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Original Article

TM2D1 contributes the epithelial-mesenchymal transition of hepatocellular carcinoma via modulating AKT/β-catenin axis

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Abstract: Various epidemiology studies showed the correlation between Alzheimer's disease (AD) and low incidence of cancer. However, the etiology underlying etiology of AD-related carcinogenesis remains largely elusive. Our study focused on characterizing the role of TM2D1 (TM2 domain containing 1) in hepatocellular carcinoma. TM2D1 is also known as β-amyloid peptide binding protein and is critical to the pathogenesis of AD. We found that TM2D1 is increasingly expressed in HCC tumors relative to the peritumoral tissues of the matched tumors and high TM2D1 expression predicts unfavorable clinical outcomes. TM2D1 overexpression induced HCC cell proliferation, migration and invasion, which was related to the epithelial-mesenchymal transition (EMT) observed in these cells. Conversely, TM2D1 depletion led to opposite phenotype in HCC. Mechanistically, we found that TM2D1 promoted Akt and β-catenin hyper-activation, which corresponded with molecular marker change in EMT signaling pathway. Taken together, our results indicated that TM2D1 played an important role in the EMT process in HCC cells by activating AKT and β-catenin signaling and may become a promising therapeutic target in HCC.

Keywords: Hepatocellular carcinoma, EMT, TM2D1, AKT/β-catenin, prognostic marker

Introduction

Hepatocellular carcinoma (HCC) remains to be one of the most prevalent life-threatening cancers worldwide [1]. Surgical resection is still the cornerstone of curing HCC patients. However, despite the radical resection, approximate 60% to 70% of patients experience recurrence or distant metastasis within 5 years [2]. Hence, it is necessary to better understand the molecular mechanisms of the HCC invasion and metastasis properties, which is expected to lead to better treatment strategies for this deadly cancer. Epithelial-mesenchymal transition (EMT) is a transdifferentiation process that epithelial cells lose its polarity and gain mesenchymal-cell like motile ability, which implicates enhanced cell invasion and epithelial plasticity. EMT program correlates with the HCC recurrence and metastasis [3, 4]. Thus, elucidation of the underlying molecular mechanism of EMT may ultimately help in the development of innovative therapeutic strategies against HCC recurrence/metastasis.

Alzheimer's disease (AD) is a common neurodegenerative disease worldwide. Intriguingly, a series of population-based epidemiologic studies have shown the inverse correlation between the incidence of AD and cancer [5-9]. Therefore,

a better understanding of this inverse correlation may provide us some new therapeutic avenues in cancer. β-amyloid (Aβ) is the well-known initiation factor in AD pathogenesis, which is the product of amyloid precursor protein (APP) proteolysis by β- or γ-secretase [10, 11]. The relationship between Aβ or APP and various cancers (including HCC) has been partially reported [12, 13]. TM2D1 (TM2 Domain Containing 1) contains a structural module related to G protein-coupled receptor superfamily which has seven transmembrane domains and can bind β-amyloid protein [14]. Considering the epidemiology of inverse relationship between AD and cancer, we speculate that TM2D1 may have important effects in cancer including HCC.

In this present study, we firstly analyzed whether TM2D1 affected cell proliferation, migration and invasion in HCC. Additionally, we probed the prognostic significance of TM2D1 for the HCC patients. Finally, we revealed the molecular mechanism by which TM2D1 may promote HCC tumorigenesis.

Materials and methods

Patients and specimens

In order to explore the expression of TM2D1, 20 matched samples of liver cancer resection specimen were collected from Zhongshan Hospital of Fudan University between January and March in 2018. To determine the relationship between TM2D1 and patients prognosis, 195 HCC patients were enrolled between January 2012 and December 2012. Approval for the use of human subjects was provided by the research ethics committee of Zhongshan Hospital of Fudan University. Informed consent was obtained from all the patients. Recurrence free survival (RFS) is the interval between surgery and the tumor recurrence of patients. Overall survival (OS) represents the time between the surgery and the death.

Cell lines and cell culture

HCC cell lines SMMC-7721, Hep3B, Huh7 and HepG2 were obtained from the cell bank at the Institute of Biochemistry and Cell Biology, China Academy of Science (Shanghai, China). L02, MHCC97L, MHCC97H and HCCLM3 cell lines were previously generated and preserved by our institute. All the up-mentioned cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) containing with 10% fetal bovine serum (Gibco) at 37°C under 5% CO_2.

RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

We extracted total RNA by RNeasy mini kit (Qiagen) according to the manufacturer's protocols. SuperScript III Platinum SYBR green one-step qRT-PCR kit (Thermo Fisher) was used to qualify the target genes. The endogenous control in the experiment was GAPDH. The primers used are listed as follows: TM2D1, F: 5'-TGTCCTGTGGTTCGTCTCAGT-3' R: 5'-GGTTCTTGCGTAGCGTCATTT-3'; E-Cadherin, F: 5'-GTAGGAAGGCACAGCCTGTC-3' R: 5'-CAGCAAGAGCAGCAGAATCA-3'; N-Cadherin, F: 5'-GAGCATGCCAAGTTCCTGAT-3' R: 5'-TGGCCACTGTGCTTACTGAA-3'; vimentin, F: 5'-CTGCAGGACTCGGTGGACTT-3' R: 5'-GAAGCGGTCATTCAGCTCCT-3'; SNAIL, F: 5'-TCTGAGGCCAAGGATCTCCA-3' R: 5'-GTGGCTTCGGATGTGCATCT-3'; Twist, F: 5'-GCCGACGACAGCCTGAGCAA-3' R: 5'-CGCCACAGCCCGCAGACTTC-3'; CCDN1, F: 5'-GCTGCGAAGTGGAAACCATC-3' R: 5'-CCTCCTTCTGCACACATTTGAA-3'; MYC, F: 5'-GGCTCCTGGCAAAAGGTCA-3' R: 5'-CTGCGTAGTTGTGCTGATGT-3'; JUN, F: 5'-TCCAAGTGCCGAAAAAGGAAG-3' R: 5'-CGAGTTCTGAGCTTTCAAGGT-3'; GAPDH, F: 5'-ATGGGGAAGGTGAAGGT-3' R: 5'-AAGCTTCCCGTTCTCAG-3'.

The ΔCq method was used to calculate the relative quantities of mRNA expression. The equation is listed as follows: $2^{-\Delta Ct}$ (ΔCt = Ct[target gene]-Ct[GAPDH]).

Western blot analysis

RIPA lysis buffer (Beyotime, China) containing PMSF (Roche, Germany) was used to lyse the cells. AKT activator SC-79 and inhibitor MK-2206 were purchased from MCE (USA). Protein concentration was qualified with the BCA protein kit (Beyotime, China). After qualified the protein by BCA protein kit (Beyotime, China), 30 μg of protein samples were loaded to run SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. Different primary antibodies were incubated for detection. They were rabbit anti-human antibodies against TM2D1 (1:500, Abcam, USA), AKT (1:800, CST, USA), pAKT (1:1000, Abcam,

USA), ERK (1:500, Abcam, USA), pERK (1:750, Abcam, USA), P65 (1:500, Abcam, USA), pP65 (1:500, Pharmingen, USA), E-Cadherin (1:1000, Abcam, USA), N-Cadherin (1:800, Abcam, USA), Fibronection (1:750, Abcam, USA), Vimentin (1:800, Abcam, USA), GSK3β (1:1000, Abcam, USA), pGSK3β (1:750, CST, USA), active β-catenin (1:800, Abcam, USA), β-catenin (1:1000, Abcam, USA), Cyclin D1 (1:1000, Abcam, USA), and GAPDH (1:2000, Proteintech, China) After incubation at 4°C for 12 hours and another incubation for 2 hours with the secondary antibody (1:5000, Jackson ImmunoResearch Labs, West Grove, PA, USA), the membranes were scanned.

Cell proliferation, colony formation, migration and invasion assays

Cell proliferation was determined by the cell counting kit-8 (CCK8; Dojindo) assays. 5×10^3 cells were aliquoted into 96-well plates and cultured overnight at 37°C with 5% CO_2. After waiting for proper intervention and time duration, CCK8 solution (10 μl) was injected into each well. By measuring the absorbance at 450 nm, cell concentration and growth rate can be calculated.

A colony formation assay was conducted to further evaluate cell proliferation. Cells were cultured into 6-well plates at the concentration of 1×10^3 cells per well with DMEM containing 1% FBS. The medium was changed every 4 days. After two weeks, the colonies were fixed with ice-cold 4% paraformaldehyde and further analyzed by crystal violet staining.

Evaluation of cell migration and invasion ability was performed through wound healing and transwell assays. In invasion tests, 1×10^5 cells were seeded in the upper chamber with a Matrigel-coated membrane (dilution: 1:6). The lower chamber was supplemented with DMEM containing 10% FBS to serve as chemoattractant. Waiting for incubation of 24 or 48 hours at 37°C, we fixed the cells that invaded the opposite surface of the membrane with 4% methanol and stained with crystal violet. We randomly counted ten 200 × microscopic fields for further analysis of each sample.

In the wound healing assays, cells were seeded and grown to 80-90% confluence in 6-well plates. After removing the medium, we starched a straight line by a 200 μl pipette tip on the cell monolayers. Each plate was rinsed twice with PBS, thus removing the floating cells. The cells were cultured in The serum-free medium. By observing under a microscope at 0 h and 24 h after scratching, the wound was evaluated.

Cell transfection

Cells were transfected using the retroviral vector pMSCV for stable knockdown (KD) of TM2D1 or negative control (Mock). Retroviruses are generated by packaging 293T cells. The sequence of pLKO.1-shRNA targeting the TM2D1 mRNA was CCGGGCTACGCAAGAACCAGTTAACCTCGAGGTTAACTGGTTCTTGCGTAGCTTTTTTG for shTM2D1-1, CCGGAGCCCATATCTTGCCGAAATGCTCGAGCATTTCGGCAAGATATGGGCTTTTTTTG for shTM2D1-2 and CCGGGACCTCAAAGTGGGACAATATCTCGAGATATTGTCCCACTTTGAGGTCTTTTTTG for shTM2D1-3. Cells were infected with retrovirus and puromycin selection was performed for 3 days before further test.

Tissue microarray (TMA) and immunohistochemistry (IHC)

Tissue microarrays are immunohistochemically stained by the avidin-biotin-peroxidase complex method. After pretreating the slides following IHC protocols, primary anti-human-TM2D1 antibodies were applied and incubated overnight at 4°C overnight following rehydration and microwave antigen retrieval. Subsequently, horseradish-peroxidase secondary antibody was incubated for half an hour at 37°C. Slides were then stained with 3'3-diaminobenzidine tetrahydrochloride. Mayer's hematoxylin was used for counterstaining. As mentioned previously, TM2D1 staining intensity was assessed under microscope and stratified as either strong or weak [15].

Nude mice xenograft model

Six-week-old nude male mice were obtained from the Chinese Science Academy (Shanghai, China) to set up the xenograft model in specific-pathogen-free (SPF) condition. To evaluate the proliferation ability, 5×10^6 cancer cells were injected subcutaneously into the left upper ventral area of nude mice. The mice were sacrificed about 4-5 weeks later, waiting the volume of tumor reaching around 100 mm^3. The tumor

tissues were divided into 2 × 1 × 1 mm^3 slices using the aseptic technique and then injected into the liver with a 27-gauge needle. At six weeks, each group of mice was euthanized by anesthesia overdose and the visceral organs, including the lungs and livers, were collected. The tumor size was measured and recorded every week using a vernier caliper, calculated by the equation: tumor volume (mm^3) = (L × W^2)/2, where L represented long axis and W = represents the short axis. Five weeks after tumor tissue xenograft, the mice were euthanized, and tumor tissues and lung tissues were harvested for hematoxylin and eosin staining.

Statistical analysis

Statistical analysis was conducted using SPSS 22.0 software (IBM) and R 3.2. One-way analysis of variance (ANOVA) or Student t test was used for the Statistical Inference of continuous data. Continuous variables were presented as the mean ± SEM. In contrast, categorical data were analyzed by χ^2 test or Fisher's exact test. Kaplan-Meier survival method and log-rank test were applied to judge the relationships between TM2D1 expression level and OS or TTR. The independent prognostic factors of HCC patients were analyzed by univariate and multivariate Cox regression analysis to determine the independent prognostic factor for HCC patients. Statistical significance was cut by $P < 0.05$ with two tails.

Results

TM2D1 over-expression in human HCC

To explore the potential role of TM2D1 in HCC, we first identified TM2D1 messenger RNA (mRNA) and protein expression of TM2D1 in 7 established HCC lines and one non-neoplastic hepatic cell lines (L02) determined. After 96 hours of cell culture, we found that all HCC cell lines tested over-expressed both mRNA and protein levels of TM2D1 compared with the non-neoplastic hepatic cell line (**Figure 1A**). In addition, we also evaluated the mRNA expression of TM2D1 in 20 pairs of HCC tumors and peritumoral tissues. Fourteen of twenty (70%) patients showed high level of TM2D1 mRNA in tumors than peritumoral tissues (**Figure 1B**). In accordance with mRNA expression levels, we also found that TM2D1 protein levels were higher in tumor samples compared to peritumoral tissues in most cases (**Figure 1C**). To confirm our findings, we also checked three clinical datasets from GEO database and found that compared with peritumoral tissues, the expression level of TM2D1 in HCC tumors were always higher ($P < 0.05$, **Figure 1D**).

High expression of TM2D1 was correlated with poor prognosis of liver cancer patients

To evaluate the role of TM2D1 in the survival of liver cancer patients, we constructed a TMA with 195 paired tumor-adjacent tissues and the corresponding tumors from patients who received radical hepatectomy in Zhongshan Hospital of Fudan University were performed IHC. The 195 patients were dichotomized as TM2D1 low (n = 109) and TM2D1 high (n = 86) according to the IHC data (**Figure 1E**). High expression of TM2D1 was significantly correlated with larger tumor size ($P = 0.008$) and the presence of microvascular invasion ($P < 0.001$) (**Table 1**). High TM2D1 expression was significantly correlated with the poor overall survival and recurrence-free survival after radical hepatectomy (OS and RFS, $P < 0.001$, **Figure 1F** and **1G**) in univariate Cox regression analysis. Multivariate Cox regression of all significant variables in univariate analyses confirmed that high TM2D1 expression was the independent prognostic factor for recurrence-free survival (hazard ratio (HR) = 1.702, 95% confidence interval (CI): 1.142-2.528, $P = 0.009$) and overall survival HR = 1.908, 95% CI: 1.204-3.024, $P = 0.006$) (**Table 2**). Taken together, our TMA results showed that high TM2D1 expression was an independent indicator for unfavorable prognosis after radical surgery in HCC patients.

TM2D1 enhanced the in vitro proliferation and invasion of HCC cell

Next, we aimed to characterize the function of TM2D1 in HCC cells. We picked two representative HCC cell lines, HCCLM3 and Huh7, where TM2D1 was abundantly expressed. We infected these cell lines with three independent TM2D1 shRNAs and among them, two shRNAs showed significant downregulation of TM2D1 on both mRNA and protein levels. In these cells, knockdown of TM2D1 reduced HCCLM3 and Huh7 cell proliferation. Conversely, we also overexpressed TM2D1 in two representative HCC cell lines (Hep3B and MHCC97L) with relatively lower endogenous TM2D1. Our qRT-PCR and

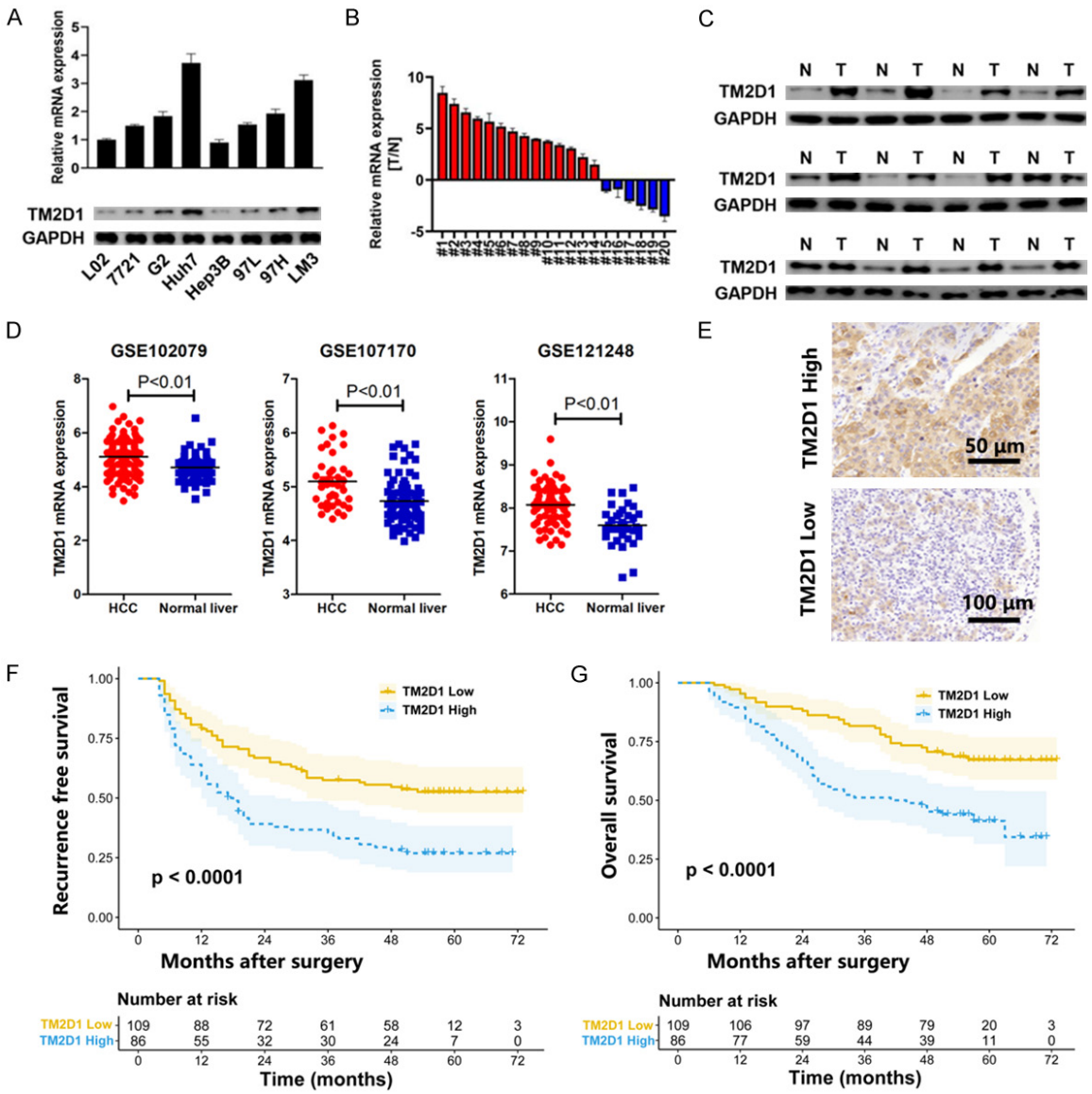

Figure 1. TM2D1 is upregulated in hepatocellular carcinoma (HCC) and correlated with poor prognosis. A. TM2D1 mRNA and protein expression in seven established HCC cell lines and one normal human hepatic cell line. B. Compared with paired adjacent normal liver tissues of 20 HCC patients, the fold change of tumor TM2D1 mRNA expression. C. Representative protein expression of TM2D1 in 12 matched HCC tumors (T) and adjacent peritumoral liver tissues (P). D. TM2D1 mRNA levels in three clinical datasets from GEO databases. E. Typical immunohistochemistry (IHC) images of HCC specimens, which was defined as high (strong) or low (weak) expression staining for TM2D1 in this TMA. F. RFS of patients with different levels of TM2D1 expression (Kaplan-Meier analysis). G. OS of HCC patients with different levels of TM2D1 expression (Kaplan-Meier analysis).

WB analysis confirmed the TM2D1 overexpression in both cell lines (**Figure 2A**). Next, we examined the cell proliferation as a function of time and found that TM2D1 overexpression promoted cell proliferation (**Figure 2B**). In addition, clonogenic assays showed that TM2D1 depletion by multiple independent shRNAs led to decreased colony formation in HCCLM3 and Huh7 cells. On the other hand, TM2D1 overexpression resulted in increased colony formation for Hep3B and MHCC97L cells (**Figure 2C**).

TM2D1 triggers epithelial-mesenchymal transition in vitro

Given the profound cell proliferation phenotype observed with TM2D1 knockdown or overexpression, we next decided to analyze the func-

Table 1. Clinicopathologic characteristics of the 195 HCC patients by the TM2D1 expression

Parameters	TM2D1 expression		*P value*
	Low (n = 109)	High (n = 86)	
Sex			
Male	91	77	0.225
Female	18	9	
Age			
< 50	52	32	0.142
≥ 50	57	54	
HBsAg			
Negative	20	19	0.516
Positive	89	67	
Tumor number			
Single	96	68	0.088
Multiple	13	18	
Tumor size (cm)			
< 5	76	43	0.005
≥ 5	33	43	
AFP (ng/ml)			
< 400	71	55	0.864
≥ 400	38	31	
Microvascular invasion			
Absent	92	50	< 0.001
Present	17	36	
Edmondson stage			
I-II	85	57	0.068
III-IV	24	29	
Tumor encapsulation			
Complete	55	39	0.478
None	54	47	

HBsAg, hepatitis B surface antigen; AFP, alpha fetoprotein.

tion of TM2D1 in HCC cell migration and invasion. For migration, we conducted a wound healing assay and found that TM2D1 knockdown in HCCLM3 and Huh7 cells led to decreased cell migration (**Figure 3A**). Conversely, overexpression of TM2D1 caused increased cell migration in Hep3B and MHCC97L cells (**Figure 3A**). Next, we also examined the invasion ability of these cells upon TM2D1 gene manipulations. Consistently, whereas TM2D1 depletion decreased cell invasion, its overexpression led to significantly enhanced cell invasion in multiple HCC cell lines (**Figure 3B**). Therefore, our results demonstrated that TM2D1 promotes cell migration and invasion of HCC cell lines.

Recent studies established that the EMT usually occurred during the invasion and metastasis of many categories of cancers, as well as HCC. In view of our results showing that TM2D1 regulates migration and invasion, we intended to determine the potential effect of TM2D1 on some recognized EMT markers inclusive of E-cadherin, N-cadherin, Vimentin, Snail and Twist. By performing qRT-PCR, we found that knockdown of TM2D1 resulted in an increase in the expression of E-cadherin, while the expression of N-cadherin, Vimentin, Snail and Twist decreased, suggesting that TM2D1 plays an essential role in the HCC EMT process (**Figure 3C**). In accordance with qRT-PCR results, protein expression level of these important EMT genes correlated closely with mRNA levels upon TM2D1 depletion in both HCCLM3 and Huh7 cells (**Figure 3D**). In contrast, overexpression of TM2D1 resulted in a decrease in E-cadherin expression, while the mRNA and protein levels of N-cadherin, Vimentin, Snail and Twist increased (**Figure 3C** and **3D**). To sum up, our results suggest that TM2D1 promoted EMT in HCC.

TM2D1 promotes the growth of HCC xenograft tumor in vivo

Next, in order to further evaluate the significance of TM2D1 in HCC tumorigenesis *in vivo*, an equal amount of HCC cells (5×10^6) infected with either TM2D1-Knockdown (KD), overexpression (OE) or respective controls (mock) were implanted via subcutaneous injection and then the corresponding small tumor tissues were transplanted into the mice liver orthotopically. Consistent with the *in vitro* observation of cell proliferation-defect phenotype with TM2D1 knockdown cells, mice transplanted with tumor tissue derived from LM3 cells carrying TM2D1 knockdown formed significantly smaller tumors than control cells after 5 weeks significantly. In contrast, mice transplanted with tumor tissue derived from 3B cells harboring TM2D1 OE developed significantly larger tumors than control cells ($P < 0.05$, **Figure 4A**). HE staining was also performed to examine the potential lung metastasis in mice at the necropsy. In LM3 cells that displayed high baseline TM2D1, lung metastasis was detected, the phenotype abrogated by TM2D1 knockdown in these cells. On the other hand, Hep3B cells did not show obvious lung metastasis, but tumor tissues derived from Hep3B cells with TM2D1 overexpression in-

Table 2. Univariable and multivariable Cox regression for the recurrence-free survival and overall survival for HCC patients

Variables	RFS		OS	
	HR (95% CI)	*p*	HR (95% CI)	*P*
Univariate analyses				
Sex (Male vs. female)	1.519 (0.852-2.708)	0.156	1.896 (0.916-3.933)	0.085
Age (≥ 50 vs. < 50 years)	1.180 (0.809-1.720)	0.390	1.213 (0.784-1.977)	0.385
HBsAg (Positive vs. negative)	1.207 (0.745-1.958)	0.445	0.950 (0.558-1.616)	0.849
Tumor number (Multiple vs. single)	1.768 (1.116-2.799)	0.015	1.725 (1.034-2.877)	0.037
Tumor size (≥ 5 vs. < 5 cm)	1.825 (1.259-2.646)	0.001	1.931 (1.261-2.958)	0.002
AFP (≥ 400 vs. < 400 ng/ml)	1.489 (1.023-2.169)	0.038	1.409 (0.912-2.175)	0.122
Microvascular invasion (Present vs. absent)	1.868 (1.276-2.753)	0.002	2.152 (1.389-3.333)	0.001
Edmondson stage (III-IV vs. I-II)	1.346 (0.903-2.006)	0.145	1.430 (0.909-2.249)	0.121
Tumor encapsulation (None vs. complete)	1.556 (1.069-2.266)	0.021	1.294 (0.843-1.984)	0.238
TM2D1 (High vs. low)	2.074 (1.429-3.009)	< 0.001	2.382 (1.544-3.675)	< 0.001
Multivariate analyses				
Tumor number (Multiple vs. single)	1.584 (0.965-2.600)	0.069	1.407 (0.822-2.408)	0.213
Tumor size (≥ 5 vs. < 5 cm)	1.481 (0.993-2.209)	0.054	1.573 (1.007-2.457)	0.047
AFP (≥ 400 vs. < 400 ng/ml)	1.489 (1.009-2.197)	0.045	NA	NA
Microvascular invasion (Present vs. absent)	1.142 (0.733-1.779)	0.557	1.429 (0.876-2.331)	0.153
Tumor encapsulation (None vs. complete)	1.594 (1.090-2.330)	0.016	NA	NA
TM2D1 (High vs. low)	1.702 (1.142-2.538)	0.009	1.908 (1.204-3.024)	0.006

RFS recurrence free survival, OS overall survival, NA not available, P < 0.05 was regarded as statistically significant; *p* value was calculated using Cox proportional hazards regression.

duced obvious lung metastasis (**Figure 4B**). This indicated that TM2D1 depletion leads to a reduction of tumor growth and metastasis *in vivo*. We also performed immunohistochemical staining in subcutaneous xenograft tumor models and found that in the LM3 cell line xenograft model with TM2D1 knockdown, E-cadherin expression increased and N-cadherin decreased markedly. Conversely, in 3B cell line xenograft model with TM2D1 overexpression, E-cadherin expression decreased and N-cadherin increased significantly (**Figure 4C**). This phenomenon indicated TM2D1 promoted HCC EMT process *in vivo*.

TM2D1 induces HCC cell proliferation, invasion and EMT via AKT phosphorylation

Our above results suggested that TM2D1 promoted the malignant behavior of the HCC. However, the basic mechanism of TM2D1 regulating HCC is still elusive. To this end, we used a Cignal Finder Reporter Array to unravel the downstream signaling of TM2D1 and found the only significant change affected by TM2D1 depletion is PI3K/AKT signaling (P < 0.05, **Figure 5A**). To confirm this regulation, we performed a series of western blots by examining total and phosphorylation forms of AKT, p65, ERK, and JNK by using TM2D1 depletion and TM2D1 overexpression. Among them, AKT phosphorylation was the only marker that was downregulated by TM2D1 depletion and upregulated upon TM2D1 overexpression in HCC, suggesting that TM2D1 regulation on AKT activation is specific (**Figure 5B**). To further validate these findings, we used the AKT activator (SC-79) [16] in TM2D1-KD HCCLM3 and Huh7 and used AKT inhibitor (MK-2206) in TM2D1-OE Hep3B and MHCC97L to investigate their impact on cell proliferation and invasion. As expected, the AKT activator could reverse the inhibition of proliferation and invasion after TM2D1-KD. To examine whether AKT activation is the critical downstream signaling mediating the effect of TM2D1 in HCC cell proliferation and invasion phenotype, we implemented AKT inhibitor (MK-2206) in HCC cells overexpressing TM2D1 (TM2D1-OE). We found that whereas TM2D1 OE can induce increased cell proliferation and invasion, the phenotype can be completely abrogated by treating these cells with MK-2206 (P < 0.05, **Figure 5C** and **5D**). Next, we tested EMT markers (E-Cadherin, N-Cadherin and

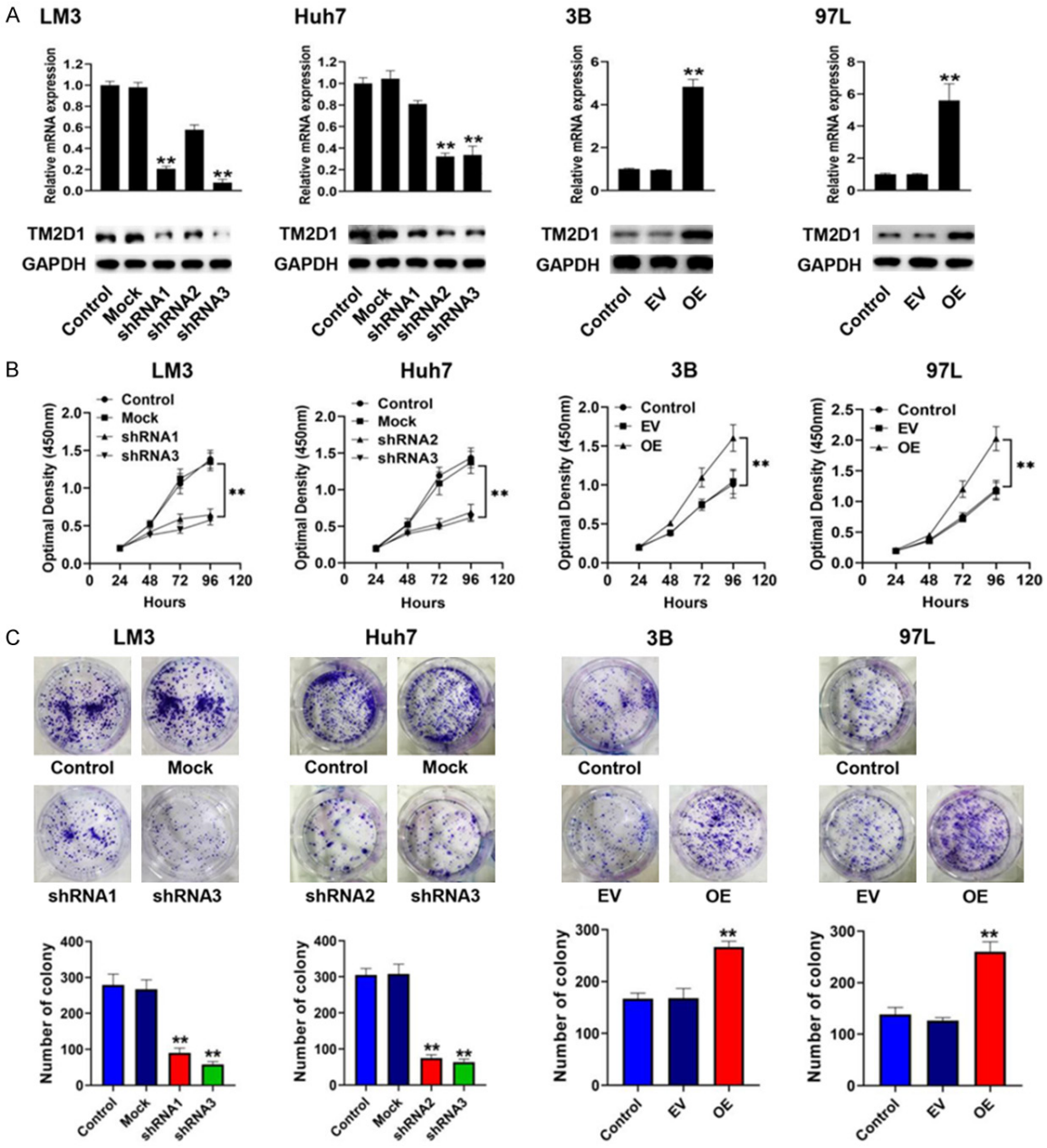

Figure 2. TM2D1 promoted proliferation and migration of liver cancer cells. A. TM2D1 mRNA and protein expression in HCC cell lines after KD or OE. B. The proliferation of HCC cell lines after TM2D1 KD or OE was evaluated by CCK-8 assays. C. Proliferation of HCC cell lines after TM2D1 KD or OE evaluated by colony-formation assays. All *in vitro* experiments were performed three times; ** means $P < 0.05$.

Vimentin) in the designated cells by Western-blot after treated with AKT activator (SC-79) and AKT inhibitor (MK-2206). The epithelial marker (E-Cadherin) was upregulated after TM2D1-KD and downregulated again after further SC-79 treatment in HCCLM3 and Huh7. E-Cadherin was downregulated after TM2D1-KD and was rescued by further MK2206 treatment in Hep3B and MHCC97L. The opposite result was observed in the mesenchymal markers (N-Cadherin and Vimentin) (**Figure 5E**). In all, our results above show that AKT is an important downstream element of TM2D1 contributing to HCC proliferation, invasion and EMT.

TM2D1 induced EMT process by activating AKT/GSK3β/β-catenin pathway

Since AKT functions in the upstream of β-catenin signaling, we decided to explore the

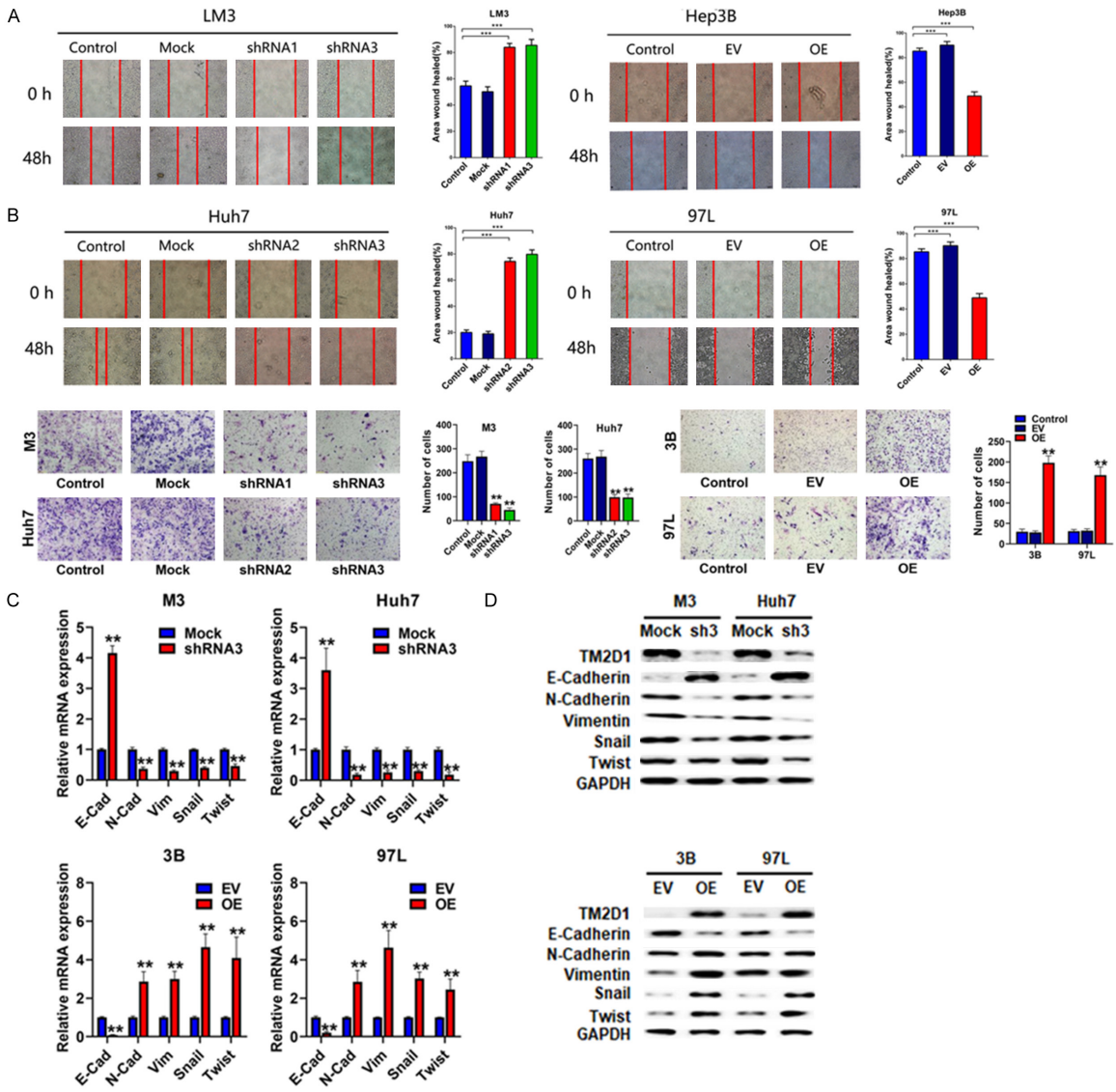

Figure 3. TM2D1 promoted migration and invasion of liver cancer. A. The migration of HCC cell lines after TM2D1 KD or OE were evaluated by wound healing assay. B. Invasion of HCC cell lines after TM2D1 KD or OE evaluated by transwell assays. C, D. The effect of TM2D1 KD or OE on the expression of epithelial and mesenchymal markers by qRT-PCR (C) and western blot (D). All *in vitro* experiments were performed three times; ** means $P < 0.05$.

consequence of TM2D1 on β-catenin activation. We analyzed the protein level of GSK3β, β-catenin, c-MYC and CCND1 in the HCC cell lines after KD or OE of TM2D1 by western-blot. We found the expression GSK3β was negatively regulated by TM2D1 expression, whereas β-catenin positively correlated with the expression of TM2D1. As the markers of β-catenin activation by TM2D1, cyclin D1 and c-Myc were found to be upregulated after TM2D1 overexpression (**Figure 6A**). Conversely, TM2D1 depletion led to a decreased protein expression of β-catenin, cyclin D1 and c-Myc in HCC. In order to further examine the subcellular localization of β-catenin protein, we isolated the protein in either nuclear and cytoplasm and found that TM2D1 depletion decreased nuclear localization of β-catenin and its overexpression caused the opposite phenotype, the results fitting in the canonical view that the nuclear β-catenin is critical for activating transcription of cyclin D1 and c-Myc as observed by our findings (**Figure 6B**). Immunofluorescence analysis also showed that the nuclear translocation of β-catenin from the cytoplasm in TM2D1-knockdown cells decreased and β-catenin translocation from

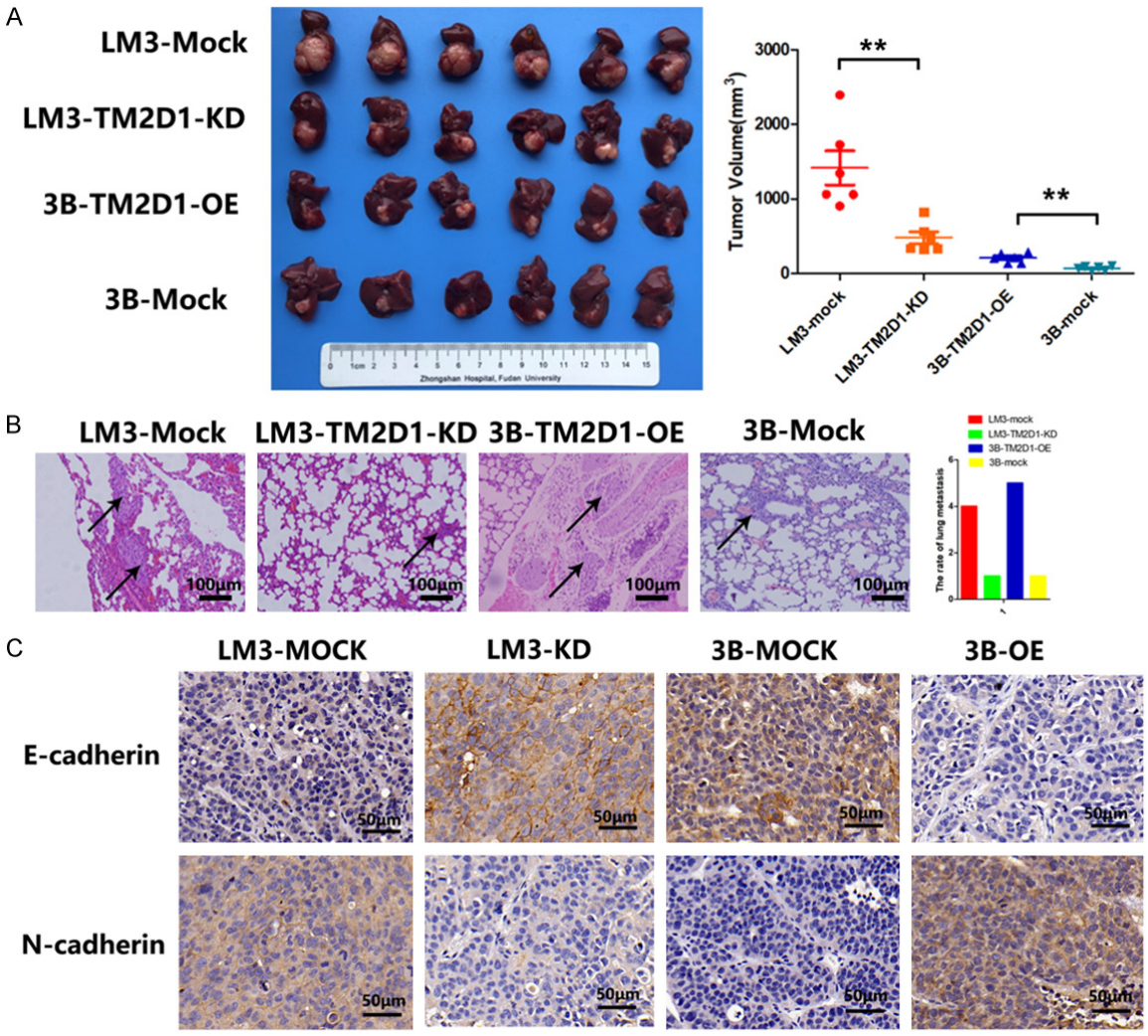

Figure 4. The function of TM2D1 in HCC cell lines in vivo. A. The effect of TM2D1 KD or OE on tumor growth was evaluated *in vivo*. B. The effect of TM2D1 KD or OE on tumor metastasis was evaluated via pulmonary metastasis model. ** indicated $P < 0.05$, arrow indicated lung metastases. C. The effect of TM2D1 KD or OE on EMT process was evaluated via cell line xenograft tumor models.

the cytoplasm toward and into the nucleus increased in TM2D1-overexpression cells (**Figure 6C**). In order to further test the function of β-catenin in EMT of HCC cells regulated by TM2D1, E-Cadherin, N-Cadherin and Vimentin levels were examined by Western-blot and the functional experiment was carried out in the HCC cells after β-catenin OE or KD. Overexpression of β-catenin reversed the effect of TM2D1-KD induced EMT marker expression in HCCLM3 and Huh7 (**Figure 6D**). Conversely, knocking-down the β-catenin reversed the effect of TM2D1-OE induced EMT in Hep3B and MHCC97L (**Figure 6D**). The CCK-8 assay and transwell analysis showed that the β-catenin OE in TM2D1-KD HCCLM3 and Huh7 could rescue the inhibited proliferation and invasion ability. KD of β-catenin in TM2D1-OE Hep3B and MHCC97L could suppress the upregulated ability of proliferation and invasion ($P < 0.05$, **Figure 6E** and **6F**). In summary, our results indicated that through activating AKT/β-catenin signaling pathway, TM2D1 accelerated HCC cell growth and EMT.

Discussion

In this study, we discovered TM2D1 as a new tumor-promoting gene in HCC. It correlates with the enhanced proliferation and invasion ability. In addition, it can induce EMT in HCC cells. Mechanistically, TM2D1 activates AKT/β-

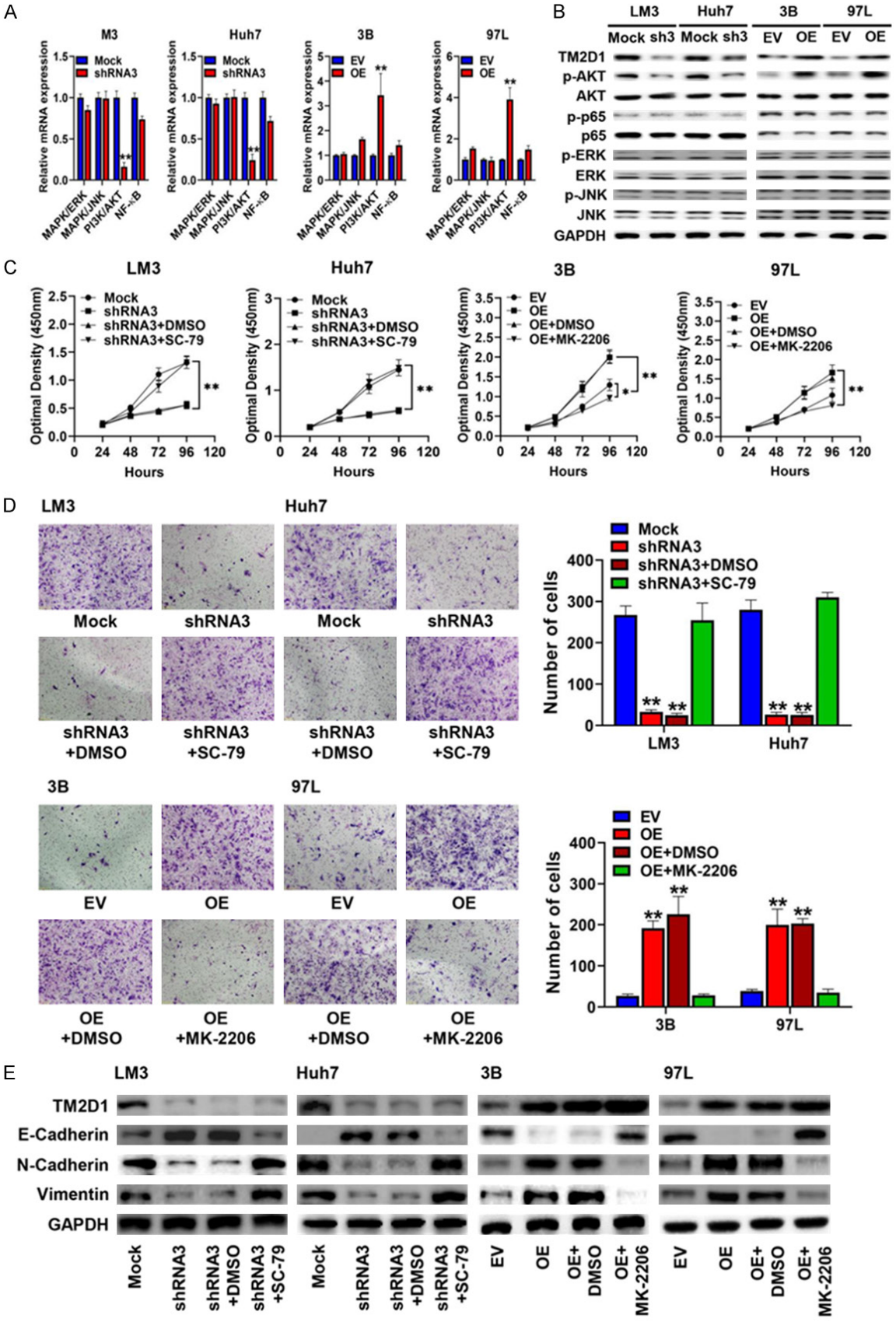
A
M3
Huh7
3B
97L
Relative mRNA expression
Mock
shRNA3
EV
OE
MAPK/ERK
MAPK/JNK
PI3K/AKT
NF-κB
B
LM3
Huh7
3B
97L
Mock sh3 Mock sh3 EV OE EV OE
TM2D1
p-AKT
AKT
p-p65
p65
p-ERK
ERK
p-JNK
JNK
GAPDH
C
LM3
Huh7
3B
97L
Optimal Density (450nm)
Hours
Mock
shRNA3
shRNA3+DMSO
shRNA3+SC-79
EV
OE
OE+DMSO
OE+MK-2206
D
LM3
Huh7
Mock
shRNA3
shRNA3 +DMSO
shRNA3 +SC-79
Number of cells
3B
97L
EV
OE
OE +DMSO
OE +MK-2206
E
LM3
Huh7
3B
97L
TM2D1
E-Cadherin
N-Cadherin
Vimentin
GAPDH
Mock
shRNA3
shRNA3 +DMSO
shRNA3 +SC-79
EV
OE
OE+ DMSO
OE+ MK-2206

Figure 5. TM2D1 induced epithelial-mesenchymal transition (EMT) via enhancing AKT phosphorylation *in vitro*. A. Cignal Finder Reporter Array identified the downstream signaling regulated by TM2D1. B. The effect of TM2D1 KD or OE on the activation status of AKT, p65, ERK, and JNK in HCC cells by western blot. C. Effect of AKT activator (SC-79) and AKT inhibitor (MK-2206) on cell proliferation after TM2D1 KD or OE. D. Effect of AKT activator (SC-79) and AKT inhibitor (MK-2206) on cell invasion after TM2D1 KD or OE. E. Effect of AKT activator (SC-79) and AKT inhibitor (MK-2206) on the expression of EMT markers after TM2D1 KD or OE. All *in vitro* experiments were performed three times; ** means $P < 0.05$.

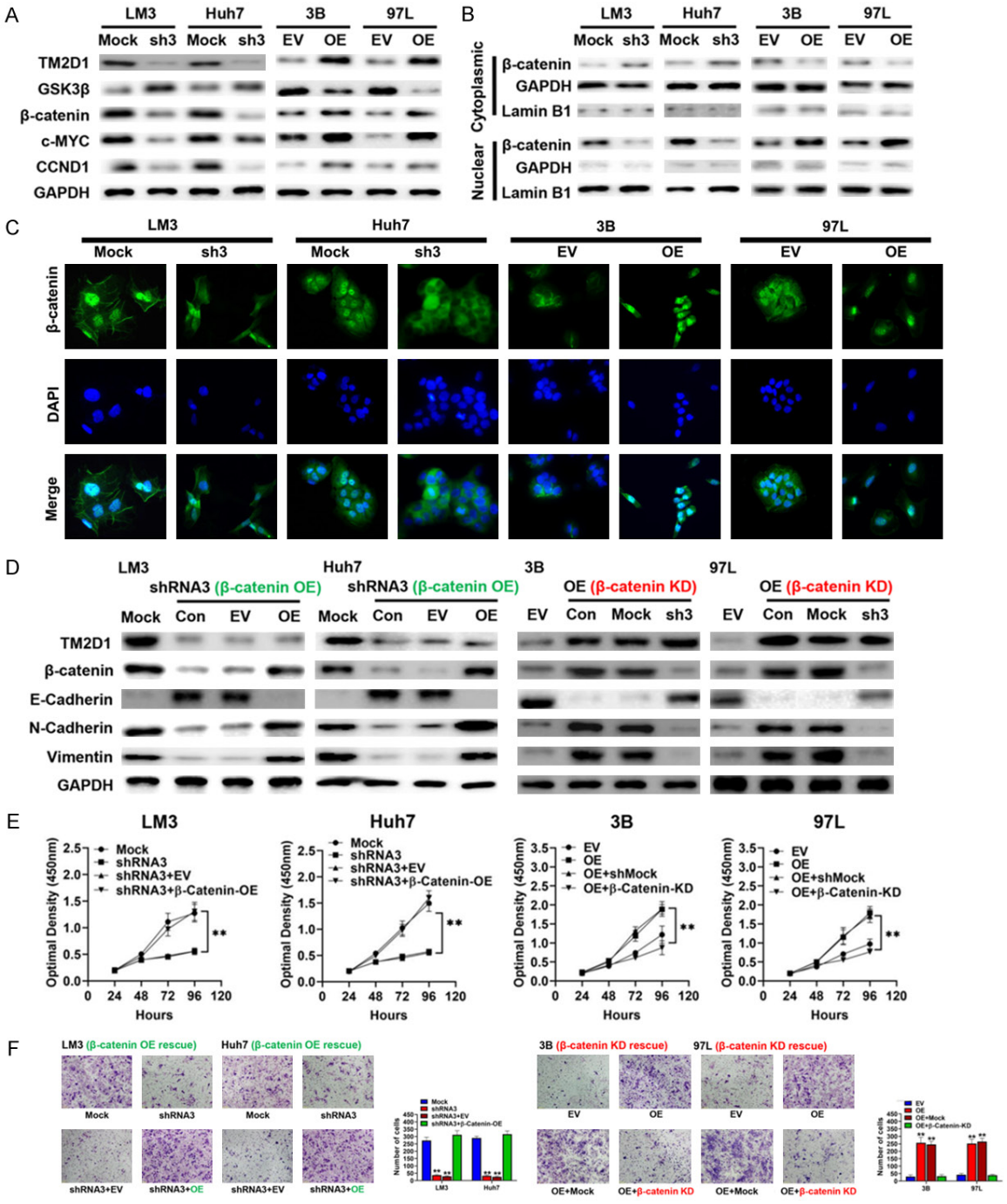

Figure 6. TM2D1 induced epithelial-mesenchymal transition (EMT) by activating AKT/GSK3β/β-catenin axis. A. The effect of TM2D1 KD or OE on the expression of Wnt pathway markers in HCC cells by western blot. B. The effect of TM2D1 KD or OE on β-catenin levels in the cytoplasm and nuclear of HCC cells. C. TM2D1 promotes the nuclear translocation of β-catenin in HCC cells. D. The effect of TM2D1 KD or OE on β-catenin protein level in HCC cells. E.

The effect of β-catenin rescue on cell proliferation in TM2D1 KD or OE cells using CCK-8. F. Using Matrigel invasion assay, the effect of β-catenin rescue on cell invasion was analyzed in TM2D1 KD or OE cells. All in vitro experiments were performed three times; ** means $P < 0.05$.

catenin, therefore contributing to its oncogenic phenotype in HCC.

TM2D1 might participate in the AD because of its close relation with the β-amyloid, the most well-known pathogenic factor of the AD. It has been reported that TM2D1 could bound Aβ with high affinity and specificity. TM2D1 regulated neuronal apoptosis in a caspase-dependent manner initiated by Aβ [17, 18]. Recently, several studies have demonstrated that TM2D1 also associated with clinical outcomes of various cancer patients. Wallbillich et al. identified 9 genes including TM2D1 by LASSCO machine learning algorithm to calculate a transcriptomic risk score for squamous cell carcinoma of the cervix and they found that TRS could identify patients at various risk of mortality [19]. Wu et al. constructed a 25-gene signature including TM2D1 to assigned early-stage lung patients to either high- or low-risk groups and found this 25-gene signature could successfully predicted patients' overall survival [20]. For liver cancer, a highly diverse collection of liver cancer datasets at both genome and transcriptome levels was integrated to constructed robust gene signatures by Zhang et al., which may be important in liver cancer research. Specifically, TM2D1 appeared in at least 14 of 19 liver cancer signatures [21]. However, the detailed mechanism of TM2D1 in cancer progression and metastasis has not been previously reported yet. Our results show that the TM2D1 is highly expressed in HCC and participated in the EMT process of HCC cells, thus linking important pathogenic factor in AD with HCC disease progression.

EMT is an important process in carcinogenesis, invasion and metastasis [22], which induces attenuating epithelial cell intercellular adhesion and boosting the migration and invasion ability. The transition is involved in the HCC progression and correlates with the poor prognosis [3, 4]. To reveal the previously uncharacterized function of TM2D1 in EMT and HCC progression, here we implement gain-of-function as well as loss-of-function studies for TM2D1 in multiple HCC cell lines. We found that mesenchymal markers (Vimentin and N-cadherin) were upregulated at mRNA and protein levels in HCC cell lines significantly followed by TM2D1 overexpression. At the same time, as an epithelial marker, E-cadherin decreased significantly. The opposite regulation was observed at the mRNA or protein levels in the TM2D1 KD cell lines. Our data proved that TM2D1 induces the invasion of HCC cell lines and further validate that the TM2D1 triggers EMT in cell lines, which plays a crucial role in HCC invasion and metastasis. To our knowledge, this is the first study that established the important role of TM2D1 in HCC. To further understand the underlying molecular mechanism, we implemented the reporter system that includes the reported EMT related pathway such as MAPK/ERK, MAPK/JNK, PI3K/AKT and NF-κB in HCC cell line with either TM2D1 overexpression or knockdown. We found the only significant change by either TM2D1 overexpression or knockdown is the PI3K/AKT signaling and the role of this signaling pathway in TM2D1 induced EMT was validated by rescue experiments. Our follow-up expe, pression facilitated the bound of β-amyloid and induced decreased level of extracellular β-amyloid [17]. Previous literatures have demonstrated that extracellular β-amyloid inhibited Akt phosphorylation, thus suppressing the activation of Akt pathway and inhibiting death-inducing proteins, including GSK-3t [23, 24]. Therefore, decreased extracellular β-amyloid will contribute Akt phosphorylation, thus contributing cell survival and cell growth. However, the specific interaction model about β-amyloid and Akt is still mysterious [23]. In the previous reports, AKT/β-catenin is crucial in various cancers [25-30]. Akt can negatively regulate GSK-3β by inducing Ser9 phosphorylation [31]. GSK-3β acts as an important part in β-catenin phosphorylation and degradation and further prevents β-catenin from nucleus translocation. Therefore, it blocks the formation of the complex of β-catenin and the TCF transcription factor family members, which inhibits the β-catenin-responsive gene expression, such as E-cadherin, and induce EMT [32, 33].

However, there are still some limitations in this study. First, we need to further explore the

detailed regulatory network that connects TM2D1 with p-AKT, GSK3-β and nuclear β-catenin signaling in HCC. Second, it is important to determine the upstream signaling responsible for TM2D1 overexpression in HCC. Third, the clinical significance of TM2D1 in both AD and HCC calls for further validation in a large cohort. Finally, the relationship among Aβ, APP and TM2D1 need to be further investigated.

All in all, the present study reveals that TM2D1 promotes HCC cell proliferation, migration and EMT by modulating AKT and β-catenin. TM2D1 is a promising prognostic indicator and treatment approach for HCC.

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Disclosure of conflict of interest

None.

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