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TCP10L negatively regulates alpha-fetoprotein expression in hepatocellular carcinoma

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Running title: TCP10L transcriptionally regulates AFP

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ABSTRACT

Alpha-fetoprotein (AFP) is one of the most commonly used and reliable biomarkers for Hepatocellular carcinoma（HCC）. However, the underlying mechanism of AFP expression in HCC is poorly understood. In this study, we found that TCP10L, a gene specifically expressed in the liver, is down-regulated in HCC and that its expression inversely correlates with AFP expression. Moreover, overexpression of TCP10L suppresses AFP expression whereas knockdown of TCP10L increases AFP expression, suggesting that TCP10L might be a negative regulator of AFP. We found that TCP10L is associated with the AFP promoter and inhibits AFP promoter-driven transcriptional activity. Taken together, these results indicate that TCP10L negatively regulates AFP expression in HCC and that it could be a potential prognostic marker and therapeutic target for HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related deaths, ranking sixth among new cases worldwide (1). A more terrible feature of hepatocellular carcinoma is that it lacks typical symptoms in the early stage. Most patients have reached an advanced stage when at initial diagnosis, and the surgical treatment including partial liver resection and liver transplantation are not satisfactory (2, 3). In addition, the high incidence of recurrence and metastasis compromises the long-term therapeutic effect of surgical treatment, which makes the early diagnosis and treatment of this tumor ineffective (4). Therefore, it is critical to elucidate the molecular mechanisms underlying HCC development and find potential novel molecular targets thus developing new strategies for HCC treatment.

Alfa-fetoprotein (AFP) is a polypeptide of approximately 600 amino acids and was first detected by electrophoresis in human fetal serum. AFP is expressed at high levels in the fetal liver and visceral endoderm of the yolk sac (5). Shortly after birth, the AFP gene is dramatically repressed and is normally expressed at extremely low levels in the adult liver. However, AFP can be reactivated during liver regeneration and hepatocellular carcinogenesis. Importantly, AFP is a secretory protein and changes in its blood level make it an important marker for HCC diagnosis and patient prognosis that is widely used in clinical practice. However, the underlying mechanism of AFP re-expression in HCC remains largely unknown.

Five distinct regulatory elements that govern AFP expression are found within 7.6 kb

of DNA upstream of the AFP transcription start site: a 250-bp tissue-specific promoter, a repressor domain which lies within 1 kb of the AFP transcription start site that is at least partially responsible for the decrease in AFP gene expression in the adult liver, and three independent enhancers located 2.5, 5.0, and 6.5 kb upstream of the AFP promoter that are essential for AFP transcription *in vivo* and continue to be active in the adult liver(6). Several known liver-enriched factors that positively or negatively regulate AFP transcription have been identified. Hepatocyte nuclear factors (HNFs) \ nuclear factor 1 (NF-1) and CAAT/enhancer binding protein (C/EBP), which mediate the transcription of most of the liver-specific genes, have been shown to bind to a region at 120 from the transcription start site (+1) promoter and are capable of activating AFP gene transcription both *in vitro* and *in vivo*(7,8). Likewise, transcriptional repressors involved in postnatal AFP silencing have been identified. For example, zinc finger and BTB domain containing 20 (ZBTB20) directly binds to a region of the AFP promoter between −104/−86 and downregulates the activity of the AFP promoter (9, 10). Zinc-fingers and homeoboxes 2 (ZHX2) suppresses the activity of HNF1 transcription factors at the level of the AFP promoter (11). p53 mediates AFP repression by competing with HNF3 for binding to DNA in the repressor region −838/−250 of the AFP gene and altering its chromatin structure(12,13). Therefore, postnatal repression of AFP transcription may involve the combinatorial action of multiple distinct repressors. In summary, the physiological mechanism of AFP repression in the postpartum liver is not well understood, including the involved trans-acting repressors and the corresponding

cis-acting elements.

T-complex protein 10A homolog 2 (TCP10L) was first cloned by Chen Z. et al. in 2003 through a differentially displayed hybrid from a human liver cDNA library. It is specifically expressed in the liver and testis in normal human tissues (14, 15). TCP10L contains a putative leucine zipper (LZ) domain, which is required for homodimerization and heterodimerization with other LZ-containing proteins (16-19). Recently, we reported that TCP10L is a potential tumor suppressor in HCC (20). TCP10L stabilizes the MAD1 protein level through direct interaction, and they cooperatively regulate cell cycle progression (19).

In this study, we aim to explain the relationship between AFP and TCP10L in clinical HCC samples, and to discover the molecular mechanism of how TCP10L regulates AFP by using liver cancer cell lines. Due to the important role of AFP in liver cancer diagnosis, the study of TCP10L will provide some new evidence and support for the clinical diagnosis and treatment of liver cancer.

RESULTS

TCP10L expression is negatively associated with AFP level

Previously, we found that the TCP10L expression level is downregulated in HCC (20). To understand the clinical significance of this finding, we further analyzed the relationship between the expression level of TCP10L mRNA and clinical pathological features of HCC. Based on the expression level of TCP10L in their HCC tissues, 102 patients were divided into two groups: low TCP10L expression and moderate or high TCP10L expression. As shown in Table 1, low expression of TCP10L significantly correlated with high serum AFP values ($P = 0.024$). No other correlations were found between TCP10L expression and other clinical pathological features, suggesting that TCP10L expression may negatively correlate with the AFP level.

To further confirm the relationship between the expression levels of TCP10L and AFP in HCC, we analyzed TCP10L and AFP mRNA expression in paired tissues from another 106 HCC patients using quantitative real-time PCR (qRT-PCR). As shown in Figures 1A and B, TCP10L was significantly downregulated in 50% of the HCC specimens compared to the corresponding control tissues (53 of 106 cases showed a more than two-fold decrease in TCP10L mRNA compared to the normal adjacent liver tissue). Furthermore, the expression level of AFP was dramatically increased in most of the HCC tissues, which is consistent with previous results. Importantly, statistical analysis indicated that TCP10L expression negatively correlated with AFP expression (Pearson correlation coefficient, $r = -0.2198$, $P < 0.001$) (Figure 1C). Interestingly, a

guiding dotted line in Figure 1C indicated that low TCP10L expression was significantly negatively correlated with AFP expression (Pearson correlation coefficient, $r = -0.5594$, $P < 0.001$). Moreover, the protein levels of TCP10L and AFP in 12 paired HCC specimens also displayed a significant inverse correlation as determined by western blot (Figure 1D). These results indicate that the expression level of TCP10L negatively correlates with the AFP level in HCC, suggesting that TCP10L may be a negative regulator of AFP.

TCP10L suppresses AFP expression in HCC cells

To investigate the potential functional relationship between TCP10L and AFP, we generated stably-overexpressed Flag-tagged TCP10L in Hep3B cells which expressed AFP at a high level and investigated the expression of AFP by western blot, ELISA and qRT-PCR. As shown in Figure 2A, the protein level of AFP dramatically decreased in stably-overexpressed Flag-tagged TCP10L Hep3B cells compared to the control cells. The TCP10L protein contains a putative leucine zipper (LZ) domain, which is required for it to suppress gene transcription (15). Next, we generated the stably-overexpressed leucine zipper domain deleted mutant TCP10L-ΔLZ and examined the AFP expression level. As shown in Figure 2A, TCP10L-ΔLZ overexpression failed to inhibit AFP expression in Hep3B cells, suggesting that TCP10L suppresses AFP expression through its leucine zipper domain. The AFP level in cell culture medium was also examined in control, TCP10L overexpressed and TCP10L-ΔLZ overexpressed Hep3B cells by ELISA. As shown in Figure 2B, compared to the control and TCP10L-ΔLZ

overexpressed Hep3B cells, the AFP level in TCP10L overexpressed cell culture medium was dramatically reduced. AFP mRNA levels were then examined in control, TCP10L overexpressed and TCP10L-ΔLZ overexpressed Hep3B cells by qRT-PCR. As shown in Figure 2C, compared to the control and TCP10L-ΔLZ overexpressed Hep3B cells, the AFP mRNA level in TCP10L overexpressed cells was also dramatically reduced. Taken together, these results indicate that TCP10L suppresses AFP expression in HCC cells through its leucine zipper domain.

Next, we knocked down the expression of TCP10L using shRNA in HCC cells and examined the AFP level. A lentiviral vector expressing the TCP10L shRNA construct (named Lenti-S3) and a control vector containing a non-targeting sequence (named Lenti-NS) were transfected into Hep3B and HepG2 cells. As shown in Figure 2D, compared to the control cells, the TCP10L protein was efficiently suppressed in TCP10L shRNA infected Hep3B and HepG2 cells. In contrast, the AFP protein dramatically increased in TCP10L shRNA infected Hep3B and HepG2 cells compared to the control cells as determined by western blot. Furthermore, compared to the control cells, the AFP level in the culture medium of TCP10L shRNA infected Hep3B and HepG2 cells was also dramatically increased as determined by ELISA (Figure 2E). Next, AFP mRNA levels were examined in control and TCP10L shRNA infected Hep3B and HepG2 cells by qRT-PCR. As shown in Figure 2F, compared to the control shRNA infected Hep3B and HepG2 cells, the AFP mRNA level in TCP10L shRNA infected cells was also dramatically increased. These results indicate that downregulation of

158 TCP10L enhances the expression of AFP.

159 ***TCP10L suppresses AFP transcription through association with the AFP promoter***

160 Previously, we reported that TCP10L is located in the nucleus and functions as a
161 transcriptional repressor (21); therefore, we assessed whether TCP10L represses
162 transcription of AFP. The promoter region (-2069~+27 bp) of the human AFP gene was
163 cloned from human genomic DNA and inserted into the pGL3-basic vector to detect
164 AFP transcription using the luciferase assay. As shown in Figure 3A, compared to the
165 control cells and cells with TCP10L-ΔLZ overexpression, the transcriptional activity of
166 the AFP promoter dramatically decreased in cells with TCP10L overexpression.
167 Moreover, the inhibition of AFP promoter driven transcriptional activity by TCP10L
168 was dose dependent (Figure 3B), suggesting that TCP10L might repress the activity of
169 the AFP promoter through its leucine zipper domain.

170 Next, TCP10L response regions in the AFP promoter were mapped. Two AFP mutants,
171 namely, P1 (-2069~-1069 bp) and P2 (-1069~+27 bp), were constructed (Figure 3C)
172 and subsequently co-transfected into Hep3B cells with pCMV-Myc-TCP10L. The
173 luciferase reporter assay indicated that overexpression of TCP10L decreased the
174 activity of the luciferase reporter harboring the P2 (-1069~+27 bp), but not the P1 (-
175 2069~-1069 bp) mutant (Figure 3D), suggesting that the TCP10L response region might
176 be located in the -1069 to 27 region of the AFP promoter. We then tested whether
177 TCP10L directly associates with the AFP promoter region using the ChIP assay. Hep3B
178 cells that stably expressed exogenous Flag-TCP10L were lysed and

immunoprecipitated using an anti-Flag antibody or an IgG control. The nucleotide sequence of the −981 to +12 nt region of the AFP promoter is shown in Figure 3E and various length fragments (160 bp, 176 bp, 243 bp, 166 bp, 168 bp, and 193 bp) of AFP P (-981/+12) were amplified using the underlined primers. As shown in Figure 3F, nt -981/-821 of AFP was amplified in the samples pulled down with Flag antibodies but not with the IgG control by RT-PCR. We did not detect any PCR products for the other DNA fragments. ChIP-quantitative polymerase chain reaction (qPCR) confirmed that endogenous TCP10L was enriched around this fragment (Figure 3G). These data together demonstrate that TCP10L binds to the AFP promoter and that the responsive region is located between 981 bp and 821 bp upstream of the ATG start codon of AFP.

DISCUSSION

In the present study, we found that TCP10L associates with the promoter region of AFP and suppresses its expression. Moreover, TCP10L is downregulated in HCC, and the expression of TCP10L is negatively correlated with AFP, suggesting that TCP10L might negatively regulate AFP expression in HCC and play an important role in HCC development.

Transcription repressors play important roles in tissue-specific and temporal gene expression (22, 23). Our previous results indicate that TCP10L is a transcription inhibitor and that it is expressed exclusively in the liver and testis. In this work, we found that TCP10L represses the activity of the AFP promoter and that this repression depends on the leucine zipper domain of TCP10L. These results provide the first direct evidence that TCP10L acts on the AFP promoter and negatively regulates AFP expression.

The AFP promoter contains binding sites for ubiquitous and tissue-specific transcription factors, such as HNF-1/NF-1, nkx2.8, fetoprotein transcription factor (FTF) and FoxA. There is a DNA binding site located at -135 of the AFP promoter for transcriptional repressor COUP-TF (24, 25). Additionally, p53 mediates AFP repression by competing with HNF3 for DNA binding in the repressor region of the AFP gene (-838 to -250). The region between −1010 and −838 is required for Afr2-regulated AFP expression during liver regeneration (26, 27). Our results show that TCP10L interacts with the AFP promoter (-981 to -821) and represses AFP gene transcription. Therefore, we

hypothesize that TCP10L might interact with other transcription factors to exert its biological effects. Through a typical leucine zipper (ZIP) motif, TCP10L could form homodimers or heterodimers with other ZIP motif-containing proteins (15). Our data shows that TCP10L represses the activity of the AFP promoter via the leucine zipper domain. We hypothesize that TCP10L might repress gene transcription via heterodimerization with other ZIP motif-containing proteins. Nevertheless, future studies are necessary to investigate whether other cofactors are also involved in this transcriptional repression. Overall, these results emphasize the complexity of AFP repression and provide further evidence that postnatal AFP shut-off requires many transcription factors.

The relationship between liver cell proliferation and AFP gene transcription has been noted but is not yet fully explored (28, 29). Postnatal AFP repression in the liver is coincident with the cessation of hepatocyte cell division, and AFP reactivation is often related to hepatocyte proliferation (30). Thus, it would be interesting to determine whether AFP reactivation in HCC reflects decreased TCP10L levels or activity. Whether AFP re-expression in TCP10L-knockdown cells leads to enhanced susceptibility for hepatocellular carcinogenesis remains under investigation. Our studies provide a better understanding of the function and regulatory role of TCP10L in HCC development and may also provide new targets for drug development.

MATERIALS AND METHODS

HCC specimens and cell lines

Primary HCC and the corresponding non-tumorous liver tissues were freshly collected from HCC patients who underwent a hepatectomy at the Zhongshan Hospital (Shanghai, China). Samples were snap-frozen in liquid nitrogen immediately after surgery and stored at -80°C until further use. The human HCC cell lines Hep3B (AFP positive) and HepG2 (AFP positive) were cultured in Dulbecco's Modified Eagle Medium (Gibco BRL, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, USA) and 100U/ml of penicillin at 37°C with 5% carbon dioxide.

Generation of stable cell lines

For the generation of stable cell lines, we purchased lentiviral stocks from GenePharma (Shanghai, China) that were produced by co-transfection of 293T cells with a recombinant lentiviral shRNA vector (pGLV-U6-Puro) containing the TCP10L target sequence (siRNA: 5'-CAGAAGAGCAACACCUACUTT-3') or the negative control (siRNA: 5'-UUCUCCGAACGUGUCACGU-3'). Stably transduced Hep3B or HepG2 cells were selected using puromycin, with a minimum concentration of puromycin being added to kill the untransduced cells. HCC cells with TCP10L knockdown were isolated by FACS sorting and stable knockdown was confirmed by green fluorescence and western blotting. Stable control colonies were also generated in parallel.

The TCP10L cDNA and a leucine zipper deleted mutant (amino acids 72 to 108) were subcloned into the mammalian expression vector SBP-Vector (Invitrogen, USA) which

contained a Flag tag and neomycin resistance gene to establish stable transfectants. After selection with G418 and detection by western blotting, 2 randomly selected colonies expressing TCP10L-Flag (named Hep3B-T1 and Hep3B-T2) were combined. Likewise, 2 randomly selected colonies expressing TCP10L-LZ-Flag (named Hep3B-ΔLZ1 and Hep3B-ΔLZ2) were combined. Control colonies were generated in parallel (named Hep3B-C1 and Hep3B-C2).

Luciferase reporter assays

In a 24-well plate, Hep3B cells were co-transfected with pGL3 containing the AFP promoter, *Renilla* luciferase plasmid and the TCP10L expression plasmid or an empty vector control using the Lipofectamine 2000 reagent (Invitrogen, USA). Cells were harvested 30h after transfection. Relative luciferase activity was determined using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega, USA). Experiments were repeated three times. The relative light units were measured by a luminometer (Promega, USA). The results are representative of the three independent experiments.

Chromatin Immunoprecipitation (ChIP) Assay

Physical associations between mammalian TCP10L and the AFP promoter were analyzed using a ChIP assay kit (Millipore, USA). The *in vivo* ChIP assay was performed using Hep3B cells. Cross linked protein-DNA complexes were immunoprecipitated with Flag antibodies. Isolated DNA was subjected to RT-PCR and analyzed by performing agarose gel electrophoresis, staining with ethidium bromide,

and then visualizing using ultraviolet (UV) light. DNA fragments were recovered by phenol/chloroform extraction and amplified through qRT-PCR. The primers used were listed in supplementary Table S1.

Other materials and methods are listed in the supplementary materials and methods.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

FIGURE LEGENDS

Figure 1. The expression of AFP and TCP10L are negatively correlated in HCC.

(A, B) The mRNA levels of AFP and TCP10L were analyzed in 106 HCC samples together with their corresponding non-cancerous tissues by quantitative RT-PCR. The columns show the fold change of AFP (red) and TCP10L (blue) mRNA levels in HCC samples compared to the corresponding adjacent noncancerous liver tissue, with beta-2-microglobulin serving as an internal control. (C) The scatter plot shows the correlation of TCP10L and AFP expression. The data was analyzed using a paired f-test, and correlation analysis was performed using Pearson's correlation test. The red line represents the correlation in 106 pairs of HCC samples. A guiding dotted line indicates the correlation between AFP and low TCP10L expression in 53 pairs of HCC samples. (D) The protein levels of AFP and TCP10L were analyzed in 12 HCC samples together with their corresponding non-cancerous tissues by western blot using the indicated antibodies, with β-actin serving as the loading control.

Figure 2. TCP10L suppresses AFP expression in HCC cells.

(A) The protein level of AFP in Hep3B cells stably expressing FLAG-TCP10L (T1, T2) and FLAG-TCP10L-ΔLZ (ΔLZ1, ΔLZ2) and in control cells (C1, C2) was examined by western blot using the indicated antibodies, with β-actin serving as the loading control. (B) The AFP level in media was measured by ELISA. The mean values (± S.D.) are shown. **P < 0.01. (C) The mRNA level of AFP was measured by qRT-PCR. The mRNA level of β2-MG was used for normalization. The mean values (± S.D.) are shown. **P < 0.01. (D) The protein levels of AFP and TCP10L in TCP10L knockdown (Lenti-S3) and control cells (Lenti-NS) were examined by western blot using the indicated antibodies. β-actin was used as a loading control. (E) The AFP level in media was measured by ELISA. The mean values (± S.D.) are shown. **P < 0.01. (F) The mRNA level of AFP in TCP10L knockdown (Lenti-S3) and control cells (Lenti-NS) was measured by qRT-PCR. The mRNA level of β2-MG was used for normalization. The mean values (± S.D.) are shown. **P < 0.01.

Figure 3. TCP10L associates with the AFP promoter and inhibits its transcriptional activity.

(A) TCP10 suppressed the transcriptional activity of the AFP promoter. Control vector, Myc-TCP10L or Myc-TCP10L–ΔLZ were co-transfected with the AFP full-length promoter (PGL3-AFP) into Hep3B cells, and the transcriptional activity of the AFP promoter was examined by luciferase reporter assays. Reporter activity values are expressed as the mean ± S.D., **P < 0.01. (B) Relative reporter activities were analyzed in Hep3B cells co-transfected with the AFP promoter and different dosages of Myc-TCP10L. Reporter activity values are expressed as the mean ± S.D., **P < 0.01. (C) Schematic view of the luciferase reporter constructs containing various regions of the 5'-flanking region of the AFP gene. (D) Hep3B cells were transfected with Myc-TCP10L and the AFP reporter constructs as indicated. Reporter activity values are expressed as the mean ± S.D., **P < 0.01. (E) The nucleotide sequences of the 5'-flanking region (-981/+12) of the AFP gene and the primers F1-F6 and R1-R6 used in ChIP-PCR and ChIP-qRT-PCR analysis are underlined. (F, G) TCP10L associated with the -981/-821 region of the AFP promoter. TCP10L promoter regions were pulled down by chromatin immunoprecipitation using the indicated antibodies, followed by real time PCR (F) or PCR (G) using the indicated primers.

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Figure 1

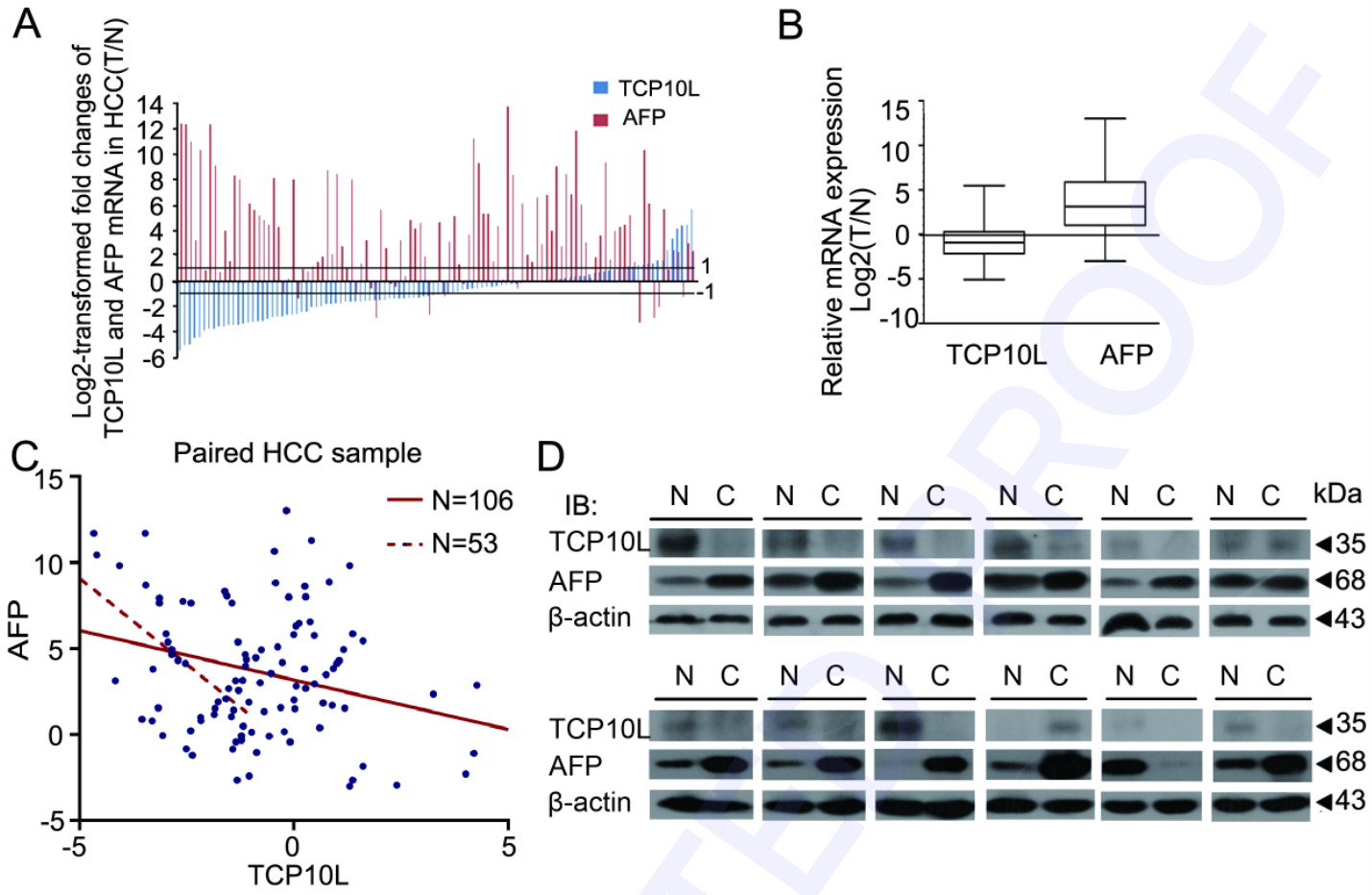

Fig. 1.

Figure 2

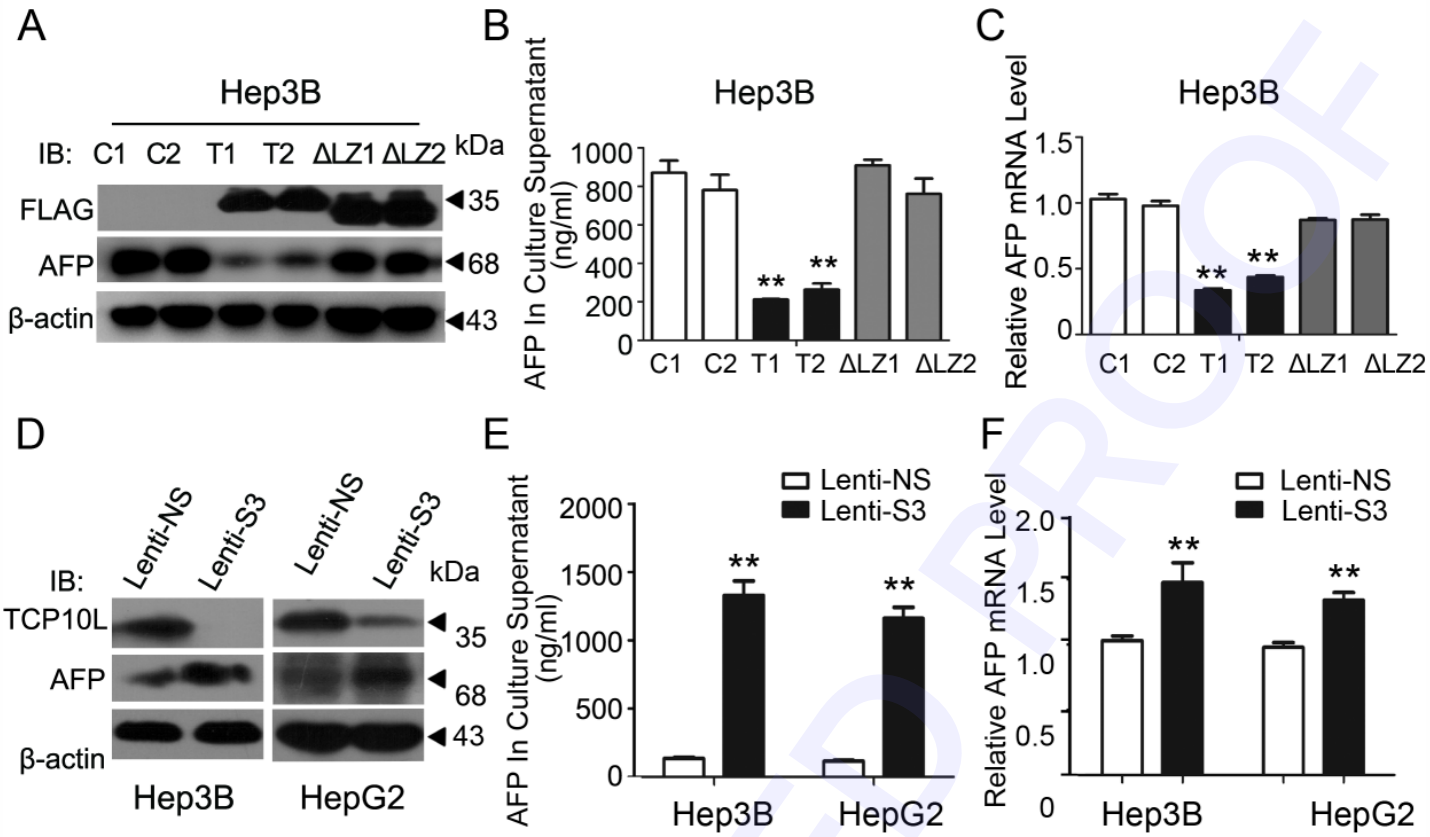

Fig. 2.

Figure 3

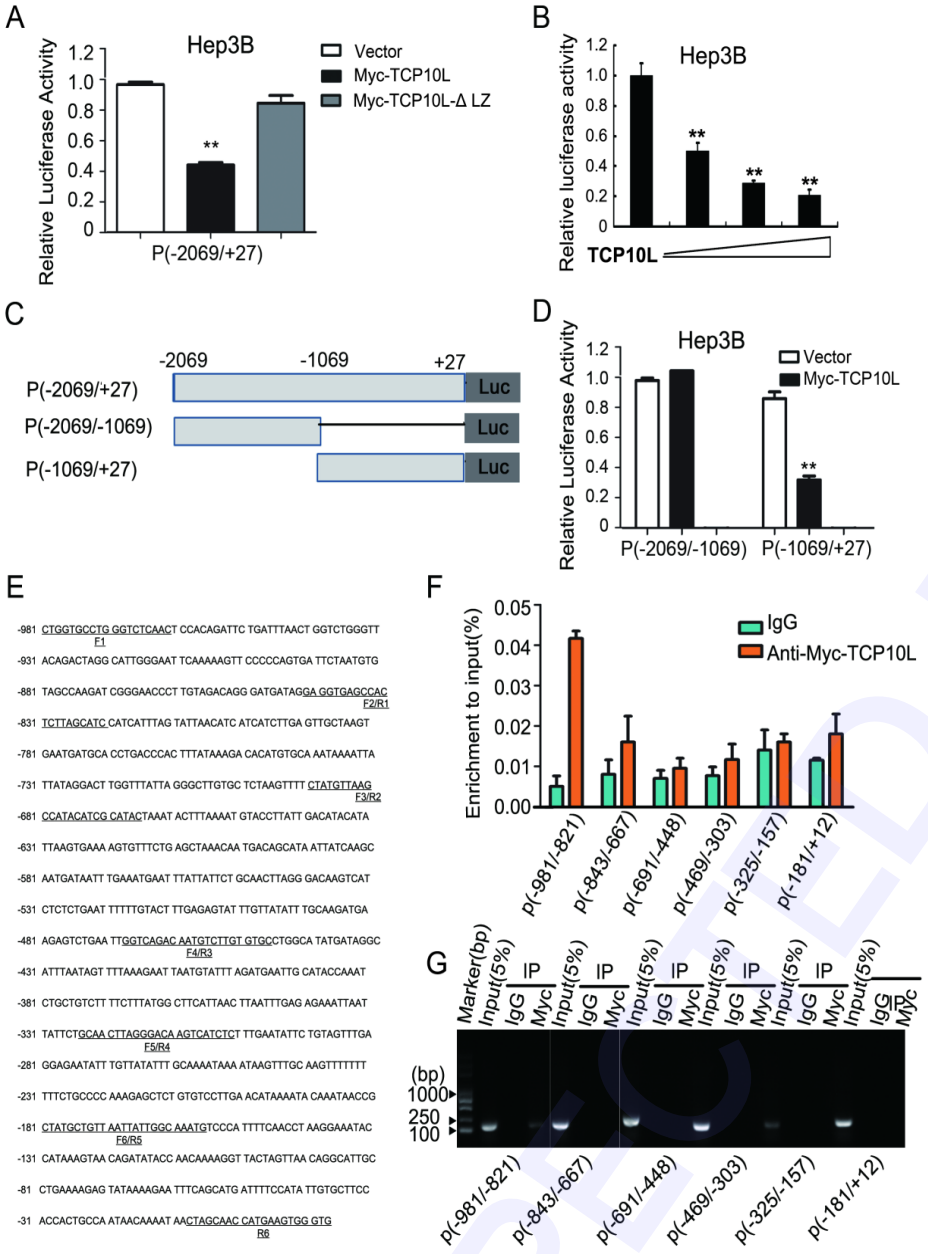

Fig. 3.

Table 1

Correlation of the clinic-pathological findings with TCP10L Downexpression in 102 cases of Human HCC

Pathological characteristic	TCP10L expression (T/N)			P value
	Down-regulation (no. of case)	Up-regulation (no. of case)	Down-regulation proportion (%)	
Sex				
Male	50	31	61.73%(50/81)	0.677
Female	14	7	66.67%(14/21)	
Age(years)				
≤50	21	10	67.74%(21/31)	0.490
>50	43	28	60.56%(43/71)	
Family History				
(+)	3	1	75.00%(3/4)	0.605
(-)	61	37	62.24%(61/98)	
Hepatitis History				
(-)	29	13	69.05%(29/42)	0.271
(+)	35	25	58.33%(35/60)	
HBsAg				
Negative	16	9	64.00%(16/25)	0.847
Positive	47	29	61.84%(47/76)	
AFP				
Negative(≤20ng/ml)	24	23	51.06%(24/47)	0.024*
Positive(>20ng/ml)	40	15	72.73 %(40/55)	
Tumor Number				
(single)	52	33	61.18%(52/85)	0.464
(multiple)	12	5	70.59%(12/17)	
Tumor Size(Φ,cm)				
≤5	27	21	56.25%(27/48)	0.201
>5	37	17	68.52%(37/54)	
Tumor Encapsulation				
Absent	39	18	68.42%(39/57)	0.182
Present	25	20	55.56%(25/45)	
Portal Vein Tumor Thrombus				
No	36	26	58.06%(36/62)	0.259
Yes	27	12	69.23%(27/39)	
Pathological Differentiation				
I-II	45	29	60.81%(45/74)	0.511
III-IV	19	9	67.86%(19/28)	
TNM Clinical Stage				
I	29	18	61.70%(29/47)	0.746
II-III	35	19	64.81%(35/54)	
Survival(d)				
≤1000	24	14	63.16%(24/38)	0.537
>1000	15	12	55.56%(15/27)	

Pearson Chi-square test was used

* Represents p values with significant difference

HCC, hepatocellular carcinoma

Table-S1: Sequences of Primer Sequences

Gene	Primer Sequences	
	Forward	Reverse
AFP	5’-CAGCCAAAGTGAAGAGGGAAGAC-3’	5’-AGCAGCCCAAAGAAGAATTGTAGG-3’
TCP10L	5’-CAAAGATCGTCATCTAACAATTC-3’	5’-CTAAGTGGA ACTTTATCTGAATC-3’
β2-MG	5’-ATGAGTATGCCTGCCGTGTGAAC-3’	5’-TGTGGAGCAACCTGCTCAGATAC-3’
AFP1P (-1301/-1179)	5'-CTGGTGCCTGGGTCTCAAC-3'	5'-GATGCTAAGAGTGGCTCACCTC-3'
AFP2P (-99/+56)	5'-GAGGTGAGCCACTCTTAGCATC-3'	5'-GTATGCGATGTATGGCTTAACATAG-3'
AFP3P (-415/-54)	5'-CTATGTTAAGCCATACATCGCATAC-3'	5'-GCACACAAGACATTGTCTGACC-3'
AFP4P (-796/-397)	5'-GGTCAGACAATGTCTTGTGTGC-3'	5'-GAGATGACTTGTCCCTAAGTTGC-3'
AFP5P (-796/-397)	5'-GCAACTTAGGGACAAGTCATCTC-3'	5'-CATTTGCCAATAATTAACAGCATAG-3'
AFP6P (-796/-397)	5'-CTATGCTGTTAATTATTGGCAAATG-3'	5'-CACCCACTTCATGGTTGCTAG-3'

Supplementary materials and methods

Western blot and antibodies

Protein samples were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membranes (Amersham). After blocking in phosphate-buffered saline/Tween-20 containing 5% non-fat milk at room temperature for 20 minutes, the membranes were incubated at 4°C overnight with primary antibodies against AFP (1:500, cat. no. ab133617, Abcam), β-actin (1:5000, cat. no. 254061, ABmart), TCP10L (1:1000, cat. no. 13709-1-AP, Proteintech) and Flag (1:3000, cat. no. 293674, ABmart). Subsequently, the membranes were incubated with anti-rabbit antibodies (1:3000, cat. no. 128689, Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 1h. Proteins were visualized using enhanced chemiluminescence reagents (cat. no. 20140708, 7sea biotech).

Real-time PCR

Total RNA was extracted from tissues or cultured cells using the TRIzol reagent (Toyobo, Japan). RNA was subjected to reverse transcription using a reverse transcription kit (Toyobo, Japan), which was followed by qRT-PCR analysis using a SYBR Green Supermix kit (Toyobo, Japan). The specificity of the PCR product was confirmed by melting curve analysis and the gene expression levels were normalized to the β2-MG housekeeping gene. Each sample was processed in triplicate independently and the relative quantification was calculated using the formula $2^{-\Delta Ct}$. The primers used for amplification were listed in Table S1.

Measurement of AFP in culture medium

ELISA was used to measure the level of AFP secreted into culture medium using the ACCESS® immuno-assay system (Beckman Coulter, USA). Cells were cultured in 6-well plates for 72h. Media were collected and the cell number/well was determined. The amount of protein secreted was expressed as ng/ml. Each experiment was repeated at least three times and the results are representative of the three independent experiments.

Statistical analysis

GraphPad Prism (GraphPad Software, San Diego, CA) was used for data analysis. The relationship between AFP and TCP10L in HCC was analyzed using Stata 10. Statistical analyses were performed using Student's two tailed t-tests. Values of *P < 0.05 and **P < 0.01 were considered statistically significant.