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Original Article

TRIM66 knockdown inhibits cell growth, but induces cell cycle arrest and apoptosis of hepatocellular carcinoma cells

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Abstract: TRIM66, a member of tripartite motif (TRIM)-containing proteins, has been reported as an oncogene in osteosarcoma and lung cancer. However, the expression and biological function of TRIM66 in hepatocellular carcinoma (HCC) is still unclear. In this study, we found that mRNA and protein levels of TRIM66 were elevated in HCC tissues compared with normal tissues. Knockdown of TRIM66 inhibited the proliferation of HCC cells *in vitro* and the growth of xenograft tumors *in vivo*. Inhibition of TRIM66 in HCC cells significantly induced cell arrested in G0/G1 phase and cell apoptosis. Moreover, we tried to investigate the underlying mechanism. We found that TRIM66 knockdown affected the protein levels of cell cycle-related proteins (Cyclin D1 and p27) and cell apoptosis-related proteins (Bcl-2, Bax, Cleaved Caspase3). Furthermore, TRIM66 knockdown led to a significant decrease in the AKT phosphorylation. Our data indicates that TRIM66 could be a potential diagnosis marker for HCC and work as anoncogene by targeting AKT signaling.

Keywords: TRIM66, proliferation, cell cycle, apoptosis, AKT, HCC

Introduction

Hepatocellular carcinoma (HCC) has become a major health problem as it is one of the most common malignancies and the third leading cause of cancer-related deaths worldwide [1, 2]. There are more than 700,000 new cases diagnosed annually. More than 80% of HCC occur in developing countries, especially in Asia [3]. Many risk factors have been described, including hepatitis B or C viral infections, alcohol-related cirrhosis and non-alcoholic steatohepatitis [4-6]. The five-year survival of patients with HCC is extremely low because most cases are diagnosed at a late stage with severe metastases [7], which limits the curative efficacy of liver resection or transplantation. Therefore, elucidation of the molecular mechanisms underlying the HCC pathogenesis will facilitate the development of new diagnostic markers and effective therapy for this disease.

TRIM66, also known as transcription intermediary factor 1γ (TIF-1γ), belongs the family of tripartite motif (TRIM)-containing proteins, one of the subfamilies of the RING type E3 ubiquitin ligases [8]. TRIM family proteins are known to regulate a variety of biological processes, including cell growth, cell apoptosis, development, innate immunity and tumorigenesis [9]. Recent studies have revealed the abnormal expression of members of TRIM family protein in various cancers, such as gastric cancer (TRIM31 [10, 11]), prostate cancer (TRIM68 [12]), breast cancer (TRIM24 [13, 14], TRIM25 [15] and TRIM27 [16]) and chronic myelomonocytic leukaemia (TRIM33 [17]). It has been reported that TRIM66 is increased in osteosarcoma and lung cancer, and serves as oncogenes in both cancers [18, 19]. However, the expression and functions of TRIM66 in HCC still to be explored.

In the present work, expression of TRIM66 mRNA and protein was increased in HCC tissues compared with that in normal tissues. Knockdown of TRIM66 decreased HCC proliferation *in vitro* and *in vivo*, probably by blocking the cell cycle progression and inducing cell

apoptosis. The expression levels of cell cycle and cell apoptosis related protein were also investigated. Our study sought to elucidate the regulatory mechanism of TRIM66 in the development of HCC and provide new insight in exploring molecular targets for the treatment of this disease.

Materials and methods

Patients and tissue samples

HCC tissues and paired noncancerous tissues were collected from 30 patients with HCC admitted to Seventh People's Hospital (Shanghai, China) from January 2012 to January 2013. The study was approved by the independent ethics committee, Seventh People's Hospital. Written informed consent was obtained from all patients according to the ethics committee guidelines.

Cell culture

All cell lines were obtained from cell bank of Shanghai biology institute, Chinese Academy of Science (Shanghai, China) and maintained in a humidified atmosphere containing 5% CO_2 at 37°C. HEK293T, SK-Hep-1, MHCC-97L, MHCC-97H, BEL-7404 and HepG2 cells were cultured in Dulbecco's modified Eagles medium (DMEM). The SMMC-7721 cells were cultured in RPMI1640. All media (Invitrogen, Carlsbad, CA, USA) were supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) 100 mg/ml of penicillin-streptomycin.

Western blotting

The tissue samples or cultured cells were lysed in RIPA buffer (Beyotime, Shanghai, China). Amounts of extracted proteins were measured using the BCA method (Thermo Fisher Scientific, Rockford, IL, USA) and kept at -80°C until Western blotting analysis. Equal amount of proteins (25 μg) were resolved by 10% SDS-PAGE and transferred to NC membrane (Millipore, Bredford, MA, USA). After blocked with 5% skim milk for 1 h at room temperature, membrane was probed with the following primary antibodies: anti-TRIM66 and anti-Cleaved Caspase3 (Abcam; Cambridge, MA, USA); anti-Bcl-2 and Bax (Santa Cruz Biotech.; Santa Cruz, CA, USA); and anti-CyclinD1, anti-p27, anti-AKT and p-AKT, 1:15000 GAPDH (Cell Signaling Technology; Danvers, MA, USA) at 4°C overnight. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Beyotime) and visualized with enhanced chemiluminescence detection system (Millipore).

Immunohistochemical staining (IHC)

IHC staining was performed on paraffin tissue sections (5 μm) of HCC and noncancerous specimens as previously described [18].

RNA isolation, cDNA preparation, and real-time quantitative PCR analysis

Total RNA was extracted from tissue samples or cells with TRIZOL according to the manufacturer's protocols (Invitrogen). One microgram of total RNA was used for reverse transcription reaction using MuLV reverse transcriptase (Promega, Madison, WI, USA) with a random hexamer. Gene expression was quantified by real-timequantitative PCR with SYBR Green PCR mix (Invitrogen) on an ABI Prism 7300 RT-PCR System (Applied Biosystems, Foster City, CA, USA). GAPDH was served as an internal control. Real-time PCR was performed in triplicate for each sample. The following primer sequences were used: 5'-GCCCTCTGTGCTACTTACTC-3' (forward) and 5'-GCTGGTTGTGGGTTACTCTC-3' (reverse) for TRIM66; 5'-CACCCACTCCTCCACCTTTG-3' (forward) and 5'-CCACCACCCTGTTGCTGTAG-3' (reverse) for GAPDH.

Lentivirus-mediated shRNA (small hairpin RNA) knockdown

shRNAs targeting TRIM66 and a scrambled shRNA were cloned into a lentiviral vector (PLKO.1, Addgene, Cambridge, MA, USA). To produce the lentiviruses, lentiviral plasmids were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen) together with the lentiviral helper plasmids, psPAX2 and pMD2G (Addgene). Viruses were collected at 48 h after transfection and used to infect MHCC-97H and SK-Hep-1 cells. After 48 h, the knockdown efficiency of lentiviral shRNA in cells was then determined by real-time PCR analysis. The most efficient shRNA sequences were as follows: shTRIM66, CCGGGAGCATCAGAGCCTTCAATCTCGAGGAATACCTCATCTTTCCTCTTTTTTTC; scrambled shRNA, CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTTC. MHCC-97H stable cell

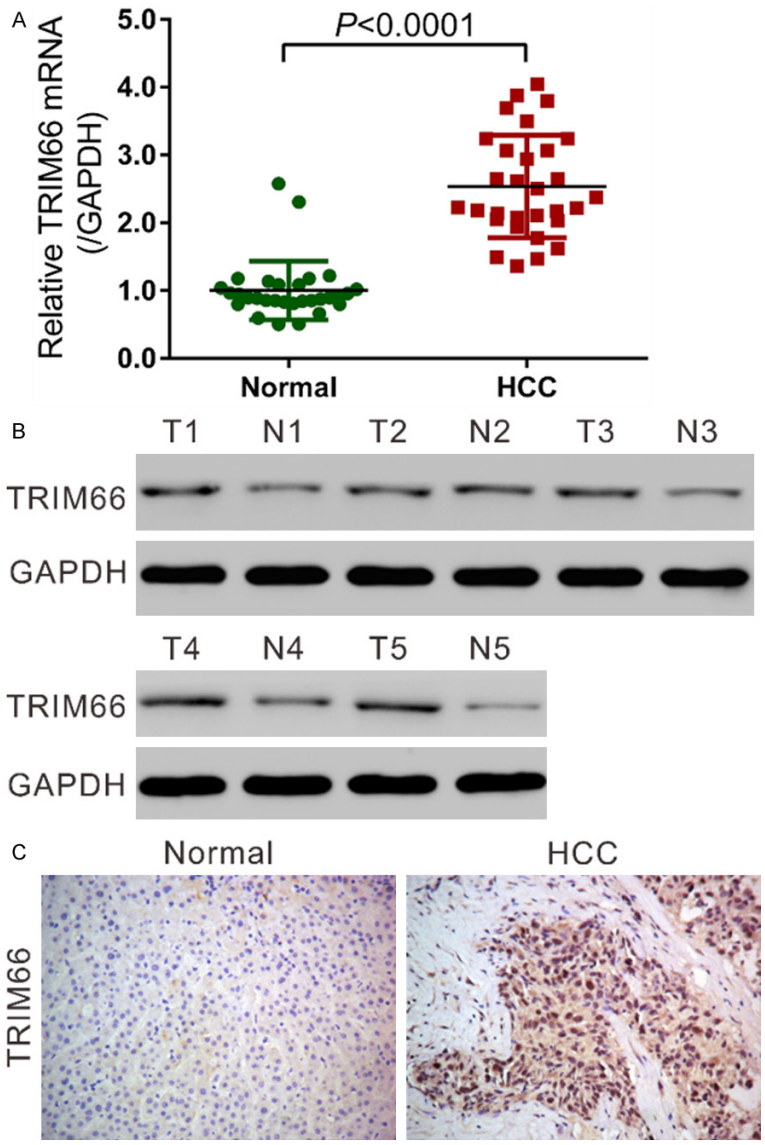


Figure 1. TRIM66 is overexpressed in HCC tissues. A. A total of 30 pairs of HCC and noncancerous tissues were collected from January 2012 to January 2013. Total RNA was extracted from tissues samples and real-time PCR was performed to analyzed TRIM66 mRNA expression. GAPDH was served as internal control. B. TRIM66 protein expression in HCC (T1, T2, T3, T4 and T5) and noncancerous tissues (N1, N2, N3, N4 and N5). C. TRIM66 expression was evaluated by IHC in HCC and noncancerous tissues.

line was constructed by puromycin (Sigma, St. Louis, MO, USA) selection.

Cell proliferation assay

Cell proliferation was assessed by CCK-8 Assay (Dojindo Lab, Kumamoto, Japan) according to manufacturer's protocol. Briefly, cells were seeded onto 96-well plates at a density of 5.0×10^3 per well and cultured overnight. Cells were then infected with TRIM66 shRNA or control shRNA lentivirus. At 0 h, 24 h, 48 h and 72 h after viral infection, 100 μl 10% CCK8 solution was added to each well. The plates were incubated at 37°C for 1 h. The optical density (OD) was determined at 450 nm with microplate reader microplate reader (BioRad, Richmond, CA, USA).

In vivo tumor growth assay

For subcutaneous implantation, 10 four-week old male BALB/c nude mice (Shanghai Experimental Animal Center, Shanghai, China) were randomly divided into a TRIM66 knockdown group and a control group with five mice per group. A total of 2.0×10^6 MHCC-97H cells stably infected with TRIM66 shRNA or control shRNA were injected into the right flank of nude mice. Tumor length and width were measured with calipers once every three days. Tumor volume was calculated based on the formula: Volume = (width^2×length)/2. At 45 days after implantation, the mice were euthanized, and tumors were weighed. All animal experiments were approved by the Institutional Animal Care and Use Committees at Seventh People's Hospital (Shanghai, China).

Cell cycle and apoptosis analysis

Cells were seeded onto 6-well plates at a density of 5.0×10^5 per well. After cultured overnight, cells were infected with TRIM66 shRNA or control shRNA lentivirus. After another 48 h, cells were collected and subjected to cell cycle and apoptosis analysis using flow cytometry.

The cell cycle distributions were analyzed by propidium iodide (PI) staining. Collected cells were washed with ice-cold PBS and fixed with ice-cold 75% ethanol at -20°C. The fixed cells

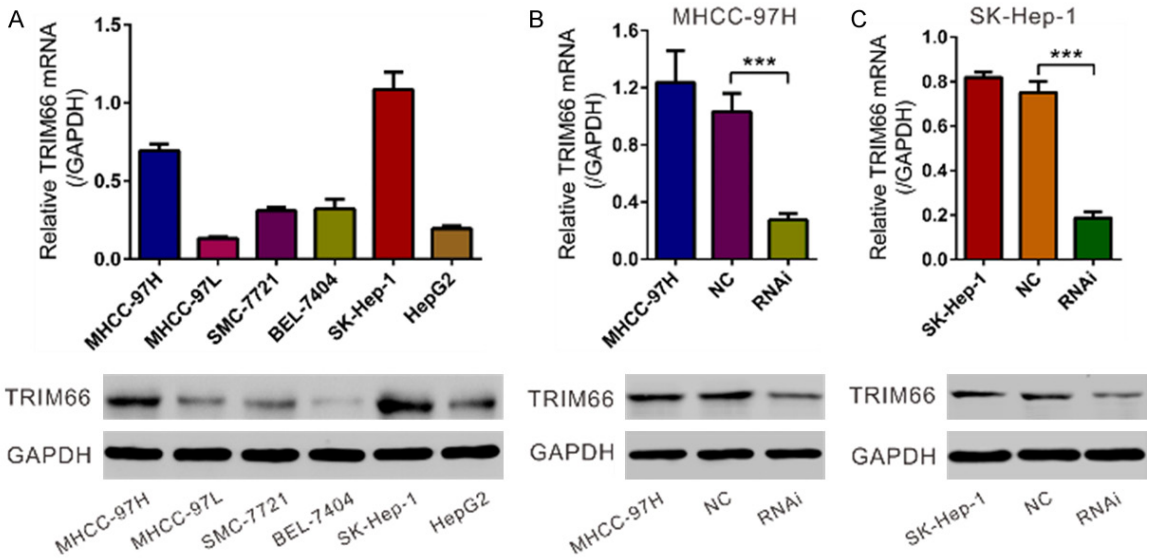


Figure 2. Lentivirus mediated RNAi down regulated TRIM66 expression in HCC cells. A. TRIM66 mRNA and protein levels in 6 HCC cell lines were analyzed by real-time PCR (upper panel) and Western blotting (lower panel), respectively. B, C. MHCC-97H cells and SK-Hep-1 cells were infected with TRIM66 shRNA (RNAi) or scrambled shRNA (NC) virus. Expression of TRIM66 in both cell lines was analyzed by real-time PCR (upper panel) and Western blotting (lower panel) at 48 h after viral infection. ***P<0.001.

were washed with PBS and incubated with 100 μg/mL PI and 10 μg/mL RNase A for 30 min at 37°C. DNA content was analyzed on a FACSCalibur system (BD Biosciences, San Jose, CA, USA).

For cell apoptosis analysis, collected cells were washed twice with PBS and incubated with APC-conjugated Annexin V and PI (eBioscience, San Diego, CA, USA) for 15 min. Cellular fluorescence was measured by FACSCalibur system (BD Biosciences).

Gene set enrichment analysis (GSEA)

Gene expression data for the LIHC (liver hepatocellular carcinoma) projects were obtained at The Cancer Genome Atlas website (TCGA, https://tcga-data.nci.nih.gov/tcga/). GSEA was carried out to analyze pathways associated with TRIM66 expression as previously described [18].

Statistical analysis

Data were represented as the means ± standard deviations (SD) from at least three independent experiments. All statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad, San Diego, CA, USA). Student's t test was used to estimate statistical significance values. Differences were considered to be statistically significant for values of P<0.05.

Results

TRIM66 was overexpressed in human HCC

By real-time PCR carried out in 30 HCC and matched adjacent non-tumorous liver tissues, we found that TRIM66 mRNA expression was significantly higher in HCC tissues than in non-tumorous liver tissues (P<0.0001, **Figure 1A**). The overexpression of TRIM66 was further confirmed by Western blotting (**Figure 1B**) and immunohistochemical staining (**Figure 1C**) at protein level.

Lentivirus mediated RNAi down regulated TRIM66 expression in HCC cells

To investigate the effect of TRIM66 on HCC, TRIM66 was knocked down in HCC cells using lentivirus mediated RNAi. The TRIM66 shRNA (RNAi) or scrambled shRNA (NC) were infected into MHCC-97H and SK-Hep-1 cells, which had relative higher TRIM66 expression (**Figure 2A**). After 48 h, the effect of shRNA on the endogenous expression of TRIM66 was determined by real-time PCR and Western blotting

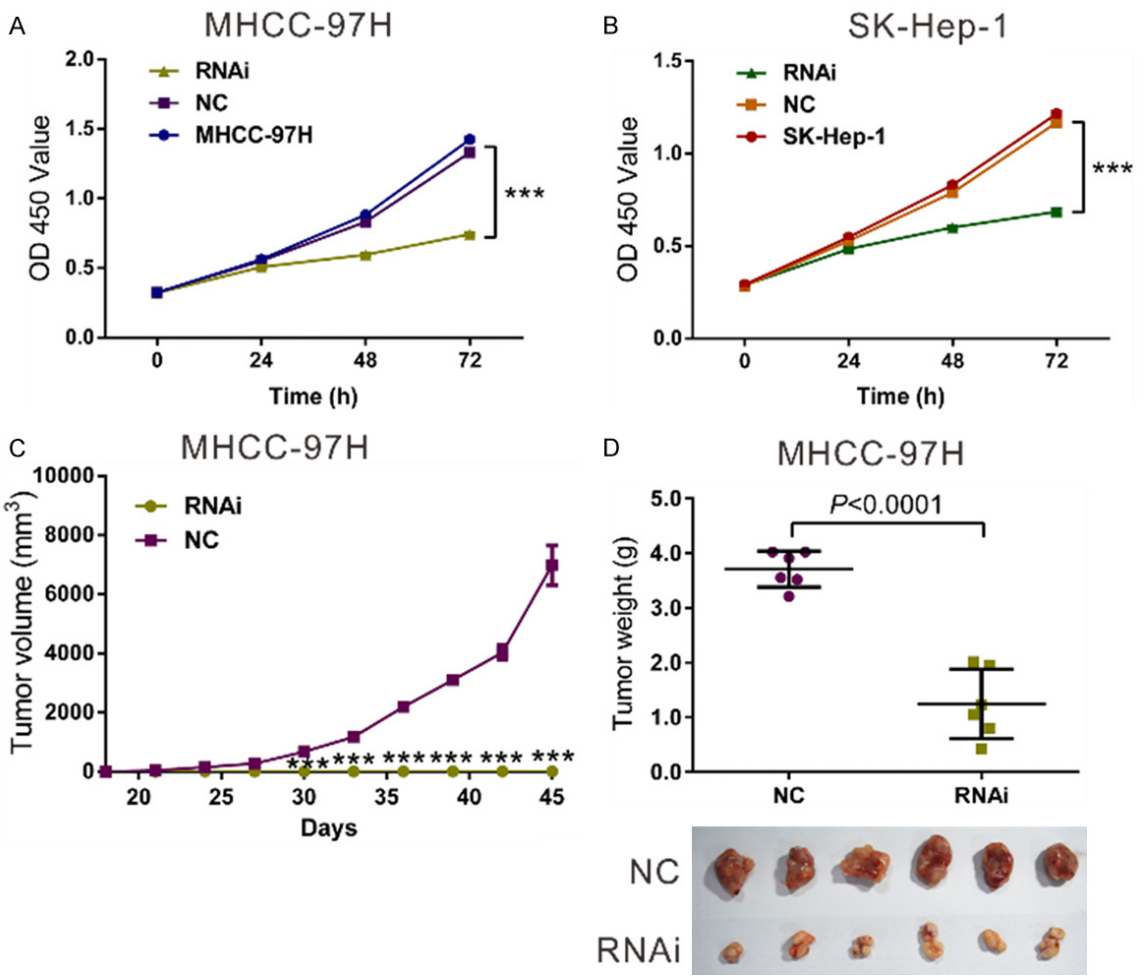


Figure 3. Effect of TRIM66 knockdown on HCC cell proliferation *in vitro* and *in vivo*. (A, B) Cell proliferation of MHCC-97H (A) and SK-Hep-1 (B) cells infected with TRIM66 shRNA (RNAi) or scrambled shRNA (NC) virus was detected by CCK-8. (C, D) MHCC-97H cells stably transfected with TRIM66 shRNA (RNAi) or scrambled shRNA (NC) were subcutaneously injected in nude mice (n=6 per group). Tumor volume was evaluated for 45 days (C). At day 45 after implantation, mice were sacrificed, and tumors were imaged and weighted. ***P<0.001.

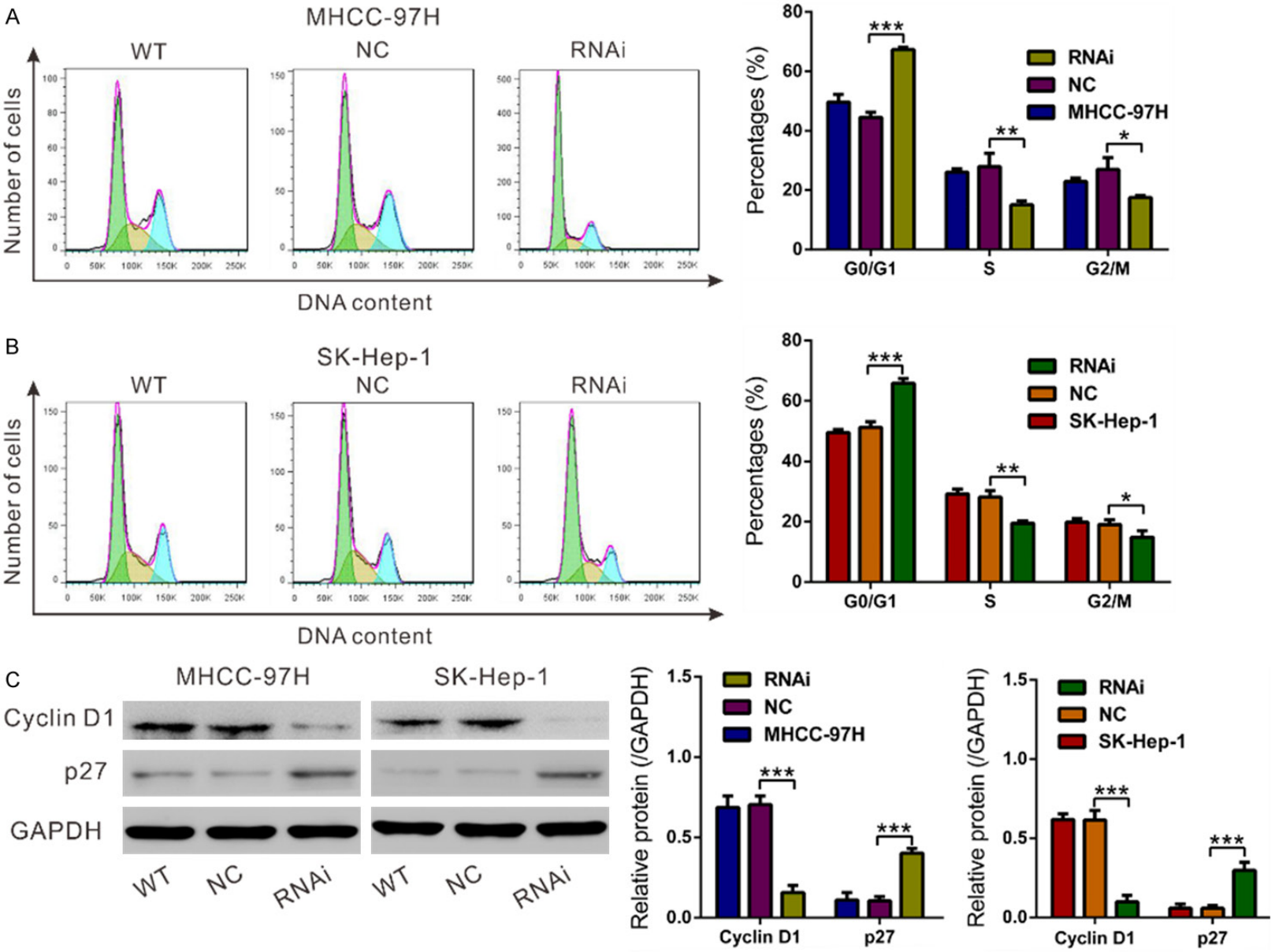
analysis. As shown in **Figure 2B** and **2C**, TRIM66 expression was effectively knocked down by TRIM66 shRNA (RNAi) infection in both MHCC-97H and SK-Hep-1 cells with a silencing efficiency of approximately 70% (P<0.001).

Inhibiting TRIM66 suppressed the proliferation of HCC cells in vitro

The effect of TRIM66 knockdown on HCC cell proliferation was analyzed by CCK-8 assay. As shown in **Figure 3A** and **3B**, there was no significant difference between wild-type and scrambled shRNA (NC) viral infected cells. The proliferation of MHCC-97H and SK-Hep-1 cells infected with TRIM66 shRNA lentivirus was time-dependently decreased compared with control cells (wild-type and NC). The significant inhibitory effects were observed from 48 h. At 72 h after viral infection, TRIM66 knockdown led to a 44.2% and 38.7% reduction in proliferation rate of MHCC-97H and SK-Hep-1 cells, respectively (P<0.001). These data indicate the role of TRIM66 on the *in vitro* proliferation of HCC cells.

TRIM66 knockdown inhibited HCC cell growth in nude mice xenograft model

To determine whether knockdown of TRIM66 could reduce tumor growth *in vivo*, MHCC-97H cells stable transfected with scramble shRNA control (NC) or TRIM66 shRNA (RNAi) were subcutaneously injected in nude mice. As shown in


A
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MHCC-97H
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Cyclin D1
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RNAi
Relative protein (/GAPDH)
RNAi
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MHCC-97H
Cyclin D1
p27
Relative protein (/GAPDH)
RNAi
NC
SK-Hep-1
Cyclin D1
p27

Figure 4. TRIM66 silencing induced G0/G1 phase cell cycle arrest. (A, B) Flow cytometry analysis of cell cycle progression in MHCC-97H (A) and SK-Hep-1 (B) cells after TRIM66 shRNA (RNAi) or scrambled shRNA (NC) virus infection. (C) Western blotting showed that TRIM66 silencing influenced the expression of cell cycle-related proteins (Cyclin D1 and p27). **P*<0.05, ***P*<0.01 and ****P*<0.001.

Figure 3C, the tumors formed from RNAi cells grew significantly slower than those from NC cells in nude mice (*P*<0.001). After 45 days, the weight of tumors with TRIM66 silence was 33.5% of that of control tumors (*P*<0.0001, **Figure 3D**). These results suggest TRIM66 knockdown suppresses HCC cell growth *in vivo*.

Suppressing TRIM66 expression induced cell cycle arrest of HCC cells

The effect of TRIM66 on the cell cycle progression was analyzed by PI staining and flow cytometry. As shown in **Figure 4A**, the mean percentage of TRIM66-silenced MHCC-97H cells in the G0/G1 phase was significantly increased (*P*<0.001), but notably decreased in both S (*P*<0.01) and G2/M phases (*P*<0.05) compared with control cells. Similarly, G0/G1 phase arrest was observed in TRIM66-silenced SK-Hep-1 cells (**Figure 4B**).

We then detected the expression of cell cycle-related proteins. We found that the Cyclin D1 (a factor required for transition from G0/G1 to S phase of cell cycle [20]) expression were markedly decreased, while p27 (an inhibitory factor for cell cycle progression of mammalian cells [21]) expression was notably increased in TRIM66-silenced cells compared with matched controls (**Figure 4C**).

Inhibiting TRIM66 induced cell apoptosis of HCC cells

Cell apoptosis was also estimated using flow cytometry with Annexin V-APC and PI staining. The percentage of MHCC-97H cells undergoing early apoptosis was significantly increased in TRIM66-sliienced cells (28.97% ± 1.81%) compared with wild-type (3.67% ± 0.64%) and NC cells (4.03% ± 0.60%; **Figure 5A**, *P*<0.001). Similarly, increased cell apoptosis was observed in TRIM66-silenced SK-Hep-1 cells (**Figure 5B**).

Western blot analysis was then done to detect the alteration of apoptosis-related proteins. We found that lentivirus-mediated shRNA targeting TRIM66 in both HCC cell lines markedly decreased the protein level of Bcl-2 (an apoptosis-inhibitory factor [22]), while notably increased the protein level of Bax (an apoptosis-promoting protein [22]) and Cleaved Caspase3 (an apoptosis marker [23]; **Figure 5C**). Down-regulation of TRIM66 might induce apoptosis in HCC cells through the reduction of Bcl-2 protein expression and the increase of Bax expression.

TRIM66 and PI3K/AKT signaling

To explore TRIM66 associated pathways, we performed GSEA analysis on TCGA LIHC cohort, and we found that PI3K/AKT pathway is strongly associated with TRIM66 higher expression (**Figure 6A**). The inhibitory effect of TRIM66 knockdown on AKT phosphorylation was assessed by Western blotting analysis (**Figure 6B**).

Discussion

TRIM family proteins contain a RING domain, one or two B-boxes and a coiled-coil region and play critical roles in a broad spectrum of biological processes [9]. Altered expression of TRIM family proteins has been linked to several cancers [10-18]. TRIM66, a member of TRIM family protein, was reported overexpressed in osteosarcoma and could be used predict the prognosis of patients with osteosarcoma [18]. Here, we demonstrated that TRIM66 was overexpressed in HCC tissues as compared to normal tissues (**Figure 1**). Our data suggested the possible clinical value of TRIM66 in the diagnosis of HCC although further investigations with larger number of cases and more detailed clinical data are needed.

Then we investigated the functions of TRIM66 in HCC cells by inhibiting its expression with lentivirus mediated RNAi (**Figures 2**-**5**). Our results indicate that TRIM66 knockdown significantly inhibited HCC cell growth *in vitro* and *in vivo* through inducing cell cycle arrest and cell apoptosis. These data matched well with a previous study on osteosarcoma cells [18], suggesting that TRIM66 is a potential oncogene regardless of tissue types.

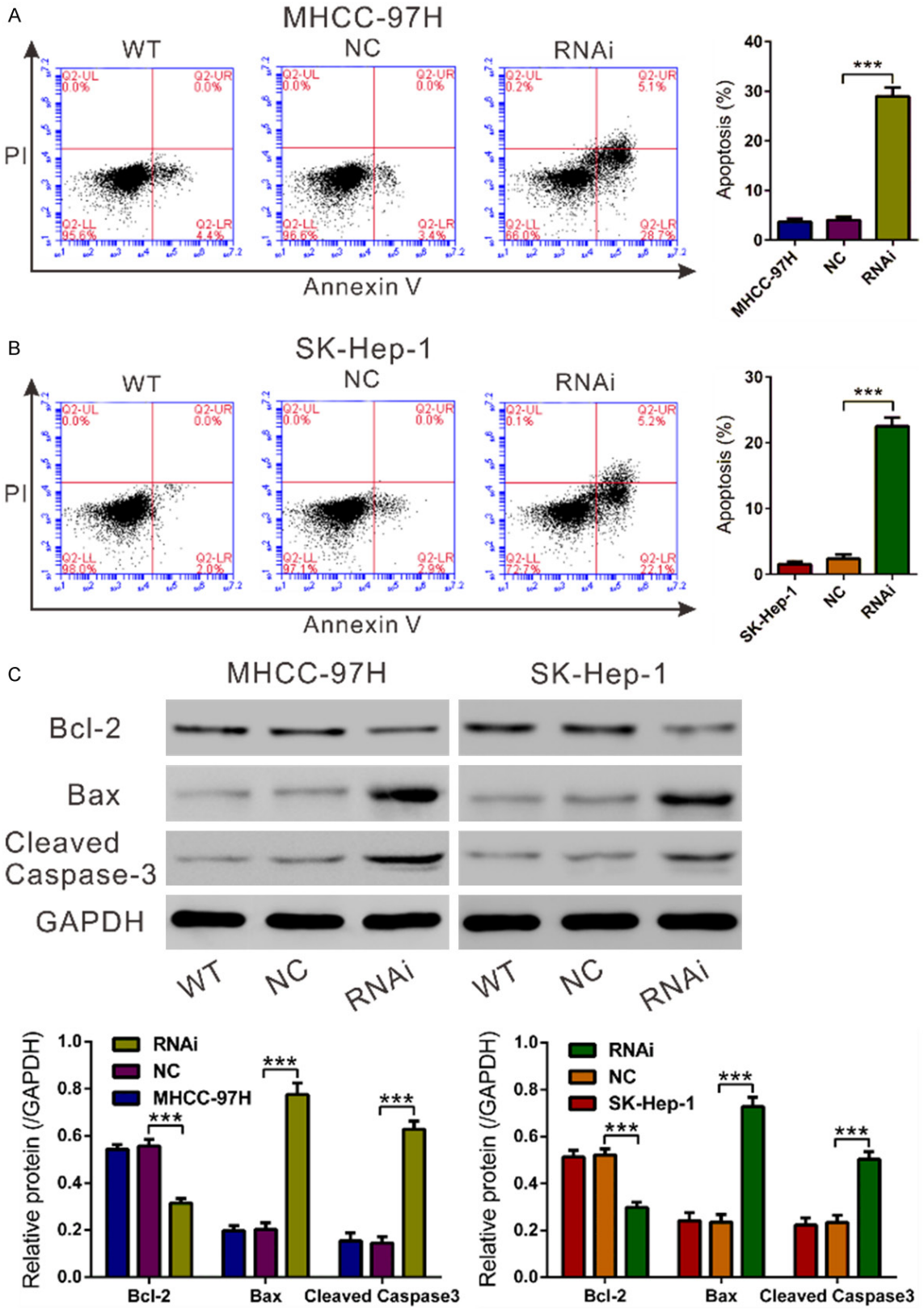

Figure 5. Inhibiting TRIM66 induced cell apoptosis of HCC cells. (A, B) Flow cytometry analysis of cell apoptosis in MHCC-97H (A) and SK-Hep-1 (B) cells after TRIM66 shRNA (RNAi) or scrambled shRNA (NC) virus infection. (C) Western blotting showed that TRIM66 silencing affected the expression of apoptosis-related proteins, including Bcl-2, Bax and Cleaved Caspase3. ***P<0.001.

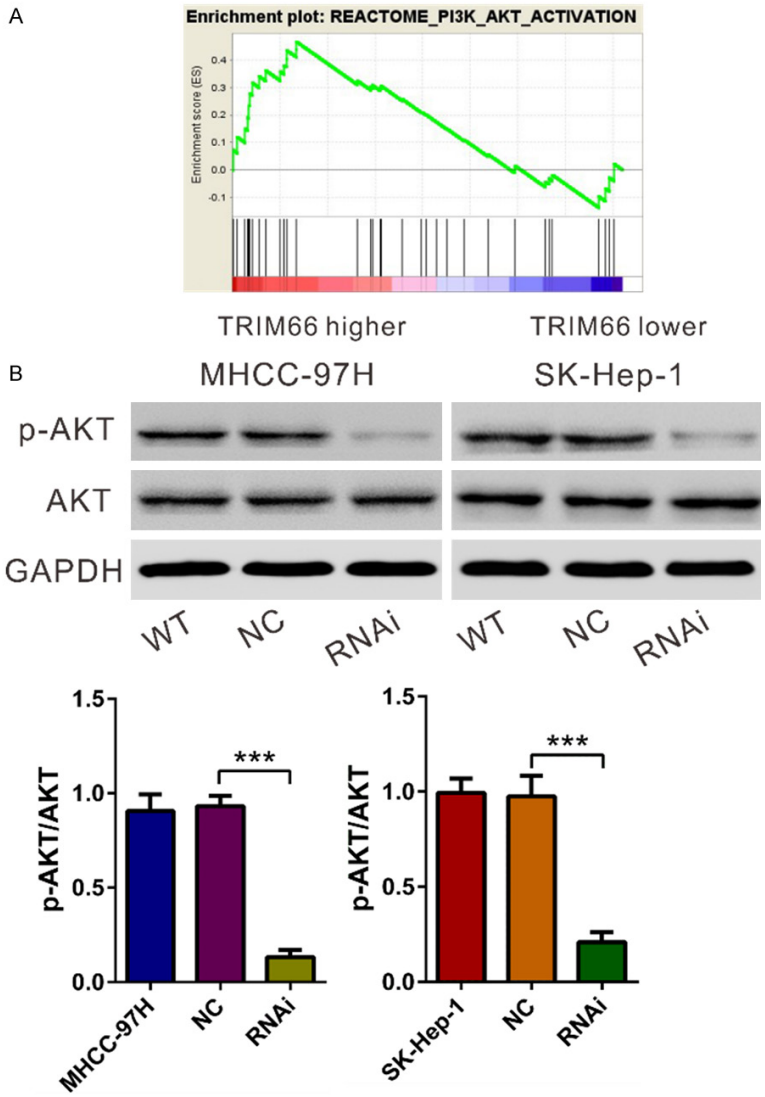

Figure 6. TRIM66 and PI3K/AKT signaling. A. Gene Set Enrichment Analysis (GSEA) identified that the PI3K/AKT signaling pathway was significant associated with TRIM66 expression. B. Western blotting showed that TRIM66 silencing significantly reduced AKT phosphorylation. ***P<0.001.

We further investigated the possible molecular mechanism that TRIM66 involved in cell cycle and cell apoptosis regulation. Cylcin D1 is a well-known factor for cell cycle transition from G0/G1 to S phase [20], while p27 exerted inhibitory effect for cell cycle progression [21]. In the current study, we observed a significant decrease of AKT phosphorylation (**Figure 6**) and Cyclin D1 expression, and a remarkable increase of p27 expression (**Figure 4C**) in TRIM66-silenced HCC cells. GSEA is a powerful method to identify the possible pathways associated with genes expression [24]. Our GSEA results of TCGA LIHC cohort showed that the PI3/AKT pathway was strongly associated with TRIM66 expression (**Figure 6A**), which was further validated by Western blotting analysis in TRIM66-silenced HCC cells. It is well known that the PI3/AKT pathway plays a key role in G1/S cell cycle progression via increasing Cyclin D1 expression and decreasing p27 expression [25]. Our data indicated that TRIM66 may promote HCC cell growth via regulating AKT signaling pathway. Moreover, the Bcl-2 family proteins are critical regulators of mitochondrial pathway of apoptosis [22]. In the mitochondrial pathway of apoptosis, pro-Caspase3 is cleaved, which is an apoptosis marker [23]. Here, TRIM66 knockdown in HCC cells significantly decreased Bcl-2 expression, but increased Bax and Cleaved Caspase3 expression (**Figure 5C**). Our data suggested that TRIM66 may regulate mitochondrial pathway of apoptosis in HCC cells.

In conclusion, our findings indicate that high expression of TRIM66 in HCC may promote cell proliferation via promoting cell cycle progression and inhibiting cell apoptosis, thus contributing to the development of HCC. TRIM66 might regulate these biological progresses through AKT signaling. Our study provides a theoretical basis and new insights into the treatment of HCC.

Acknowledgements

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Disclosure of conflict of interest

None.

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