

Biological effect of ETV4 and the underlying mechanism of its regulatory effect on epithelial-mesenchymal transition in intrahepatic cholangiocarcinoma cells

LI LIU, YONG FENG, XUELIAN XIANG, MENGTAO XU and GUODU TANG

Department of Gastroenterology, The First Affiliated Hospital of Guangxi Medical University,
Nanning, Guangxi Zhuang Autonomous Region 530021, P.R. China

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Abstract. Intrahepatic cholangiocarcinoma (ICC) is a highly invasive malignant tumor. The prognosis of patients with ICC after radical surgical resection remains poor, due to local infiltration, distant metastasis, a high recurrence rate and lack of effective treatment strategies. E26 transformation-specific sequence variant 4 (ETV4) is a pro-carcinogenic factor that is upregulated in several tumors; however, the role of ETV4 in ICC is relatively unknown. The present study aimed to determine the role of ETV4 in the Hccc9810 ICC cell line and to assess how it contributes to epithelial-mesenchymal transition (EMT) in ICC. Hccc9810 cells were infected with lentiviruses to construct stable ETV4-overexpressing cells, stable ETV4 knockdown cells and corresponding control groups. The Cell Counting Kit-8 and Transwell assays were used to quantify cell proliferation, invasion and migration, and the effects on cell cycle progression and apoptosis were detected by flow cytometry. ETV4 was identified as a driver of cell growth, invasion, migration and cell cycle progression, while restraining apoptosis in Hccc9810 cells. Reverse transcription-quantitative PCR and western blotting revealed that increased ETV4 levels may drive EMT by triggering the TGF- β 1/Smad signaling pathway. This cascade, in turn, may foster tumor cell proliferation, migration, invasion and cell cycle advancement, and hinder apoptosis.

Introduction

Intrahepatic cholangiocarcinoma (ICC) is a malignant tumor originating from the epithelium of the secondary bile duct and its branches. Chronic hepatitis and cirrhosis, inflammatory diseases of the biliary tract and hepatobiliary flukes increase the risk of ICC (1). Notably, 5-30% of all primary liver cancer cases are ICC, the incidence of which has increased in the past 30 years (2). ICC is associated with fatal outcomes in most patients. Regarding treatment of ICC, 20-30% of patients are eligible for surgical resection, which is currently the best treatment option; after surgical resection, capecitabine is typically used as an adjuvant therapy, and the median survival time among these patients is reported to be 53 months (3). For 70-80% of patients with local infiltration or distant metastasis, systemic treatment may delay disease progression, but once metastasis has occurred, survival is still limited to ~1 year (3). Therefore, it is crucial to understand the drivers of metastasis in ICC.

The E26 transformation-specific sequence (ETS) family is a large group of transcription factors, which includes ETS variant 4 (ETV4). This particular variant belongs to the polyomavirus enhancer activator 3 subfamily, which serves a crucial role in tumor advancement and spread by controlling various cellular processes, such as the cell cycle, apoptosis, epithelial-mesenchymal transition (EMT), cell migration, invasion, tumor stem cell characteristics and resistance to chemotherapy (4). ETV4 is upregulated in a number of tumor tissues; for example, ETV4 is highly expressed in malignant tumors, such as pancreatic ductal adenocarcinoma (PDAC) (5) and prostate cancer (6), and can act as a pro-carcinogenic factor. In comparison to other tumors, research on ETV4 in ICC is limited (7).

Epithelial cells undergo a transformation known as EMT, enabling them to acquire mesenchymal traits that promote migration and invasion into surrounding cells and the extracellular matrix. Dysregulated signaling pathways, hypoxia and interactions within the tumor microenvironment trigger the initiation and progression of EMT; this results in the disruption of epithelial cell polarity and intercellular adhesion, and ultimately enhances migratory capabilities towards adjacent cells and penetration into the extracellular matrix (8).

Correspondence to: Professor Guodu Tang, Department of Gastroenterology, The First Affiliated Hospital of Guangxi Medical University, 22 Shuangyong Road, Qingxiu, Nanning, Guangxi Zhuang Autonomous Region 530021, P.R. China
E-mail: tangguodu@stu.gxmu.edu.cn

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EMT is characterized by rearrangement of the cytoskeleton (such as actin recombination), cellular motility and invasiveness, increased cell-related protein hydrolysis activity and reprogramming of gene expression (9). EMT leads to *in situ* invasion or distant metastasis of tumors, and is considered a key mechanism for epithelial cells to assume a malignant phenotype. Given that it is considered the primary driver of tumor metastasis and recurrence, inhibiting EMT is considered important in the development of chemotherapeutic drugs for treating malignant tumors (10,11).

TGF- β 1 is a multifunctional regulator responsible for stimulating EMT. Smad2/3 and Smad4 form a trimeric complex as transcription factors that participate in regulating the expression of EMT-related genes (12), inhibiting E-cadherin synthesis, promoting the expression of interstitial cytokines, such as Vimentin and N-cadherin, reducing cell polarity, weakening epithelial features of tumor cells and enhancing interstitial features, all of which are promoters of tumor invasion and metastasis (13). EMT is also common in cholangiocarcinoma with high invasiveness and recurrence (14-16). The role of ETV4 in ICC cells and its connection to EMT warrants additional investigation. The present study aimed to investigate the biological impact of ETV4 in ICC and the underlying mechanism by which ETV4 influences EMT.

Materials and methods

Cell culture and cell lines. The human ICC Hccc9810 cell line was obtained from iCell Bioscience, Inc. The cells were cultured in RPMI-1640 medium (Dalian Meilun Biology Technology Co., Ltd.) supplemented with 10% fetal bovine serum [FBS; Cyagen Biosciences (Guangzhou) Inc.] at 37°C in a 5% CO₂ cell incubator.

Lentivirus infection. The lentivirus packaging process was completed using the 2nd generation self-inactivating lentiviral packaging system developed by Shanghai GeneChem Co., Ltd. The human ETV4 sequence (NM_001986) was obtained from the cDNA library of Shanghai GeneChem Co., Ltd. The following primers were used for PCR amplification of the target gene from the template plasmid to obtain gene fragments: ETV4, forward 5'-AGGTGCGACTCTAGAGGATCCCCGCCACCATG GAGCGGAGGATGAAAGC-3', reverse 5'-TCCTTG TAG TCCATACCGTAAGAGTAGCCACCCTTGGGGC-3'. The GV492 (Ubi-MCS-3FLAG-CBh-gcGFP-IRES-puromycin) lentiviral vector plasmid (Shanghai GeneChem Co., Ltd.) and the ETV4 gene sequence were digested using *Age*I and *Nhe*I restriction enzymes, and complete cloning was performed using the in-fusion recombination method. The recombinant vector was detected by Sanger sequencing. 293T cells (American Type Culture Collection) were used as the interim cell line in the present study. Lentiviral plasmids were transfected with GeneChem transfection reagent (Shanghai GeneChem Co., Ltd.) at the following quantities into 293T cells in a 10-cm cell culture dish: Empty GV492 vector plasmid (negative control) or GV492 vector plasmid containing ETV4, 20 μ g; PHelper 1.0 vector plasmid (Shanghai GeneChem Co., Ltd.), 15 μ g; PHelper 2.0 vector plasmid (Shanghai GeneChem Co., Ltd.), 10 μ g. The 293T cells were placed in a 37°C cell culture incubator with 5% CO₂. After culturing for 6-8 h, the culture

medium (DMEM; Corning, Inc.) containing the transfection system mixture was discarded. PBS solution (Beijing Solarbio Science & Technology Co., Ltd.) was used to wash the cells, followed by the addition of 20 ml cell culture medium containing 10% FBS (Beijing Diyi Biotechnology Co., Ltd.). The cells were then cultured at 37°C for 48 h in an incubator containing 5% CO₂. After 48 h, the supernatant of the 293T cells was collected and was centrifuged at 4,000 x g for 10 min at 4°C to remove cell debris and impurities. Subsequently, the supernatant was filtered with a 0.45- μ m filter and centrifuged at 90,000 x g for 2 h at 4°C. The supernatant was discarded, and any remaining liquid on the tube wall was removed. Virus preservation solution (Shanghai GeneChem Co., Ltd.) was added, gently resuspended, and centrifuged at 14,000 x g for 5 min at 4°C. The supernatant was then collected as a sample for safety and titer testing.

Lentiviral vectors expressing a short hairpin RNA (shRNA) targeting the ETV4 gene sequence (5'-GTCCCTTG TCCCACTTGGAT-3') or the negative control (5'-TTCTCC GAACGTGTCACGT-3') were synthesized using the GV493 (hU6-MCS-CBh-gcGFP-IRES- puromycin) vector (Shanghai GeneChem Co., Ltd.); the recombinant vector was detected by Sanger sequencing. 293T cells were used as the interim cell line in the present study. Lentiviral plasmids were transfected with GeneChem transfection reagent at the following quantities into 293T cells in a 10 cm cell culture dish: GV493 vector plasmid containing shRNA, 20 μ g; PHelper 1.0 vector plasmid, 15 μ g; PHelper 2.0 vector plasmid, 10 μ g. The 293T cells were placed in a 37°C cell culture incubator with 5% CO₂. After culturing for 6 h, the culture medium containing the transfection system mixture was discarded, and the cells were washed with PBS solution. Subsequently, 20 ml cell culture medium containing 10% FBS was slowly added, and the cells were cultured at 37°C for 48 h in an incubator containing 5% CO₂. A total of 48 h post-transfection, the supernatant of the 293T cells was collected. The supernatant was centrifuged at 4,000 x g for 10 min at 4°C to remove cell debris and impurities. Subsequently, the supernatant was filtered through a 0.45- μ m filter and then centrifuged at 90,000 x g for 2 h at 4°C. The supernatant was discarded, and the remaining liquid on the tube wall was removed. Virus preservation solution was added to the tube, gently resuspended, and centrifuged at 14,000 x g for 5 minutes at 4°C. The supernatant was then taken as a sample for safety and titer testing.

For Hccc9810 cell infection, the multiplicity of infection for the overexpression group was 30, and that for the knock-down group was 10. The cells were cultured in an incubator with 5% CO₂ at 37°C. The medium was changed 16 h after infection with the virus stock solution, and the infection efficiency was observed at 96 h. Stable cell lines were screened and created using a puromycin working solution of 2 μ g/ml, with a maintenance concentration of 1 μ g/ml puromycin. The overexpression group was named LV-ETV4 and its negative control group (empty vector) was named CON335. The knock-down group was named LV-ETV4-RNAi and its negative control group was named CON313. The non-transfected group comprised normal Hccc9810 cells.

Cell proliferation assay. Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8) assay (Dalian Meilun Biology

Technology Co., Ltd.) according to the manufacturer's instructions. Briefly, the cell suspension was inoculated into a 96-well plate at a density of 5×10^3 cells/well and cultured overnight at 37°C in a 5% CO_2 cell incubator. Subsequently, $10 \mu\text{l}$ CCK-8 solution was added to each well, followed by a 2-h incubation period. The optical density of the solution in each well was then measured using a microplate reader at 450 nm at 0, 24, 48, 72 and 96 h (17).

Cell migration and invasion assays. Cell migration was evaluated using Transwell plates (pore size, $8 \mu\text{m}$) that were not coated in Matrigel, whereas the cell invasion assay was carried out on Transwell plates that were precoated with Matrigel (cat. no. 354480; Corning, Inc.). Transfected cells were digested and centrifuged at $100 \times g$ for 5 min at room temperature before being suspended in serum-free medium and added to the upper chamber of the Transwell plate at a density of 1×10^5 cells/chamber (migration) or 8×10^4 cells/chamber (invasion). The lower chamber was filled with $500 \mu\text{l}$ medium containing 10% FBS. After a 24-h incubation period at 37°C in a 5% CO_2 cell incubator, cells that had migrated or invaded through the membrane were fixed with methanol solution (>99.5%; Chengdu Chemical Co., Ltd.) for 30 min and stained with 1% crystal violet solution (Beijing Solarbio Science & Technology Co., Ltd.) for 60 min at room temperature, and images were captured and semi-quantified under a light inverted microscope (12,18).

Flow cytometric detection of cell cycle progression and apoptosis. According to the manufacturer's instructions, the Cell Cycle Staining Kit [cat. no. CCS012; Multi Sciences (Lianke) Biotech Co., Ltd.] was used to assess cell cycle progression. Briefly, infected cells underwent digestion with EDTA-free trypsin (Beijing Solarbio Science & Technology Co., Ltd.) and centrifugation at $100 \times g$ for 5 min at room temperature to prepare a cell suspension with a concentration of 1×10^6 cells/ml, and were then washed with phosphate-buffered saline (PBS). Subsequently, cells were centrifuged at $100 \times g$ for 5 min at room temperature and the supernatant was discarded; 1 ml DNA staining solution, which contained PI and RNase A, and $10 \mu\text{l}$ permeabilization solution were then applied to the cells, accompanied by vortex oscillation and a 30-min incubation at room temperature. Flow cytometric analysis (BD FACSVerser; BD Biosciences) of the cell cycle was conducted utilizing the lowest loading speed configuration. FlowJo (10.8.1; FlowJo, LLC) was used to analyze cell cycle progression.

According to the manufacturer's instructions, the Annexin V-allophycocyanin (APC)/propidium iodide (PI) cell apoptosis detection kit (Jiangsu Kaiji Biotechnology Co., Ltd.) was used to assess apoptosis. After digestion of the cells with EDTA-free trypsin, they were centrifuged at $100 \times g$ for 5 min at room temperature to prepare a cell suspension with a concentration of 5×10^5 cells/ml, and washed twice with PBS. Subsequently, cells were centrifuged at $100 \times g$ for 5 min at room temperature and the supernatant was discarded; $500 \mu\text{l}$ binding buffer was then added to the cells, and single cell suspension samples were obtained, which were incubated with $5 \mu\text{l}$ Annexin V-APC ($0.49 \mu\text{g/ml}$) and $5 \mu\text{l}$ PI ($0.98 \mu\text{g/ml}$) in the dark at room temperature for 5-10 min prior to flow

Table I. List of primer sequences.

Gene	Sequence, 5'-3'
ETV4	F: GGTGGCTGGTGAGCGTTA R: CCAAGTGGGACAAAGGGA
E-cadherin	F: AATCTGAAAGCGGCTGATACTG R: GCGATTGCCCATTCGTT
N-cadherin	F: TGA CTCCAACGGGGACTGC R: CAAACATCAGCACAAGGATAAGC
Vimentin	F: GCCAGGCAAAGCAGGAGTC R: CACGAAGGTGACGAGCCATT
TGF- β 1	F: GGGTGGCTCACGCCTGTAA R: GCTGGTCTCAAATGCCTGGAT
Smad4	F: ATGAGGTTATGGTTCTGGGTGG R: GAATGAGGTCTTACGGTGGGTG
Smad7	F: TGGTGCCTGGTGGCATACT R: AAGCCATTCCTGAGGTAGA
GAPDH	F: AAGGTCGGAGTCAACGGAT R: CCTGGAAGATGGTGTATGGG

ETV4, E26 transformation-specific sequence variant 4; F, forward; R, reverse.

cytometric analysis (BD FACSVerser). FlowJo (10.8.1) was used to analyze apoptosis.

Reverse transcription-quantitative (RT-qPCR). According to the manufacturers' instructions, RT-qPCR analysis of gene transcription levels in the cells was performed. Total RNA was extracted from the cells using the RNA Easy RNA extraction kit (Beyotime Institute of Biotechnology), and a cDNA RT kit (PrimeScript™ RT Master Mix Perfect Real-time; Takara Biotechnology Co., Ltd.) was used to convert RNA into cDNA. cDNA was then amplified by fluorescence qPCR using specific primers (Nanning GenSys Biotechnology Co., Ltd.) and a qPCR kit (TB Green® Premix Ex Taq™ Tli RnaseH Plus; Takara Biotechnology Co., Ltd.). The qPCR program included preheating at 95°C for 30 sec; followed by 40 cycles of heating at 95°C for 5 sec, and cooling and extension at 60°C for 34 sec; and final dissolution curve procedures (95°C for 15 sec, 60°C for 60 sec and 95°C for 15 sec). The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta\text{C}_q}$ method (19). The primer sequences used for qPCR analysis are listed in Table I, and GAPDH was used as the reference gene (20,21).

Inhibitor amygdalin and activator SRI-011381 hydrochloride treatment. Cells (5×10^5 cells/ml) were seeded in a 6-well plate (2ml/well) and allowed to reach ~80% confluence. Subsequently, the TGF- β /Smad inhibitor amygdalin (MedChemExpress), at a working concentration of $400 \mu\text{g/ml}$ (22), was added to the LV-ETV4 group, forming the LV-ETV4 + amygdalin group. In addition, the TGF- β activator SRI-011381 hydrochloride (MedChemExpress) solution, at a working concentration of $10 \mu\text{M}$ (23), was added to the LV-ETV4-RNAi group, creating the LV-ETV4-RNAi + SRI-011381 group. These experimental groups, along with cells from the CON335, LV-ETV4,

CON313 and LV-ETV4-RNAi groups, were then placed in a 95% humidity and 5% CO₂ incubator at 37°C for 24 h.

Western blot analysis. The cells were rinsed with PBS, and were incubated with a mixture of RIPA lysis buffer and PMSF (both from Beijing Solarbio Science & Technology Co., Ltd.) on ice for 20 min. Subsequently, the cell lysates were centrifuged at 12,000 x g for 20 min at 4°C, and the total protein concentration was quantified by ultraviolet spectrophotometry. The samples containing protein (50 µg protein/lane) were then subjected to SDS-PAGE on 8% gels in protein loading buffer and were heated to 95-100°C for electrophoresis. The separated proteins were transferred to a PVDF membrane. Blocking was performed using 5% skim milk powder at room temperature for 1 h, prior to incubation with the following primary antibodies at 4°C for 18 h: Anti-Vimentin (cat. no. ab92547; 1:1,000), anti-TGF-β1 (cat. no. ab215715; 1:1,000), anti-Smad4 (cat. no. ab40759; 1:1,000), anti-GAPDH (cat. no. ab181603; 1:5,000) (all from Abcam), anti-E-cadherin (cat no. 20874-1-AP; 1:5,000), anti-N-cadherin (cat no. 22018-1-AP; 1:2,000) (both from Wuhan Sanying Biotechnology), anti-ETV4 (cat. no. PA5-99226; 1:1,000) and anti-Smad7 (cat. no. 701940; 1:1,000) (both from Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, the membrane was washed three times with 1X Tris-buffered saline-0.1% Tween 20 buffer (TBST) and incubated with a fluorescent DyLight™ 800-conjugated secondary antibody (anti-rabbit; cat. no. 5151; 1:10,000; Cell Signaling Technology, Inc.) for 1 h at room temperature. The membrane was washed a further three times with TBST and protein bands were detected using an Odyssey membrane scanner (LI-COR Biosciences). ImageJ (1.46; National Institutes of Health) was used to semi-quantify band staining intensities, and the relative expression of these proteins was determined (17). GAPDH was used as an internal reference protein.

Statistical analysis. All experiments were performed at least three times, and the data are presented as the mean ± standard deviation. The data were analyzed using SPSS Statistics 27 (IBM Corporation). Unpaired Student's t-test was used for comparing the mean between two groups, whereas one-way ANOVA followed by Tukey's post-hoc test was used for comparisons among multiple groups. The Wilcoxon rank-sum test was used to compare two groups that did not conform to normal distribution. Bar plots were prepared using GraphPad Prism 8.0 (Dotmatics). P<0.05 considered to indicate a statistically significant difference.

Results

Overexpression of ETV4 promotes Hccc9810 cell proliferation, migration and invasion. Lentiviral infection was used to construct stable ETV4-overexpressing cells, and the infection efficiency was verified through RT-qPCR and western blot analysis (Fig. 1A and B). Notably, the ETV4 antibody is a polyclonal antibody; the thicker band located at 54 kDa was used for western blot analysis of ETV4. No significant variation was observed in ETV4 expression between Hccc9810 cells and the negative control group CON335 (Fig. 1B-D). Subsequently, the CON335 and LV-ETV4 groups underwent

subsequent experiments. For the CCK-8 experiment, cells were incubated for 24, 48, 72 and 96 h. The LV-ETV4 group showed an increase in cell proliferation compared with that in the CON335 group, thus suggesting that ETV4 overexpression may promote cell proliferation (Fig. 1E). In the Transwell experiment, after incubating cells for 24 h, the invasion and migration of cells in the LV-ETV4 group were increased compared with those in the CON335 group (Fig. 1F).

Knocking down ETV4 inhibits Hccc9810 cell proliferation, migration and invasion. Lentiviral infection was also used to construct stable cells with ETV4 knockdown, and the infection efficiency was verified through RT-qPCR and western blot analysis (Fig. 1A and B). No significant variation was observed in ETV4 expression between Hccc9810 cells and the negative control group CON313 (Fig. 1B-D). Subsequently, the CON313 and LV-ETV4-RNAi groups underwent subsequent experiments. For the CCK-8 experiment, cells were incubated for 24, 48, 72 and 96 h. The LV-ETV4-RNAi group exhibited lower cell proliferation than the CON313 group, indicating that knocking down ETV4 may inhibit cell proliferation (Fig. 1E). In the Transwell experiment, after incubating cells for 24 h, the invasion and migration of cells in the LV-ETV4-RNAi group were decreased compared with those in the CON313 group (Fig. 1F).

Effects of overexpression and knockdown of ETV4 on cell cycle progression and apoptosis. Flow cytometry was used to analyze the effects of overexpression and knockdown of ETV4 on cell cycle progression. The results showed that there were more cells in the S (25.1 vs. 8.88%) and G₂ phases (11.7 vs. 4.70%), and fewer in the G₁ phase (64.1 vs. 85.2%) of the cell cycle in the LV-ETV4 group compared with in the CON335 group (Fig. 2A). This finding suggested that overexpression of ETV4 promoted cell differentiation i.e., transition from the G₁ to S phase, and accelerated cell proliferation. By contrast, the LV-ETV4-RNAi group exhibited a significant increase in the number of cells in the G₁ phase of the cell cycle (94.8 vs. 85.4%), whereas the proportion of cells in the S (2.68 vs. 7.92%) and G₂ phases (3.23 vs. 5.87%) was decreased compared with in the CON313 group (Fig. 2B). This finding suggested that the cells in the LV-ETV4-RNAi group were arrested in the G₁ phase, leading to cell cycle arrest and reduced cell proliferation. In addition, the LV-ETV4 group exhibited reduced apoptosis compared with that in the CON335 group (5.21 vs. 7.61%), whereas the LV-ETV4-RNAi group exhibited increased apoptosis compared with that in the CON313 group (16.99 vs. 9.78%) (Fig. 3).

ETV4 overexpression promotes EMT, whereas ETV4 knockdown inhibits EMT. The LV-ETV4 group exhibited reduced mRNA and protein expression levels of E-cadherin, and increased mRNA and protein expression levels of N-cadherin and Vimentin compared with those in the CON335 group, thus indicating that EMT was stimulated (Fig. 4A and B). By contrast, the LV-ETV4-RNAi group exhibited significantly increased mRNA and protein expression levels of E-cadherin, and reduced mRNA and protein expression levels of N-cadherin and Vimentin compared with those in the CON313 group, thus suggesting that EMT was

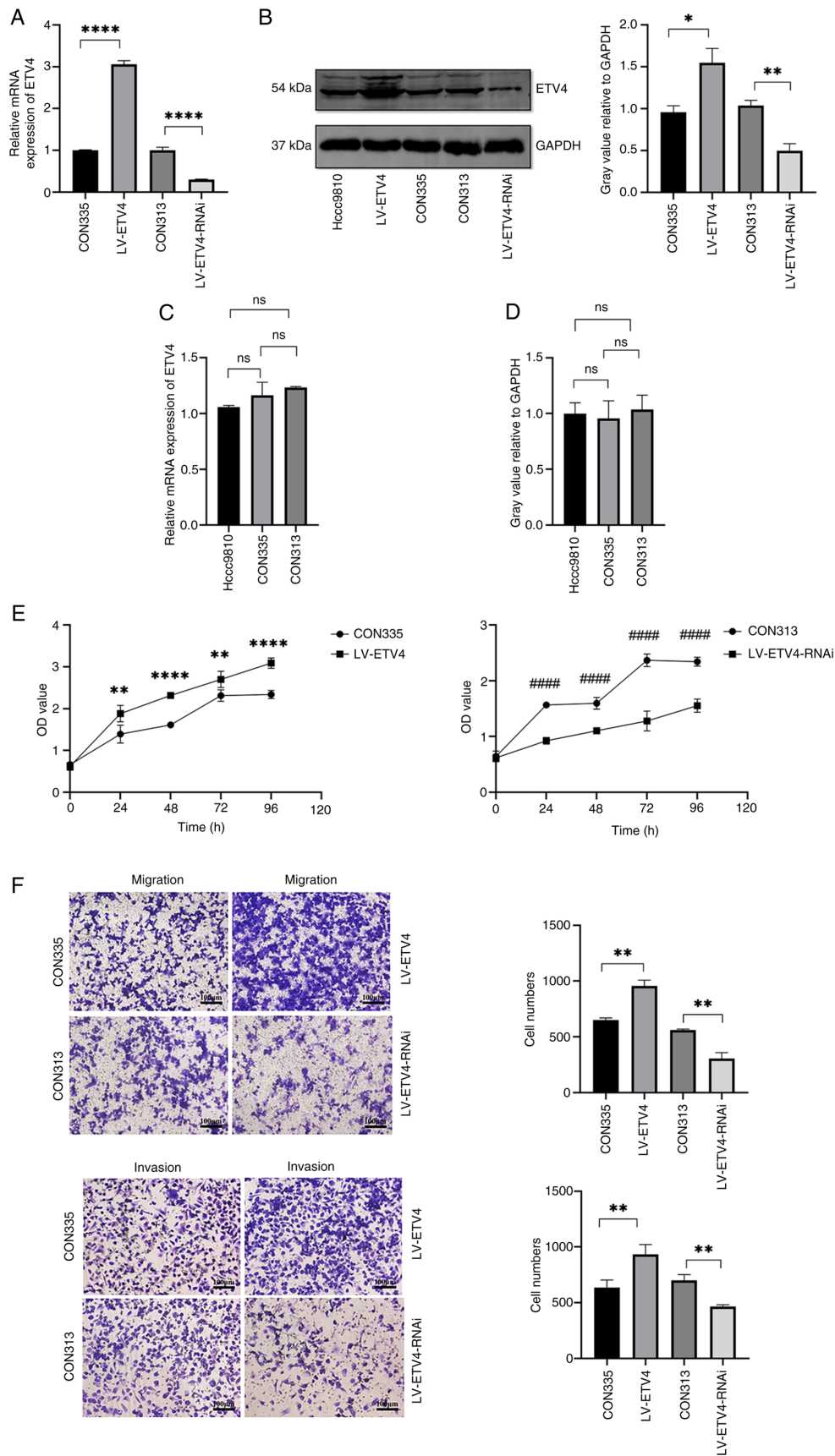


Figure 1. Effects of overexpression and knockdown of ETV4 on the proliferation, migration and invasion of Hccc9810 cells. Verification of the lentiviral infection efficiency of LV-ETV4 and LV-ETV4-RNAi using (A) RT-qPCR and (B) western blot analysis. (C) RT-qPCR and (D) western blot analysis showed no difference in the expression levels of ETV4 mRNA and protein expression among the Hccc9810, CON335 and CON313 groups. (E) LV-ETV4 promoted cell proliferation, whereas LV-ETV4-RNAi inhibited cell proliferation. (F) LV-ETV4 promoted cell migration and invasion, whereas LV-ETV4-RNAi inhibited cell migration and invasion (scale bar, 100 μ m). * P <0.05, ** P <0.01, **** P <0.0001 vs. CON335 or as indicated; ### P <0.0001 vs. CON313. ETV4, E26 transformation-specific sequence variant 4; LV-ETV4, ETV4 overexpression; LV-ETV4-RNAi, ETV4 knockdown; ns, not significant; ns, not significant; RT-qPCR, reverse transcription-quantitative PCR.

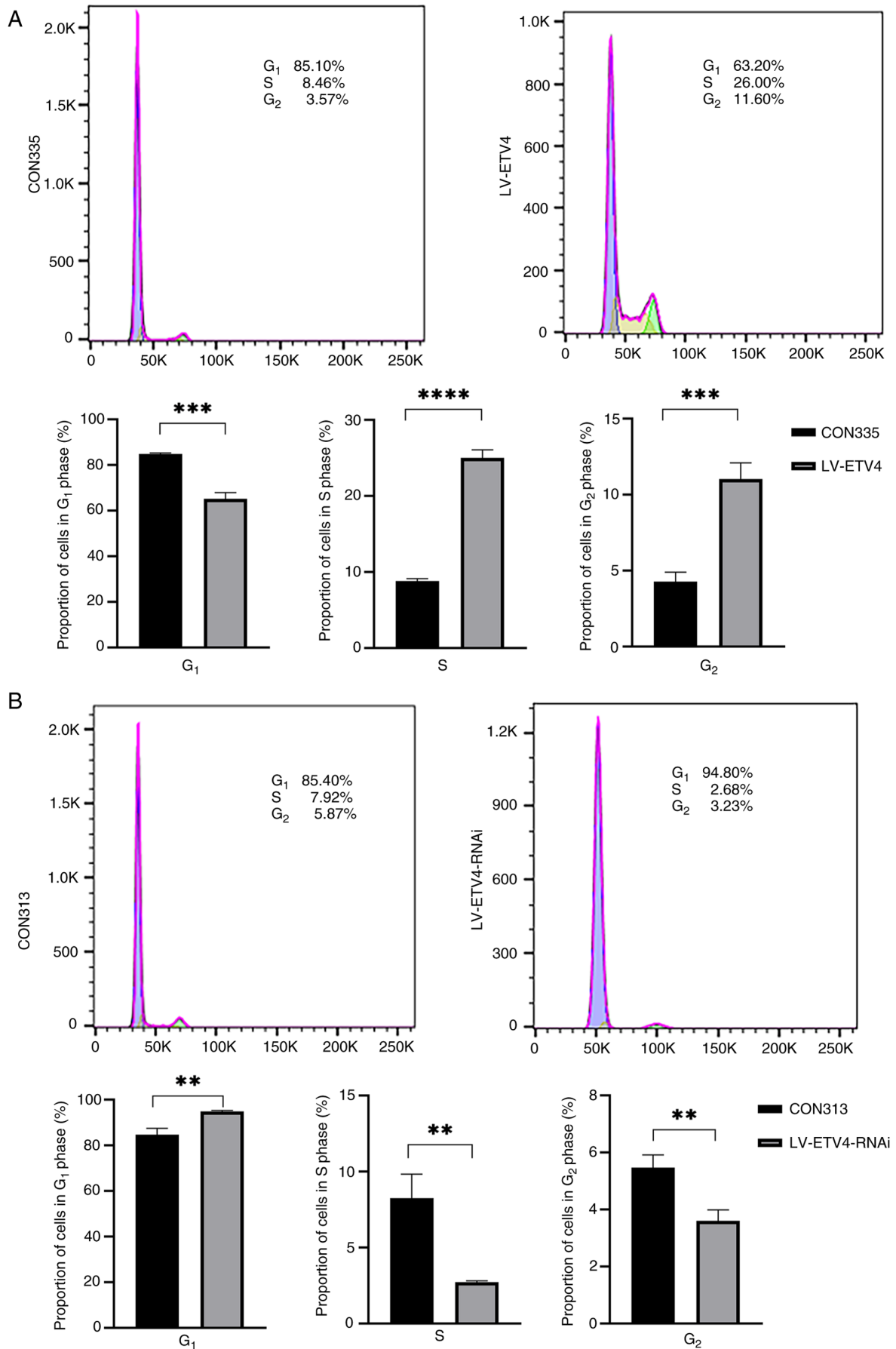


Figure 2. Effects of overexpression and knockdown of ETV4 on cell cycle progression. (A) LV-ETV4 increased the proportion of cells in the S and G₂ phases, promoting cell cycle differentiation from G₁ to S phase. (B) LV-ETV4-RNAi increased the proportion of cells in the G₁ phase, and decreased the proportion of cells in the S and G₂ phases. **P<0.01, ***P<0.001, ****P<0.0001. ETV4, E26 transformation-specific sequence variant 4; LV-ETV4, ETV4 overexpression; LV-ETV4-RNAi, ETV4 knockdown.

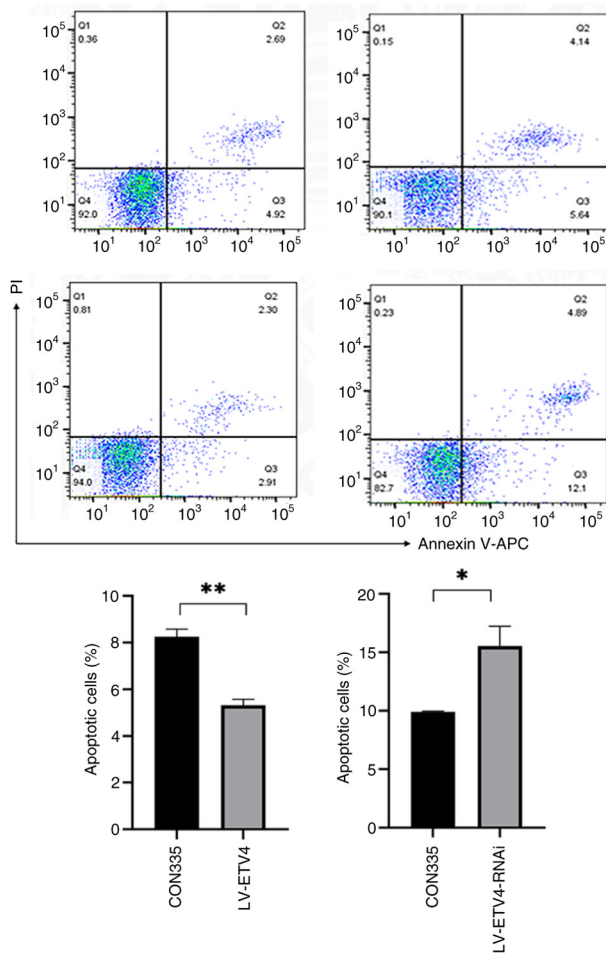


Figure 3. Effects of overexpression and knockdown of ETV4 on the apoptosis of Hccc98-10 cells. LV-ETV4 decreased apoptosis, whereas LV-ETV4-RNAi increased apoptosis. * $P < 0.05$, ** $P < 0.01$. APC, allophycocyanin; ETV4, E26 transformation-specific sequence variant 4; LV-ETV4, ETV4 overexpression; LV-ETV4-RNAi, ETV4 knockdown; PI, propidium iodide.

inhibited (Fig. 4A and B). These findings indicated that overexpression and knockdown of ETV4 had an impact on EMT.

ETV4 regulates EMT through the TGF- β 1/Smad signaling pathway. The results of RT-qPCR showed that, in the LV-ETV4 group, the mRNA expression levels of TGF- β 1 and Smad4 were increased, whereas those of Smad7 were decreased compared with those in the CON335 group (Fig. 5A). By contrast, in the LV-ETV4-RNAi group, the mRNA expression levels of TGF- β 1 and Smad4 were decreased, whereas those of Smad7 were increased compared with those in the CON313 group (Fig. 5A). Similarly, western blot analysis showed upregulated protein expression levels of TGF- β 1 and Smad4, and downregulation of Smad7 in the LV-ETV4 group compared with those in the CON335 group (Fig. 5B). In addition, in the LV-ETV4-RNAi group, the protein expression levels of TGF- β 1 and Smad4 were decreased, whereas those of Smad7 were increased compared with those in the CON313 group (Fig. 5B). Subsequently, treatment with an inhibitor (amygdalin) and an activator (SRI-011381 hydrochloride) of the TGF- β 1/Smad signaling pathway was used to validate the regulatory effect of ETV4 on EMT through western blot analysis.

In the LV-ETV4 group, E-cadherin expression was decreased, whereas N-cadherin and Vimentin expression levels were increased compared with in the CON335 group (Fig. 6A). Conversely, in the LV-ETV4 + amygdalin group, E-cadherin expression was increased, while N-cadherin and Vimentin expression was decreased in comparison to the LV-ETV4 group. Similarly, in the LV-ETV4-RNAi group, E-cadherin expression was increased, while N-cadherin and Vimentin expression was decreased compared with in the CON313 group. Finally, in the LV-ETV4-RNAi + SRI-011381 group, E-cadherin expression was decreased, while N-cadherin and Vimentin expression was increased compared with in the LV-ETV4-RNAi group. Furthermore, in the LV-ETV4 group, the expression levels of TGF- β 1 and Smad4 were increased, whereas the expression of Smad7 was decreased in comparison to the CON335 group (Fig. 6B). Conversely, in the LV-ETV4 + amygdalin group, the expression of TGF- β 1 and Smad4 was decreased, while the expression of Smad7 was increased compared with in the LV-ETV4 group. Similarly, in the LV-ETV4-RNAi group, the expression of TGF- β 1 and Smad4 was decreased, while the expression of Smad7 was increased compared with in the CON313 group. Finally, in the LV-ETV4-RNAi + SRI-011381 group, the expression of TGF- β 1 and Smad4 was increased, while the expression of Smad7 was decreased compared with in the LV-ETV4-RNAi group. These findings indicated that ETV4 may regulate EMT through the TGF- β 1/Smad signaling pathway.

Discussion

The present study investigated the effects of ETV4 on proliferation, migration, invasion, cell cycle progression, apoptosis and EMT activation in Hccc9810 ICC cells. The findings indicated that ETV4 modulated the EMT process through acting on the TGF- β 1/Smad signaling pathway.

As a member of the ETS transcription factor family, ETV4 can target related genes, such as dishevelled segment polarity protein 2, and matrix metalloproteinases (MMP1, MMP2, MMP7, MMP9, MMP13, MMP24 and MMP25), thus influencing disease progression in numerous types of cancer (24). Studies have shown that ETV4 enhances the proliferation, infiltration and metastasis of tumors. Overexpression of ETV4 in hepatitis B-related liver cancer has been reported to promote the invasion and migration of cancer cells, whereas knocking down ETV4 can weaken the ability of these cancer cells to invade and migrate (25). Similarly, knocking down ETV4 in glioblastoma multiforme cells was found to reduce the transcriptional activation of epithelial membrane protein 1, thereby inhibiting PI3K/AKT/mTOR signaling and promoting autophagy and apoptosis (26). Silencing ETV4 in ASPC1 and Colo357 pancreatic cancer cell lines has been reported to decrease cell proliferation by 55.3 and 38.9%, respectively. Conversely, overexpression of ETV4 in BXPC3 human pancreatic adenocarcinoma cells was shown to result in a 46.8% increase in cell proliferation compared with control cells. Further mechanistic investigations revealed that ETV4 specifically controls cyclin D1 expression, facilitating a swift transition from the G₁ to S phase of the cell cycle and enhancing cell proliferation (27). However, to the best of our knowledge, the function of ETV4 in ICC has not been fully elucidated.

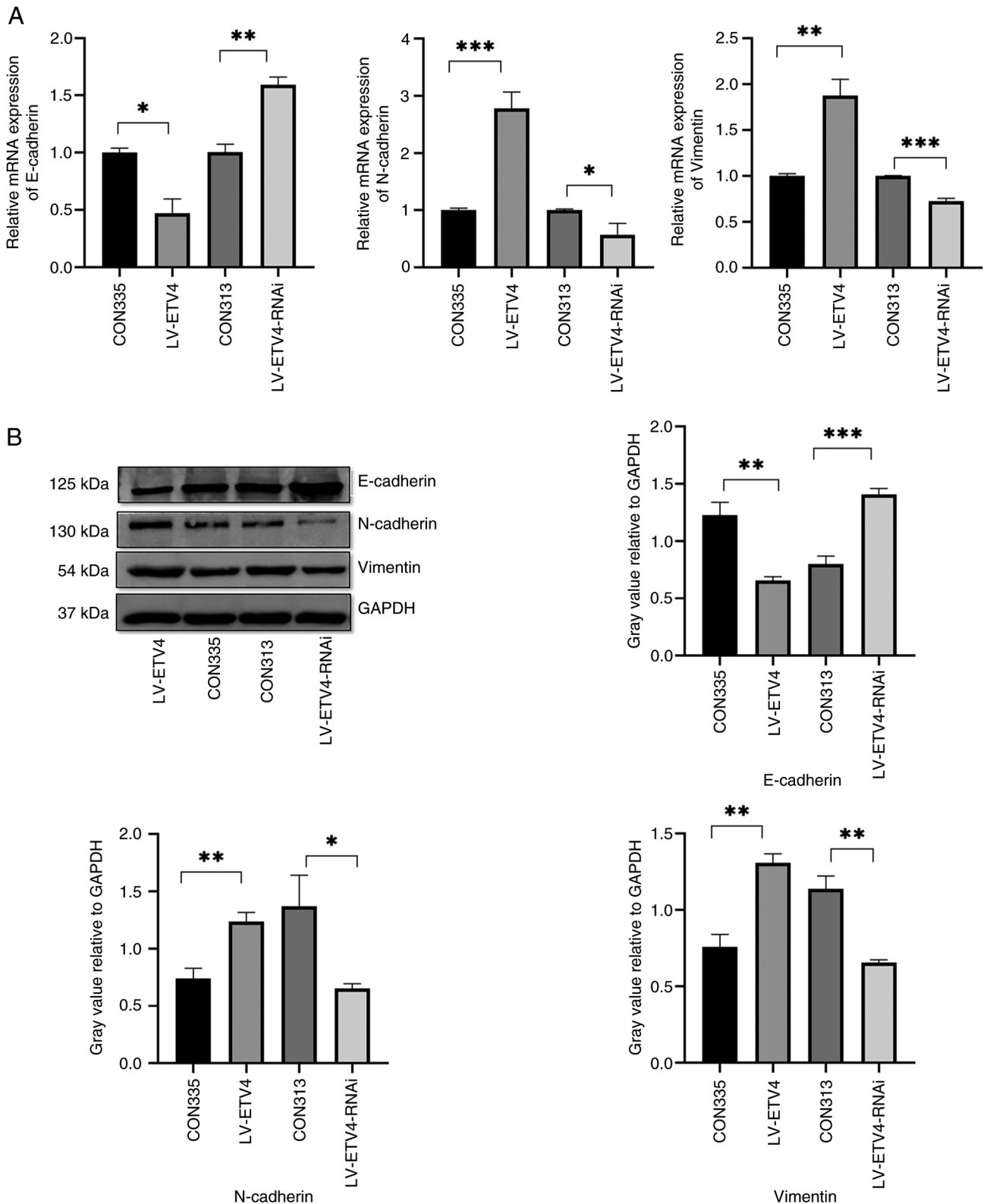


Figure 4. Effects of overexpression and knockdown of ETV4 on epithelial-mesenchymal transition in Hccc9810 cells. (A) Reverse transcription-quantitative PCR results showed that the expression levels of E-cadherin were decreased in the LV-ETV4 group, whereas the expression of N-cadherin and Vimentin was increased. The expression levels of E-cadherin were increased in the LV-ETV4-RNAi group, whereas the expression of N-cadherin and Vimentin was decreased. (B) Western blotting showed similar results. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ETV4, E26 transformation-specific sequence variant 4; LV-ETV4, ETV4 overexpression; LV-ETV4-RNAi, ETV4 knockdown.

The present study revealed that ETV4 overexpression in ICC increased cell proliferation, migration and invasion, and reduced apoptosis. By contrast, ETV4 knockdown had the

opposite effect. Apoptosis was detected in ICC cells transfected with LV-ETV4-RNAi; however, no sub- G_1 fraction was observed by flow cytometry in these cells. This discrepancy

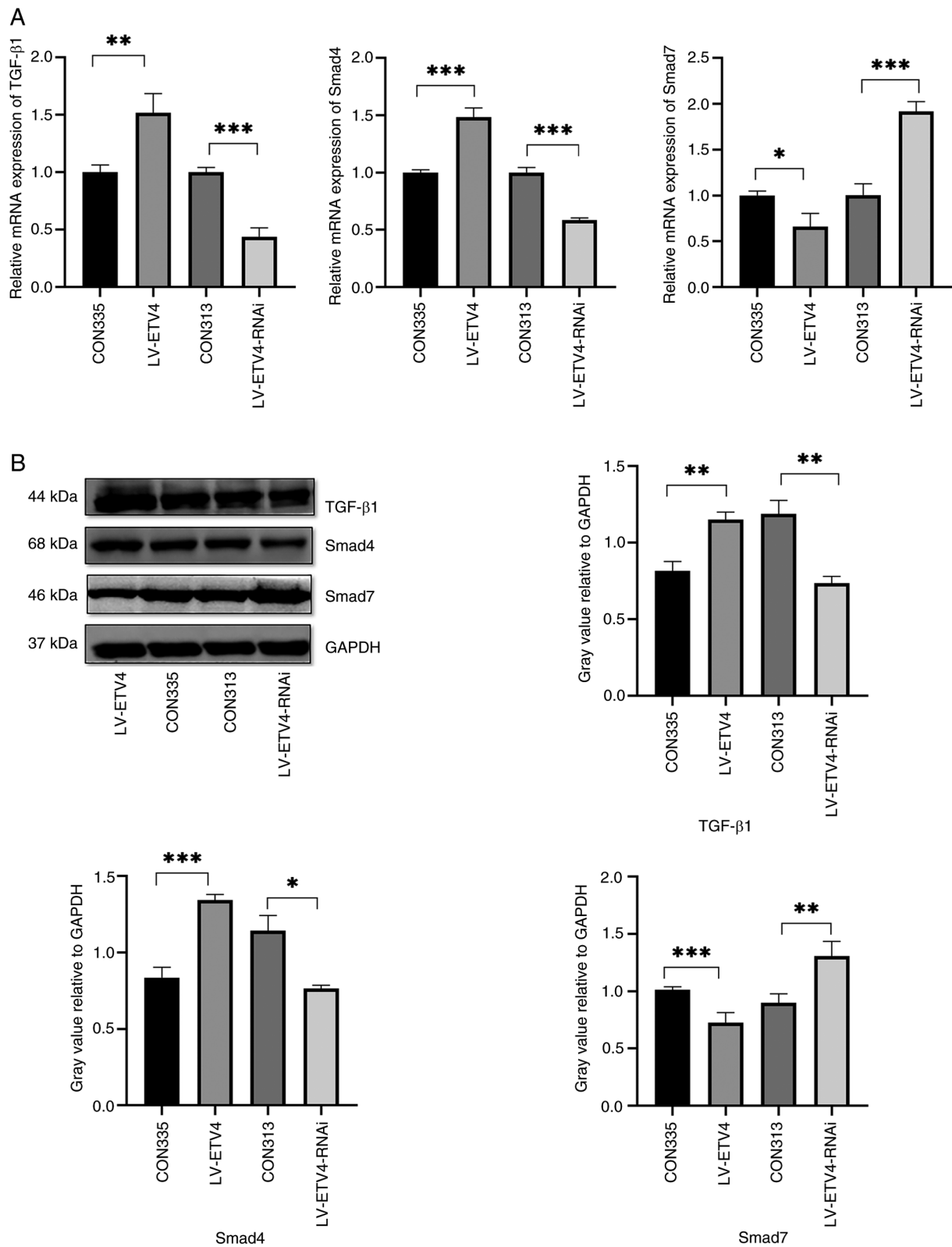


Figure 5. LV-ETV4 activates the TGF-β1/Smad signaling pathway, whereas LV-ETV4-RNAi inhibits the TGF-β1/Smad signaling pathway. (A) Reverse transcription-quantitative PCR results showed that in the LV-ETV4 group, the mRNA expression levels of TGF-β1 and Smad4 were increased, whereas the mRNA expression levels of Smad7 were decreased. In the LV-ETV4-RNAi group, the mRNA expression levels of TGF-β1 and Smad4 were decreased, whereas those of Smad7 were increased. (B) Western blotting showed similar results. *P<0.05, **P<0.01, ***P<0.001. ETV4, E26 transformation-specific sequence variant 4; LV-ETV4, ETV4 overexpression; LV-ETV4-RNAi, ETV4 knockdown.

may be attributed to the removal of the supernatant containing apoptotic cells during cell collection, as well as the possibility that cell cycle arrest did not necessarily coincide with apoptosis.

It is important to note that apoptosis and cell cycle progression can occur concurrently, or that cell cycle arrest may precede apoptosis. Further experiments are required to validate these

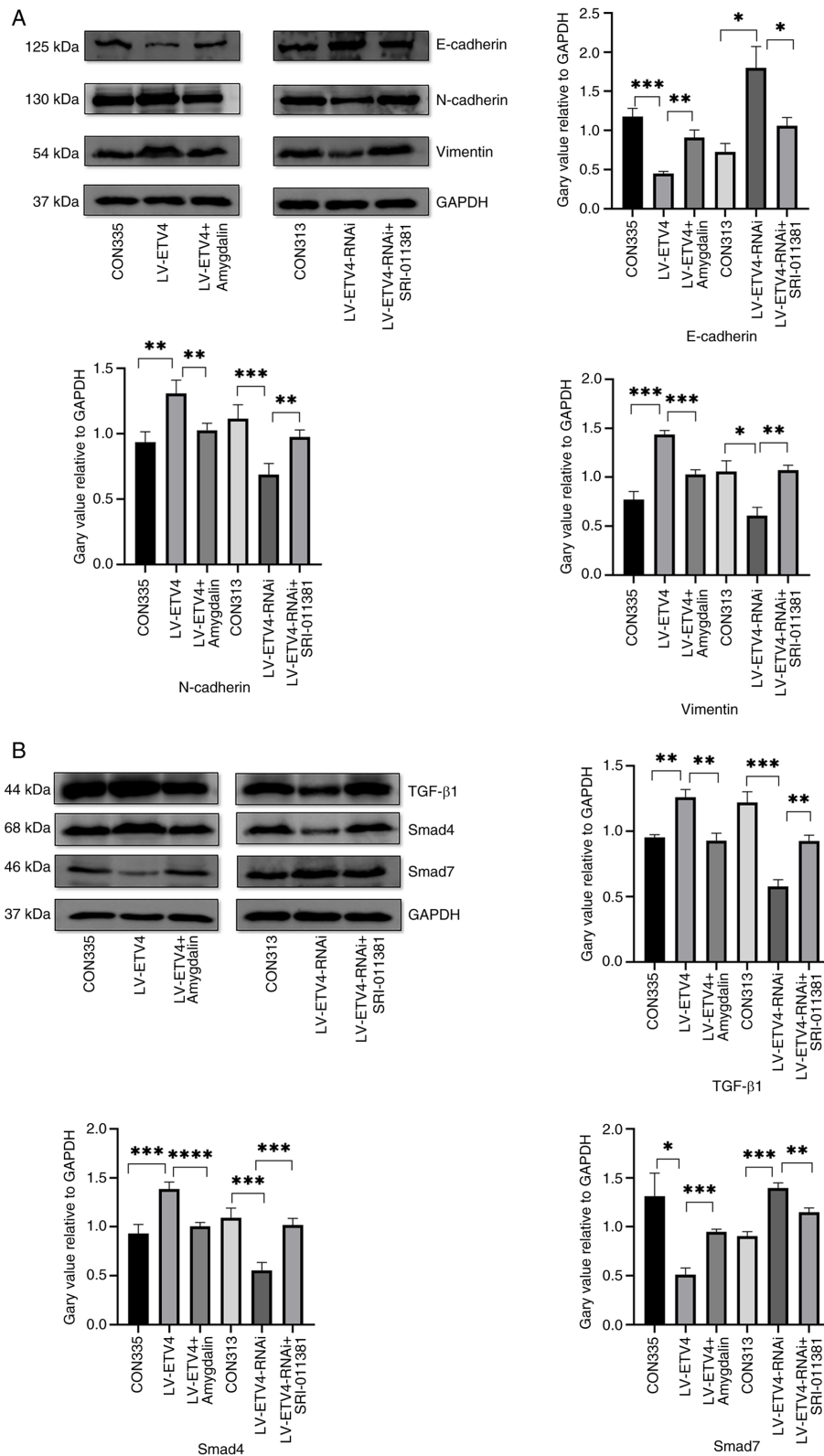


Figure 6. Validation of the results using the TGF- β 1/Smad signaling pathway inhibitor amygdalin and activator SRI-011381 hydrochloride. (A) In the LV-ETV4 group, the expression levels of E-cadherin were decreased, whereas those of N-cadherin and Vimentin were increased compared with those in the CON335 group. In the LV-ETV4 + amygdalin group, E-cadherin expression was increased, whereas N-cadherin and Vimentin expression was decreased compared with those in the LV-ETV4 group. In the LV-ETV4-RNAi group, E-cadherin expression was increased, whereas N-cadherin and Vimentin expression was decreased compared with those in the CON313 group. In the LV-ETV4-RNAi + SRI-011381 group, E-cadherin expression was decreased, while N-cadherin and Vimentin expression was increased compared with those in the LV-ETV4-RNAi group. (B) In LV-ETV4 group, the expression of TGF- β 1 and Smad4 was increased, while the expression of Smad7 was decreased compared with those in the CON335 group. In the LV-ETV4 + amygdalin group, the expression of TGF- β 1 and Smad4 was decreased, while the expression of Smad7 was increased compared with those in the LV-ETV4 group. In the LV-ETV4-RNAi group, the expression of TGF- β 1 and Smad4 was decreased, while the expression of Smad7 was increased compared with those in the CON313 group. In the LV-ETV4-RNAi + SRI-011381 group, the expression of TGF- β 1 and Smad4 was increased, while the expression of Smad7 was decreased compared with those in the LV-ETV4-RNAi group. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001. ETV4, E26 transformation-specific sequence variant 4; LV-ETV4, ETV4 overexpression; LV-ETV4-RNAi, ETV4 knockdown.

findings. Conversely, the LV-ETV4 group, which exhibited reduced apoptosis, did not show a sub-G1 peak. Given the evidence of the cancer regulatory activity of ETV4 (25-27), it appears that ETV4 may act as a pro-carcinogenic factor in ICC and a key driver of ICC infiltration or metastasis.

EMT is characterized by the absence of the epithelial marker E-cadherin and the elevated expression of the mesenchymal marker Vimentin (28). This transition is associated with the loss of epithelial cell polarity, disruption of epithelial cell contacts, alterations in cell adhesion molecules and cytoskeletal components, as well as the degradation of certain extracellular matrix and basement membrane constituents (29). Through EMT, tumor cells are able to breach the histological barriers, enabling invasion, separation and metastasis from primary sites to adjacent or distant normal tissues. E-cadherin serves a crucial role in facilitating this process (30). The absence of E-cadherin in cancer leads to metastasis and diffusion (31), and activation of several EMT transcription factors, such as Twist and β -catenin (32). A significant characteristic of the EMT is the occurrence of 'cadherin alteration', in which there is a decrease in E-cadherin expression and an increase in N-cadherin expression (33). N-cadherin serves as a marker for active EMT, and its abnormal expression is strongly linked to multiple facets of malignant tumor advancement, including transformation, adhesion, apoptosis, angiogenesis, invasion and metastasis (34). Vimentin is another indicator of the mesenchymal transition within the EMT (35), and it serves a crucial role in regulating the cellular mechanics necessary for coordinating mechanical sensing, transduction, signaling pathways, motility and inflammatory responses (36), as well as for maintaining cell integrity and combating cellular stress. Overexpression of Vimentin has been observed in numerous epithelial malignancies, such as prostate cancer, gastrointestinal tumors, central nervous system tumors, breast cancer and malignant melanoma. The excessive levels of Vimentin in cancer have been strongly associated with the proliferation and invasion of tumors (37). In the present study, markers of EMT, including E-cadherin, N-cadherin and Vimentin, were detected. The results from both RT-qPCR and western blot analysis revealed the downregulation of E-cadherin, along with the upregulation of N-cadherin and Vimentin, in the group overexpressing ETV4 compared with in the negative control group. These findings suggested that the overexpression of ETV4 may have the potential to trigger EMT. Conversely, a knockdown in ETV4 led to an increase in E-cadherin expression, and a decrease in N-cadherin and Vimentin levels in comparison to the respective negative control group, suggesting that ETV4 knockdown has the ability to suppress the EMT process. These findings indicated that ETV4 may promote EMT in ICC, leading to peritumoral infiltration or distant metastasis. ETV4 has been reported to activate B3GNT3 and mediate TGF- β 1 signal transduction, thereby promoting EMT in liver cancer (38). In colon adenocarcinoma, knocking out ETV4 has been reported to reduce EMT (39), which is consistent with the present study.

TGF- β signaling promotes EMT in cancer cells, facilitating cancer growth and advancement in later stages (40). Upon activation of the TGF- β signaling pathway, cancer cells undergo EMT, resulting in the loss of their epithelial characteristics, such as polarity and cell-cell contact, and the gain of

migratory and invasive capabilities to adopt a mesenchymal phenotype (41). These transformed cells are able to penetrate nearby tissues, lymphatic vessels and blood vessels, leading to distant metastasis. Consequently, TGF- β signaling-induced EMT serves a critical role in the progression of tumor metastasis. In the canonical TGF- β 1/Smad pathway, Smad2/3 binds to Smad4 to form a heteromeric complex, which then enters the nucleus to regulate gene transcription (42). At the same time, TGF- β 1 is also regulated by inhibitory Smads, such as Smad7 (43).

As a vital element in the TGF- β 1/Smad signaling pathway, the absence of Smad4 on its own does not initiate tumor formation. Notably, it can promote tumor progression initiated by other genes or act as a critical initiator by interrupting the cellular DNA damage response and repair mechanisms, thereby increasing genomic instability, suggesting its distinct roles in different types of cancer (44). In a mouse liver cancer model, knocking down Smad4 significantly inhibited tumor development, and the expression levels of EMT-inducible factors Snail and Twist1 were also significantly reduced (45). Knocking down Smad4 in NMuMG breast epithelial cells has been shown to effectively block TGF- β -induced EMT, restore E-cadherin levels, and hinder the induction of N-cadherin and morphological changes (46). Furthermore, knocking down Smad4 by 95% was revealed to effectively block TGF- β -induced EMT (47). Spirulina phycocyanin extract and its active components can suppress the EMT process in endometrial cancer via targeting the TGF- β 1/Smad4 signaling pathway (48). The TGF- β /Smad4 signal can also inhibit the transcription of Kruppel-like factor 5 (KLF5) through Snail; when KLF5 levels are low, sex determining region Y box protein 4 shifts from being a tumor promoter to a suppressor. Consequently, TGF- β /Smad4 effectively induces lethal EMT in PDAC (49). These reports have indicated that the TGF- β signal can affect EMT in a Smad4-dependent manner (44).

Smad7 acts as a potent endogenous inhibitor of TGF- β signaling, which leads to the inhibition of downstream protein activation in the TGF- β signaling pathway, disrupting signal transduction (43). Smad7 competes with Smad4 to associate with the receptor-regulated Smads and recruits the NEDD4-like E3 ubiquitin protein ligase to activate the receptor-regulated Smads, leading to their polyubiquitination and proteasomal degradation (50). The overexpression of microRNA-21 has been shown to promote TGF- β -induced EMT by inhibiting Smad7/p-Smad7 and activating Smad3/p-Smad3 (51). The results of RT-qPCR and western blot analysis in the present study demonstrated that, in the LV-ETV4 group, there was an increase in the expression levels of TGF- β 1 and Smad4, alongside a decrease in Smad7 expression. Conversely, the LV-ETV4-RNAi group displayed a decrease in the expression levels of TGF- β 1 and Smad4, with a corresponding increase in Smad7 expression. These results suggested that activation of ETV4 may trigger the TGF- β 1/Smad signaling pathway, whereas suppressing ETV4 hinders it, thus aligning with an earlier liver cancer study (38). Furthermore, the evaluation of E-cadherin, N-cadherin and Vimentin levels through RT-qPCR and western blot analysis revealed that ETV4 may mediate EMT via the TGF- β 1/Smad pathway. Application of the TGF- β 1/Smad pathway inhibitor amygdalin or activator SRI-011381 hydrochloride aided in confirming the

observations regarding the regulatory impact of ETV4 on EMT.

The present study mainly focused on the effects and mechanisms of ETV4 on ICC cells, providing novel insights into ICC progression and providing a basis for further clinical research on ETV4. EMT is a complex process involving numerous signaling pathways. However, it should be noted that the present study has certain limitations. The findings regarding TGF- β 1/Smad signaling-induced EMT were based only on *in vitro* experiments. Additional data on the expression of ETV4 in clinical patient tissue, and validation studies using animal models are needed. There is also a lack of validation in other ICC cell lines and the effects on other downstream genes/proteins of ETV4, including cell cycle-related proteins such as cyclin D1, and apoptosis-related genes such as Bax/Bcl-2. Our forthcoming studies will be centered on comprehensive exploration, using both clinical and animal studies, to unravel the downstream genes/proteins such as cyclin D1 and Bax/Bcl-2, regulated by ETV4. In addition, we aim to elucidate the synergistic or antagonistic mechanisms involving the TGF- β 1/Smad signaling pathway and other pathways.

In conclusion, the present study showed that ETV4 may act as a cancer inducer in ICC, which could enhance the proliferation, migration and invasion, and inhibit the apoptosis of Hccc9810 cells. Notably, ETV4-induced increases in N-cadherin and Vimentin expression, along with the decrease in E-cadherin, may initiate EMT via the TGF- β 1/Smad signaling pathway.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

GT contributed to the conception of the study. LL performed the experiments, contributed to the data analyses and wrote the manuscript. YF, XX and MX helped perform the experiments and the analysis with constructive discussions. GT and LL confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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