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TCP10L acts as a tumor suppressor by inhibiting cell proliferation in hepatocellular carcinoma

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ABSTRACT

TCP10L (T-complex 10 (mouse)-like) has been identified as a liver and testis-specific gene. Although a potential transcriptional suppression function of *TCP10L* has been reported previously, biological function of this gene still remains largely elusive. In this study, we reported for the first time that *TCP10L* was significantly down-regulated in clinical hepatocellular carcinoma (HCC) samples when compared to the corresponding non-tumorous liver tissues. Furthermore, *TCP10L* expression was highly correlated with advanced cases exceeding the Milan criteria. Overexpression of *TCP10L* in HCC cells suppressed colony formation, inhibited cell cycle progression through G0/G1 phase, and attenuated cell growth *in vivo*. Consistently, silencing of *TCP10L* promoted cell cycle progression and cell growth. Therefore, our study has revealed a novel suppressor role of TCP10L in HCC, by inhibiting proliferation of HCC cells, which may facilitate the diagnosis and molecular therapy in HCC.

1. Introduction

Hepatocellular carcinoma (HCC), one of the most common solid malignancies worldwide, ranks as the second leading cause of cancer death for males, and the sixth leading cause for females [1]. In clinic, Surgery, including partial liver resection and liver transplantation, remains the most effective treatment for HCC. However, surgery is feasible for only a small fraction of patients with localized disease, and liver transplantation is severely limited by the availability of liver donors. In addition, the high incidence of recurrence and metastasis after surgical treatment compromise the long-term therapeutic effect [2]. For the majority of cases of HCC, systemic chemotherapy was one of the few treatment alternatives, but provided only marginal benefit in the past, largely due to the extreme chemoresistance of HCC. Sorafenib is the first molecular inhibitor to be approved by the FDA for the treatment of advanced HCC, and this advancement in the treatment of HCC has brought excitement to the field of systemic therapy, which pushes the studies searching for potential molecular targets and drug candidates [3].

Liver development and maintenance of hepatic structure and functions rely on the precise temporal and spatial expression regulation of liver-specific proteins in hepatocytes, including albumin, α1-antitrypsin, alpha-fetoprotein (AFP), etc. Abnormal expressions and activities of these proteins in neoplastic hepatocytes are often observed during the development of multiple liver diseases, providing potential biomarkers for clinical diagnosis [4]. For example, AFP, normally silent in the adult liver but is frequently reactivated in HCC, is the most widely used biomarker of HCC [5]. In addition, studies on these liver-specific proteins also provide insights into the physiological and pathological functions of hepatocytes.

T-complex 10 (mouse)-like (*TCP10L*) was firstly cloned in our lab, and our previous work revealed a liver and testis-specific expression of *TCP10L* in normal human tissues [6]. Through a typical leucine zipper (ZIP) motif, TCP10L could form homodimers or heterodimers with other ZIP motif-containing proteins [7–9]. In addition, TCP10L exerted a transcriptional suppressive activity *in vitro* [6]. However, the biological function of this factor *in vivo* has never been reported.

In this study, we found that the expression of *TCP10L* was significantly down-regulated in HCC. Forced expression of *TCP10L*, as well as silencing of endogenous *TCP10L* further revealed its anti-proliferation effect on HCC cells both *in vitro* and *in vivo*. Therefore, our findings suggested a tumor suppressor role of TCP10L in HCC, by regulating cell growth, which provided a potential candidate for molecular targeted therapy in HCC.

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2. Materials and methods

2.1. HCC specimens and cell line

Primary HCC tissues and the corresponding non-tumorous liver tissues were freshly collected from HCC patients who underwent hepatectomy at Zhongshan Hospital (Shanghai, China). Written informed consent was obtained from each patient before tissue acquisition. Samples were snap-frozen in liquid nitrogen immediately after surgery, and stored at −80 °C for further use. Human SMMC-7721 HCC cell line was purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), and was cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (PAA) at 37 °C in a humidified incubator containing 5% CO_2.

2.2. Quantitative real time PCR (qPCR)

Total RNA was extracted from HCC samples or cells with Trizol reagent (Invitrogen) and the first-strand cDNA was synthesized using ReverTra Ace qPCR RT Kit (TOYOBO) following the manufacturers' instructions. PCR amplification reactions were performed by an iCycler detection system (Bio-Rad). Gene expression levels were normalized to that of the housekeeping gene β2-microglobulin (*β2-MG*). Primers for human *TCP10L* were: sense, 5′-CAAAGAT CGTCATCTAACAATTC-3′, and antisense, 5′-CTAAGTGGAACTTTATC TGAATC-3′. Primers for *β2-MG* were: sense, 5′-ATGAGTATGCCTGCC GTGTGAAC-3′, and antisense, 5′-TGTGGAGCAACCTGCTCAGATAC-3′. The relative *TCP10L* mRNA expression in paired HCC samples was calculated as previously described [10].

2.3. Generation and purification of rabbit polyclonal antibody

Generation of polyclonal antibody against TCP10L was performed according to the reference with modifications [11]. Briefly, GST-TCP10L and his-TCP10L fusion proteins were expressed and purified as previously described [8,9]. New Zealand white rabbits (Shanghai Slac, China) were immunized with 1 mL purified GST-TCP10L (1 mg/mL) emulsified with 1 mL Freund's complete adjuvant (Sigma) via intradermal injection on the back and proximal limbs. 4 weeks after the first injection, half dose of same antigen mixed with Freund's incomplete adjuvant (Sigma) was injected into the rabbits twice at interval of 2 weeks. Another 2 weeks later, antisera were harvested by carotid artery bloodletting and purified by HiTrap NHS-actived HP column (GE Healthcare) filled with 1 mg/mL his-TCP10L fusion protein following the manufacturer's instructions. The antibody specificity was verified by Western blot.

2.4. Western blot

Protein samples separated by SDS–PAGE were electro-transferred onto nitrocellulose membranes (Amersham). After blocking, membranes were incubated with specific antibodies at 4 °C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000, Santa Cruz). Immunoreactivities were visualized by enhanced chemiluminescence reagents (Santa Cruz). Antibodies used were: anti-β-actin (1:5000, Sigma), anti-myc (1:5000, Sigma), and anti-TCP10L (1:1000).

2.5. Colony formation assay and selection of stable transfectants

Cells were grown to 80% confluence, then were transfected with pcDNA3.1a(−)-*TCP10L* or control vector pcDNA3.1a(−) using Plus and Lipofectamine reagent (Invitrogen), following the manufacturer's instructions. Cells were harvested 24 h post-transfection for Western blot analysis, or portioned into new 60-mm dishes in triplicate at the indicated densities and subjected to a 2-weeks' selection with G418 at a final concentration of 800 μg/mL (Invitrogen). Visible colonies were stained by crystal violet dye in a colony formation assay. To generate stable cell lines, independent colonies were picked and subjected to Western blot analysis for TCP10L expression.

2.6. Knocking down of TCP10L

Transient knocking down of *TCP10L* was conducted by transfecting cells with annealed double-stranded small interfering RNA (siRNA) (Genechem, Shanghai, China) against *TCP10L* (GenBank ID: NM_144659, base 2376–2394, and base 704–722) at a final concentration of 100 nM using Lipofectamine 2000 reagent (Invitrogen). A random scrambled siRNA was used as control. A long-term silencing of *TCP10L* was mediated by infection with lentivirus harboring a *TCP10L*-specific siRNA sequence. Independent colonies were picked and subjected to qPCR and Western blot to evaluate the knocking down effect.

2.7. Flow cytometry analysis

Cells were harvested, washed by phosphate-buffered saline (PBS) and then incubated with propidium iodide (50 μg/mL) together with RNase (100 μg/mL) diluted in PBS containing 0.03% TritonX-100 for 10 min. DNA content in the cells was assessed by flow cytometry (FACS Calibur, BD Biosciences) and the cell-phase distribution was analyzed by ModFit software.

2.8. Cell proliferation assay

Cells were harvested and seeded in 96-well plates at density of 1.0×10^3 cells per well. During the culture period, cells were subjected to 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H – tetrazolium (MTS) assay (Promega) according to the manufacturer's instructions. Spectrophotometric absorbance at 490 nm was read by a microtiter reader (HITACHI).

2.9. Xenograft mouse model

Female BALb/c nude mice (Shanghai Slac, China) at the age of 3–4 weeks were bred and housed under our institutional pathogen-free mouse facilities. 4×10^6 cells suspended in 200 μL PBS were subcutaneously injected into the right upper flanks of the mice. The tumor sizes were measured every 3 days with calipers. Tumor volume was calculated using the formula: volume (mm^3) = $(a \times b^2)/2$ (where a = length, b = width). Mice were sacrificed 4 weeks after cell inoculation. Tumors were dissected, photographed, weighted and snap-frozen in liquid nitrogen for further analysis.

2.10. Ki67 staining

Frozen xenografts were sectioned (10 μm thick) and fixed with ice-cold acetone for 10 min. After blocked with 10% normal goat serum at 37 °C for 30 min, sections were incubated with anti-Ki67 antibody (1:100, Invitrogen) at 4 °C overnight, followed by incubation with Alexa Fluor 488-conjugated secondary antibody (1:500, Molecular Probes) and DAPI (1:1000, Sigma). Image acquisitions were performed by a LEICA DC500 camera on a microscope equipped with LEICA DMRA2 fluorescent optics under 10 × 10 magnification. For each group, 18 randomly selected fields from 5 tumor tissues were used to calculate the mean number of Ki67-positive staining cells using Image J software (NIH).

2.11. Statistical analysis

All experiments were repeated at least three times. Data are expressed as mean ± standard deviation (SD). Comparisons of quantitative data were analyzed by Student's *t*-test. Categorical data were analyzed by Fisher's exact test. A *p*-value lower than 0.05 is considered significant.

2.12. Additional information

All animal studies were conducted under approval by the Institutional Animal Care and Use Committee of Fudan University.

3. Results

3.1. TCP10L was down-regulated in clinical HCC samples

The liver-specific expression of *TCP10L* suggested its involvement in liver physiology and/or pathology. In this work, we first examined the expression of *TCP10L* in 102 pairs of HCC samples by qPCR. Fig. 1A demonstrated the $\log_2$-transformed fold changes of *TCP10L* mRNA expression ratio of T/N (Tumor tissues/non-tumorous liver tissues), and a 2-fold threshold was set for significant changes in *TCP10L* expression. As shown in Fig. 1A, the expression of *TCP10L* was significantly reduced in 64 of 102 (62.75%) HCC tissues compared to their non-tumorous counterparts. Twenty-three of 102 (22.55%) cases showed no alteration, and only 15 of 102 (14.70%) cases showed slight up-regulation of *TCP10L* in HCC tissues, which revealed a differential expression pattern of *TCP10L* between normal liver and tumor tissues (Fig. 1A, $p < 0.001$, paired student's *t* test). As shown in Supplementary Table S1, statistical analysis revealed that reduced *TCP10L* expression was correlated with increased tumor size ($p = 0.012$). Additionally, patients exceeding the Milan criteria, which is accepted as gold standard to assess suitability in patients with cirrhosis and HCC for orthotopic liver transplantation (OLT) [12], were more likely to have an reduced *TCP10L* expression ($p = 0.022$).

To further analyze the protein expression of TCP10L, we generated polyclonal antibody against TCP10L. In HCC cell line SMMC-7721, endogenous TCP10L protein was detected as a 34 kDa band, which disappeared when the antibody was pre-incubated with excess recombinant his-TCP10L fusion protein overnight before use, identifying the specificity of this anti-TCP10L antibody (Fig. 1B). Next, proteins extracted from 9 paired HCC samples were subjected to Western blot. As shown in Fig. 1C, the expression of TCP10L was obviously decreased in 6 of 9 (66.66%) HCC tissues compared with their normal counterparts, which was consistent with the qPCR results. Therefore, downregulation of TCP10L in HCC clinical samples was revealed at either mRNA or protein level, suggesting its suppressive role in HCC.

3.2. Forced TCP10L expression inhibited HCC cell growth

Then, we investigated the effect of TCP10L expression in HCC cells. SMMC-7721were transfected with pcDNA3.1a(−)-*TCP10L* plasmid or control vector, respectively. After 24 h, the transfectants were subjected to colony formation assay, and TCP10L expression was confirmed by Western blot (Fig. 2A). As shown in Fig. 2A, overexpression of TCP10L resulted in a significant reduction in the number of viable colonies, indicating a suppressive effect of TCP10L on HCC cell growth (Fig. 2A, left panel).

To get further insights into TCP10L function, we then generated stable transfectants in SMMC-7721. 7721-C and 7721-T were two stable clones selected from cells transfected with pcDNA3.1a(−) and pcDNA3.1a(−)-*TCP10L*, respectively. The growth of stable clones was assessed by MTS assay. As shown in Fig. 2B, in a 5-day culture period, 7721-T showed an obvious decreased cell growth rate compared with control 7721-C cells.

Further cell cycle analysis revealed that, overexpression of TCP10L induced a significant decrease in the fraction of cells in S-phase (18% vs 27%, $p < 0.001$), and a concomitant increase in

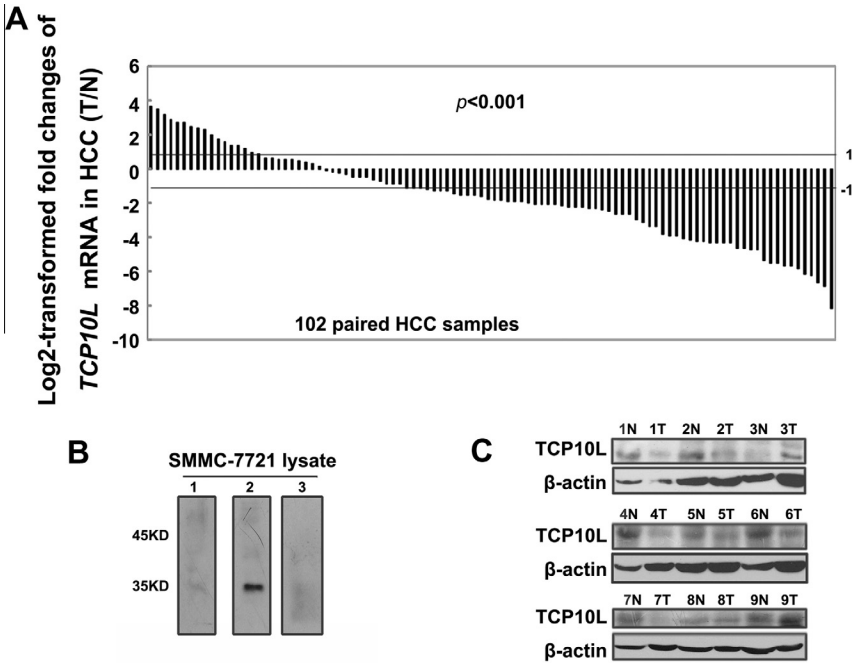

Fig. 1. Downregulation of TCP10L in human HCC samples. (A) Log_2-transformed fold changes of *TCP10L* mRNA in tumor samples (T) relative to corresponding non-tumorous tissues (N). (B) Determination of anti-TCP10L antibody specificity. Lysates of SMMC-7721 were subjected to SDS–PAGE Western blot and probed with pre-immune sera (lane 1), anti-TCP10L antibody (lane 2) and anti-TCP10L antibody pre-blocked by 1 μg/mL recombinant his-TCP10L fusion protein (lane 3), respectively. (C) Protein expression of TCP10L in 9 paired HCC samples were analyzed by Western blot with anti-TCP10L polyclonal antibody. β-Actin was used as a loading control.

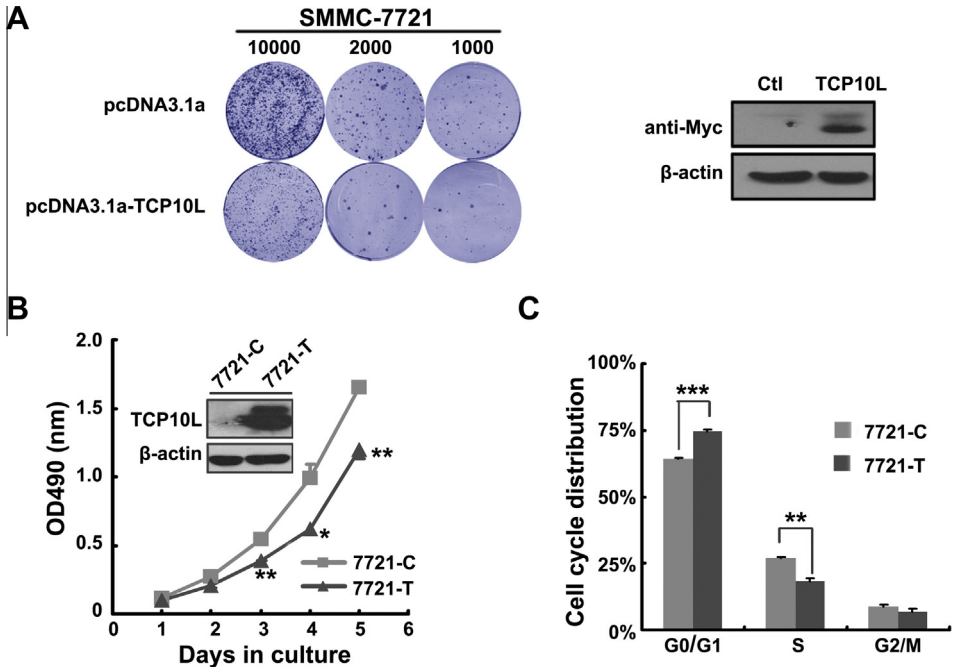

Fig. 2. TCP10L inhibited proliferation of HCC cells *in vitro*. (A) Representative images of colony formation assay in SMMC-7721 at indicated cell densities. Expressions of TCP10L were verified by Western blot (right panel). (B) Cell proliferation of stable clones was examined by MTS assay. TCP10L expression was identified by Western blot using anti-Myc antibody. β-Actin was used as a loading control. Cell growth curve was expressed as absorbance at 490 nm read by a microtiter reader. (C) Cell cycle analysis. Cells were harvested and stained with propidium iodide before being subjected to flow cytometric analysis. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

the fraction of cells in G0/G1-phase (75% vs 64%, $p < 0.01$) (Fig. 2C). This indicated that overexpression of TCP10L could induce G0/G1 arrest in HCC cells.

3.3. TCP10L attenuated tumorigenicity of HCC cells in vivo

To explore the effect of TCP10L on HCC cell *in vivo*, 4×10^6 cells of 7721-C and 7721-T were subcutaneously inoculated into the upper flank of female nude mice, respectively. Tumor volumes were measured every 3 days and tumor weights were recorded after the xenografts were dissected from the sacrificed mice. We found that the tumors derived from 7721-T cells were much smaller than those of the control xenografts in size (508 ± 87 vs 873 ± 366 cm^3, $p < 0.05$) and in weight (0.35 ± 0.11 vs 0.61 ± 0.15 g, $p < 0.001$) (Fig. 3A and B). Furthermore, staining of the proliferation marker Ki67 demonstrated the reduced growth capability of 7721-T cells *in vivo* when compared to 7721-C cells, which contributed to the decreased tumorigenicity of TCP10L-overexpressing HCC cells (Fig. 3C).

3.4. Silencing of endogenous TCP10L promoted HCC cell growth

To confirm the growth inhibitory effect of TCP10L on HCC cells, loss-of-function RNA interference (RNAi) was employed. Two effective siRNAs (S1 and S2) were used to knock down endogenous *TCP10L* in SMMC-7721 cells (Fig. 4A, left panel). When compared with control group (NS), knockdown of *TCP10L* in SMMC-7721 induced a significant increase in S-phase cell fraction and a concomitant decrease in G0/G1-phase cell fraction (Fig. 4A, right panel).

The core sequences of siRNAs were further constructed into lentiviral vector. Cell clones infected with lentivirus haboring NS siRNA sequence were used as control cells, defined as Lenti-NS, whereas Lenti-S1 and Lenti-S2 were two clones infected with S1 and S2-harboring lentivirus, respectively. The knockdown effect of lentivirus was detected through qPCR and Western blot (Fig. 4B, left panel), and cell growth was examined by MTS assay. As shown in Fig. 4B (right panel), both Lenti-S1 and Lenti-S2 grew faster than Lenti-NS.

Next, the effect of *TCP10L* silencing on tumorigenicity was assessed. Consistent with the *in vitro* result, TCP10L knockdown promoted tumor growth in nude mice, resulting in tumors with increased size and weight compared with controls (Fig. 4C and D). Taken together, these data suggested that manipulating endogenous *TCP10L* might affect HCC development.

4. Discussion

HCC development is a multi-step process, accompanied by accumulation of gene expression alterations and cell activity abnormalities [13]. Previous work has identified *TCP10L* as a liver and testis-specific gene [6]. Herein, we firstly found that TCP10L was significantly down-regulated in clinical HCC tissues ($p < 0.001$) (Fig. 1). More importantly, reduced expression of TCP10L was associated with advanced cases with larger tumor size. Milan criteria, which set the upper limits of tumor size and number, have been widely applied around the world in the selection of patients with HCC for liver transplantation. It is also useful predictor of clinical outcome in patient post-OLT [12]. Our data revealed that patients exceeding Milan criteria are more likely to have decreased *TCP10L* expression, indicating a potential prognostic value of *TCP10L*.

Based on the remarkable expression alteration of TCP10L in HCC, we hypothesized that it may play a suppressor role in HCC development. To identify this hypothesis, manipulation of TCP10L expression was performed, and the effects were examined both *in vitro* and *in vivo*. Transient overexpression of TCP10L in SMMC-7721 reduced the number of viable colonies (Fig. 2A), and stable expression of TCP10L in SMMC-7721 led to decreased cell growth rate and G0/G1 arrest *in vitro* (Fig. 2B and C), which were consistent with the impaired xenograft growth *in vivo* (Fig. 3A and B). The Ki67 antigen is detected in proliferating cells in all phases of the cell division cycle, but is absent in resting cells (G0

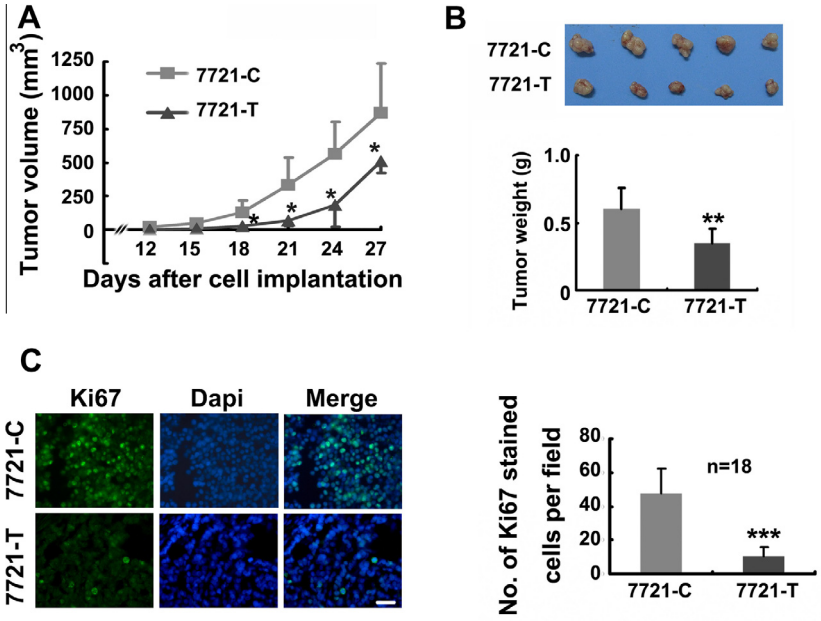

Fig. 3. TCP10L attenuated HCC cell growth *in vivo*. (A) Inhibition of tumor growth by overexpression of TCP10L. Both 7721-C and 7721-T cells were subcutaneously inoculated into the nude mice. Tumor sizes were measured with a caliper every 3 days and calculated as described in Section 2. (B) Tumor weights recorded after dissection. (C) Representative images of Ki67 staining of tumor tissues from the xenografts (left panel) and calculation of Ki67-positive cells in tumor sections (right panel). Scale bar, 50 μm. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

phase) [14]. In the tumor xenografts of control mice, we observed an intensive nuclear staining of Ki67, indicating the robust cell division. In contrast, Ki67 signals were rarely detected in tumor tissues derived from TCP10L-overexpressing HCC cells, which suggested a retarded growth of these cells *in vivo* (Fig. 3C). To avoid pleiotropic effect in forced expression system, we also knocked down the endogenous expression of *TCP10L* in SMMC-7721. Consistently, lentivirus-mediated silencing of TCP10L exhibited a promoting effect on HCC cell growth both *in vitro* and *in vivo* (Fig. 4). All these findings indicated the tumor suppressor role of TCP10L through inhibiting cell proliferation, providing reasonable explanation for the inverse correlation between *TCP10L* expression and tumor size in clinical HCC samples.

Although TCP10L induced cell growth suppression and G0/G1 arrest, no apoptosis was observed (data not shown), which excludes the proapoptotic function of TCP10L in the development of HCC. Previous work has identified a leucine zipper (ZIP) motif in TCP10L, and homodimerized TCP10L showed a transcriptional repressive function in a ZIP motif-dependent manner [6,9]. ZIP motif exists in numerous transcription factors such as c-myc and AIF3 [15,16]. Recently, many transcription factors containing ZIP motif have found to be associated with oncogenesis, mainly due to their function in regulating cell cycle [17]. Dimerization through ZIP motif is indispensable for the biological functions of these factors [18]. Thus we hypothesized that TCP10L might exert the anti-proliferation role via ZIP-dependent transcriptional activity. Additionally, TCP10L could also heterodimerize with Mad4 and Dapk3, other two ZIP motif-containing proteins [7–9]. Mad4, belonging to Mad family, is able to antagonize functions of Myc family to reduce cell proliferation and play a role in G0/G1 arrest [19,20]. Dapk3, belonging to Death-associated protein kinase (Dapk) family, is characterized as a pro-apoptotic serine/threonine kinase [21]. Thus, Mad4 rather than Dapk3, is more likely to be involved in the growth suppression by TCP10L in HCC cells. However, this hypothesis remains to be experimentally identified.

Synthesis of liver-specific proteins is regulated predominantly at transcriptional level by sets of liver enriched transcription factors (LETFs). Up to date, several members of LETFs have been identified, including CCAAT/enhancer binding protein (C/EBP) family, D binding protein (DBP) and hepatocytes nuclear factor (HNF) family [22,23]. These LETFs were found to bind to the promoter and enhancer regions of genes expressed in terminally-differentiated hepatocytes. They are also able to regulate each other, form a hierarchy regulatory network of liver gene expression, and play an important role in maintaining the balance between cell proliferation and differentiation. Decrease of expression and loss of function were frequently reported for these LETFs during hepatocarcinogenesis [24,25]. Considering the decreased expression of TCP10L in HCC, and its suppressive effect on HCC cell growth, identification of the specific LETFs modulating the expression of TCP10L in liver will lead to a better understanding of the function and regulation of TCP10L in HCC development. Furthermore, TCP10L might belong to one of these families, for example, C/EBP or DBP family which contains a basic region and ZIP motif. Thus the DNA binding domain and transcriptional activation domain of TCP10L should be clearly characterized to confirm this supposition. Therefore, further studies investigating the sequence and structure features of TCP10L will facilitate our comprehensive understanding on the sophisticated regulation network among the liver-specific proteins and LETFs members.

In conclusion, we have identified a tumor suppressor role of TCP10L through inhibition of HCC cell proliferation. Further exploration of the transcriptional mechanisms accounting for the expression alteration of TCP10L in HCC, and the mechanisms underlying the suppressive effect on HCC cell growth by TCP10L will provide comprehensive understanding of this molecule in the development of HCC, which might contribute to the improvement of diagnosis and molecular targeted therapy in HCC.

Conflict of interest

The authors declare no conflict of interest.

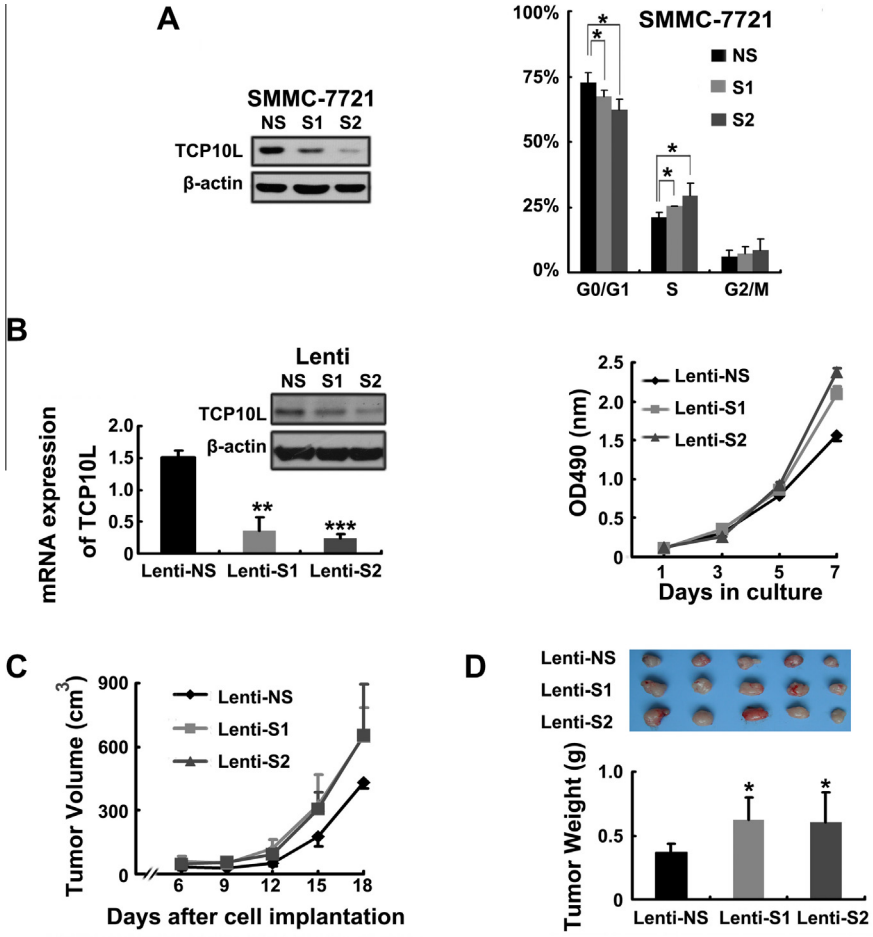

Fig. 4. Silencing of TCP10L in HCC cells accelerated cell growth *in vitro* and promoted tumorigenesis *in vivo*. (A) Cell cycle analysis after endogenous expression of *TCP10L* was knocked down in SMMC-7721 (right panel). Two specific siRNA fragments targeting *TCP10L* were used to knock down the endogenous expression of *TCP10L* in SMMC-7721, and the effect was verified by Western blot using anti-TCP10L antibody (left panel). (B) Effect of TCP10L knockdown on cell proliferation of SMMC-7721. Stably silencing of TCP10L in 7721 cells was achieved by infecting parental cells with lentivirus harboring effective siRNAs. The efficiency of knockdown was examined by qPCR and Western blot (left panel). (C) Effect of *TCP10L* knockdown on the growth of SMMC-7721 xenografts. Five mice were used in each group. (D) Pictures of the dissected tumors from mice (upper panel) and the average tumor weights of each group (lower panel). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.049.

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