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Original Article

TDP-43 induces EMT and promotes hepatocellular carcinoma metastasis via activating Wnt/β-catenin signaling pathway

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Abstract: The trans-activation response DNA-binding protein of 43 kDa (TDP-43) is a nuclear protein that has been shown to be involved in the growth and metastasis of breast cancer, neuroblastoma, and melanoma. However, the effect of TDP-43 on hepatocellular carcinoma (HCC) metastasis remains unclear. Here, we demonstrated that TDP-43 was highly upregulated in both clinical samples and cell lines of HCC. Moreover, knockdown and overexpression of TDP-43 efficiently affected the proliferation and metastasis of HCC cells as well as the expression of some proteins associated with epithelial-mesenchymal transition (EMT) and Wnt/β-catenin signaling pathway. Furthermore, activation of the Wnt/β-catenin pathway by LiCl restored the effect of TDP-43 knockdown on EMT and HCC cells, whereas inhibition of the Wnt/β-catenin pathway by XAV939 negated the effect of TDP-43 overexpression. Importantly, we found that TDP-43 protein could interact with *GSK3β* mRNA and regulate the level of GSK3β protein translation. Taken together, our findings suggest that TDP-43 may activate the Wnt/β-catenin pathway by targeting the inhibition of GSK3β protein translation, thus inducing the proliferation and metastasis of HCC cells, which supports its potential value as a therapeutic target for the treatment of metastatic HCC.

Keywords: TDP-43, HCC metastasis, EMT, GSK3β, Wnt/β-catenin signaling pathway

Introduction

Hepatocellular carcinoma (HCC) is a primary hepatic malignancy with severe morbidity and mortality, which has ranked fifth in all cancer incidences and has become the second leading cause of cancer-related deaths worldwide [1, 2]. Despite recent advances in comprehensive surgical treatment, the clinical outcomes in patients with HCC remain poor, with a five-year survival rate of less than 30% [3, 4]. Although there are currently many clinical treatments for HCC, such as surgical resection, liver transplantation, liver targeted therapy, and systemic therapy, the mortality rate in HCC patients is still very high [5]. In addition, about 70% of HCC patients undergoing surgery are at a risk of recurrence and metastasis within 5 years [6], which has been perplexing many clinicians. Therefore, it is urgent to explore the molecular mechanism of HCC metastasis and seek novel potential and effective therapeutic targets for improving the survival rate in HCC patients.

Recently, a growing number of findings suggested that epithelial-mesenchymal transformation (EMT) was a critical process for tumor cells to gain invasive and motile properties, and the modulation of intercellular adhesion components, such as E-cadherin, N-cadherin, and Vimentin, was the leading cause of HCC metastasis [7-9]. Moreover, increasing evidence has indicated that abnormal activation of Wnt/β-catenin signaling contributed to the EMT initiation and formation of invasive carcinoma [10-12]. β-catenin is a key signal transducer during

the Wnt/β-catenin signaling pathway. The stabilization and nuclear translocation of β-catenin further initiates the transcription of target genes, such as *MYC*, *MYB*, *CJUN*, and *CYCD1*, thereby accelerating the malignant proliferation and metastasis of normal cells [11-13]. β-catenin is usually phosphorylated and degraded by the β-catenin destruction complex composed of glycogen synthase kinase 3β (GSK3β), Axin, and adenomatous polyposis (APC) to prevent its stabilization in the cytoplasm under physiological conditions [12, 14]. A serine/threonine protein kinase GSK3β is the key kinase of the destruction complex. It phosphorylates β-catenin by adding a phosphate group to its N-terminal serine/threonine residue. After the covalent modification by an E3 ubiquitin ligase β-TRCP, the phosphorylated β-catenin is further recognized and degraded by the proteasome [14-16]. Furthermore, when the GSK3β binding sites of Axin or APC protein or the GSK3β phosphorylation site of β-catenin were mutated, β-catenin could not be phosphorylated [17, 18], and growth factors such as insulin and IGF-1 could mediate the inhibition of GSK3β through the PI3K/Akt pathway, which could lead to an imbalance in the phosphorylation and degradation of β-catenin [19, 20]. Therefore, GSK3β plays a critical regulatory role in maintaining intracellular β-catenin dynamic balance, which makes it a novel target for the treatment of metastatic tumors.

The trans-activation response DNA-binding protein of 43 kDa (TDP-43) is a human nuclear protein that is highly conserved among species and ubiquitously expressed in various tissues in mammals and invertebrates [21]. It was initially identified as a protein that bound to the TAR DNA of the long terminal repeat (LTR) region of the human immunodeficiency virus 1 (HIV-1) gene and participated in the process of gene transcriptional regulation [22]. Structurally, TDP-43 is similar to other members of the family of heterogeneous ribonucleoproteins (hnRNP), which contains an N-terminal region and two RNA recognition motifs (RRM1 and RRM2), which are mainly used for interactions with other proteins and bind to specific target RNAs, respectively [23, 24]. It is also involved in the processes of gene expression, including transcription, pre-mRNA splicing, regulation of mRNA stability, and mRNA transport and translation [24-26].

Most previous studies have focused on the relations between abnormal TDP-43 expression and neurodegenerative diseases. Abnormal TDP-43 distribution in the neuronal cytoplasm always results in its hyperphosphorylated and ubiquitinated form that is cleaved to produce C-terminal fragments, forming tau-negative and ubiquitin-positive inclusions (FTLD-U) in the damaged brain tissue, which is characteristic of many neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) and frontotemporal lobe degeneration (FTLD) [27, 28]. With respect to cancer, we found that TDP-43 was involved in promoting the occurrence and development of human cancer. In triple-negative breast cancer (TNBC), TDP-43 can mediate alternative splicing of serine/arginine-rich splicing factor 3 (SRSF3), a proto-oncogene of breast cancer, to promote tumor cell invasion and spread [29]. Glucose is the main energy source in tumor cells, and knockdown of TDP-43 can reduce melanoma cell proliferation and metastasis by down-regulating the expression of type 4 glucose transporter (GLUT4) [30]. Additionally, high TDP-43 expression in neuroblastoma and breast cancer was associated with tumor cell growth and patient prognosis [31].

In the present study, we aimed to examine the expression of TDP-43 in HCC and describe the molecular mechanism by which TDP-43 promotes HCC cell proliferation and metastasis. We reported that the activation of the Wnt/β-catenin signaling pathway may be executed via TDP-43 targeting inhibition of GSK3β post-transcriptional translation. Overall, our study demonstrated that TDP-43 acts as a novel carcinogenic factor in HCC, indicating that it may be a new potential target for HCC therapy.

Materials and methods

Tissue samples

In this study, all clinical HCC tissues (58 cases) samples were provided by the First Affiliated Hospital of Wenzhou Medical University. Before conducting this study, we ensured that all patients signed a written informed consent form. And our study obtained the approval of the ethics committee of the First Affiliated Hospital of Wenzhou Medical University according to the Declaration of Helsinki Principles.

Cell lines and cell culture

Three human HCC cell lines-HCCLM3, MHCC-97H and MHCC97L cells were purchased from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China) and the human normal liver cell line LO2 was obtained from Chinese Academy of Sciences. All cell lines were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 1% streptomycin and penicillin (Sigma-Aldrich, USA) in a humidified incubator with 5% CO_2 at 37°C. Additionally, conventional tests were conducted to confirm the absence of mycoplasma during our experimental study.

Cell proliferation assay

For cell proliferation assay, Cell Counting-8 (CCK-8) Kit (Dojindo Laboratories, Japan) was performed to evaluate the cell proliferation ability at 0 h, 24 h, 48 h, 72 h and 96 h according to the manufacturer's instructions. Briefly, cells were plated into 96-well plates at a final concentration of approximately 2×10^3 cells/well, and 10 μL CCK-8 reagent was added into each well every 24 h, then the cells were incubated in a humidified incubator with 5% CO_2 at 37°C for 2 h. The absorbance of the cell sample was measured at 450 nm.

Cell invasion and migration assay

For cell invasion assay, we used 24-well transwell chambers (Corning, USA, #3422) with 8-μm pore size to assess the cell invasion and migration ability. Firstly, the upper chamber was coated with BioCoat Matrigel (BD Biosciences, USA, #354234) in advance, then 2.5×10^5 cells were seeded into the upper chamber with 100 μL serum-free medium, while the lower chamber was added with 700 μL culture medium containing 20% FBS to induce invading HCC cells, and the cells were cultured in a humidified incubator with 5% CO_2 at 37°C. After 72 h, the upper chamber was gently rubbed with a cotton swab, cells that had invaded to the underside of the insert membrane were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet (Beyotime, Shanghai, China) for 15 min. Migration ability was also examined by transwell insets using the same methods without the supplement of Matrigel. Finally, the number of cell invasion and migration were counted in five randomly selected fields (×100) under the microscope.

Wound healing assay

For wound healing assay, approximately 1×10^6 cells were seeded into 6-well plates cultured at 37°C, 5% CO_2 overnight. Then the cell monolayer wound was gently scraped with 200 μl pipette tip. After scratching, cells were washed 3 times with phosphate-buffered saline (PBS; Gibco, USA) and cultured in DMEM with 2% FBS to attract cell migration. Next, cells were photographed using a microscope every 24 h in the streaking area to quantify the closure of the wound-preserving area. The denuded area closure was calculated by (Denuded distance 0 h - Denuded distance Endpoint)/Denuded distance 0 h.

Plasmid, lentivirus construction and cell transfection

Lipofectamine 3000 Transfection Reagent (Invitrogen, USA) was used for cell transfection according to the manufacturer's recommendation. Plasmid and RNA interference lentivirus were packed and purchased from GeneChem, Shanghai, China. To overexpress TDP-43 in MHCC97L cell line, we established the experimental group (transfected with pcDNA-TDP-43 plasmid, TDP-43) and the control group (transfected with pcDNA empty plasmid, Vector). To knockdown TDP-43, stably infected cell lines HCCLM3 and MHCC97H with RNA interference lentivirus LV-shNC and LV-shTDP-43 were isolated by puromycin (300 μg/ml, Life Technologies, Gibco, USA) selection. The total proteins were extracted from the cells, and the expression of TDP-43 was confirmed by western blot. The sequence of shRNA was as follows: shTDP-43 5'-ccgggcAATAGACAGTTAGAAAGAActcgagTTCTTTCTAACTGTCTATTgctttttg-3'.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated with Trizol reagent (Invitrogen, USA) according to the manufacturer's protocols and quantified by Nanodrop spectrophotometry. And cDNA was synthesized from 1 μg total RNA of each sample using the PrimeScript RT reagent kit (Takara, China) according to the manufacturer's instructions. The CFX Connect TM real-time system (Bio-

Rod, Hercules, CA, USA) was conducted to perform Quantitative real-time polymerase chain reaction with SYBR Green kit (Takara, Dalian, China) following the manufacture' s guidelines. All expression Ct values of target genes were analyzed by the 2-ΔΔCT methods. The specific primers (Sangon, Shanghai, China) used for the study are as follows: TDP-43-qF: 5'-CCCCCTACCCCTTTGTCAAC-3', TDP-43-qR: 5'-GCTCACTGGTGAACTCTGCT-3'; GSK3β-qF: 5'-CCACATGCTCGGATTCAGGC-3', GSK3β-qR: 5'-TGTCCACGGTCTCCAGCATTAGTAT-3'; GAPDH-qF: 5'-CAACGTGTCAGTGGTGGACCTG-3', GAPDH-qR: 5'-GTGTCGCTGTTGAAGTCAGAGGAG-3'.

Western blot analysis

For western blot assay, whole cells or fresh tissues were washed 3 times with cold PBS, lysed in RIPA buffer (Beyotime, Shanghai, China) complemented with protease inhibitor cocktail (Sigma, USA) at 4°C for 10 min. Concentrations of protein were obtained using a bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China) based on manufacturer's instructions. Then, proteins (20 μg per well) were separated by 8%-12% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., USA). The membrane was blocked with 5% skim milk for 2 h at room temperature and then incubated at 4°C overnight with the following primary antibodies: TDP-43 (1:1000, Abcam, ab41881), E-cadherin (1:1000, Abcam, ab1416), N-cadherin (1:1000, Cell signaling Technology, #13116), CyclinD1 (1:1000, Cell signaling Technology, #2978), c-Myc (1:1000, Cell signaling Technology, #5606), GSK3β (1:1000, Abcam, ab12456), Vimentin (1:1000, Abcam, ab92547) and β-catenin (1:1000, Abcam, ab32572). Then, the strips were incubated in HRP-conjugated corresponding secondary antibodies (1:1000) for 2 h at room temperature. After washing the strips 3 times with Tris-buffered salin-Tween (TBST), the bands were visualized with an enhanced chemiluminescence (ECL) system. The expressions of relative proteins were quantified and analyzed by the Image J software. GAPDH (1:1000, Cell Signaling Technology, #5174) was used as an internal reference.

RNA-immunoprecipitation (RIP)

The experiment was carried out according to the previously published method. First, 6 dishes (10 cm) of MHCC97H cells were collected with cell scrapers. The cells were slightly scraped in 10 mL pre-cooled PBS, collected by centrifugation and cleaved with polysome lysis buffer. Protein A+G agarose beads were then incubated with 5 μL of rabbit anti-TDP-43 (Abcam, ab41881) or normal IgG (Beyotime, China, #A7016) for 1 h. The cell lysate was preserved overnight at -80°C. After centrifugation, the protein G agarose-antibody complex was incubated with cell lysate at 4°C overnight. Next day, the agarose beads were washed 6 times with 1 mL NT 2 buffer (containing 1 M urea, 0.1% SDS, 150 mM NaCl). And the proteins were digested in NT 2 buffer with 180 μg protease K at 55°C. Then 1 mL Trizol reagent was added to extract RNA and 20 μg glycogen was supplemented with isopropanol to precipitate ribonucleic acid. To avoid DNA contamination, we used a reverse transcription kit (Takara, China, #RR047A) to remove DNA.

Immunofluorescence

For immunofluorescence assay, cells were cultured in 24-well plate cell slides for 48 h. Firstly, the cells were fixed with 4% paraformaldehyde for 30 min, then washed the cells three times with cold phosphate-buffered saline (PBS) and permeabilized the cells with 0.5% Tritonx-100 for 15 min. Next, after washing with PBS 3 times, cells were blocked with 0.5% BSA for 2 h. We incubated the cells with primary antibodies, such as rabbit anti-Vimentin (1:200, Abcam, ab92547) and rabbit anti-E-cadherin (1:200, Abcam, ab1416) for 12 h, and then incubated with the secondary antibodies Alexa Fluor 594 donkey anti-mouse IgG (Invitrogen, USA) for 2 h, and the nuclear was stained with DAPI for 5 min. Finally, the analysis was performed using confocal microscope (Nikon A1, Japan).

Immunohistochemistry and H&E staining assays

The expression level of TDP-43 in HCC tissues and normal tissues was evaluated by immunohistochemistry (IHC) assay. Tissue sample slides were dewaxed in xylene and dehydrated in graded ethanol. After washing 3 times with PBS, antigen retrieval was performed in sodium citrate buffer, and endogenous peroxide was inhibited with 3% H_2O_2. Then the slides were treated with 3% BSA for 30 minutes, and the samples were incubated at 4°C overnight with

1:200 dilution of anti-TDP-43 antibody. Slides were then incubated with biotinylated goat anti-rabbit secondary antibody (Dako, Denmark) for 30 minutes at room temperature, and then subjected to a color reaction using DAB working solution (Dako, Denmark). After counterstaining with hematoxylin for 2 minutes, the slides were sealed with neutral resin. In the end, the images of IHC staining were photographed under a microscope and the analysis of immunostaining results was evaluated by two pathologists as described previously [32].

Xenograft mouse model

For xenograft mouse model. A total of 12 healthy male BALB/C-nu nude mice at the age of 4-6 weeks weighing 18-20 g were provided by the Experimental Animal Center of Wenzhou Medical University. Firstly, nude mice were fed and observed under SPF standard feeding conditions for two weeks before their use in the study. 2×10^6 cells (HCCLM3-LV-shNC, HCCLM3-LV-shTDP-43) were seeded and resuspended in 100 μL PBS/Matrigel (BD Biosciences, 1:1 mixture), and then the cells were injected into the hepatic lobe of these mice (n=6 per group) respectively. After 7 weeks of injection, the nude mice were sacrificed by cervical dislocation, and then weighed the weight of the tumors. Liver and lung tissues were collected and processed for histological and cytological evaluation. All animal experiments were approved by the institutional animal committee of Wenzhou Medical University for medical laboratory animal sciences.

Statistical analysis

All statistical data were presented as the mean ± SD. T-test was used for statistical analysis of two groups of independent samples, and one-way ANOVA test was used for statistical analysis of more than two groups of samples. Statistical significance was assessed in Pad Prism 6.0 software, and *P* values >0.05 were considered that there is no significant difference between the two groups, *P* values <0.05 were considered statistically significant. ****P< 0.0001, ***P<0.001, **P<0.01, *P<0.05, and N. S. means P>0.05. All acquired data come from independent experiments at least 3 times.

Results

Increased level of TDP-43 expression highly correlates with HCC progression according to the analysis of the TCGA database

To investigate the correlation between TDP-43 expression and clinical characteristics of HCC, clinical data associated with the progression and survival of HCC patients were downloaded from the TCGA database for further analysis. The expression level of TDP-43 was elevated in 370 HCC tissues compared with that in 50 normal liver tissue samples according to the TCGA database, and the results indicated that TDP-43 was significantly overexpressed in HCC (**Figure 1A**). In addition, TDP-43 was positively correlated with the degree of malignancy of HCC, including tumor stage and grade as well as lymph node metastasis (**Figure 1B-D**). Furthermore, patients with high expression level of TDP-43 had a significantly poorer survival rate than those with low/medium patients based on the Kaplan-Meier analysis (**Figure 1E**). These data demonstrated the clinical significance of TDP-43 expression, suggesting that it might be a critical regulator in HCC progression.

TDP-43 is overexpressed in clinical HCC tissues and HCC cell lines

To further confirm the high expression level of TDP-43 in human HCC samples, immunohistochemical (IHC) staining analysis was used to identify the expression of TDP-43 in 48 paired clinical HCC tissue samples. IHC staining showed that TDP-43, was predominantly localized in the nucleus in HCC cells and it was significantly upregulated in HCC tissues compared with paratumoral tissues (**Figure 2A**, **2B**). Similarly, western blot (WB) analysis of TDP-43 expression in 10 paired fresh HCC tissue samples also demonstrated that TDP-43 level was significantly higher in HCC tissues (**Figure 2C**). Additionally, WB analysis showed that the expression of TDP-43 was significantly higher in HCC cell lines HCCLM3 and MHCC97H with high invasive and metastatic potentials, than in MHCC97L cells with low metastatic potential, while the lowest level was observed in the normal human liver cell line LO2 (**Figure 2D**). Collectively, our data indicate that TDP-43 is a potential promoter of HCC metastasis.

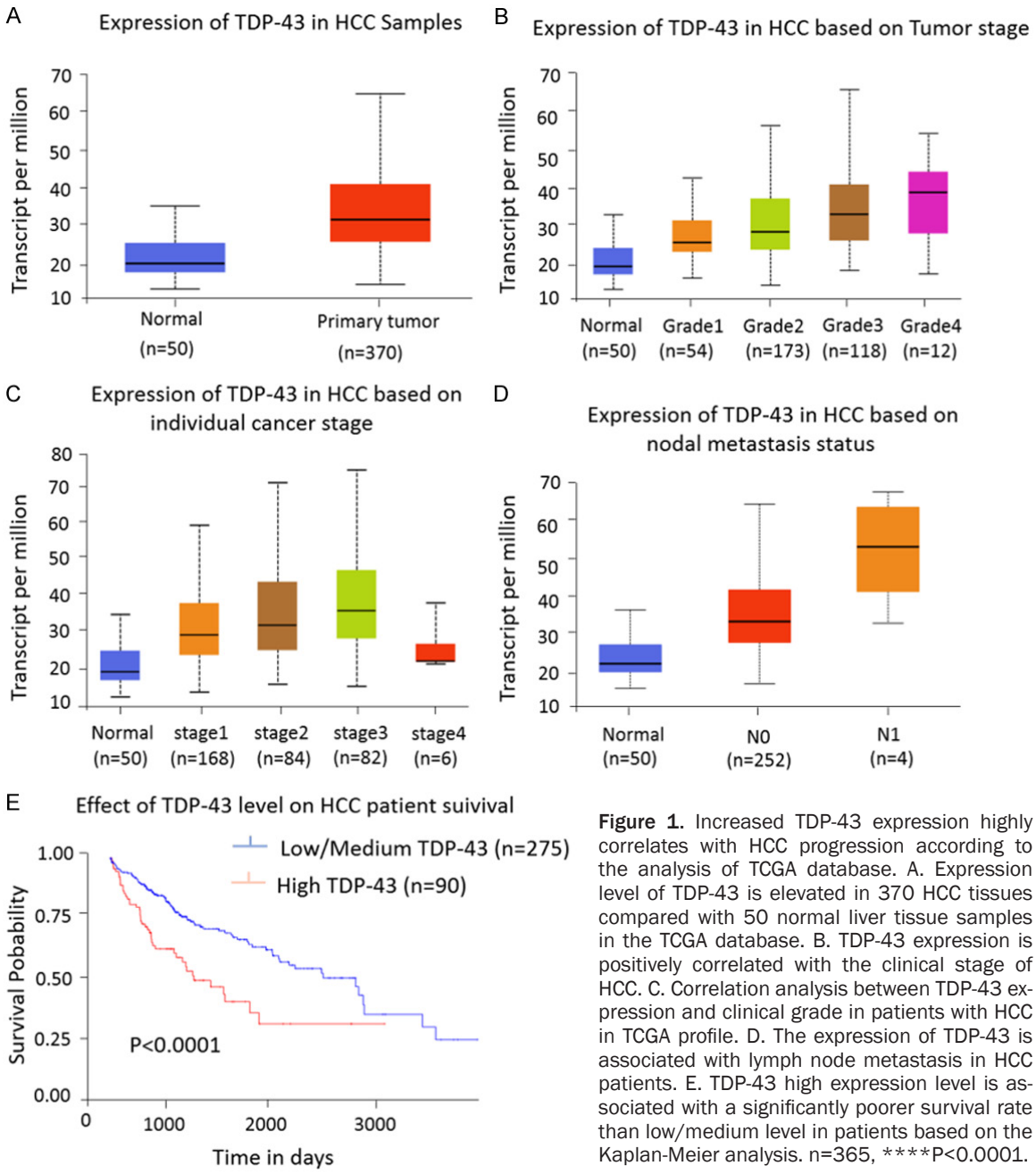

Figure 1. Increased TDP-43 expression highly correlates with HCC progression according to the analysis of TCGA database. A. Expression level of TDP-43 is elevated in 370 HCC tissues compared with 50 normal liver tissue samples in the TCGA database. B. TDP-43 expression is positively correlated with the clinical stage of HCC. C. Correlation analysis between TDP-43 expression and clinical grade in patients with HCC in TCGA profile. D. The expression of TDP-43 is associated with lymph node metastasis in HCC patients. E. TDP-43 high expression level is associated with a significantly poorer survival rate than low/medium level in patients based on the Kaplan-Meier analysis. n=365, ****P<0.0001.

Downregulation of TDP-43 inhibits HCC progression in vivo and in vitro

Since high expression of TDP-43 was detected in HCCLM3 and MHCC97H cell lines (**Figure 2D**), RNA interference lentivirus was used for a stable knock down TDP-43 in these cells. Similarly, the TDP-43 cDNA expression vector was transfected into the MHCC97L cell line for TDP-43 overexpression (**Figure 3A**). Then we found that TDP-43 knockdown inhibited the proliferation and migration of HCC cells according to CCK-8 and wound healing assay results, while its overexpression in MHCC97L cells significantly enhanced their proliferation and migration capacities (**Figure 3B-E**). Furthermore, transwell assay showed that TDP-43 knockdown in HCC cells had significantly lowered their migratory and invasive capacities compared to those in than parental cells. In contrast, TDP-43 upregulation greatly accelerated migration and invasion potentials in MHCC97L

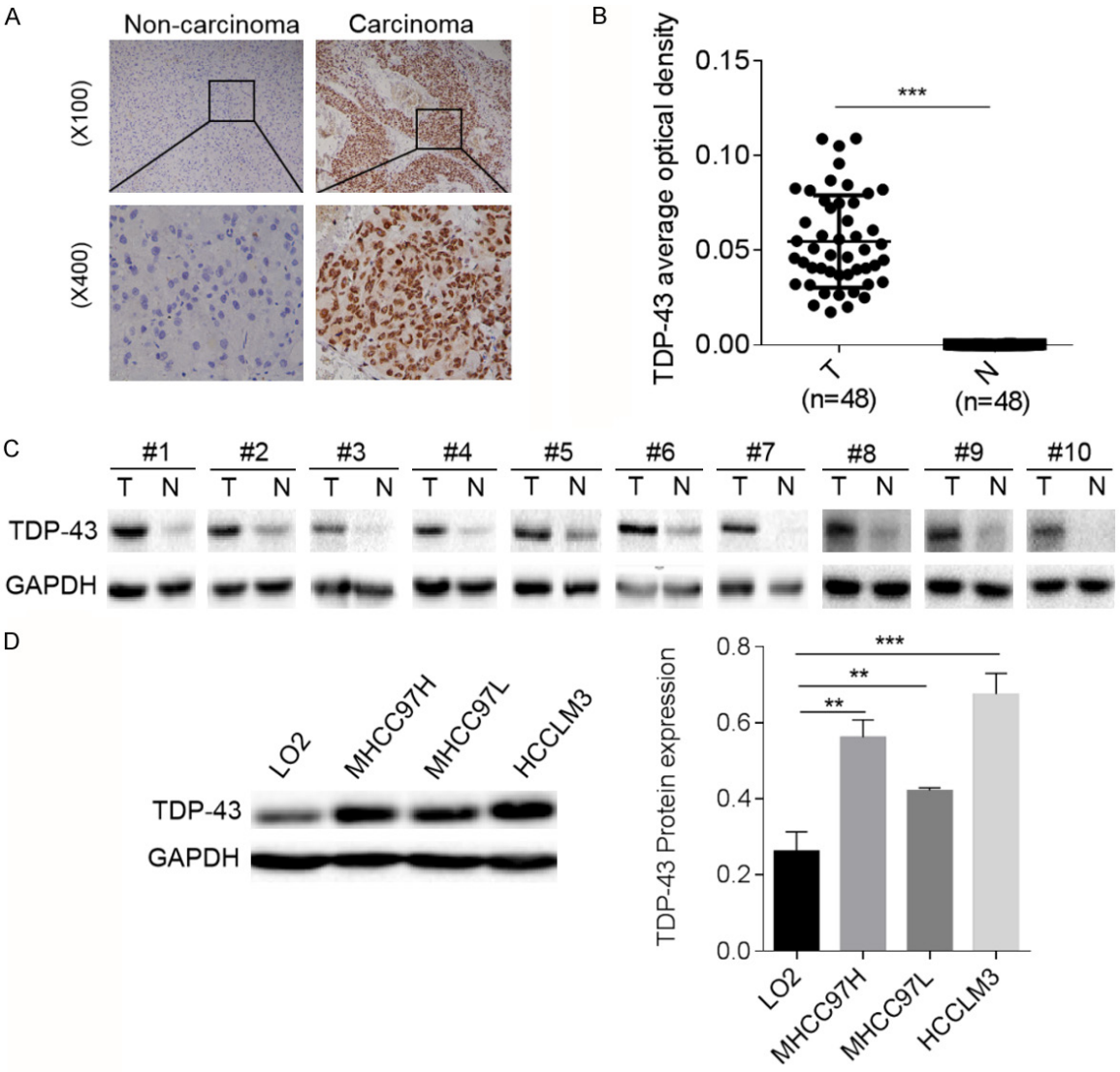

Figure 2. TDP-43 is overexpressed in HCC issues and HCC cell lines. A. Representative IHC staining of TDP-43 expression in HCC and matched paratumoral tissues from the same case. B. TDP-43 expression levels in HCC and peritumoral tissues evaluated by relative IOD value. C. WB analysis of TDP-43 expression levels in 10 matched pairs of HCC and paratumoral tissues. D. Expression level of TDP-43 in normal liver cell and different HCC cell lines. **P<0.01, ***P<0.001. HCC, hepatocellular carcinoma; IHC, immunohistochemistry; N, peritumor; T, tumor; WB, western blot.

cells (**Figure 3F-I**). In the orthotopic xenograft model, the statistical analysis of tumor weight showed that the ability of tumor proliferation in the control group was higher than that in the TDP-43 knockdown group (**Figure 3J**), and the incidence of lung metastasis in the TDP-43 knockdown group was also lower than that in the HCCLM3-shNC group (**Figure 3K**). These results indicate that the downregulation of TDP-43 suppresses the proliferation and metastasis of HCC cells in both vivo and vitro.

Elevated TDP-43 levels promote EMT in HCC cells

Previous studies have shown that EMT was a crucial initial stage in promoting the invasion and metastasis of HCC cells [33, 34]. In this study, we presumed that TDP-43 was involved in EMT events during HCC progression. We confirmed this assumption showing that the expression of mesenchymal makers N-cadherin and Vimentin were remarkably inhibited in the

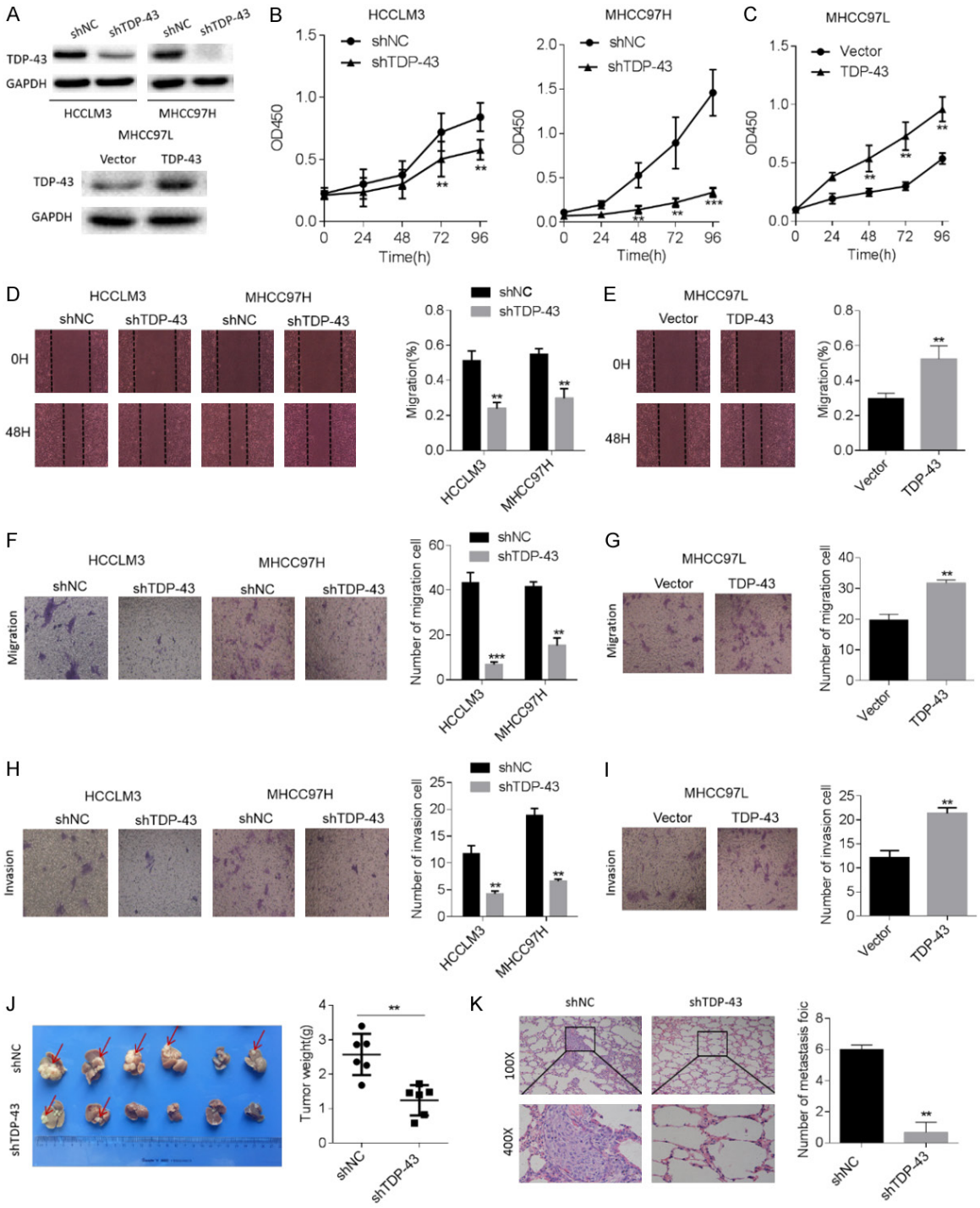

Figure 3. Down-regulation of TDP-43 inhibits HCC progression in vivo and in vitro. A. HCCLM3 and MHCC97H cells are transfected with short hairpin RNAs (shRNAs) while MHCC97L cells are transfected with TDP-43 overexpression plasmids. Efficiencies of TDP-43 knockdown and overexpression are validated using WB assay. B, C. Evaluations of the influence of TD-43 on proliferation of HCCLM3, MHCC97H, and MHCC97L cells using CCK-8 assay. D, E. Evaluations of the influence of TDP-43 on migration activities of HCCLM3, MHCC97H, and MHCC97L cells using wound healing assay. F, G. Evaluations of the influence of TDP-43 on migration activities of HCCLM3, MHCC97H, and MHCC97L cells using Transwell assay. H, I. Evaluations of the influence of TD-43 on invasion activities of HCCLM3, MHCC97H, and MHCC97L cells using Matrigel invasion assay. J. Establishment of the orthotopic xenograft model with BALB/C-nu nude mice by in situ hepatic injection with shTDP-43 or shNC cells. Weights of xenograft tumors in two groups are measured. K. The effect of TDP-43 knockdown on lung metastasis in mice is observed using in situ hepatic injection, and the number of metastatic lung nodules was examined using H&E staining. *P<0.05, **P<0.01, ***P<0.001. HCC, hepatocellular carcinoma; H&E, haematoxylin and eosin; WB, western blot.

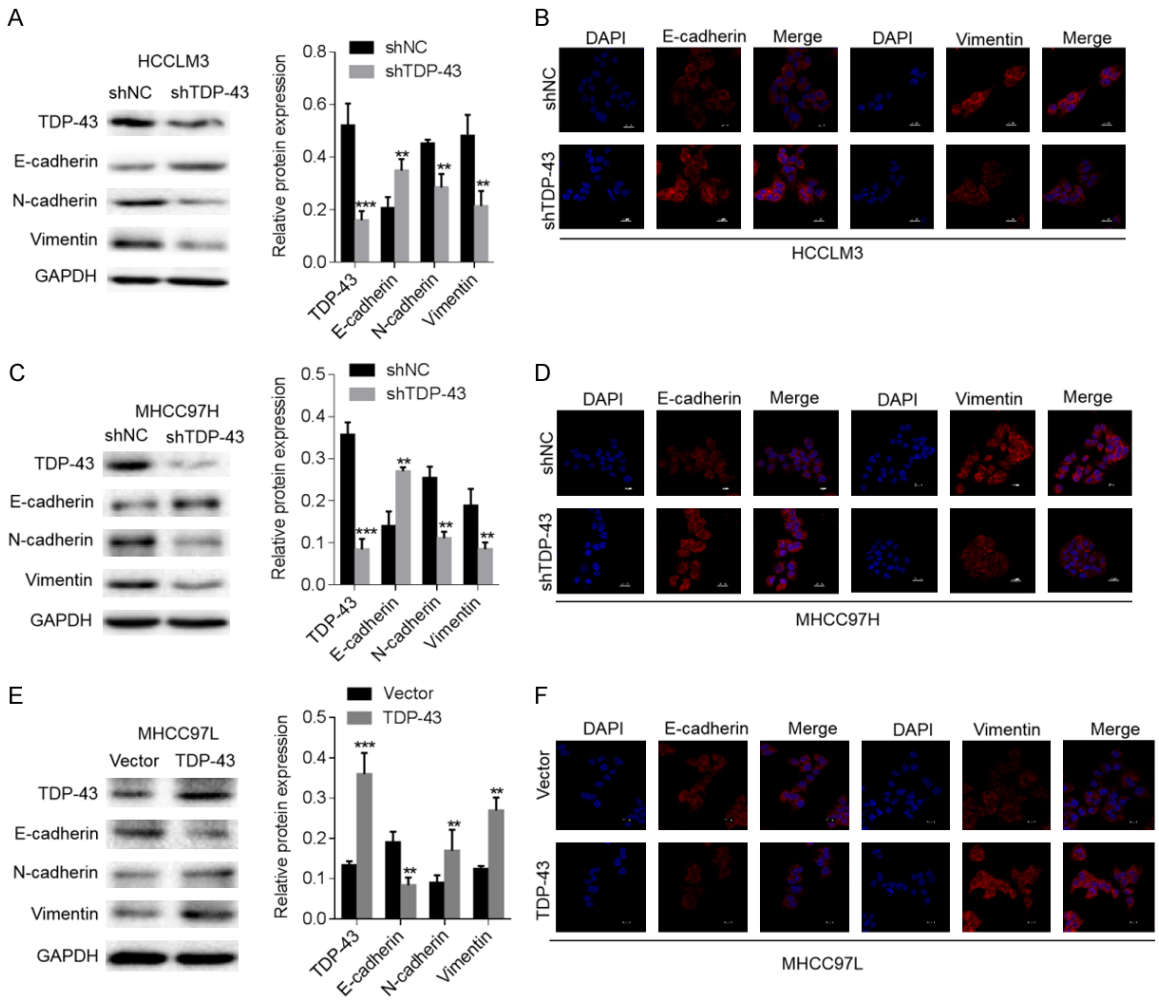

Figure 4. Elevated TDP-43 levels promote cellular EMT in HCC cells. A. Expressions of EMT-related markers in HCCLM3 cells with downregulated TDP-43 assayed using WB assay. B. Expressions of E-cadherin and Vimentin in HCCLM3 cells with TDP-43 knockdown assayed using immunofluorescence staining. C. Expressions of EMT-related markers in MHCC97H cells with TDP-43 downregulation assayed using WB. D. Expressions of E-cadherin and Vimentin in MHCC97H cells with TDP-43 knockdown assayed using immunofluorescence staining. E. Expressions of EMT-related markers in MHCC97L cells with TDP-43 overexpression assayed using WB. F. Expressions of E-cadherin and Vimentin in MHCC97L cells with TDP-43 overexpression assayed using immunofluorescence staining. **P<0.01, ***P<0.001; scale bar 25 μm. EMT, epithelial-mesenchymal transformation; HCC, hepatocellular carcinoma; WB, western blot.

shTDP-43 group in HCCLM3 and MHCC97H cell lines, while E-cadherin expression was reversed as assayed by WB analysis. In addition, the shTDP-43 group in HCCLM3 and MHCC97H had shown increased levels of E-cadherin and decreased levels of Vimentin (**Figure 4A-D**), which was consistent with the results of WB analysis. The WB and immunofluorescence (IF) analysis of the TDP-43 overexpression group of MHCC97L cells showed a totally opposite result in contrast to that observed in the TDP-43 knockdown cells (**Figure 4E**, **4F**). Taken together, these results indicate that TDP-43 can induce EMT, thereby contributing to HCC progression.

TDP-43 induces EMT and promotes HCC progression by activating the Wnt/β-catenin signaling pathway

The Wnt/β-catenin signaling is one of the classical pathways related to tumorigenesis and metastasis of HCC; therefore, we investigated whether TDP-43 mediated EMT through the Wnt/β-catenin pathway. WB analysis was used to test the levels of related proteins in the Wnt/β-catenin pathway. We observed decreased expression level of β-catenin and its downstream genes CyclinD1 and c-Myc in the shTDP-43 group in HCCLM3 and MHCC97H cells

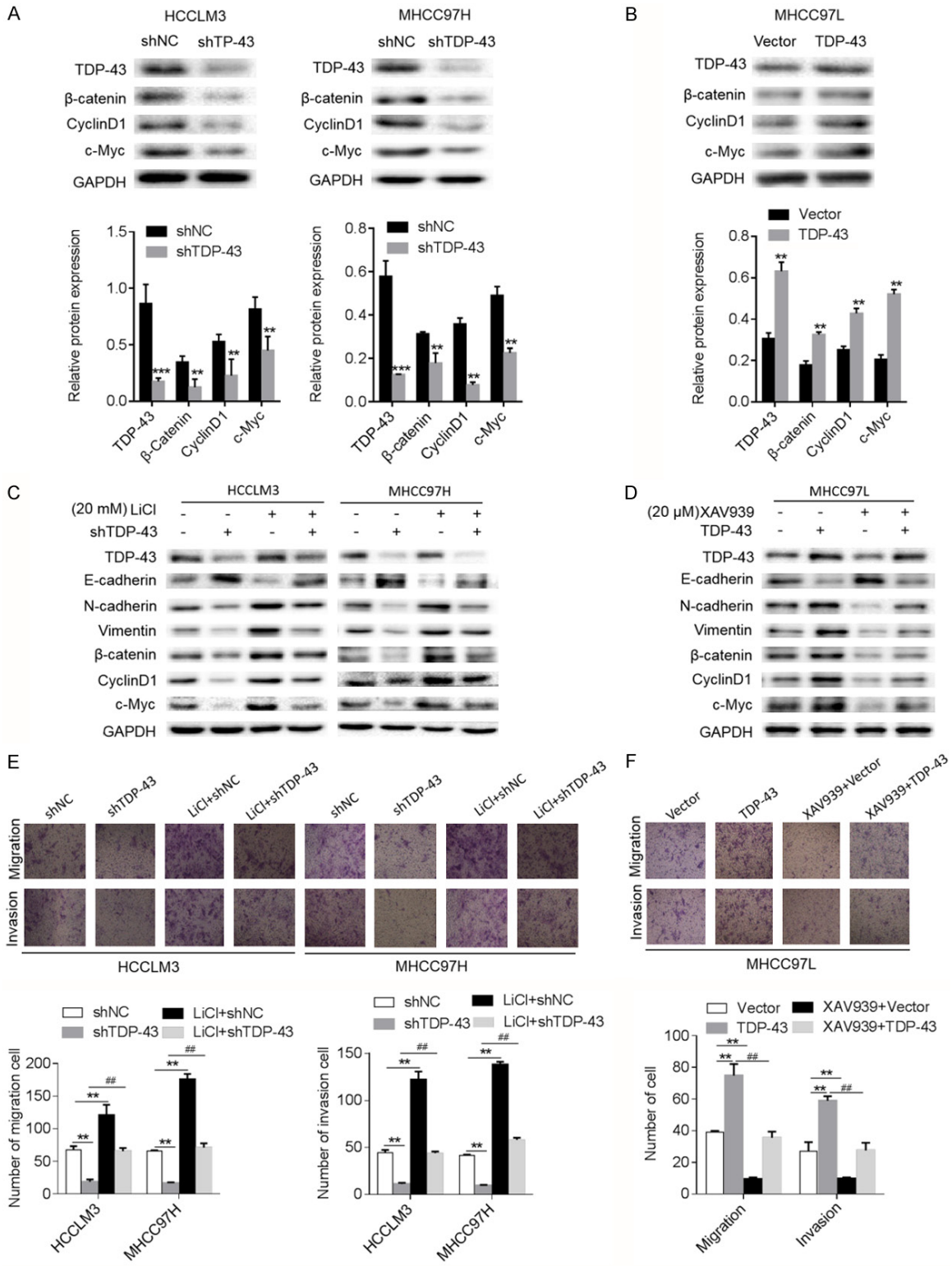

Figure 5. TDP-43 induces EMT and promotes HCC progression via activation of Wnt/β-catenin signaling pathway. A. Expression of Wnt/β-catenin pathway target proteins (β-catenin, c-Myc and CyclinD1) in HCCLM3 and MHCC97H cells with downregulated TDP-43 assayed using WB. B. Expressions of Wnt/β-catenin pathway targets (β-catenin, c-Myc and CyclinD1) in MHCC97L cells with overexpressed TDP-43 assayed using WB. C. The protein levels of EMT makers and Wnt/β-catenin pathway target proteins in the TDP-43 knockdown cells treated with 20 mM LiCl are assessed using WB assay. D. The protein levels of EMT makers and Wnt/β-catenin pathway target proteins in the TDP-43 overexpressing cells treated with 20 μM XAV939 are assessed using WB assay. **P<0.01, ***P<0.001. E.

Evaluations of the influence of TDP-43 knockdown on migration and invasion activities in HCC cells treated with 20 mM LiCl using Transwell assay. **P<0.01 vs. shNC group, ##P<0.01 vs. LiCl-treated TDP-43 knockdown. F. Evaluations of the influence of TDP-43 overexpression on migration and invasion activities of HCC cells treated with 20 μM XAV939 using Transwell assay. **P<0.01 vs. Vector group, ##P<0.01 vs. XAV939-treated TDP-43 overexpression. HCC, hepatocellular carcinoma; WB, western blot.

(**Figure 5A**). Conversely, an increased expression of these proteins compared with that in the control groups of cells was observed in TDP-43 overexpressing MHCC97L cells (**Figure 5B**). Overall, to further confirm the hypothesis that TDP-43 induces the occurrence of EMT by activating the Wnt/β-catenin pathway, we used the agonist LiCl (SIGMA, USA, #213233, 20 mM, 24 h) and inhibitor XAV939 (ApexBio Technology, USA, #1877, 20 μM, 24 h) to intervene TDP-43 knockdown and overexpression in cells, respectively. First, using the pathway agonist, we found that the inhibition of the Wnt/β-catenin pathway and EMT induced by TDP-43 knockdown in HCC cells was restored by LiCl (**Figure 5C**). Conversely, the intervention using XAV939 significantly impaired the activation of TDP-43 overexpression in MHCC97L cells in the Wnt/β-catenin pathway and EMT (**Figure 5D**). Additionally, transwell assay analysis indicated that the application of LiCl could significantly reverse the suppression of TDP-43 knockdown caused migration and invasion of HCC cells (**Figure 5E**), while the treatment with XAV939 significantly counteracted MHCC97L cell migration and invasion promoted by TDP-43 overexpression (**Figure 5F**). Together, these results demonstrate that up-regulation of TDP-43 activates the Wnt/β-catenin pathway, which in turn promotes EMT, invasion, and metastasis of HCC cells.

TDP-43 activates the Wnt/β-catenin pathway probably by targeting the inhibition of GSK3β protein translation

Known as a RNA-binding protein, TDP-43 can recognize and bind specific RNA through its RNA recognition motif RRM [35, 36]. Recently, RNA sequencing and genomic analysis have found that many genes associated with Wnt signaling, including GSK3β and APC, may be candidate target mRNAs regulated by TDP-43 [37, 38]. First, we speculated that TDP-43 may activate the Wnt/β-catenin signaling pathway by targeting the regulation of GSK3β. RNA immunoprecipitation (RIP) assay analysis indicated that there was an interaction between TDP-43 protein and *GSK3β* mRNA in MHCC97H cells (**Figure 6A**). Furthermore, qRT-PCR analysis showed that there was no significant change in *GSKβ* mRNA expression after knockdown and overexpression of TDP-43 in HCC cells, respectively (**Figure 6B**, **6C**). However, WB analysis indicated that TDP-43 knockdown could significantly upregulate the expression of GSK-3β protein, while overexpression of TDP-43 could downregulate the level of GSK3β protein (**Figure 6D**, **6E**). Our study indicates that the activation of the Wnt/β-catenin pathway by TDP-43 may be executed through targeted inhibition of GSK3β protein translation.

Discussion

Despite recent considerable achievements made in the diagnosis and treatment of HCC, the clinical outcome in HCC patients remains poor because of the lack of timely diagnosis, high risk of recurrence, and metastasis [39]. Therefore, in-depth elucidation of the specific molecular mechanism of HCC invasion and metastasis remains of great significance. In this study, we focused on the TDP-43 role in promoting HCC metastasis in order to explore the mechanism of the HCC pathogenesis. We found that TDP-43 was highly expressed in HCC and was able to promote EMT and the invasion and metastasis of HCC cells by activating the Wnt/β-catenin signaling pathway. More importantly, we further revealed that TDP-43 may activate the Wnt signaling pathway by targeted regulation of GSK3β protein translation. Our results may provide new research ideas and exploration directions for effective diagnosis and targeted therapy for HCC.

TDP-43, encoded by the human TARDBP gene, is not only a DNA/RNA binding protein abundantly expressed in nearly all tissues, such as the heart, liver, kidney, spleen, bone, and brain, but also a nuclear factor involved in the regulation of mRNA transcription and alternative splicing [21, 22]. In recent years, it has been reported that TDP-43 was the major component of characteristic tau-negative and ubiquitin-positive inclusions in ALS and FTLD, and its function in motoneurons associated with ALS

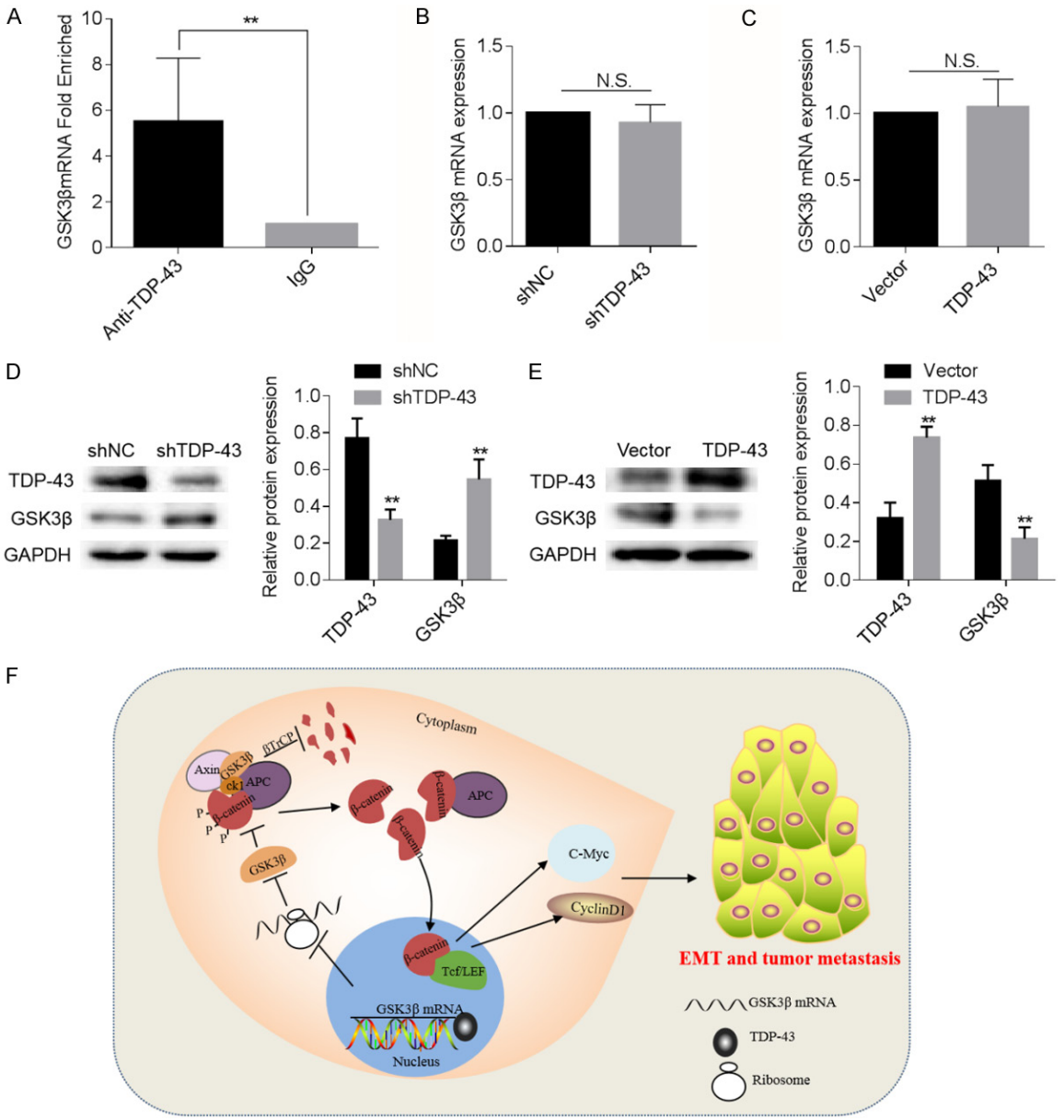

Figure 6. TDP-43 activates Wnt/β-catenin pathway probably by inhibiting the GSK3β protein translation. A. Interaction between TDP-43 protein and *GSK3β* mRNA was analyzed using RIP assay. B. Expression level of *GSK3β* mRNA in MHCC97H cells with TDP-43 knockdown was assessed using qRT-PCR assay. C. Expression level of *GSK3β* mRNA in MHCC97L cells with overexpressed TDP-43 is assessed using qRT-PCR assay. D. Expression level of GSK3β protein in MHCC97H cells with TDP-43 knockdown is assessed using WB assay. E. Expression level of GSK3β protein in MHCC97L cells with TDP-43 overexpression is assessed using WB assay. F. Proposed mechanistic model of TDP-43 promoting HCC development and metastasis. **P<0.01, N. S. indicated P>0.05. RIP, RNA immunoprecipitation.

and FTLD has been widely studied [27, 28, 40]. Interestingly, several studies have suggested the functional role of TDP-43 in human cancer. For example, mutations in the TARDBP gene increase susceptibility to Euclid's sarcoma [41]. High TDP-43 expression level can regulate the glycolysis level in HCC cells through the TDP-43/miR-520/PFKP axis [42]. Low TDP-43 expression can reduce the proliferation, migration, and invasion of tumor cells in non-small cell lung cancer [43]. In this study, we explored the role of TDP-43 in HCC metastasis. First, according to the analysis of HCC patient data in TCGA database, we found that TDP-43 was highly expressed in HCC and had a significant clinical correlation with HCC stage, lymph node

metastasis, and patient survival rate. IHC and WB analyses further confirmed the increased expression of TDP-43 in human HCC tissues and cell lines, indicating that it may be a carcinogenic factor involved in the occurrence and development of HCC. In order to support this hypothesis, we performed a series of *in vitro* and *in vivo* experiments after stable knockdown and overexpression of TDP-43 in different HCC cell lines. It was proved that the low and high expression of TDP-43 could lead to the weakening and enhancement of proliferation, invasion, and migration of HCC cells, respectively. Strikingly, our results showed that TDP-43 played an important role in promoting HCC metastasis, which was consistent with the carcinogenic function of TDP-43 previously shown in melanoma and neuroblastoma [30, 31]. Although some studies have confirmed the relationship between TDP-43 expression and human tumorigenesis, the molecular mechanism by which TDP-43 promotes HCC metastasis remains unclear and needs to be further explored.

Tumor metastasis involves a number of processes, including tumor cells migrating from the primary tumor site and invading the extracellular matrix, spreading through blood or lymphatic circulation, and reaching distant organs [44]. Among these processes, EMT is a key initial step for tumor cells to acquire metastatic and invasive potential [45]. Therefore, we speculated that TDP-43 may promote the invasion and metastasis of HCC cells by inducing the EMT. The results of WB and IF assays showed that knockdown of TDP-43 significantly suppressed the expression of mesenchymal markers such as N-cadherin and Vimentin in HCC cell lines HCCLM3 and MHCC97H, while the expression of epithelial marker E-cadherin was significantly increased. Additionally, TDP-43 overexpression in the MHCC97L cell line led to the opposite trend in the expression of the EMT-related markers. Therefore, we confirmed that TDP-43 can induce and participate in EMT.

The Wnt/β-catenin pathway, also known as the classical Wnt signaling pathway, is essential for embryonic development and adult tissue self-renewal [14]. The abnormal activation of this pathway leads to uncontrolled cell growth and the tendency for malignant transformation. In human HCC, the typical Wnt signal can induce the interaction between intracellular E-cadherin and β-catenin to form a complex, and then induce the EMT. The positive rate of the E-cadherin/β-catenin complex is positively correlated with the metastasis of HCC and the degree of capsule infiltration [46]. Kan et al. found that about 30% of HCC cases were accompanied by overactivation of the Wnt/β-catenin signaling pathway, which could promote tumor cell proliferation and metastasis as well as sorafenib resistance [7, 47]. These findings led us to consider the potential association of TDP-43 with the Wnt/β-catenin signaling pathway. The results of the western blot assay showed that knockdown and overexpression of TDP-43 in different HCC cell lines could down-regulate and up-regulate the expression of β-catenin and its downstream target proteins c-Myc and CyclinD1, respectively. To further verify our hypothesis, LiCl (Wnt pathway agonist) and XAV939 (Wnt pathway inhibitor) were used to interfere with TDP-43 knockdown and overexpression in cell lines, respectively. Our experimental results showed that in HCCLM3 and MHCC97H cell lines, the application of LiCl could significantly activate the expression of β-catenin and restore the inhibition of TDP-43 knockdown on EMT progression and HCC migration and invasion. In the MHCC97L cell line, the treatment with XAV939 inhibited the expression of β-catenin and counteracted the promotion of EMT progression and HCC migration and invasion caused by TDP-43 overexpression. Therefore, the results of the above recovery experiment support our hypothesis that TDP-43 induces EMT to promote HCC cell invasion and metastasis by activating the Wnt/β-catenin signaling pathway.

As a RNA-binding protein, the TDP-43 protein is mainly involved in the regulation of gene expression, such as transcription, pre-mRNA splicing, mRNA transport, stability regulation and translation [24, 48]. For example, as a splicing regulator in mRNA splicing, TDP-43 can bind to mRNA, the precursor of cystic transmembrane conductance regulator (CFTR) and mediate its variable splicing. In addition, Costessi and Fiesel et al. found that TDP-43 can bind to the 3'UTR of Add2 gene encoding actin binding proteins HDAC6 and β-adducin and regulate the stability of *Add2* mRNA [49]. In contrast, Majumder et al. reported that TDP-43 could specifically inhibit the translation of several transcripts, and the combination of TDP-43 and *rac1* mRNA further promoted its bind-

ing to the FMRP/CYFIP1 complex, resulting in a decrease of rac1 protein level [50]. Considering the functional characteristics of TDP-43 mentioned above, we hypothesized that the activation of the Wnt/β-catenin signaling pathway by TDP-43 may be realized by directly targeting the expression of related genes upstream of the Wnt signaling pathway.

The key to the initiation of the Wnt signaling pathway is the accumulation of β-catenin and its translocation into the nucleus, where it forms complexes with transcription factors such as TCF/Lef. This complex further activates the transcription of downstream target genes related to the occurrence and development of malignant tumors, such as CyclinD1, c-Myc, and CD44, and promotes cell proliferation and metastasis [11, 13]. Under normal physiological conditions, β-catenin is present in the cytoplasm at a low level and can be phosphorylated by a degradation complex composed of GSK3β, protein phosphatase 2A (PP2A), casein kinase 1 (CK1), and APC, while phosphorylated β-catenin can be recognized and degraded by ubiquitin enzymes and proteasomes. When the Wnt signal is activated, the phosphorylated β-catenin is inactivated and separated from the destructive complex, resulting in the accumulation of non-phosphorylated β-catenin in the cytoplasm followed by its translocation into the nucleus. As an important component of this degradation complex, GSK3β is one of the most critical kinases involved in the phosphorylation of β-catenin [12, 14]. Interestingly, through high-throughput sequencing and genome analysis, Sephton and Hazelet et al. predicted that TDP-43 can recognize and bind to many specific target mRNAs, including some genes related to Wnt signals, such as GSK3β and APC, which makes GSK3β a potential candidate target mRNA regulated by TDP-43 [37, 38]. Therefore, we speculated that TDP-43 may activate the Wnt/β-catenin signaling pathway by targeting the regulation of GSK3β expression. First, we proved the interaction between TDP-43 protein and *GSK3β* mRNA in the MHCC97H cell line using RIP assay. Moreover, the results of western blot and qRT-PCR experiments revealed that TDP-43 knockdown and overexpression resulted in an obvious upregulation and downregulation of GSK3β protein, respectively, while the expression of *GSK3β* mRNA remained unchanged. Therefore, the interaction between TDP-43 protein and *GSK3β* mRNA leads to the following questions worthy of our in-depth exploration. First, how the TDP-43 protein regulates the post-transcriptional translation level of GSK3β protein. Second, when and where the RNA-binding protein is recruited into the novel transcript to participate in the translation regulation process. Finally, whether TDP-43 interacts with other translation-related factors and regulates the translation level of GSK3β. Therefore, continuing to explore the specific molecular mechanism of TDP-43 regulating *GSK3β* mRNA translation will be the focus of our next research work.

In this study, we proposed a mechanistic model of TDP-43 function that promotes HCC cell growth and metastasis (**Figure 6F**). TDP-43 initiates the Wnt/β-catenin signaling pathway by targeting inhibition of post-transcriptional translation of GSK3β protein, which reduces the phosphorylation and degradation of β-catenin in the cytoplasm, resulting in its translocation to the nucleus, thus activating downstream target genes CyclinD1 and c-Myc, to further induce EMT and promote the invasion and metastasis of HCC cells.

Taken together, our study is the first to reveal the significant role of TDP-43 in HCC progression, which will provide a new target for HCC diagnosis and treatment and broaden the train of thought for the study of metastatic tumors.

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Disclosure of conflict of interest

None.

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