Pre-Clinical Research Report

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
2019, Vol. 47(3) 1319–1329

DOI: 10.1177/0300060518822913
journals.sagepub.com/home/imr
SAGE

Silencing of the *TROP2* gene suppresses proliferation and invasion of hepatocellular carcinoma HepG2 cells

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Abstract

Objectives: Overexpression of human trophoblast cell surface antigen 2 (Trop2) has been observed in many cancers; however, its roles in proliferation, apoptosis, migration, and invasion of hepatocellular carcinoma (HCC) remain unclear. Thus, this study aimed to characterize the function of Trop2 in HCC.

Methods: Trop2 protein expression was detected by immunohistochemistry in HCC tissues. Cell proliferation, apoptosis, and invasion were respectively measured by CCK-8, flow cytometry, Transwell, and wound healing assays. Expression levels of epithelial–mesenchymal transition-related proteins and Trop2 protein in HCC cell lines were detected by western blotting after silencing of the *TROP2* gene.

Results: Trop2 protein was highly expressed in HCC tissues and HCC cell lines. Trop2 mRNA and protein expression levels decreased in HepG2 and HCCLM3 cells after transfection with Trop2 siRNA. Silencing of the *TROP2* gene in HepG2 and HCCLM3 cells strongly inhibited cell proliferation and migration, while enhancing cell apoptosis. Investigation of the molecular mechanism revealed that silencing of the *TROP2* gene suppressed epithelial–mesenchymal transition of HepG2 and HCCLM3 cells.

Conclusions: The results of the present study may improve understanding of the role of Trop2 in regulation of cell proliferation and invasion, and may aid in development of novel therapy for HCC.

Keywords

Trop2, hepatocellular carcinoma, invasion, proliferation, apoptosis, epithelial–mesenchymal transition, HepG2, HCCLM3, gene silencing

Date received: 19 August 2018; accepted: 12 December 2018

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, the incidence of which is substantially increasing each year; currently, HCC is ranked as the second-leading cause of digestive system cancer-related death in China.[1] Because of a lack of typical clinical manifestations, most patients with liver cancer are diagnosed in the advanced stages of the disease, and approximately 15% of patients undergo surgical treatment.[2] A high degree of metastasis is an important biological characteristic of malignant liver cancer, as well as the main cause of liver cancer death.[3] Thus, an improved understanding the mechanisms of liver cancer invasion and metastasis is imperative and may provide new insights regarding the clinical management of liver cancer.

Tumor metastasis is a complicated, multistep continuous process involving multiple relationships among cellular factors, including activation of an invasive phenotype, as well as abnormal gene expression and abnormal intracellular signaling. Trophoblast cell-surface antigen 2 (Trop2), which belongs to the tumor-associated calcium signal transducer (*TACSTD*) gene family, is a type I transmembrane protein that was originally identified in human placental trophoblastic tissue.[4] Trop2 can be cleaved into extracellular and intracellular domains by TNF-α converting enzyme; the intracellular domain then enters the cytoplasm or nucleus and exhibits biological activity.[5] Trop2 is reportedly highly expressed in various malignant tumors, while it exhibits minimal or no expression in normal tissues or tissues adjacent to tumors.[6] Overexpression of Trop2 has been associated with poor prognosis.[7,8] Additionally, Trop2 is reportedly involved in the invasion and metastasis of various carcinomas, including thyroid cancer,[9] colon cancer,[10] and prostate cancer.[11] To the best of our knowledge, no studies have been conducted regarding the possible role of Trop2 in invasion and metastasis of HCC. Thus, the aim of the present study was to characterize the function of Trop2 in HCC using *in vitro* techniques.

Materials and methods

Tissue samples

Tissue samples from the tumors of 10 patients with HCC, as well as their corresponding paracancerous tissues, were obtained at Sichuan Cancer Hospital during the period from 2015 to 2017. The present study was approved by the Ethics Review Board at The University of Electronic Science and Technology of China. All patients had given informed consent for experimental analysis of their excised tissues.

Design of *TROP2*-siRNA sequence

The nucleotide sequence of the human *TROP2* gene was obtained from GeneBank and used to design target siRNA sequences in accordance with established principles of gene silencing.[12] The target siRNA sequence and negative control sequence are shown in Table 1. The sequences were not homologous to any other human gene sequence, according to the results of BLAST analysis. The siRNA oligonucleotide molecules were synthesized by Shanghai GenePharma Technology Co., Ltd. (Shanghai, China).

Table 1. Sequences of siRNA against the *TROP2* gene

siRNA	Sequences (5′→3′)
Negative control	5′-UUCUCCGAACGUG UCACGUTT-3′
	5′-ACGUGACACGUUCG GAGAATT-3′
TROP2-siRNA	5′-CACCTTCAAGAC GTTTTTTG-3′
	5′-AGCTCAAAAAAC GTCTTGAA-3′

Cell culture and transfection

HepG2 (CL0103), HCCLM3 (CL0278), and HL7702 (CL0111) cells were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco-Invitrogen, Carlsbad, CA, USA). Cells were maintained at 37°C in an incubator with 5% CO_2 in room air. Cell transfection was performed using Lipofectamine2000 (Invitrogen), in accordance with the manufacturer's protocol, using 40 pmol/mL *TROP2*-siRNA and negative control-siRNA. The medium was changed after 6 hours. At 24 hours after transfection, cells were collected for western blotting and quantitative PCR (qPCR) analyses.

Immunohistochemical assay

Immunohistochemical staining was performed in accordance with the manufacturer's instructions. Briefly, paraffin-embedded liver tissues were cut into 4-μm-thick slices and deparaffinized with xylene. Then, antigen retrieval was performed in 3% hydrogen peroxide at room temperature for 15 minutes; sections were subsequently incubated with primary antibody (anti-Trop2 antibody, Cat. No. 90540; Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight (approximately 12 h). Sections were then washed with phosphate-buffered saline (PBS) and incubated with secondary antibody (Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 30 minutes at 37°C. Next, sections were incubated with streptavidin–biotin complex (Wuhan Boster Biological Technology, Ltd.) at 37°C for 30 minutes and then counterstained with hematoxylin. The reaction was visualized using diaminobenzidine (Wuhan Boster Biological Technology, Ltd.). Positive immunostaining was defined as the presence of brown or yellow granules in the cytoplasm or nucleus. PBS without primary antibody was used as the negative control staining condition. Five visual fields were randomly selected and assessed for immunoreactive areas at 200× magnification, using a Nikon computer image system (Nikon, Tokyo, Japan). The optical density of resulting images was analyzed by Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD, USA).

Western blotting assay

Protein samples (20 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, then transferred to a poly-vinylidene fluoride membrane (Millipore, Burlington, MA, USA) using the Bio-Rad II System (Bio-Rad, Hercules, CA, USA). Then, the membranes were blocked with 5% skim milk in Tris-buffered saline with Tween and incubated overnight (approximately 12 h) with rabbit monoclonal primary antibodies against Trop2 (Cat. No. 90540), E-cadherin (Cat. No. 3195), N-cadherin (Cat. No. 13116), vimentin (Cat. No. 5741), or β-actin (Cat. No. 4970) (1:1000 for each antibody; Cell Signaling Technology); goat anti-rabbit IgG (1:2000; Cat. No. BA1054; Wuhan Boster Biological Technology, Ltd.) was used as the secondary antibody (1 hour at room temperature). β-actin was used as inner loading control. Protein bands were detected by using an ECL chemiluminescence kit (Millipore). The protein bands were visualized by ChemiDocTMMP imaging system (Bio-Rad) and analyzed by Image-Pro Plus software.

qPCR

Total cellular RNA was extracted by Trizol reagent (Invitrogen) and reverse-transcribed to cDNA with the PrimeScript™ RT

reagent Kit (Takara Biotechnology Co., Ltd., Dalian, China). Next, qPCR was performed with the purified cDNA and SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd.) to detect the expression of Trop2 and β-actin mRNA in the samples. β-actin was used as the internal control; the data were analyzed by Bio-Rad CFX Manager software. Primer sequences (Sangon Biotech Co., Ltd, Shanghai, China) were as follows: *TROP2*, upstream primer: 5′-CCT CAT CGC CGT CAT CGT-3′, downstream primer: 5′-CGG TTC CTT TCT CAA CTC CC-3′; *β-ACTIN* upstream primer: 5′-CAC GAA ACT ACC TTC AAC TCC-3′, downstream primer: 5′-CAT ACT CCT GCT TGC TGA TC-3′.

Annexin-V/PI double-staining assay

HepG2 and HCCLM3 cells were transfected with the *TROP2*-siRNA sequence before collection for detection of apoptosis. The collected cells were resuspended in 100 μL binding buffer (1×10^5 cells) with 5 μL Annexin V-FITC and 5 μL PI (BD Pharmingen, Franklin Lakes, NJ, USA). Subsequently, cell apoptosis was detected by using a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) within 1 hour.

Cell proliferation assay

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay. Briefly, HepG2 and HCCLM3 cells were cultured in 10% CCK-8 diluted in fresh medium for 1 h, respectively. The absorbance value was measured at 450 nm on a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Transwell assay

Invasion assays were performed using Transwell chambers (Corning, Inc., Corning, NY, USA) in 24-well plates with 8.0-μm pore membranes. For invasion, 4×10^4 cells were transferred to the upper chambers in 1% serum. The lower chambers were filled with DMEM containing 10% serum. After incubation for 24 h, invaded cells on the lower surface of the Transwell were fixed with 95% alcohol and stained with crystal violet for 15 minutes. Subsequently, the invaded cells were counted under a light microscope. Before the experiment was performed, Transwell chambers were coated with Matrigel, in accordance with the manufacturer's protocol.

Wound healing assay

Cells were seeded into six-well plates at a density of 4×10^5 cells/well and grown to 70% confluence. The cell monolayer was scratched with a 200-μL pipette tip and washed with PBS three times. Subsequently, serum-free medium was used for continued incubation. Wound areas were imaged at 0 and 24 hours using an inverted microscope.

Statistical analysis

Statistical evaluation was performed by using SPSS 19.0 (SPSS, Inc., Chicago, IL, USA). Values are shown as the mean ± standard deviation (SD). Student's t-test was used to determine differences between two groups. One-way analysis of variance (ANOVA) was used to determine differences among three groups. Differences were considered statistically significant at $p < 0.05$.

Results

Expression of Trop2 was increased in HCC tissues

To investigate whether Trop2 expression is dysregulated in HCC, we assessed the endogenous levels of Trop2 in HCC tissues and

HCC cells by using immunohistochemical and western blotting assays, respectively. As shown in Figure 1a, the expression level of Trop2 protein in HCC tissues increased significantly, compared with that of adjacent tissues ($p < 0.01$). As shown in Figure 1b, the Trop2 protein expression levels in the HepG2 and HCCLM3 human hepatoma cell lines increased significantly, compared with that in the normal hepatocyte cell line HL7702 ($p < 0.01$ for both).

Transfection with TROP2-siRNA sequence inhibited expression of the TROP2 gene

HepG2 and HCCLM3 cells transfected with the *TROP2*-siRNA sequence showed inhibition of the expression of the *TROP2* gene. As shown in Figure 2a and b, the Trop2 mRNA and protein expression levels in the blank control (BC; no treatment) group of HepG2 cells showed no obvious differences compared with those in the negative control (NC; transfected with NC-siRNA sequence) group; in contrast, the Trop2 mRNA and protein expression levels in the *TROP2*-siRNA group were significantly lower than those in the BC group ($p < 0.01$ for both). As shown in Figure 2c and d, the Trop2 mRNA and protein expression levels in HCCLM3 cells were significantly lower after transfection with the *TROP2*-siRNA sequence ($p < 0.05$ and $p < 0.01$, respectively). These results indicated a high transfection efficiency in HepG2 and HCCLM3 cells.

Silencing of the TROP2 gene inhibited cell proliferation

To study the role of Trop2 in HCC development, HepG2 and HCCLM3 cells were transfected with the *TROP2*-siRNA sequence. As shown in Figure 3, the proliferation of HepG2 and HCCLM3 cells was significantly inhibited following transfection with the *TROP2*-siRNA sequence ($p < 0.01$ for both). These data demonstrated that silencing of the *TROP2* gene could effectively reduce cell proliferation in HepG2 and HCCLM3 cells.

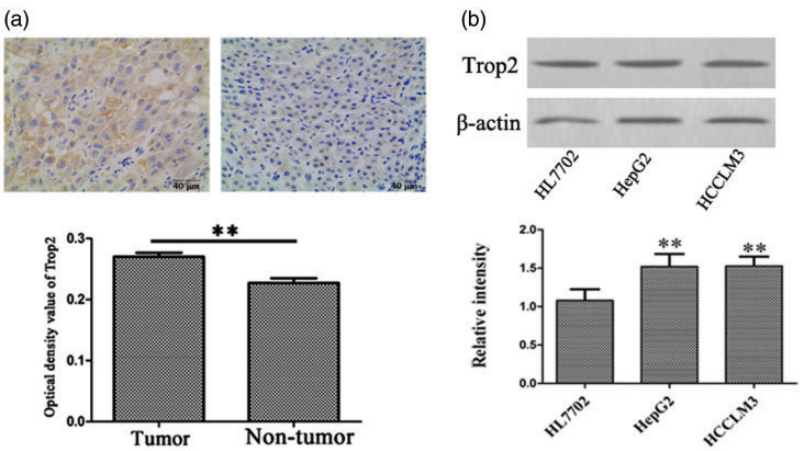

Figure 1. Increased Trop2 expression level in hepatocellular carcinoma (HCC)
(a) Immunohistochemical analysis was performed to detect the expression of Trop2 in HCC tissues and adjacent tissues; changes in the expression of Trop2 were statistically analyzed ($n = 10$). (b) Western blotting was performed to detect Trop2 protein expression levels in the HepG2 and HCCLM3 human hepatoma cell lines and the HL7702 normal hepatocyte cell line. The results are shown as mean ± standard deviation. $^{**}p < 0.01$.

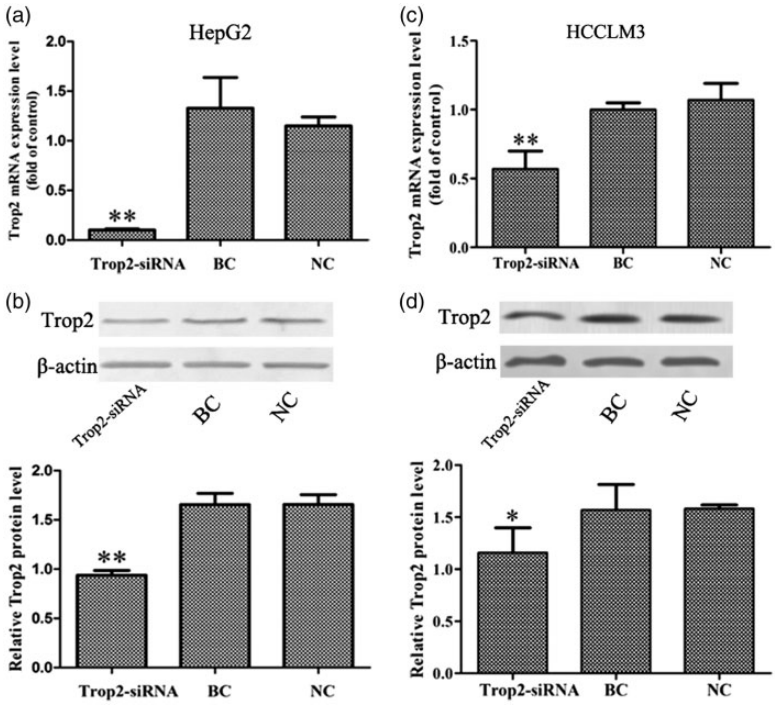

Figure 2. Expression of Trop2 is reduced following transfection with *TROP2*-siRNA sequence
(a) qPCR was performed to detect the mRNA level of Trop2 following transfection with *TROP2*-siRNA sequence (40 pmol/mL) for 24 hours in HepG2 cells. (b) Western blotting was performed to detect the protein level of Trop2 following transfection with *TROP2*-siRNA sequence (40 pmol/mL) for 24 hours in HepG2 cells. (c) qPCR was performed to detect the mRNA level of Trop2 following transfection with *TROP2*-siRNA sequence (40 pmol/mL) for 24 hours in HCCLM3 cells. (d) Western blotting was performed to detect the protein level of Trop2 following transfection with *TROP2*-siRNA sequence (40 pmol/mL) for 24 hours in HCCLM3 cells. The results are shown as mean ± standard deviation. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. BC group.

Silencing of the TROP2 gene promoted cell apoptosis

Flow cytometry results showed that the rate of apoptosis in HepG2 and HCCLM3 cells was significantly increased following transfection with the *TROP2*-siRNA sequence ($p < 0.01$ for both), whereas there was no obvious difference between BC and NC groups (Figure 4). These results indicated that silencing of the *TROP2* gene could effectively increase the rate of apoptosis in HepG2 and HCCLM3 cells.

Downregulation of TROP2 gene expression inhibited cell invasion

To further investigate the role of Trop2 in HCC migration, *TROP2*-siRNA was stably transduced into HepG2 and HCCLM3 cells. As shown in Figure 5a and c, Transwell assay analysis indicated that downregulation of *TROP2* gene expression could inhibit the migration of HepG2 and HCCLM3 cells ($p < 0.01$ for both). In addition, wound healing assay analysis showed that the downregulation of *TROP2* gene

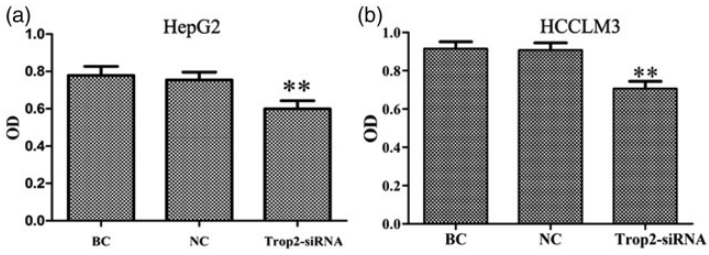

Figure 3. Downregulation of *TROP2* inhibits cell proliferation
(a) Cell viability was determined by CCK-8 assay following transfection with *TROP2*-siRNA sequence (40 pmol/mL) for 24 hours in HepG2 cells. (b) Cell viability was determined by CCK-8 assay following transfection with *TROP2*-siRNA sequence (40 pmol/mL) for 24 hours in HCCLM3 cells. The results are shown as mean ± standard deviation. $^{**}p < 0.01$ vs. BC group.

expression reduced the migratory speed of HepG2 and HCCLM3 cells (Figure 5b and d). These data suggested that Trop2 is involved in the migration of HepG2 and HCCLM3 cells.

Silencing of the TROP2 gene suppresses epithelial–mesenchymal transition

Epithelial–mesenchymal transition (EMT) is defined as the transformation of epithelial cells into spindle cells with the loss of membrane E-cadherin expression and the addition of mesenchymal markers such as vimentin, which promotes tumor initiation, progression, and metastasis.[13] We found that silencing of the *TROP2* gene increased E-cadherin expression, whereas it decreased vimentin and N-cadherin expression, in HepG2 and HCCLM3 cells (all $p < 0.05$ compared with BC group) (Figure 6).

Discussion

Invasion and metastasis are prominent features of HCC, which constitute the most important reasons for poor patient prognosis. The development of molecular targeted therapies has led to encouraging results in clinical trials over the past several years.[14,15] However, thus far, no specific targeted therapy has been developed for HCC. As an oncogene, *TROP2* expression is abnormal in many cancers; notably, *TROP2* influences cell apoptosis, invasion, and metastasis. Gu et al.[16] demonstrated that the *TROP2* gene was highly expressed in human osteosarcoma tissues and cell lines, and that the Trop2 protein could promote proliferation and migration of osteosarcoma cells through activation of the phosphoinositide-3-kinase/Akt signaling pathway. Zhao et al.[17] reported that high expression of the *TROP2* gene in gastric cancer was predictive of poor prognosis. In contrast, the loss of *TROP2* promoted carcinogenesis and EMT in squamous cell carcinoma.[18] In this study, we discovered that the Trop2 protein was highly expressed in HCC tissues and HCC cell lines. We speculated that Trop2 plays an important role in promoting carcinogenesis in HCC. Redlich et al.[19] clarified that anti-Trop2 blockade could enhance the therapeutic efficacy of ErbB3-targeted therapy in treatment of head and neck squamous cell carcinoma. Therefore, silencing of the *TROP2* gene may enable control of the development and progression of HCC.

RNA interference technology can reduce or eliminate the expression of specific genes, which may be an ideal strategy for malignant tumor therapy.[20] In this study, the expression of Trop2 mRNA and protein was effectively inhibited

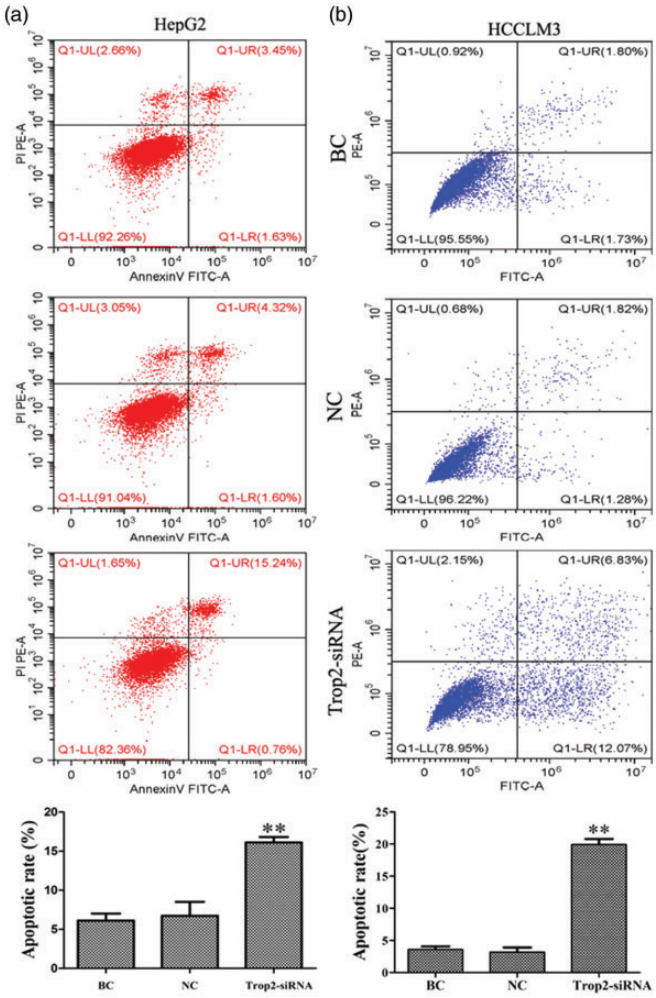

Figure 4. Downregulation of *TROP2* promotes cell apoptosis
(a) Flow cytometry was used to detect the rate of apoptosis in HepG2 cells following transfection with *TROP2*-siRNA sequence (40 pmol/mL) for 24 hours. (b) Flow cytometry was used to detect the rate of apoptosis in HCCLM3 cells following transfection with *TROP2*-siRNA sequence (40 pmol/mL) for 24 hours. The experiment was repeated three times; results are shown as mean ± standard deviation. $^{**}p < 0.01$ vs. BC group.

following transfection of the *TROP2*-siRNA sequence. This result indicated that we successfully constructed a *TROP2* gene-silencing cell model. A previous study showed that the upregulation of *TROP2* expression quantitatively promotes human tumor growth.[21] Wang et al.[22] reported inhibition of the *TROP2* gene suppresses the proliferation of laryngeal carcinoma cells via the mitogen-activated protein kinase pathway. Here, we showed that silencing of the *TROP2* gene could inhibit the proliferation of HepG2 and HCCLM3 cells. Apoptosis is one of the main pathways for cell death. Notably, our study also showed that the rate of

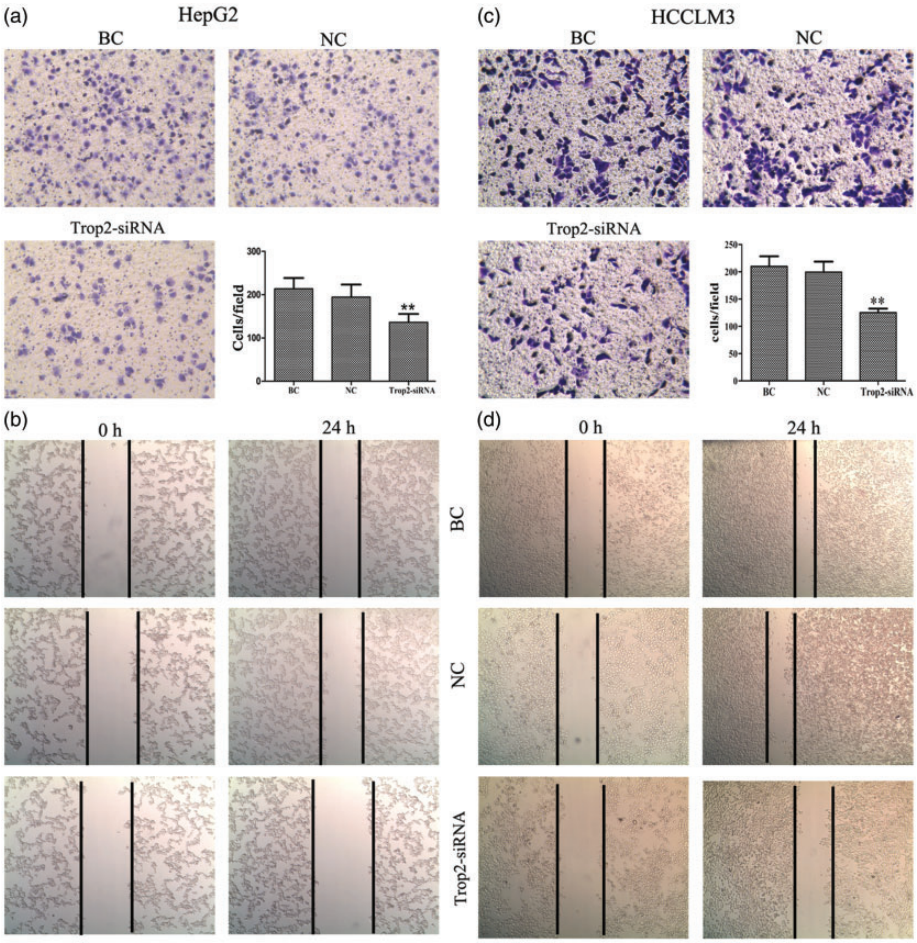

Figure 5. Knockdown of *TROP2* suppresses the migration of HepG2 and HCCLM3 cells
(a) Silencing of *TROP2* reduced the migratory capability of HepG2 cells. The histogram shows migrating cells per field. (b) Wound healing analyses of HepG2 cells. Scratches were created by a 200-μL pipette tip, and the wound areas were imaged at 0 and 24 hours with an inverted microscope. (c) The migratory capability of HCCLM3 cells was reduced following transfection with *TROP2*-siRNA sequence (40 pmol/mL). The histogram shows migrating cells per field. (d) Wound healing analyses of HCCLM3 cells. The experiment was repeated three times; results are shown as mean $\pm$ standard deviation. $^{**}p < 0.01$ vs. BC group.

apoptosis in HepG2 and HCCLM3 cells significantly increased after silencing of the *TROP2* gene.

Invasion and metastasis are signs of malignant tumors, and EMT plays an important role in this process. Multiple studies have assessed the pro-invasive role of the Trop2 protein in tumorigenesis. Gao et al.[23] demonstrated that siRNA targeting of Trop2 could suppress invasion by lung adenocarcinoma H460 cells; in contrast, siRNA targeting of Trop2 enhanced migration of liver fluke-associated cholangiocarcinoma.[24] Our data showed that the HCC invasion ability was inhibited following transfection of the *TROP2*-siRNA sequence. Trop2 has been identified as a potential biomarker for the promotion of EMT in human breast

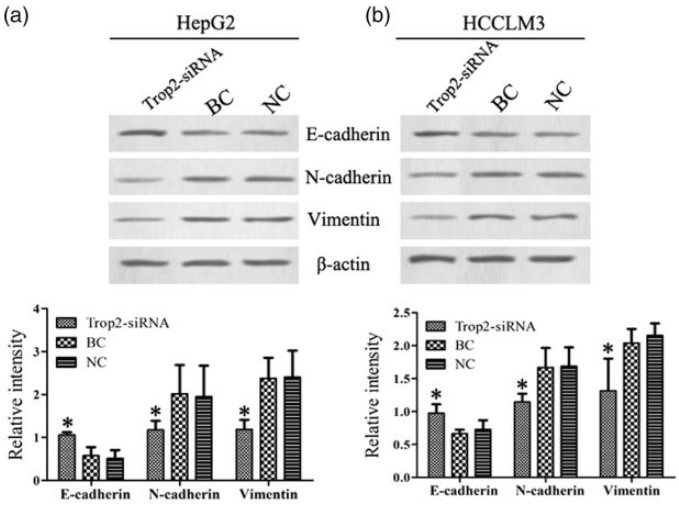

Figure 6. Knockdown of *TROP2* suppresses epithelial–mesenchymal transition
(a) Western blotting was performed to detect the protein levels of E-cadherin, vimentin, and N-cadherin following transfection with *TROP2*-siRNA sequence (40 pmol/mL) for 24 hours in HepG2 cells. (b) Western blotting was performed to detect the protein levels of E-cadherin, vimentin, and N-cadherin following transfection with *TROP2*-siRNA sequence (40 pmol/mL) for 24 hours in HCCLM3 cells. $^{*}p < 0.05$ vs. BC group.

cancer.[25] Similarly, in squamous cell carcinoma, the loss of *TROP2* promotes EMT.[18] In the present study, our results showed that the expression levels of EMT-related proteins, vimentin and N-cadherin, were significantly decreased following *TROP2* gene silencing; concurrently, the expression of E-cadherin was significantly increased. Therefore, the role of the Trop2 protein differs among various cancers. Histological classification and protein expression level should be the basis for selection of biomarkers. Additionally, the discovery of more promising therapeutic biomarkers is expected to increase the efficacy of cancer treatment.

In conclusion, we showed high expression of Trop2 mRNA and protein in HCC tissues and HCC cell lines; moreover, we demonstrated that silencing of the *TROP2* gene could inhibit cell proliferation and invasion, and could increase cell apoptosis. We also found that silencing of the *TROP2* gene suppressed EMT in HepG2 and HCCLM3 cells, potentially offering new molecular targets for treatment of HCC.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This work was supported by the grants 12A0043 from Research and development fund project of Nanchong.

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