Tropomodulin 3 promotes liver cancer progression by activating the MAPK/ERK signaling pathway

CANGUANG JIN1, ZHIKANG CHEN1, WEI SHI2 and QI LIAN1

1Department of General Surgery, Xiangya Hospital, Central South University, Changsha, Hunan 410008; 2Department of General Surgery, Benq Medical Center, Nanjing Medical University, Suzhou, Jiangsu 210019, P.R. China

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Abstract. Tropomodulin 3 (TMOD3) is a member of the pointed-end capping protein family that contributes to invasion and metastasis in several types of malignancies. TMOD3 has been found to be crucial for membranous skeleton and embryonic development; however, little is known regarding the role of TMOD3 in liver cancer progression. In addition, to the best of our knowledge, no previous studies have investigated the mechanism underlying the TMOD3-regulated promotion of liver cancer. The aim of the present study was to determine whether TMOD3 is associated with liver cancer progression. TMOD3 expression was found to be elevated in liver cancer cells and tissues. In the in vitro experiments, liver cancer cell proliferation, invasion and migration were inhibited by TMOD3 knockdown and promoted by ectopic expression of TMOD3. Furthermore, mechanistic analysis indicated that TMOD3 overexpression activated mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling and increased the levels of other targets of this pathway, including matrix metalloproteinase (MMP)2, MMP9 and cyclin D1. TMOD3 overexpression was associated with changes in liver cancer cell morphology and altered expression of epithelial and mesenchymal markers. High TMOD3 expression was hypothesized to promote epithelial-to-mesenchymal transition in liver cancer cells. In conclusion, TMOD3 was shown to promote liver cancer cell growth, invasion and migration through the MAPK/ERK signaling pathway, and it may serve as a candidate biomarker and therapeutic target in liver cancer.

Introduction

Liver cancer is one of the most common types of cancer, and is associated with a high mortality rate. With >500,000 fatalities worldwide in 2012, liver cancer is ranked as the third leading cause of cancer-related mortality (1,2). Clinically, liver cancer is characterized by its high invasiveness and incidence of recurrence. To date, numerous studies have been performed to improve the diagnosis and prognosis of liver cancer (3). Although multiple therapeutic strategies are currently available, the outcome of patients with liver cancer remains unsatisfactory (4). The poor clinical outcomes are mainly attributed to the high frequency of tumor recurrence and distant metastasis following curative surgical resection (5). Thus, identifying new molecular targets, as well as elucidating the mechanism underlying liver cancer progression, may improve available treatments and patient outcomes.

Tropomodulin 3 (TMOD3) is a ~40 kDa protein that binds the slow-growing ends of actin filaments and prevents depolymerization from the pointed ends (6,7). A previous study identified four TMOD isoforms in vertebrates (8); however, TMOD3 is a ubiquitous TMOD in non-erythroid cells, in which it regulates dynamic actin processes, such as lamellipodia protrusion and cell motility (9,10). By regulating actin dynamics in different cells, TMOD3 is involved in facilitating various processes, including determination of cell shape, cell migration and muscle contraction. It has been reported that TMOD3 serves different roles in different types of cells (11); however, the association between TMOD3 levels and cell migration is controversial, and the role of TMOD3 in epithelial cells in vivo remains elusive (12). In addition, it has been demonstrated that deletion of TMOD3 in mice caused embryonic death at E14.5-E18.5, indicating that TMOD3 may be a key factor in embryonic development (13-15). Based on its biological function in stem and progenitor cells, TMOD3 may play an important role in cancer progression. However, the role of TMOD3 in the regulation of liver cancer invasion and metastasis has not been fully elucidated.
The aim of the present study was to investigate the expression of TMOD3 in liver cancer tissues and cell lines and its role in liver cancer cell proliferation, invasion and migration, and elucidate the underlying mechanism, in order to determine whether TMOD3 may serve as a candidate biomarker and treatment target for liver cancer.

Materials and methods

Patients and samples. A total of 50 pairs of primary liver cancer (PLC) and adjacent liver tissue specimens were randomly selected from patients who had undergone hepatic resection at Xiangya Hospital (Changsha, China) between January and December 2017. The detailed clinicopathological data are presented in Table I. All cases were pathologically diagnosed by two independent pathologists. Furthermore, 30 matched fresh PLC tissues and adjacent non-tumor tissues were collected between January and August 2018 for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses. All patients or their families provided written informed consent regarding the use of their tissues for research purposes. All the patients were followed up as suggested in the REMARK guidelines (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2361579/). All experiments using human materials were approved by the Ethics Committee of Xiangya Hospital of Central South University (Changsha, China).

RNA extraction and gene expression analysis as determined by RT-qPCR analysis. Total RNA from fresh PLC, adjacent non-tumor tissues and cell lines was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. RNA quantity and quality were evaluated using a Nanodrop MA, USA) according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA by BeyoRT ™ II First-Strand cDNA Synthesis kit (Beyotime Institute of Biotechnology, Shanghai, China), and qPCR was conducted using SYBR-Green Master Mix on the Applied Biosystems QuantStudio™ 3 and 5 Real-Time PCR System (Thermo Fisher Scientific, Inc.). The primers were as follows: TMOD3 forward, 5'-TTCC CGGCAGAGAACCAGACATC-3' and reverse, 5'-CAA GAATTCCTGCAGAGTTCAC-3'; GAPDH forward, 5'-GGCACCCTGAGCTGAGAAC-3' and reverse, 5'-GTT GCCAGTGATGGCATGAG-3'. Each sample was analyzed in triplicate and the data were calculated using the 2^ΔΔCq method.

Western blot analysis. Tissues or cells were dissolved using RIPA lysis buffer supplemented with 1% phenylmethane-sulfonyl fluoride. Protein concentration was measured using a bichoninic acid protein assay kit (Beyotime Institute of Biotechnology). Next, proteins were separated by 1% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Then, the membranes were blocked with 5% skimmed milk and incubated with specific primary antibodies overnight at 4°C. Following washing, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody at room temperature for 30 min and detected using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.). Antibodies against TMOD3 (cat. no. 70-ab4606-050) were obtained from MultiSciences (Hangzhou, China). Antibodies against p-ERK (cat. no. ab126455) and ERK (cat. no. ab17942) were obtained from Abcam (Cambridge, MA, USA), and those against E-cadherin (cat. no. sc-8426), vimentin (cat. no. sc-6260) and cyclin D1 (cat. no. sc-246) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies against matrix metalloproteinase (MMP)2 (cat. no. AF0577) and MMP9 (cat. no. AF0220) were purchased from Affinity Biosciences (Cincinnati, OH, USA). The β-actin antibody (cat. no. TA-09) and corresponding secondary antibodies (cat. no. ZB-2305; cat. no. ZB-2301) were purchased from Zhongshan Golden Bridge Biotechnology (ZSGB; Beijing, China).

Immunohistochemistry (IHC). All tissues were cut into 4-µm sections, dewaxed in xylene and rehydrated in a graded ethanol series. Following heating in a microwave for antigen retrieval (12 min in sodium citrate buffer, pH6), endogenous peroxidase was inactivated with 0.3% H2O2 for 30 min and the sections were incubated with 10% normal goat serum for 30 min. The TMOD3 antibody (1:100; MultiSciences) was applied overnight in a moist chamber at 4°C, followed by incubation with the secondary antibody (ZSGB) for 30 min. The antigen-antibody interactions were detected by 3,3'-diaminobenzidine and counterstained with hematoxylin. Tissue sections were dehydrated in graded ethanols and mounted.

The immunostained sections were independently evaluated by two pathologists who were blinded to all patient clinical data. The staining intensity and the percentage of protein expression were assessed. The staining intensity of TMOD3 was graded between 0 and 3 as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. The percentage of positive cells was classified as 1 (0-25%), 2 (26-50%), 3 (51-75%) or 4 (>75%). The final score was calculated by multiplying these two scores, and the protein expression of TMOD3 in liver cancer specimens was divided into high-expression (≥4) and low-expression (<4) groups for further analysis.

Cell culture. The human liver cancer cell lines Hep3B, HepG2 and PLC/PRF5 were purchased from the American Type Culture Collection (Manassas, VA, USA). The MHCC97-H, MHCC97-L, HCCLM3 and Huh7 liver cancer cell lines and the normal liver cell line L02 were obtained from the Chinese Academy of Sciences (Shanghai, China). Cell culture was conducted according to the manufacturer's instructions and all the cell lines were cultured at 37°C in a humidified atmosphere of 5% CO2.

Construction of stable cell lines. A human TMOD3 overexpression clone lentivirus, three short hairpin RNA (shRNA) lentiviruses of TMOD3 and their control vectors were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The cells were cultured in 6-well plates prior to transfection until reaching 80-90% confluence within 24 h. Then, transfection was performed according to standard procedures. Puromycin (2 µg/ml) was used to select stable clones. The three candidate hairpin sequences for TMOD3 were as follows: 5'-CCTTTGG GAATTCGTCGAAACAC-3' (shRNA-1); 5'-AAAGAAGAGCA TTGGAGCATAAAG-3' (shRNA-2); 5'-CTCCGAGCAAT
TCTTGGGAGC-3' (shRNA-3); The efficiency of TMOD3 overexpression and knockdown were assessed by RT-qPCR and western blot analysis.

**MTT assay and colony formation assay.** For the MTT assay, 5x10^3 cells were seeded into each well of 96-well plates (6 wells/group). The cells were incubated for 0-7 days, then stained with MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and the absorbance was measured at 570 nm. For colony formation, the cells were grown in 6-well plates at a density of 5x10^2 cells/well and cultured for 14 days. Then, the number of colonies was counted following staining with 1% crystal violet solution. All studies were conducted with 3 replicates.

**Wound healing assay.** The cells were seeded into 6-well plates at a density of 1x10^5 cells/well. When grown to 90% confluence, the cells were incubated with mitomycin C (10 µg/ml) for 1 h at 37°C to suppress cell proliferation, and the cells were then starved for 24 h in serum-free medium. A 10-µl pipette tip was used to create an artificial wound. The results were observed and photographed every 12 h.

**Transwell invasion assay.** The cell invasion assay was performed in a 24-well Transwell plate. Cells were incubated with mitomycin C (10 µg/ml) for 1 h at 37°C to suppress cell proliferation, then 1x10^5 cells in 500 µl of serum-free medium were placed into the upper chamber with Matrigel-coated membranes (BD Biosciences, Franklin Lakes, NJ, USA). The lower chamber was filled with 500 µl medium supplemented with 10% fetal bovine serum. Following a 48-h incubation at 37°C, the cells that remained in the upper chambers were removed and the cells that adhered to the lower membranes were stained with 0.1% crystal violet solution. The invading cells were counted in 5 random fields per well.

**Immunofluorescence (IF).** The cells were seeded into 6-well plates with glass coverslips for 24 h. Then, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and incubated with phalloidin (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. The coverslips were counterstained with DAPI and the results were photographed under an inverted microscope.

**Statistical analysis.** Statistical analysis was conducted using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). All measurement data were expressed as the mean ± standard deviation. Student's t-test or one-way analysis of variance were used to test the statistical significance of the differences between the groups, while proportional comparisons were conducted via a Chi-squared test. P<0.05 was considered to indicate a statistically significant difference.

### Results

**TMOD3 is upregulated in liver cancer tissues.** Initially, the Oncomine Database (www.oncomine.org) was utilized to investigate TMOD3 expression in liver cancer. The results revealed that the TMOD3 mRNA levels were higher compared with those observed in normal liver tissues. The P-values recorded in the Wurmbach, Chen, Mas, Roessler 1 and 2 liver datasets were 8.27x10^-5, 1.34x10^-7, 4.81x10^-4, 9.57x10^-4 and 2.06x10^-17, respectively (Fig. 1A). Subsequently, RT-qPCR and western blotting were performed in 30 pairs of PLC samples and matched normal liver tissues. Consistently, the mRNA and protein levels of TMOD3 were significantly higher compared with those observed in the normal liver tissues (P<0.001; Fig. 1B-D). IHC was performed to further analyze TMOD3 expression in 50 paired PLC and adjacent liver specimens. As shown in Fig. 1E, TMOD3 was mainly localized in the cytoplasm. It was also positively expressed in...
the 50 PLC specimens, of which 32 (64%) exhibited a high expression and 18 (36%) exhibited a low expression (Fig. 1F). Compared with the matched non-tumor tissues, TMOD3 expression was significantly higher in liver cancer tissues (P<0.001). These results indicate that TMOD3 is upregulated in liver cancer tissues and may contribute to liver cancer progression.

**TMOD3 promotes the proliferation of liver cancer cells in vitro.** In order to study the biological functional role of TMOD3 in liver cancer, the present study conducted knockdown and overexpression experiments. First, the expression of the TMOD3 protein in the normal liver cell line L02 and in 7 liver cancer cell lines (MHCC97-H, MHCC97-L, Huh7, HepG2, PLC/PRF5, HCCLM3 and Hep3B) was evaluated. The results revealed that TMOD3 exhibited the greatest expression in Hep3B cells and the lowest in HepG2 cells, which had high and low metastatic potential, respectively (Fig. 2A and B). Stable TMOD3-overexpressing HepG2-TMOD3 cells and TMOD3-knockdown Hep3B-shTMOD3 cells were established via lentivirus transfection. The cell transfection efficiency in each cell type was confirmed by RT-qPCR and western blot analysis. Transfection of TMOD3-expressing lentivirus plasmids increased the expression of TMOD3 in HepG2 cells (P<0.001; Fig. 2C and D). Three shRNAs (shRNA1,
shRNA2 and shRNA3) were constructed to silence TMOD3 expression in Hep3B cells. The expression level of TMOD3 was determined by RT-qPCR and western blot analysis, and shRNA1 was found to be the most effective; shRNA1 was consequently selected for further experiments (Fig. 2E and F). The MTT assay demonstrated that the proliferation rate was markedly increased in HepG2-TMOD3 cells, whereas Hep3B-shTMOD3 cells exhibited the opposite effect (Fig. 3A). Consistently, in the colony formation assay, HepG2-TMOD3 cells formed more colonies, while Hep3B-shTMOD3 cells exhibited decreased clonogenic ability (P<0.01; Fig. 3B). These results suggested that TMOD3 promotes the proliferation of liver cancer cells.

**TMOD3 promotes liver cancer cell migration and invasion in vitro.** Wound healing and Transwell assays were performed to determine the impact of TMOD3 on the migration and invasion capacities of these cells. The results presented in Fig. 3C and D suggested that overexpression of TMOD3 in HepG2 cells significantly enhanced the wound healing ability and promoted cell invasion, while Hep3B-shTMOD3 cells displayed a slow wound closure rate and weak invasive abilities. Therefore, the present study demonstrated that TMOD3 promotes the migration and invasion of liver cancer cells.

**High expression of TMOD3 may promote EMT in liver cancer.** As TMOD3 is associated with actin binding and is involved in cell migration and invasion, it was hypothesized that TMOD3 may be associated with the EMT process. IF analysis revealed that ectopic expression of TMOD3 in HepG2 cells displayed fibroblast-like spindled morphology. However, TMOD3 silencing in Hep3B cells produced a cobblestone-like appearance (Fig. 4A). Western blotting was performed to determine the expression of EMT biomarkers in liver cancer cells. The results demonstrated that overexpression of TMOD3 decreased the expression of E-cadherin (epithelial marker), and resulted in high expression of TMOD3, tropomodulin 3; RT-qPCR, reverse transcription quantitative polymerase chain reaction; shRNA, short hairpin RNA.
in the upregulation of vimentin and Snail (mesenchymal markers) levels, while the opposite trend in the expression of these markers was observed in Hep3B-shTMOD3 cells (Fig. 4B). These results indicate that TMOD3 may induce EMT in liver cancer.

**TMOD3 promotes liver cancer progression by activating the MAPK/ERK signaling pathway.** To evaluate the potential regulatory mechanism of TMOD3 in promoting liver cancer development, the Gene Set Enrichment Analysis (GSEA) analysis was used to identify the pathways regulated by TMOD3. High TMOD3 levels were positively associated with Kirsten rat sarcoma viral proto-oncogene (KRAS; Fig. 4C), which has previously been defined as a key component of the MAPK/ERK signaling pathway for modulating ERK activity, suggesting that TMOD3 may regulate MAPK/ERK signaling. The MAPK/ERK pathway plays an important role in cell proliferation, migration, differentiation, and apoptosis, and it is one of the most important molecular pathways in cancer growth and metastasis (16). Western blot analysis revealed that TMOD3 overexpression increased the phosphorylation of ERK in HepG2 cells, whereas TMOD3 knockdown decreased the levels of p-ERK in Hep3B cells; however, the total level of ERK remained unchanged (Fig. 4D). In addition, the present study detected the expression of MMP2, MMP9, and cyclin D1, which are controlled by the MAPK/ERK signaling pathway and are associated with cancer cell proliferation and invasion. The results revealed that the expression of MMP2, MMP9...
and cyclin D1 were significantly increased in HepG2-TMOD3 cells and decreased in Hep3B-shTMOD3 cells (Fig. 4D). These results indicated that TMOD3 may promote liver cancer progression by activating the MAPK/ERK signaling pathway.

Discussion

Liver cancer is one of the most common and lethal cancers of the human digestive system. In the present study, the mRNA and protein levels of TMOD3 were significantly increased in liver cancer cells and tissues. Elevated TMOD3 expression was found to be significantly associated with more unfavorable clinicopathological characteristics of liver cancer. To the best of our knowledge, the present study was the first to reveal that TMOD3 overexpression may promote cancer cell proliferation, migration and invasion through the activation of the MAPK/ERK signaling pathway. In addition, the results also provided evidence that TMOD3 may enhance the EMT process. Therefore, TMOD3 may serve as a candidate prognostic biomarker and therapeutic target in human liver cancer.

TMOD3, one of the TMOD isoforms located at 15q21.2, is an important component of the cytoskeleton of brain cells (17) that can block the depolymerization of the actin filaments at the pointed end (6,18). Actin filaments are essential components of the cytoskeleton in all types of cells (19,20). F-actin has two structurally and biochemically distinct ends, namely a barbed and a pointed end. Polymerization and depolymerization occur at both ends; however, polymerization is faster at the barbed end. G-actin is continuously polymerized at the barbed end and depolymerized from the pointed end. In addition, TMOD3 can block the elongation and depolymerization of the actin filament at the pointed end. By regulating actin dynamics, TMOD3 may facilitate various processes, including determination of cell shape, cell migration and muscle contraction (11). However, TMOD3 can also sequester actin monomers or nucleate actin polymerization by binding to G-actin, although how TMOD3 affects F- and G-actin remains controversial. Our research was not sufficiently in-depth to explain how TMOD3 impacts F-actin organization in liver cancer cells, and confocal microscopy would be required to actually measure the filament length. The mechanism underlying the function of TMOD3 in actin organization requires further experimental support through image quantification and biochemical analysis. Previous studies have revealed that
TMOD3 can cap the pointed ends of actin filament, which is necessary for maintaining the actin meshwork, and is important for spindle formation and cancer cell division (21,22). In addition, Sui et al (15) reported that deletion of TMOD3 affected the fetal liver and caused embryonic death. These findings suggest that TMOD3 may play a role in cancer development. Previous studies have also revealed that TMOD3 expression was associated with prostate and bladder cancer (23,24), but no studies have yet identified the function of TMOD3 in liver cancer. We herein aimed to determine whether TMOD3 is involved in liver cancer progression and the results revealed that, when compared with adjacent non-tumor liver tissues, TMOD3 expression was significantly increased in cancer tissues. Furthermore, TMOD3 was shown to promote liver cancer cell growth, invasion and migration.

Further mechanistic studies indicated that TMOD3 promoted liver cancer progression by activating the MAPK/ERK signaling pathway. MAPK/ERK signaling, one of the most important molecular pathways in cancer development, is critical for human cancer cell proliferation, survival and dissemination (25,26). Numerous studies have confirmed the close association between MAPK/ERK signaling and liver cancer progression (27-29). The results of Gene Set Enrichment Analysis demonstrated that TMOD3 was the most closely associated with the MAPK/ERK signaling pathway, which was further verified by western blot analysis. In addition, the results also revealed that TMOD3 significantly reduced the levels of MMP2, MMP9 and cyclin D1 in Hep3B-shTMOD3 cells and increased their levels in HepG2-TMOM3 cells. p-ERK, MMP2 and MMP9 are known to promote tumor proliferation and metastasis by degrading basement membrane components (30), while cyclin D1 is required for the G1-to-S transition and plays a key role in the maintenance of the malignant phenotype (31). These findings may explain the role of TMOD3 in promoting liver cancer.

Recently, a number of studies suggested that EMT may enhance epithelial cell invasive and migratory abilities during cancer progression (32,33). Several types of cancer, such as glioma (34), lung (35) and liver cancer (36), have been found to be associated with EMT. Considering the function of TMOD3, the present study performed IF and western blot analysis to verify whether TMOD3 induced EMT in liver cancer cells. The results revealed that HepG2-TMOM3 cells exhibited an elongated morphology and decreased E-cadherin expression, but increased vimentin and Snail expression. However, the opposite effects were observed in Hep3B-TMOM3 cells. Thus, it was hypothesized that TMOD3 may induce EMT in liver cancer. In this study, TMOD3 was shown to activate MAPK/ERK signaling during liver cancer progression. It is known that MAPK/ERK signaling is associated with EMT, and loss of epithelial polarity regulated by ERK requires remodeling of the actin cytoskeleton. However, we were unable to obtain more details on the connection between actin organization and MAPK/ERK signaling, which is a limitation to our study.

There were certain limitations to the present study. First, more liver cancer specimens and experiments are required to validate the concept of TMOD3 promoting liver cancer progression. Second, further IHC analysis is required to demonstrate whether TMOD3 induces EMT in liver cancer.

Third, no confocal imaging was available to demonstrate F-actin organization and TMOD3 staining on F-actin, and confocal microscopy would be required to accurately measure filament length.

In conclusion, the present study demonstrated that TMOD3 enhanced the proliferation, migration and invasion of liver cancer cells by activating the MAPK/ERK signaling pathway, and its increased expression may be associated with EMT. Therefore, TMOD3 may serve as a potential prognostic biomarker and therapeutic target for liver cancer.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions

CJ conceived the study and wrote the manuscript. CJ and QL conducted the experiments and contributed to the data analysis. WS and QL collected the clinical samples and corresponding clinical data. ZC was involved in the conception of the study and revised the manuscript. All authors read and approved the final version of this manuscript.

Ethics approval and consent to participate

The protocols for the collection of human tissues and all experiments using human materials were approved by the Ethics Committee of Xiangya Hospital of Central South University, and written informed consent was signed by all the participants or their families prior to their inclusion in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


