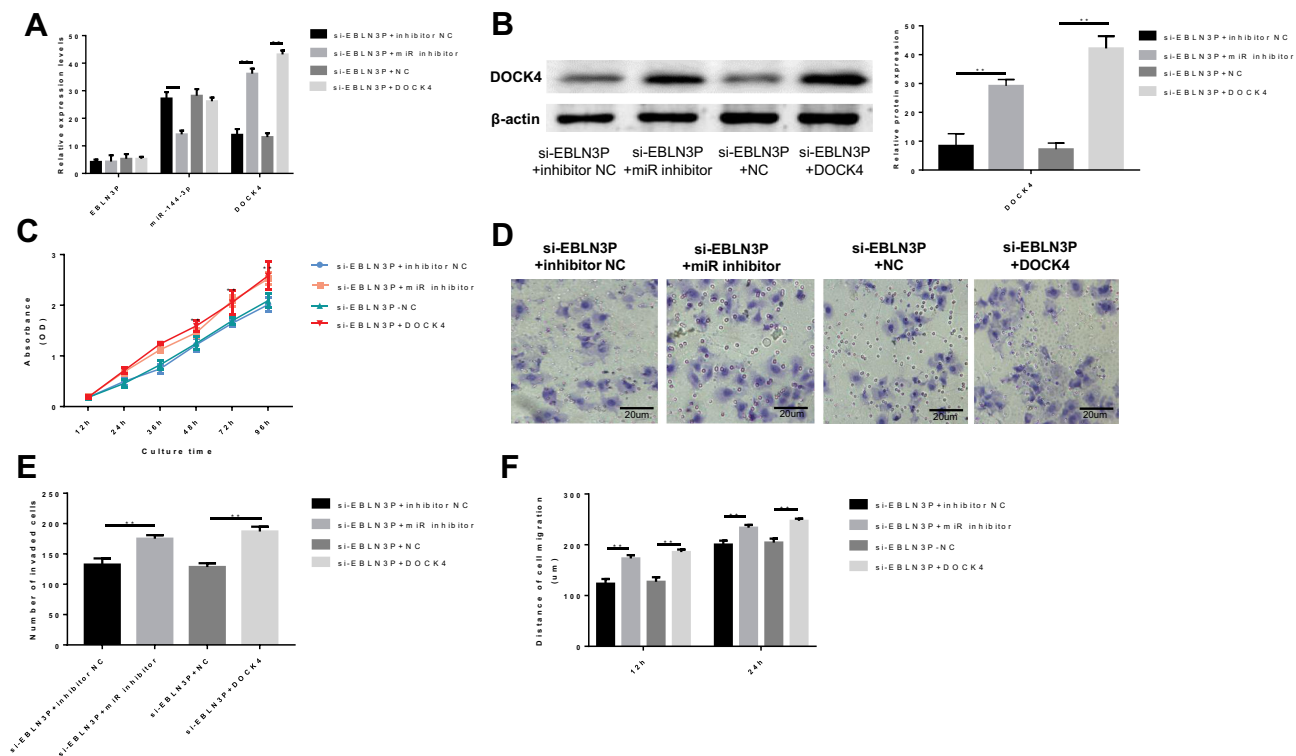


Figure 3 EBLN3P regulates liver cancer cells via the miR-144-3p/DOCK4 pathway. High mRNA (A) and protein (B) expression level of *DOCK4* was achieved by transfecting cells with pcDNA3.1-*DOCK4* or *miR-144-3p* inhibitor. Knockdown of *EBLN3P* markedly reduced cell proliferation (C), invasion (D and E) and migration (F). However, compared to transfection with sh-*EBLN3P* alone, co-transfection with sh-*EBLN3P* and *miR-144-3p* inhibitor or *DOCK4* significantly increased cell proliferation (C), invasion (D and E) and migration (F). The data are shown as the mean \pm SD. ** $P < 0.01$.

accumulating evidence has indicated that the capacities of lncRNAs to regulate complex cellular behaviors, such as cell growth and metastasis, are commonly deregulated in cancer, including liver cancer.^{28–30} Although many of potential biomarkers have been reported, specific diagnostic biomarkers for liver cancer have not yet been confirmed. *EBLN3P* is a novel lncRNA located on chromosome 9: 37,079,935–37,086,874 forward strand. The present study demonstrated that *EBLN3P* is markedly upregulated in liver cancer tissues and cell lines. In vitro, functional assays indicated that the deprivation of *EBLN3P* suppresses liver cancer cell proliferation, migration and invasion, demonstrating the potential of *EBLN3P* as a therapeutic target for liver cancer intervention. Hence, the underlying mechanism in liver cancer cell lines was next explored.

The ceRNA theory was first proposed in 2011 and was subsequently extensively accepted in the field of noncoding RNA.¹⁰ lncRNAs may serve as ceRNAs by sponging miRNAs to hinder the downregulated pathway. Bioinformatics analyses revealed that there is a conserved *miR-144-3p* binding site in *EBLN3P*. Therefore, it was postulated that *EBLN3P* can act as a ceRNA to affect *miR-144-3p*. Subsequently, it was validated

that *miR-144-3p* has a reciprocal suppressive effect on *EBLN3P* expression, and knockdown of *miR-144-3p* induced the proliferation, migration and invasion of liver cancer cells in vitro. Importantly, the dual-luciferase assay further showed that *EBLN3P* directly interacts with *miR-144-3p* to decrease its expression, suggesting that *EBLN3P* serves as an miRNA sponge that binds to and regulates *miR-144-3p* expression.

miRNAs control gene expression by binding to the 3'-UTR of the target gene, which causes mRNA cleavage or translational repression.³¹ Rho GTPases play critical roles in the initiation and progression of various tumors;³² however, the regulation of Rho GTPases in liver cancer remains largely unknown. Additionally, the role and molecular mechanism of *DOCK4*, an atypical Rho GEF,³³ in liver cancer are unknown. This study provides the first evidence that *DOCK4* is overexpressed in liver cancer and that its positive expression is associated with lymph node metastasis and a higher tumor-node-metastasis (TNM) stage. Patients with liver cancer and positive *DOCK4* expression exhibited a shorter overall survival time than patients with negative *DOCK4* expression. The present study demonstrated that *DOCK4* promoted the proliferation of liver

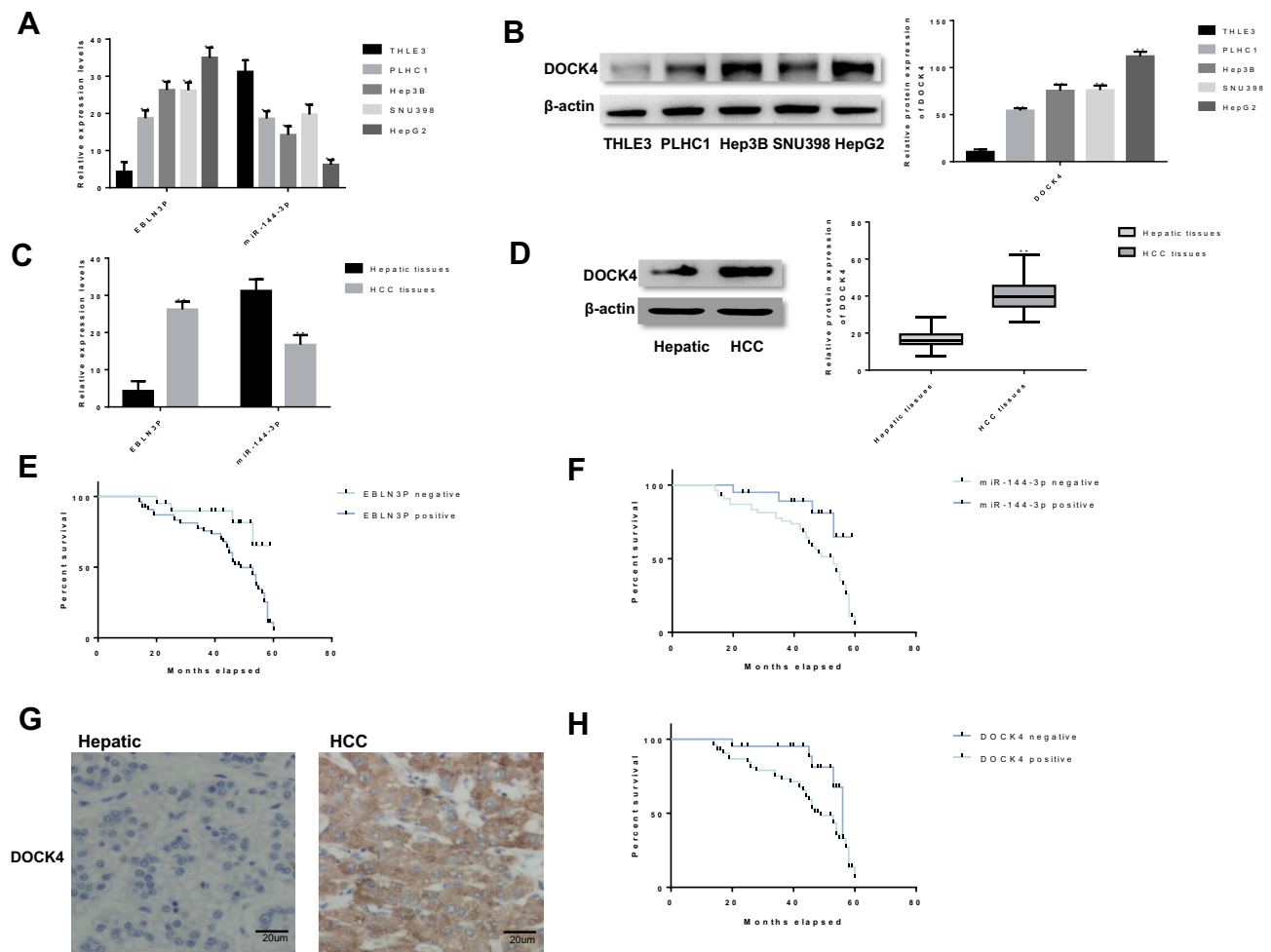


Figure 4 EBLN3P and *DOCK4* were upregulated, while *miR-144-3p* was downregulated in liver cancer tissues and cell lines. **(A)** The expression levels of *EBLN3P* and *miR-144-3p* in liver cancer cell lines (PLHC1, Hep3B, SNU398 and HepG2) and the hepatocyte cell line (THLE3) were tested by RT-qPCR. **(B)** The Western blotting results showed that *DOCK4* was expressed at a higher level in four liver cancer cell lines (PLHC1, Hep3B, SNU398 and HepG2) than in the normal human hepatic astrocyte cell line (THLE3). **(C)** The expression patterns of *EBLN3P* and *miR-144-3p* were measured in liver cancer samples and normal tissue via RT-qPCR. **(D)** The expression patterns of *DOCK4* were measured in liver cancer samples and normal tissue via Western blotting. **(E)** The association between *EBLN3P* expression and the prognosis of patients with liver cancer was explored. **(F)** The association between *miR-144-3p* expression and the prognosis of patients with liver cancer was explored. **(G)** The expression patterns of *DOCK4* were measured in liver cancer samples and normal tissue via immunohistochemistry. **(H)** The association between *DOCK4* expression and the prognosis of patients with liver cancer was explored. The data are shown as the mean \pm SD. ** $P < 0.01$.

Abbreviations: RT-qPCR, reverse transcription-quantitative PCR; miR, microRNA.

cancer cells in vitro. It was also found that *DOCK4* expression in lymph node metastases was higher than that in primary liver cancer tissues and that *DOCK4* increased the migration and invasion abilities of liver cancer cells in vitro and in vivo. As a member of the Dock family of GEFs, *DOCK4*, a typical Rac1 activator, has been reported to be a key component of the TGF- β /Smads pathway, which promotes lung ADC cell extravasation and metastasis.³⁴ In accordance with previous reports, the present data showed that *DOCK4* promoted the motility of liver cancer cells. In the present study, bioinformatics analysis revealed that *DOCK4* was regulated via the EBLN3P/miR-144-3p axis, and the expression levels of *DOCK4*

were positively correlated with the expression levels of *EBLN3P* in liver cancer cells. Moreover, the dual-luciferase assays confirmed the direct interaction between *miR-144-3p* and *DOCK4*. Furthermore, rescue experiments showed that *EBLN3P* promoted proliferation and metastasis in liver cancer via the regulation of the miR-144-3p/*DOCK4* signaling axis. Although this study reveals a novel mechanism whereby liver cancer cells are regulated by *EBLN3P* in vitro, further studies are required to fully understand the complexity of the mechanism. Future studies in model mice are needed to further verify the novel molecular mechanism of EBLN3 and value its potential as a therapeutic target.

Table 2 Expression Levels of *DOCK4* and Summary of the Clinicopathological Characteristics of Liver Cancer Patients

Variable	Patients, N	<i>DOCK4</i> (+)	<i>DOCK4</i> (-)	P-value
Liver cancer tissues	74	54	20	<0.01
Hepatic tissues	72	14	58	
Age (years)				
≤60	42	30	12	0.726 ^a
>60	32	24	8	
Sex				
Male	41	29	12	0.743 ^a
Female	33	25	8	
HbsAg				
+	67	49	18	0.872 ^a
-	7	5	2	
Distant metastasis				
+	44	37	7	<0.01
-	30	17	13	
Serum AFP (ng/mL)				
<400	46	33	13	0.162 ^a
>400	28	21	7	
TNM stage (AJCC)				
I-II	49	32	17	<0.01
III-IV	25	22	3	

Notes: ^aNo statistical significance was found with the χ^2 test/ χ^2 Goodness-of-Fit Test.

Abbreviations: DOCK, dedicator of cytokinesis protein; TNM, tumor-node-metastasis; AFP, alpha-fetoprotein.

In conclusion, the present study is the first to demonstrate that *EBLN3P* acts as a novel oncogene in liver cancer. Furthermore, *EBLN3P* acts as a ceRNA to regulate *DOCK4* expression by competitively sponging *miR-144-3p*, thereby regulating the progression of liver cancer. Therefore, the present findings provide useful information for the identification of new biomarkers for early diagnosis and therapies for liver cancer progression.

Abbreviations

ceRNA, competing endogenous RNA; *DOCK4*, dedicator of cytokinesis 4; *HOTAIR*, hox transcript antisense intergenic RNA; lncRNA, long noncoding RNA; miRNAs, microRNAs; RT-qPCR, reverse transcription-quantitative PCR, siRNA, small interfering RNA.

Funding

This study was supported by Jilin Province Science and Technology Development Projects (20160414032GH).

Disclosure

The authors report no conflicts of interest for this work.

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