Down-regulation of *TROP-2* Predicts Poor Prognosis of Hepatocellular Carcinoma Patients

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Hepatocellular carcinoma (HCC) is one of the most common and lethal cancer types worldwide, especially in Asian countries. Genetic alterations, including hyperactivation of oncogenes and loss of expression of tumor suppressor genes, greatly contribute to the initiation and progression of HCC. Here we report that down-regulation of trophoblast cell surface antigen 2 (*TROP-2*) was frequently detected in HCC. Transcriptome sequencing of non-tumor and HCC patient samples revealed down-regulation of *TROP-2* in tumor tissues. Immunohistochemical staining showed nearly undetectable levels of *TROP-2* in HCC tissues but distinct and strong staining of *TROP-2* in adjacent non-tumor tissues. The frequent down-regulation of *TROP-2* expression was further confirmed in an in-house cohort of 205 pairs of HCC patient samples (tumor and non-tumor) and in the Cancer Genome Atlas (TCGA) databases. Furthermore, the down-regulation of *TROP-2* was associated with poor overall survival of HCC patients, severe adjacent organ invasion, and poor differentiation of HCC. Using bisulfite genomic sequencing and methylation-specific polymerase chain reaction analyses, we show that higher levels of promoter methylation were detected in the DNA samples of HCC tissues (low *TROP-2* expression) than that of the non-tumor tissues (high *TROP-2* expression). *Conclusion:* Taken together, our data suggest that promoter hypermethylation contributes to the frequent down-regulation of *TROP-2* in HCC, and that *TROP-2* down-regulation predicts poor prognosis of HCC patients. (*Hepatology Communications* 2018;2:1408-1414)

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and has been rated the sixth most prevalent and the third most lethal cancer type.(1) Effective HCC therapies appear to be very limited due to the complex mechanisms of pathogenesis and heterogeneity of HCC, the 5-year survival rate of which has been reported to be lower than 20%.(2) Therefore, early detection of this disease using biomarkers with high sensitivity and specificity is in urgent need.

Trophoblast cell surface antigen 2 (*TROP-2,* also known as *TACSTD2*) is a cell surface antigen identified on trophoblast cells using monoclonal antibodies.(3) The location of the *TROP-2* gene was first mapped to the human chromosome 1p32 by Calabrese et al. in 2001 through *in situ* hybridization.(4) The *TROP-2* protein possesses a large extracellular domain of 244 amino acids, a single transmembrane region of 23 amino acids, and a 30-amino acid intracellular tail with potential serine and tyrosine phosphorylation sites.(5)

Abbreviations: BGS, bisulfite genomic sequencing; FPKM, fragments per kilobase of transcript per million mapped reads; HCC, hepatocellular carcinoma; mRNA, messenger RNA; MSP, methylation-specific PCR; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; TCGA, the Cancer Genome Atlas; TROP-2, trophoblast cell surface antigen 2.

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The expression of the *TROP-2* protein was found in most human carcinomas.[6] Following stimulation by monoclonal *TROP-2* antibodies, the intracellular calcium signals of OvCa-432 ovarian cancer cells and MCF-7 breast cancer cells were raised by up to 40% with mean latencies of 65 and 77 seconds, respectively. Therefore, *TROP-2* is regarded as a tumor-associated calcium signal transducer.[7]

The clinical significance of *TROP-2* has been implicated in various types of human carcinomas. In colorectal cancer, complementary DNA microarray and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analyses showed significantly higher expression of *TROP-2* in tumor samples than in non-tumor samples, and the high expression of *TROP-2* is associated with liver metastasis and predicts poor patient prognosis.[8,9] Similarly, the overexpression of *TROP-2* was linked to shorter overall survival, lymph node metastasis, and advanced tumor stage in pancreatic cancer patients.[10] In addition, *TROP-2* protein expression has been detected to be significantly higher in invasive prostate cancer tissues and it was suggested that *TROP-2* can promote prostate cancer cell metastasis *in vivo* by regulating the relocalization and downstream signaling pathways of integrin $\alpha 5\beta 1$.[11,12] Interestingly, in lung adenocarcinoma and head and neck squamous cell carcinoma, the expression of *TROP-2* was reported to be down-regulated in tumor tissues when compared with non-tumor tissues.[13,14] In HCC, genome-wide methylation and microarray analyses showed that the *TROP-2* gene was significantly hypermethylated in tumor tissues, hinting a loss of expression of this gene in HCC.[15] Therefore, whether the inactivation or overexpression of *TROP-2* predicts poor patient outcome could be organ and cancer type dependent.

In this study, we report that the expression of *TROP-2* is significantly down-regulated in tumor samples of HCC as indicated by our in-house cohort and the Cancer Genome Atlas (TCGA) databases. The depletion of *TROP-2* is associated with poor overall survival of patients, adjacent organ invasion, and poor differentiation of HCC. Results of bisulfite genomic sequencing (BGS) and methylation-specific PCR (MSP) analyses suggested promoter hypermethylation of *TROP-2*, providing a possible explanation for the down-regulation of *TROP-2* in HCC. Our findings could pave the way of exploiting *TROP-2* as a diagnostic and prognostic marker for HCC patients.

Materials and Methods

CLINICAL HCC TISSUE SAMPLES

HCC patient samples of tumor and adjacent non-tumor tissues were collected from the Sun Yat-Sen University Cancer Center (Guangzhou, China). All HCC patients gave written consent on the use of clinical specimens for medical research. The collection of human tissues was approved by the Committees for Ethical Review of Research Involving Human Subjects at the Sun Yat-Sen University Cancer Center.

BISULFITE GENOMIC SEQUENCING AND METHYLATION-SPECIFIC PCR

Genomic DNA was extracted from HCC patient tissue samples, followed by bisulfite DNA treatments with the EpiTECT Bisulfite Kit (Qiagen, Hilden, Germany). After the bisulfite treatment, unmethylated cytosines were converted to uracils, leaving methylated

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cytosines unchanged. For BGS, the CpG island in the *TROP-2* promoter region was predicted using an online software (MethPrimer v1.1 beta, Li Lab, Department of Urology, University of California San Francisco). The predicted CpG island was then amplified by PCR. The PCR products were subsequently cloned into pMD18-T vector and transformed into DH5α competent cells. Sanger sequencing was performed on five randomly picked colonies from each sample. After obtaining data from BGS, primers were designed for MSP to test the methylation status of the CpG island on a larger amount of HCC patient DNA samples. Sequences of primers used in this study were listed in Supporting Table S1.

IMMUNOHISTOCHEMISTRY

Paraffin-sectioned clinical samples were first dewaxed at 70°C for 1-2 hours, followed by incubation with xylene and gradient concentrations of ethanol. Quenching of endogenous peroxidase was achieved by incubation of samples in 3% H_2O_2 for 5-10 minutes. For antigen retrieval, samples were boiled for 10-15 minutes in sodium citrate buffer with 0.05% Tween-20 (pH 6.04) to remove the cross-linking of formaldehyde.

SPlink Detection Kits (Biotin-Streptavidin HRP Detection Systems; OriGene, Rockville, MD) were used for immunohistochemical staining and signal detection according to manufacturer's instructions. For signal development and visualization, 3,3'-diaminobenzidine was added to the samples. Nuclear staining was carried out by hematoxylin (DAKO, Glostrup, Denmark) staining for 1-2 minutes. Dried samples were mounted with DPX mounting media (Thermo Scientific) and analyzed under a light microscope.

QUANTITATIVE REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION

After RNA extraction using RNAiso Plus reagent (Takara, Kyoto, Japan), complementary DNA was synthesized using PrimeScript RT Master Mix (Takara). The expression levels of genes of interest were detected using the SYBR Green PCR Kit (Roche, Basel, Switzerland) on an ABI Prism 7900 System (Applied Biosystems, Foster City, CA). Relative expressions were calculated using the $2^{-\Delta\Delta Ct}$ method. 18S ribosomal RNA was used as endogenous reference.

STATISTICAL ANALYSES

Statistical analyses of clinical data were performed using SPSS version 20.0 (SPSS, Inc., Armonk, NY). The clinicopathological features of patients were analyzed using Pearson's χ^2 test for categorical variables. Kaplan-Meier plots and log-rank test were used for overall survival analysis. Student t test was applied to compare the mean values of two groups using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). Error bars represent standard deviation values. Statistical significance was defined as $P < 0.05$.

Results

TROP-2 IS FREQUENTLY DOWN-REGULATED IN HCC

To search for novel genetic markers of HCC, transcriptome Solexa sequencing on three pairs of clinical HCC samples (non-tumor and tumor) was performed. The sequencing data of the 3 HCC patients (numbered 488, 473, and 510) showed that *TROP-2* was consistently down-regulated in tumor tissues when compared with adjacent non-tumor tissues. In patient 488, the expression of *TROP-2* was down-regulated by 39.32 folds in tumor tissue (non-tumor: 7.1522 fragments per kilobase of transcript per million mapped reads [FPKM]; tumor: 0.1819 FPKM). In patient 473, the expression of *TROP-2* was down-regulated by 32.36 folds in tumor tissue (non-tumor: 2.3329 FPKM; tumor: 0.0721 FPKM). In patient 510, the expression of *TROP-2* was down-regulated by 2.59 folds in tumor tissue (non-tumor: 0.3724 FPKM; tumor: 0.1437 FPKM) (Fig. 1A). Representative images from immunohistochemical staining showed a nearly undetectable level of TROP-2 in HCC tumor tissues and high expression of TROP-2 in adjacent non-tumor tissues (n = 5) (Fig. 1B). To verify these results, *TROP-2* messenger RNA (mRNA) expression was compared between non-tumor and tumor tissues in our in-house cohort consisting of 205 pairs of clinical HCC tissues and in the TCGA databases. qRT-PCR analysis of our in-house cohort confirmed the down-regulation of *TROP-2* in HCC tumor samples ($P < 0.05$) (Fig. 1C). Among the 205 HCC patients, 56% of patients showed no less than 2-fold *TROP-2* down-regulation in tumor tissues, and 26% of patients showed no less than 10-fold *TROP-2* down-regulation in tumor tissues. Meanwhile, RNA sequencing data from the TCGA databases demonstrated that *TROP-2* mRNA

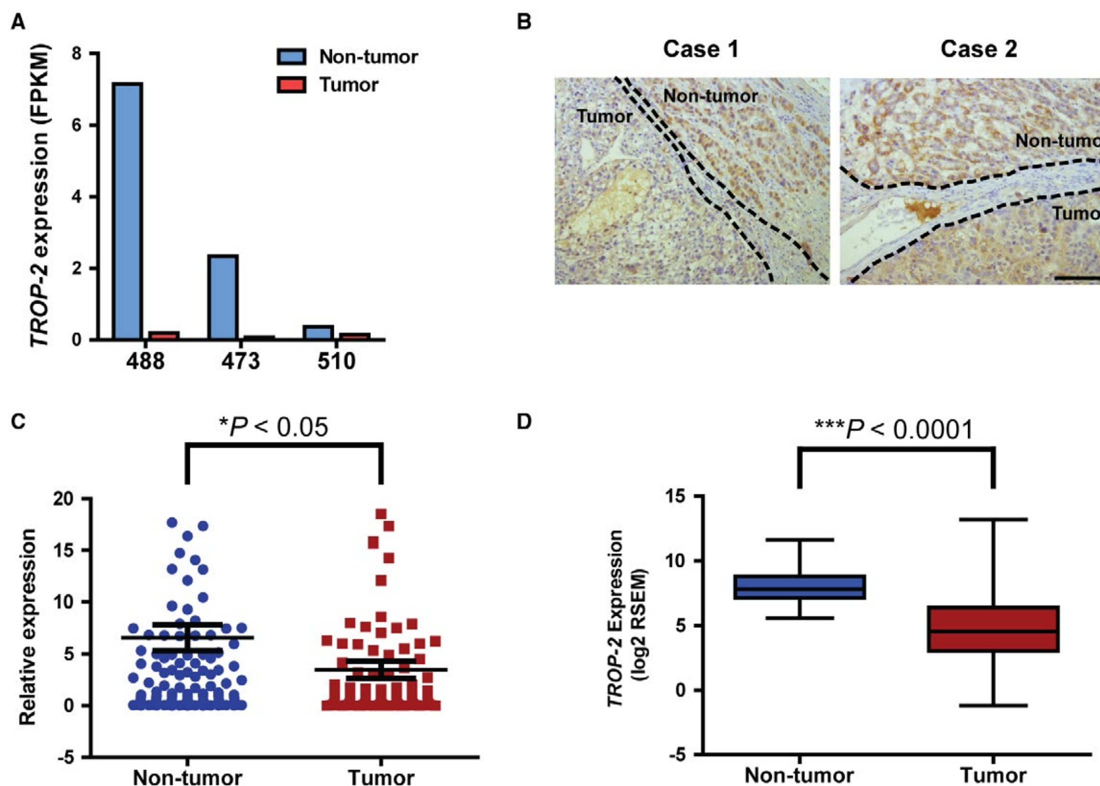

FIG. 1. *TROP-2* is frequently down-regulated in HCC. (A) Expression levels of *TROP-2* as detected by transcriptome sequencing of three pairs of HCC patient tissue samples. Results were expressed in FPKM. Numbers 488, 473, and 510 represent 3 HCC patients. (B) Representative images showing *TROP-2* protein expression in HCC patient tumor sections. Dotted lines were used to separate non-tumor and tumor regions. Scale bar, 50 μm. (C) Comparison of *TROP-2* mRNA expression between non-tumor and tumor tissues in 205 HCC patients as detected by qRT-PCR. $^{*}P < 0.05$, Student *t* test. (D) Comparison of *TROP-2* expression between non-tumor and HCC tumor samples in TCGA databases. $^{***}P < 0.0001$, Student *t* test.

expression was significantly down-regulated in HCC tumor tissues (n = 368) when compared with non-tumor tissues (n = 50; $P < 0.0001$) (Fig. 1D).

TROP-2 DOWN-REGULATION PREDICTS POOR PROGNOSIS OF HCC PATIENTS

Following validation of the frequent down-regulation of *TROP-2* in HCC, we further explored whether such down-regulation has any clinical impacts on HCC patients. Using Kaplan-Meier plots and log-rank test, we found that the down-regulation of *TROP-2* (at least 10-fold down-regulation in tumor tissues when compared with non-tumor tissues as indicated by qRT-PCR data) was tightly associated with poor overall survival of HCC patients ($P < 0.05$) (Fig. 2). In addition, clinicopathological analysis by Pearson's χ^2 test revealed that *TROP-2* down-regulation is associated with adjacent organ invasion ($P < 0.05$) and poor differentiation of HCC ($P < 0.05$) (Table 1).

PROMOTER HYPERMETHYLATION RESULTS IN *TROP-2* DOWN-REGULATION IN HCC

To examine whether promoter hypermethylation plays a role in *TROP-2* down-regulation, BGS, and MSP were used to analyze the promoter methylation status of the *TROP-2* gene. The presence and location of the CpG island within the *TROP-2* promoter region were predicted using MethPrimer software, and such analysis identified a 749-bp CpG island consisting of 79 CpG sites in the promoter region of *TROP-2* (Fig. 3A). Primers were subsequently designed to amplify and clone the genomic sequence of this CpG island from two non-tumor and two tumor samples from HCC patients. The results from BGS indicated

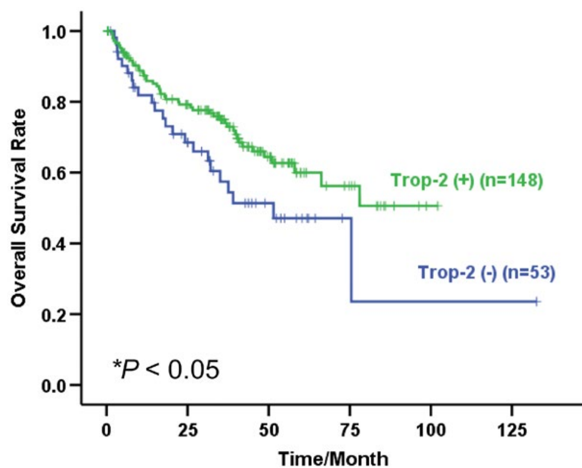

FIG. 2. Kaplan-Meier overall survival of HCC patients correlated with *TROP-2* expression. *P < 0.05, log-rank test. Abbreviations: *TROP-2* (+), patients with *TROP-2* normal expression; *TROP-2* (-), patients with *TROP-2* down-regulation.

higher levels of promoter methylation in tumor samples with low *TROP-2* expression (samples 365T and 411T) than that in non-tumor samples with high *TROP-2* expression (samples 389N and 411N), especially on CpG sites 21, 22, 23 and 38, 39, 40 (Fig. 3B). Thereafter, primers (methylated sequence- and unmethylated sequence-specific) were designed to target these six CpG sites for MSP. Five pairs of HCC DNA samples underwent MSP analysis. PCR results showed stronger bands in tumor DNA samples than that in non-tumor samples when detected with methylated sequence-specific primers, whereas non-tumor DNA samples showed stronger bands than tumor samples when detected with unmethylated sequence-specific primers (Fig. 3C).

TABLE 1. ASSOCIATION OF *TROP-2* DOWNREGULATION WITH CLINICOPATHOLOGICAL FEATURES IN 205 HCC PATIENTS

Features	Total Samples	*TROP-2* Expression: Down-regulation	*TROP-2* Expression: Normal Expression	*P* value
Sex				
Male	175	44 (25.1%)	131 (74.9%)	0.360
Female	30	9 (30%)	21 (70%)	
Age (years)				
≤ 60	167	42 (25.1%)	125 (74.9%)	0.384
> 60	38	11 (28.9%)	27 (71.1%)	
Hepatitis B surface antigen				
Negative	39	8 (20.5%)	31 (79.5%)	0.251
Positive	164	45 (27.4%)	119 (72.6%)	
Serum α-fetoprotein level (ng/mL)				
< 400	116	32 (27.6%)	84 (72.4%)	0.349
≥ 400	87	21 (24.1%)	66 (75.9%)	
Cirrhosis				
Absent	88	22 (25%)	66 (75%)	0.264
Present	41	16 (39%)	25 (61%)	
Tumor stage				
I	142	39 (27.5%)	103 (72.5%)	0.384
II + IIIa + IIIb	58	14 (24.1%)	44 (75.9%)	
Tumor size				
< 5 cm	74	18 (24.3%)	56 (75.7%)	0.383
≥ 5 cm	128	35 (27.3%)	93 (72.7%)	
Differentiation				
Low grade	123	27 (22%)	96 (78%)	< 0.05*
High grade	76	26 (34.2%)	50 (65.8%)	
Adjacent organ invasion				
Absent	160	36 (22.5%)	124 (77.5%)	< 0.05*
Present	44	16 (36.4%)	28 (63.6%)	
Recurrence				
No	79	18 (22.8%)	61 (77.2%)	0.244
Yes	124	35 (28.2%)	89 (71.8%)	

Note: Clinicopathological data of certain patients were not available. Statistical analysis was based on available data.
*P < 0.05, Pearson's χ^2 test.

Discussion

Down-regulation of expression in tumor tissue is an important indicator in the identification of candidate tumor suppressor genes. Much effort was made to search for candidate tumor suppressor genes that could serve as biomarkers for cancer diagnosis and prognosis. However, effective diagnostic markers for HCC are still lacking. We reported herein a potential tumor suppressor in HCC-*TROP-2*. The expression of *TROP-2* was significantly down-regulated in HCC tumor tissues when compared with non-tumor tissues, and promoter hypermethylation could potentially provide an explanation for such down-regulation. The loss of *TROP-2* predicts poor overall patient survival, invasion, and poor differentiation of HCC. However, the molecular mechanism of the effects of *TROP-2* leading to such patient outcomes is still unclear at this moment.

Loss of expression of genes could be attributed to multiple factors. Genetic and epigenetic alterations

FIG. 3. Promoter hypermethylation results in *TROP-2* down-regulation. (A) Illustration of the CpG island sequence and primer designs in BGS and MSP experiments. The numbers indicate the order of CpG sites within this CpG island. Arrowheads indicate the locations and directions of BGS and MSP primers. (B) BGS results of non-tumor and tumor tissue samples collected from HCC patients. The black-filled areas indicate the percentages of clones with methylation at the specified CpG site. 389N and 411N represent 2 non-tumor samples; 365T and 411T represent 2 HCC samples. (C) MSP results in five pairs of HCC patient DNA samples. Numbers 86, 219, 363, 365 and 409 represent 5 HCC patients. Abbreviations: N, non-tumor; T, tumor; TSS, transcription start site.

greatly contribute to changes in gene expression. For epigenetic alterations, DNA methylation is one of the most commonly seen mechanisms in inactivation of gene expression in cancer.[16] In fact, the hepatic methylation profile has been shown to be correlated with recurrence-free survival of HCC patients after hepatectomy.[17] As reported by Revill et al., *TROP-2* was found to be hypermethylated in HCC.[15] Therefore, by analyzing HCC patient DNA samples, we sought to confirm the cause of *TROP-2* down-regulation in HCC being promoter hypermethylation. Indeed, using BGS and MSP, we found that the *TROP-2* promoter methylation level is comparatively higher in HCC tumor tissues with low *TROP-2* expression than that in non-tumor tissues with high *TROP-2* expression (Fig. 3). However, whether promoter hypermethylation is the sole mechanism causing the loss of *TROP-2* expression in HCC remains to be elucidated. It might be interesting to further explore whether genetic alterations such as chromosomal deletion also play a role in the loss of *TROP-2* in HCC.

During the three decades since *TROP-2* was initially identified in trophoblast cells, much attention has been given to study the tumorigenic effects and predictive significance in poor patient prognosis of this gene in cancer patients. This is partly due to the important functions of this gene in the highly invasive process of blastocyst implantation.[18] Nevertheless, emerging evidence hints that there is more to it than that. Depending on the cancer types, *TROP-2* has exhibited distinct effects. In head and neck squamous cell carcinoma, the mesenchymal tumors showed decreased TROP-2 expression. Depletion of the TROP-2 protein in the cell surface stimulates the activation of epidermal growth factor receptor family member ErbB3 due to a lack of sequestering of neuregulin-1 by *TROP-2*.[14] In lung cancer, *TROP-2* expression was inhibited by DNA hypermethylation, and the loss of *TROP-2* leads to hyperactivation of insulin-like growth factor 1R

and subsequent oncogenic effects.[13] Thus, the effects of *TROP-2* in cancer development and determining patient outcome could be complex and cancer-type dependent. In this study, we found that *TROP-2* was frequently down-regulated in HCC, and the loss of *TROP-2* was associated with poor prognosis of HCC patients (Figs. 1 and 2, Table 1). This finding is encouraging and might provide support for using *TROP-2* as an effective biomarker in future diagnosis and prognosis predictions of HCC patients.

Conclusions

In conclusion, the results of RNA sequencing from TCGA databases and qRT-PCR analysis of our in-house cohort both indicated a frequent and significant down-regulation of *TROP-2* in HCC tumor tissues. DNA methylation analysis linked the down-regulation of *TROP-2* with promoter hypermethylation. Furthermore, the down-regulation of *TROP-2* could predict poor patient prognosis of HCC. These findings indicate that *TROP-2* might be a potential biomarker in predicting prognosis and aiding early cancer diagnosis of HCC patients.

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