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ORIGINAL ARTICLE

Investigating the role of transforming growth factor-beta/transforming growth factor-beta receptor in the development of hepatocellular carcinoma through the Salvador-Warts-Hippo pathway

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ABSTRACT

A key component of the Hippo signaling pathway (HSP) is a kinase cascade that connects the tumor suppressor Hippo to the oncoprotein Yki (YAP and TAZ). This oncoprotein functions as a transcriptional coactivator for target genes that play critical roles in cell proliferation and survival. The objective of this study is to evaluate the interaction between TGF-β proteins and key components of the Hippo signaling pathway in regulating hepatocellular carcinoma (HCC) cell behavior**.** One important achievement of this study was to reveal the significant inhibitory role of the HSP in the growth of HCC cells through TGF-β proteins. In the present study, we examined the expression of key proteins of the Hippo pathway in HCC cells treated with TGF-β proteins and their correlation with the Hippo signaling pathway (HSP) by immunofluorescent staining, immunoblotting method, and Real-time PCR. Both Yes- associated protein (YAP) and large tumor suppressor 1 (LATS1) were correlated with HCC cells. In the HSP, LATS1 plays as an upstream inhibitory agent of YAP. Nucleus-cytoplasm translocation of YAP1 and overexpression of LATS1 occurred in HCC cells treated with TGF-β. The nucleocytoplasmic distribution of YAP1 and overexpression of LATS1 have anti-oncogenetic roles in the incidence and progression of HCC. TGF-β in 5 ng/mL treatment resulted in a 2.5-fold increase in LATS1 expression and significant YAP1 translocation from the nucleus to the cytoplasm. We concluded that the Hippo signaling pathway mediates TGF-β/TBR-induced effects on HCC progression.

Keywords: Transforming growth factor-beta (TGF-β), Hepatocellular carcinoma (HCC), Salvador-Warts-Hippo (SWH) pathway, Hippo signaling pathway (HSP)

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Introduction

As the fifth most prevalent malignancy and the second most important cause of mortality from cancer, Hepatocellular Carcinoma (HCC) has been regarded as a major medical issue worldwide. The global pattern of HCC distribution is highly heterogeneous, which may be because of various risk factors associated with this disease, the most important of which is liver cirrhosis. In the presence of liver cirrhosis, HCC recurrence is possible even after a successful remedy. Four categories of risk factors can be identified: Toxic (aflatoxins and alcohol), Immune-related (primary biliary cirrhosis and autoimmune hepatitis), Metabolic (non-alcoholic fatty liver disease, diabetes, and hereditary hemochromatosis), and Viral (chronic hepatitis B and C) (1, 2). Thus, HCC progression can be determined after successfully eliminating causative factors of molecular and structural changes (3).

Chronic liver damage activates mesenchymal cells that are responsible for producing TGF-β which is one of the most dominant hepatic profibrogenic cytokines (4). There are also several studies reported that TGF-β plays a multifunctional role and applies biological impacts on the development of organs and tissue, cellular differentiation, apoptosis, proliferation, and survival (5). It has been hypothesized that TGF-β serves as an important connection between cirrhosis, chronic injury, and HCC in the liver (6). Numerous evidence has demonstrated the modulatory role of TGF-β in the expression of several genes associated with tumor development (7).

Moreover, its central role has been well-established in the epithelial-mesenchymal transition, an important cellular phenomenon throughout tumor metastasis. HCCs are common in cirrhotic livers where the TGF-β is highly expressed, indicating the possible role of TGF-β in HCC inception. Although TGF-β seems to actively involve in the occurrence of HCC in cirrhosis patients, the underlying mechanism is less known (8).

However, the development of a treatment standard for this malignant disease has been hampered due to its clinical heterogeneity and the absence of a molecular classification system. Today, surgery remains the main treatment for HCC so liver transplant and liver resection lead to a 5-year survival rate of almost 85%. However, these findings are reported in specific patients, e.g. cirrhotic or non-cirrhotic patients who have a well-preserved liver function without significant signs of clinically portal hypertension (9). TGF-β plays a critical role in various cellular processes, including differentiation, migration, proliferation, homeostasis, and apoptosis. One of the most significant roles of TGF-β is its modulation and suppression of immune responses, which is a key factor in HCC initiation and metastasis. TGF-β signaling cascade includes transmembrane serine/threonine kinase receptors of type I & II (TBRI & TBRII). Smads (intracellular signaling proteins) mediate cellular responses (10). Vertebrates have at least 9 Smads that are functionally divided into 3 groups of (i) receptor-activated Smads (R-Smads): Smad1, 2, 3, 5, and 8; (ii) inhibitory Smads: Smad6 and 7; and (iii) co-mediator Smads: Smad4 and 10 (11). Salvador-Warts-Hippo (SWH) or Hippo is a signaling pathway responsible for controlling the size of animal organs via regulating the apoptosis and proliferation of cells. The hippo pathway focuses on the significant overgrowth of tissue as the result of losing the Warts gene (12). Similarly, Mob, Hippo, and Salvador are tumor suppressors with the same consequences on tissue development, resulting in biochemical and functional characterization of the SWH pathway (13, 14). The evolutionarily-conserved Hippo pathway regulates yes-association protein (YAP) and its paralogue TAZ (transcriptional coactivator with PDZ-binding motif). This pathway consists of two kinases, including mammalian sterile 20-like kinase 1 and 2 (MST1/2) and large tumor suppressor homolog 1 and 2 (LATS1/2) (15). Using Salvador as a cofactor, cell-cell interaction phosphorylates LATS1/2 by activating MST1/2. Subsequently, the activation of LATS1/2 (in combination with Mob1) leads to the phosphorylation of YAP at S127 and TAZ at S89, causing proteasomal degradation of YAP/TAZ and cytoplasmic sequestering by 14-3-3 proteins (16). The HSP is also inactivated in cancer cells allowing gene modulation,while via nuclear YAP/TAZ for metastasis. Moreover, HCC cell growth and survival could be inhibited by a cyclic YAP peptide that disturbs the interaction between YAP and TEAD (17, 18). Transcriptional activity of Yki/YAP/TAZ is inhibited by Warts kinase through protein degradation, cytoplasmic retention, and nuclear export (19). There are also regulations independent of phosphorylation. Yki (TAZ and YAP within the body of mammals) can bind directly to Warts, Hippo, and the FERM-domain,

which contains an adapter protein expanded (Ex) that sequesters Yki in the cytoplasm (20). The elimination of improper differentiating neurons during development has been shown to require SWH pathway activation through promoting expanded (ex) levels (21). This study aimed to investigate the impact of TGF-β1 treatment on the expression and activity of Hippo signaling pathway components, specifically YAP1 and LATS1, in HCC cells. Additionally, we aimed to evaluate the role of the Hippo signaling pathway in mediating TGF-β1-induced inhibition of HCC cell growth.

Methods

Cell culture

To prepare cellosaurus cell lines (SMMC-7721, Bel-7402, HepG2, and QGY-7703) were grown in Roswell Park Memorial Institute (RPMI)-1640 medium consisting of 10-mM Hydroxyethylpiperazine Ethane Sulfonic Acid (HEPES) buffer, 10 % fetal bovine serum, 2 mM glutamine, 100 µg/mL streptomycin, and 100 units/mL of penicillin at a CO2 atmosphere of 5% and a temperature of 37 °C.

Examination of cellular proliferation

The proliferation of HCC cells was assessed using Cell Counting Kit 8 (WST-8 / CCK8). After being seeded into a 96-well plate (3×10^3 in each well), human HCC cells were incubated by TGF-β in various concentrations (0, 1, 2.5, 5, and 10 ng/mL) for 48 hours based on previous experimental studies (22). The incubation of the 96-well plate was conducted at 37°C for 48 hours. Each well received 10 µl of Cell Counting Kit-8 (CCK-8), and the absorbance was quantified at 450 nm by a multi-label plate reader (Synergy HTX Multi-Mode Reader).

Real-time polymerase chain reaction (RT-PCR)

Real-time PCR was performed to measure the mRNA transcript level of genes mRNA in the cells. RNA was extracted from all the samples using QIAzol reagent (Cat.No 79306) according to Qiagen protocol (RNeasy Mini Kit). Briefly, based on the recommended protocol, 20 µg of total RNA was used for each reaction, and the cDNA samples prepared were in a final volume of 84 µl , enabling sensitive real-time kit protocol and oligo (dT) primers. Each PCR reaction is performed using the PCR master mix and SYBR Green of Real Q Plus 2x Master Mix Green – Ampliqon – according to the manufacturer's protocol (CAT NO: A325402). Forty cycles were considered for each Real-Time PCR and temperatures of each cycle were set at 94.00 ˚C for 30 S, 58.00 ˚C for 30 S, and 72.00 ˚C for 30 S. Specific forward and reverse primer sequences were designed for the genes studied using NCBI as shown in Table 1. Data were analyzed based on delta-delta CT from the device, and the normalization of data was done by 18S rRNA as a control gene. The primers for genes and housekeeping genes were designed using a verified BLAST program in NCBI databases. Forward and reverse primer sequences for the aforementioned genes are listed in (Table 1).

Table 1. PCR and Real-time Quantitative Reverse Transcription-PCR (Real-Time qRT-PCR) (5' to 3')

Sample	Gene	Primer	
Core components of the pathway	LATS1	FP	AAATTGGAACGCCTCATAAGGCC
		RP	TCGCTCGAGGATCTGGTTACTC
	YAP1	FP	TAGCCTGCGTTAGCCAGTAA
		RP	TCATGCCTAGTCAACTGGCTGT
Control	18S rRNA	FP	GAAGCCAGATCCTTCGCAATC
		RP	AGCTGTTGTCATACGTCTCATTG

LATS1: Large tumor suppressor expression, YAP1: Yes-associated protein 1, 18S rRNA: 18S ribosomal RNA, FP: Forward primer, RP: Reverse primer

The qRT-PCR was performed with the sense and antisense primers using a 7500 Real-time PCR System. The $2^{-\Delta\Delta CT}$ was calculated for the obtained data (23, 24) (Table 1). To evaluate the quality and yield of RNA, the A260/A280 and A260/A230 ratios of RNA samples were measured using a Nano-Drop® ND-1000 spectrophotometer (NanoDrop Technologies). The integrity of the RNA and the presence of DNA contamination were assessed through the visualization of 28S and 18S rRNA on an agarose gel, which was

stained with ethidium bromide. To identify any potential DNA contamination, PCR was performed utilizing primers specific to 18S rRNA, specifically the Quantum RNA Universal 18S Internal Standards primers (Ambion). The PCR reaction mixture included 1 µL of RNA input (ranging from 20-200 ng), 0.6 U of Taq DNA Polymerase (Fermentas), 2.5 µL of 18S PCR Primer Pair (Ambion), 2 mM MgCl2, 0.2 mM of each dNTP (Invitrogen), 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM (NH4)2SO4, and 0.01% (v/v) Tween 20.

The thermal cycling conditions consisted of an initial denaturation step at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds, concluding with a final extension at 72°C for 5 minutes. The resulting PCR products were analyzed on agarose gels and stained with ethidium bromide.

The Assay for colony formation and apoptosis detection

Cells seeding process was performed using a 6-well plate and the cells were cultured for 2 weeks. The colonies were stained with crystal violet and then put under a microscope (LABOMED Co; USA) (Figure 1). A density of 5×10^2 cells per well was considered for cell seeding, and cell apoptosis was determined using the flow cytometry technique. Cells seeded in a 6-well plate (2×10^5 per well) were treated by TGF-β for 48 hours and a Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (CAT NO: A2214-SIGMA) was used to analyze the apoptotic cells.

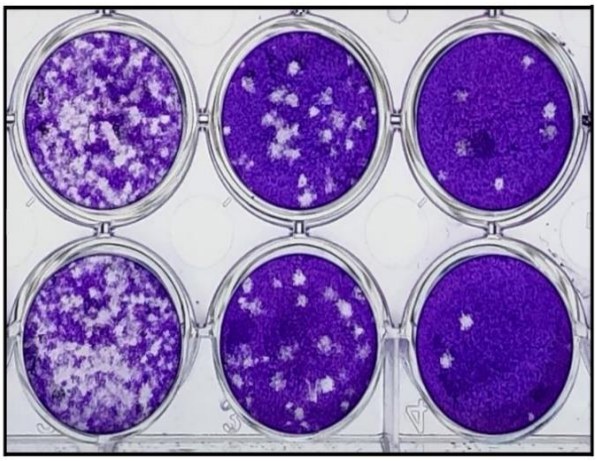


Figure 1. The Assay for colony formation and apoptosis detection.

Immunofluorescent staining

The diagnosis of abnormal cells was performed using immunofluorescence (IF) gens (proteins) in cells (25). YAP1 immunofluorescent staining in SMMC-7721 cells was carried out after 48 hours of treatment with various concentrations of TGF-β. Briefly, for IF analysis, cells were cultured on coverslips and subjected to treatment with various concentrations of TGF-β (0, 5, and 10 ng/mL) for 48 hours. Following treatment, the cells were fixed using 200 µl of 4% paraformaldehyde (PFA) for 15 minutes, after which, they were washed three times with phosphate-buffered saline (PBS) at pH 7.4, with each wash lasting 5 minutes. Subsequently, a blocking buffer composed of 3% fetal bovine serum (FBS), 1% goat serum, and 0.1% Triton X-100 was applied to each well for 1.5 hours at room temperature. The cells were then incubated overnight at 4°C with a primary antibody against YAP1, diluted in the blocking buffer. Following this incubation, the cells were treated with Cy3-conjugated goat anti-rabbit IgG (H+L) for 1 hour at room temperature. Finally, the cells were stained with DAPI for 10 minutes at room temperature and subsequently examined under a fluorescence microscope.

Immunoblotting

Moreover, the immunoblotting method, which uses antibody-based probes to acquire special data on target proteins from complex samples, was used for protein detection (26). Relative protein expression was analyzed with a Java-based image processing program (ImageJ) using Plugins/Fractal Analysis/FracLac V. 2.5. Mouse monoclonal antibodies of LATS1/WARTS and YAP1 were purchased from Custom Antibody Services. Sufficient amounts of Anti-phospho-YAP1 (pTyr357), phospho-LATS1/LATS2 (Ser909, Ser872), and an anti-IL-20 monoclonal antibody (7E), and anti-mouse IgG, horseradish peroxidase (HRP)-linked antibodies were prepared from Immuno-Precise Company, Netherlands. Besides and in line with the aims of this study, Smad2/3 antibody (C-8), β-actin antibodies, and mouse monoclonal antibodies Bcl-2-associated X protein were prepared from ImmunoPrecise Company. Human TGF β with various concentrations 0, 1, 2.5, 5, and 10 ng/mL was utilized to analyze reagents recombinant and immunofluorescence. After seeding and culturing into 6-well plates, the cells were rinsed and fixed in phosphate-buffered saline and

4% polyformaldehyde, respectively. Smad2/3 solution with a concentration of 1:100 wad was then introduced, and plate incubation was performed at 4 °C, over the night. After rinsing, FITC-labeled anti-mouse secondary antibodies designed for fluorescent-based usage were introduced and incubated for 2 h. The cells were then placed under a confocal microscope after counterstaining by diamidino-2-phenylindole (DAPI).

RNA interference (RNAi)

Lipofectamine™ 3000 transfection reagent was used for cell transfection in 6-well plates. Cell harvesting was done 48 hours after transfection for real-time PCR or immunoblotting purposes. Table (2) shows the sequences by which the gene synthesis process was performed.

Statistical analysis

Data were analyzed in SPSS V27 software. Data analysis was performed using student t-test and ANOVA, and the data was presented as mean ± SD. A significance level of 0.05 was considered (Table 2).

Table 2. Process of gene synthesis according to Sigma and Gene Chem Financial Corp company method (5' to 3')

Gene	Sequence
Smad2	GAGGUCGCCUGCAAUAUGAdTdT
siRNA	UCAUGUUGAAGCCGAACUCdTdT
Smad3	CAUGCACGCAGGUTCUCCAdTdT
siRNA	UGCAGAAGCUGCGUCTAUGdTdT
LATS1	GCATUUGAAGUCACUAGUAdTdT
siRNA	UACUACUGAAGUCAACUGCdTdT

Small Interfering RNA (siRNA)

Results

Cell viability using CCK-8 assay

Cell viability was determined using CCK-8 assay, and cell treatment was performed for 48 hours using TGF-β concentrations of 0, 1.0, 2.5, 5.0, and 10.0 ng/mL. The results of this study showed that TGF-β suppressed HCC cell growth.

The biological role of TGF-β in HCC was examined by evaluating its efficacy on the viability of QGY-7703, HepG2, Bel-7402, and SMMC-7721 cells. CCK-8 assay showed that TGF-β suppressed the QGY-7703, HepG2, Bel-7402, and SMMC-7721 cells, as shown in Figure (2). Also, the cells treated with TGF-β resulted in fewer colonies than those without TGF-β (Figure 2).

Apoptosis assay with PI, Annexin V, and Immunoblotting technique

Apoptosis assays of HCC cells were conducted after 48 hours to detect the cellular events related to cell death, and cell treatment was performed using TGF-β at two concentrations of 5 and 10 ng/mL. The results showed no clear variation in cell apoptosis rates compared to the control. As previously mentioned, the immunoblotting technique was applied to evaluate the expression levels of Bcl-2 and Bax proteins. The results showed that the change in the expression level of Bcl-2-associated X protein was insignificant (Figure 3). It was also revealed that TGF-β suppressed the proliferative capacity of HCC cells, but no apoptosis promotion was observed (Figure 4).

Immunofluorescence staining results

The cellular localization of YAP was examined by immunofluorescence staining. Increased phosphorylation of YAP1 was consistent with the translocation of nucleocytoplasmic YAP1 treated by TGF-β following 48 h (Figure 5). Activation of the HSP was associated with higher LATS1 expression and YAP1 phosphorylation on certain serine remnants (12, 27)

Western blotting

Accordingly, the expression level of LATS1 and YAP1 was examined in SMMC-7721 and Bel-7402 cells treated with TGF-β (0, 1, 2.5, 5, and 10 ng/mL). The results of the immunoblotting assay indicated a higher expression level of LATS1 in SMMC-7721 and BEL-7402 cells, but the total YAP1 expression remained unchanged (Figure 6). Based on the results, TGF-β causes the HSP to be activated in HCCs. In both BEL-7402 and SMMC-7721 cells, no significant association was observed in the relative expression of TGF-β and YAP1 ($P>0.05$).

mRNA and siRNA expression level

However, the results showed no significant changes in expression levels of YAP1 and LATS1 mRNA by real-time PCR. The expression of LATS1 and YAP1 mRNA was not affected by TGF-β with 5ng/mL. YAP activity can be determined through its cellular localization and phosphorylation status. The phosphorylation of YAP causes its nucleus-to-

cytoplasm translocation (27). Phosphorylated LATS1 can also regulate the phosphorylation status of YAP. Accordingly, we investigated YAP1 phosphorylation at anti-IL-20 monoclonal antibody (7E) and anti-phospho-YAP1 (pTyr357), and LATS1 phosphorylation at Phospho-LATS1/LATS2 (Ser909, Ser872) in TGF-β-treated (5ng/mL) SMMC7721 cells. The results showed a time-dependent increase in the phosphorylation level of LATS1 at Phospho-LATS1/LATS2 (Ser909, Ser872) and YAP1 at anti-IL-20 monoclonal antibody (7E) after incubation with TGF-β, but the increase in the phosphorylation level of YAP1 at Anti-phospho-YAP1 (pTyr357) only observed in the first 2 hours (Figure 7).

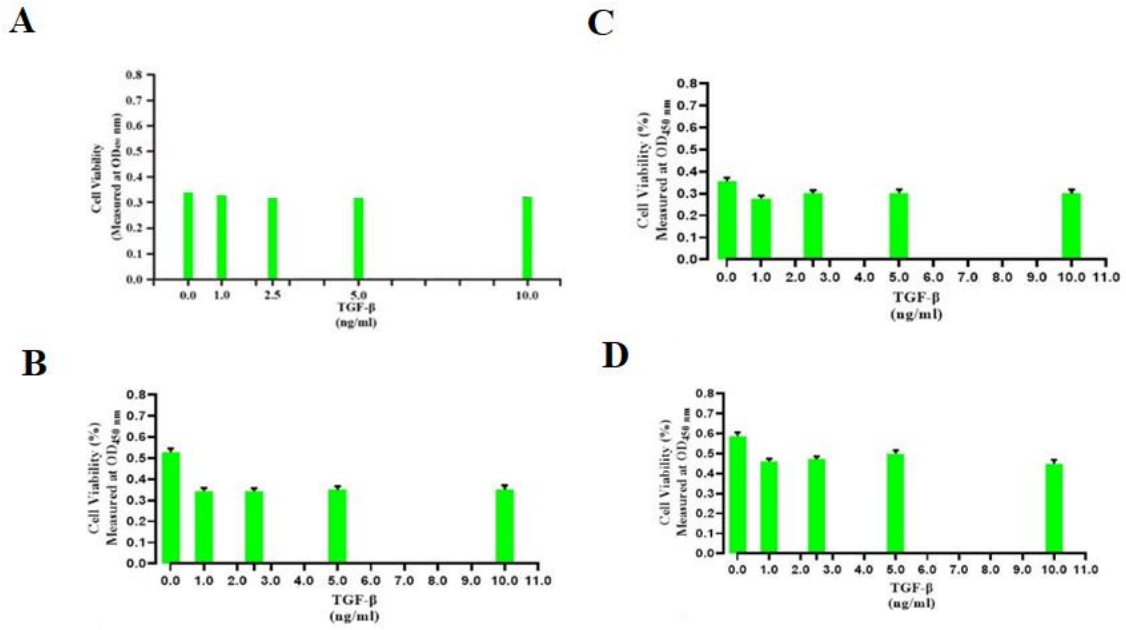
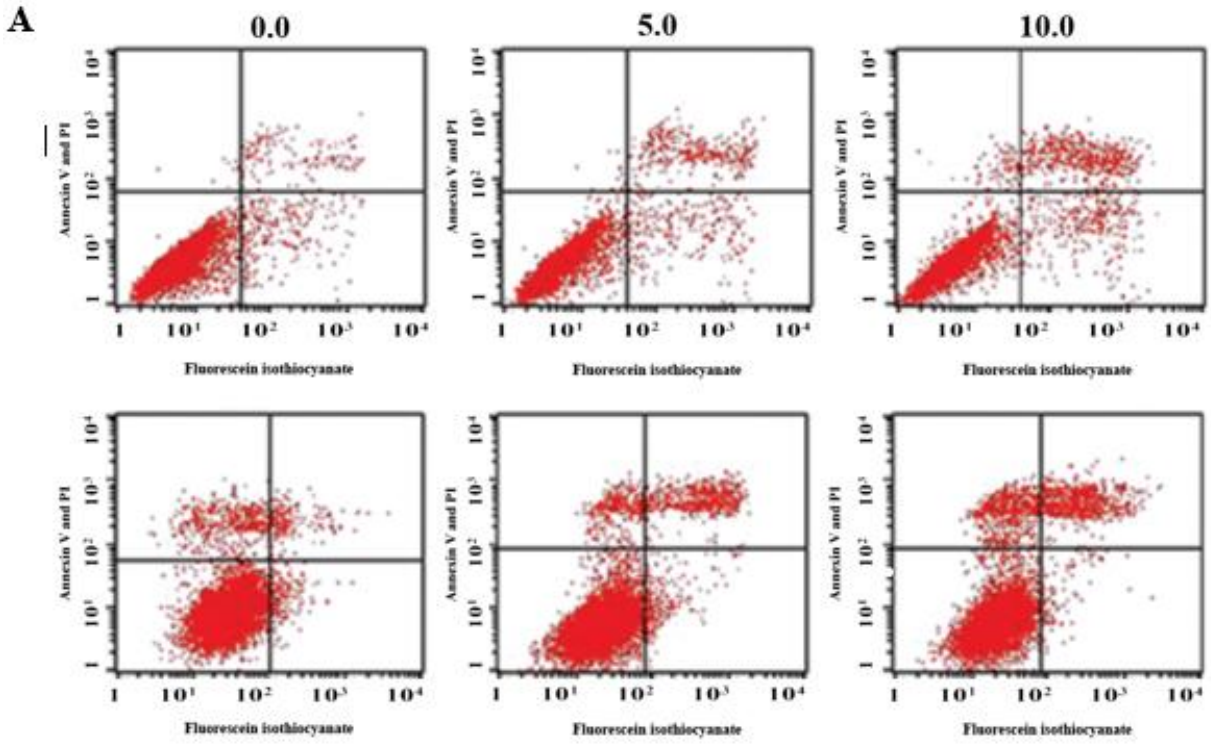


Figure 2. Inhibitory effect of TGF-β on HCC cell growth. Cell viability was assessed using the CCK-8 assay after treatment with TGF-β concentrations of 0, 1.0, 2.5, 5.0, and 10.0 ng/mL for 48 hours. A) QGY-7703 Cells (P>0.05); B) BEL-7402 Cells (P<0.05); C) SMMC-7721 Cells (P<0.05); D) Hep G2 Cells (P<0.05).



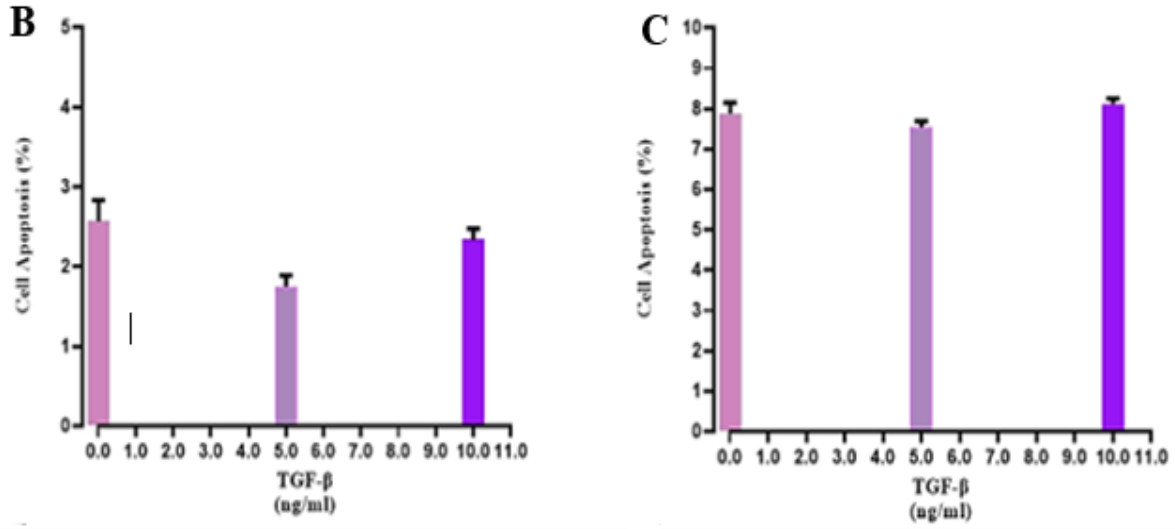


Figure 3. A) Apoptosis assay using PI and Annexin V showed that TGF-β inhibited the proliferation of HCC cells without affecting apoptosis. In the assay, the lower row corresponds to SMMC-7721, and the upper row to Bel-70402. B) Represents Bel-7402, while C) Refers to SMMC-7721. TGF-β treatment for 48 hours was applied to SMMC-7721(B) and BEL-7402 cells (C) at concentrations of 0, 5, and 10 ng/Ml .No significant correlation was observed between TGF-β and cell apoptosis (P > 0.05).

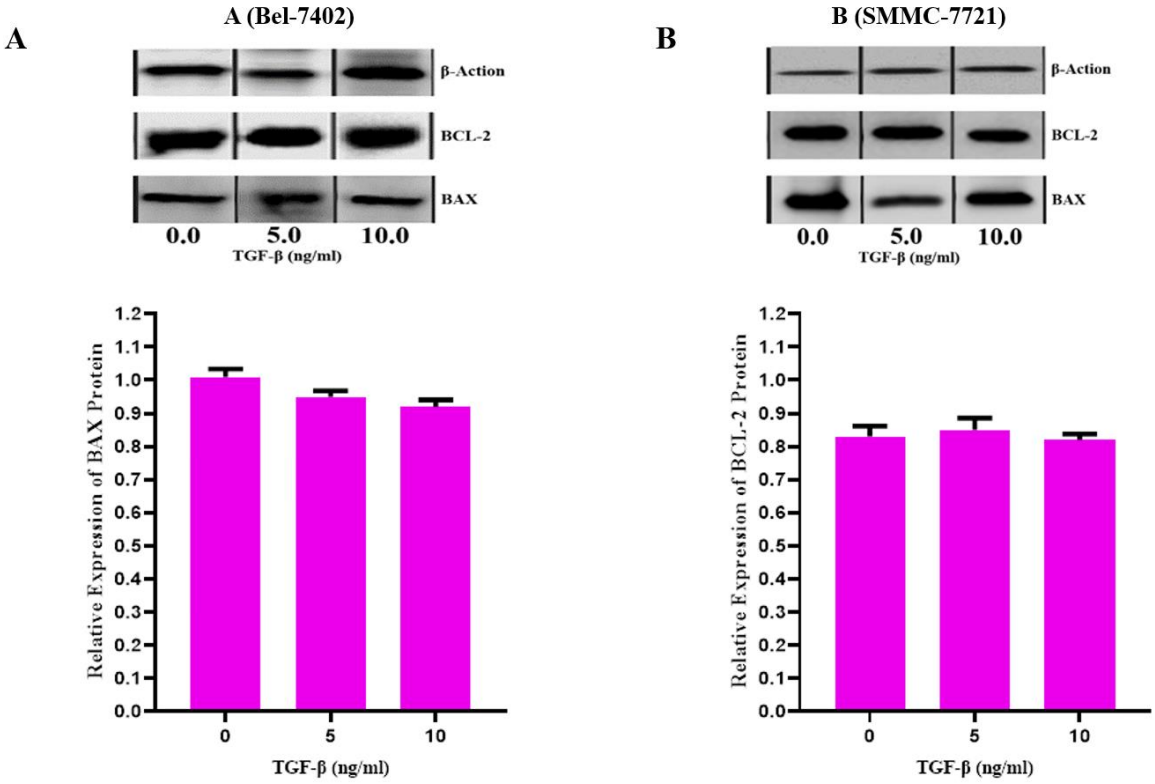


Figure 4. Immunoblotting assay to assess the expression levels of Bax and Bcl-2. A) cell line of SMMC-7721, and B) hepatocarcinoma cell line of Bel-7402.

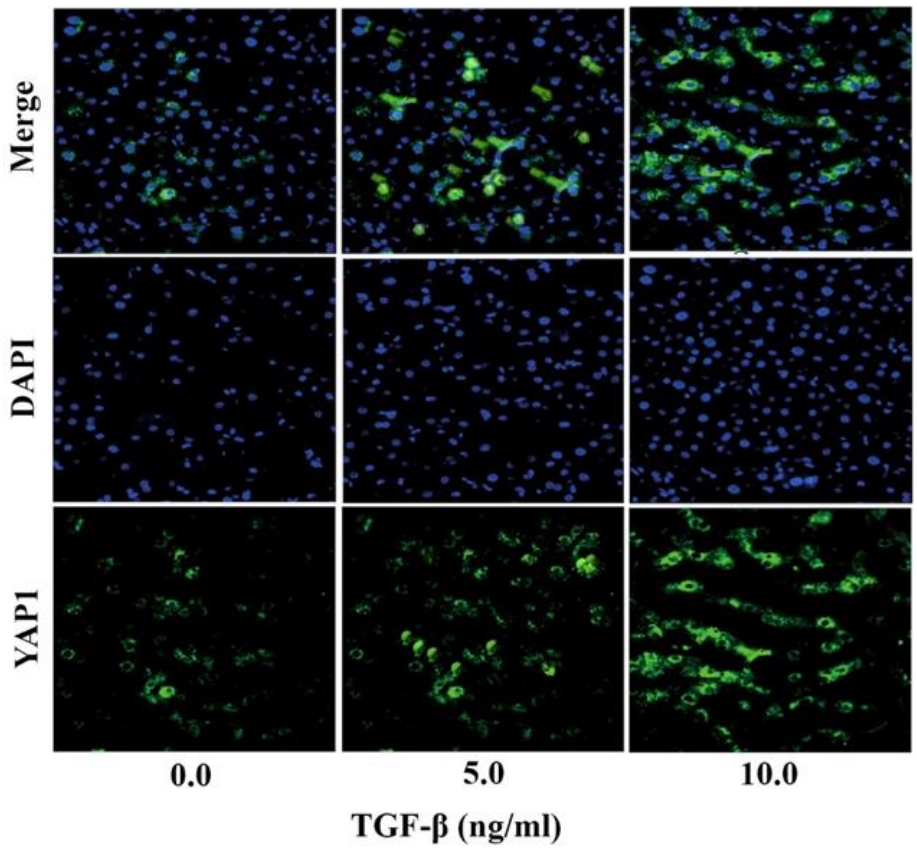
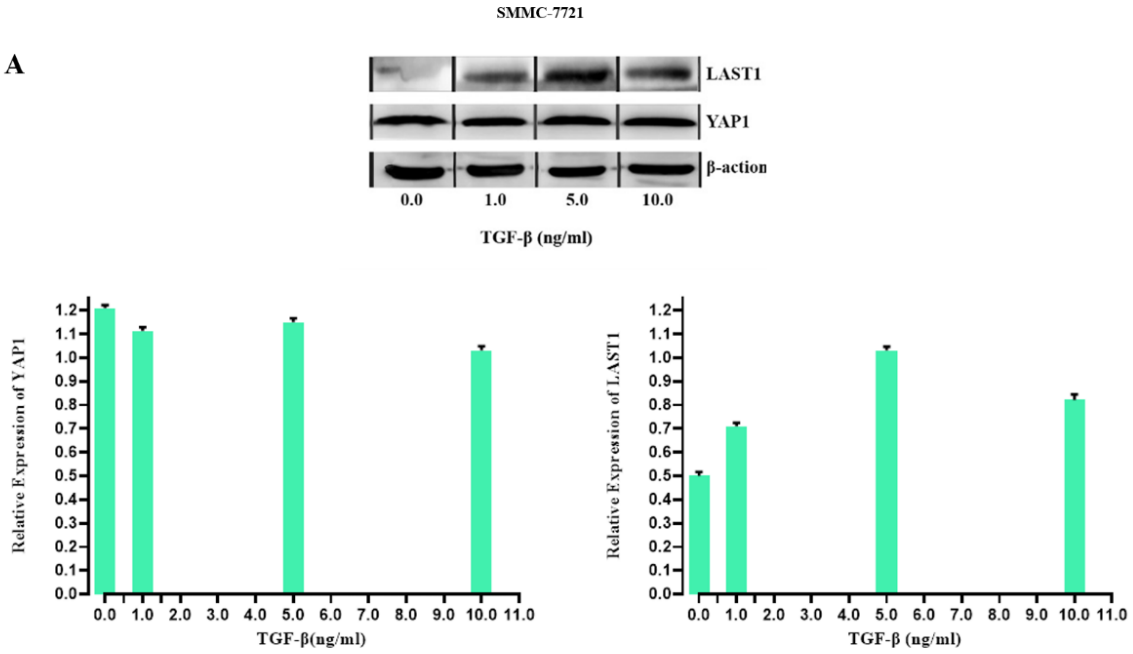


Figure 5. YAP1 immunofluorescent staining in SMMC-7721 cells after 48 hours of treatment with various concentrations of TGF-β.



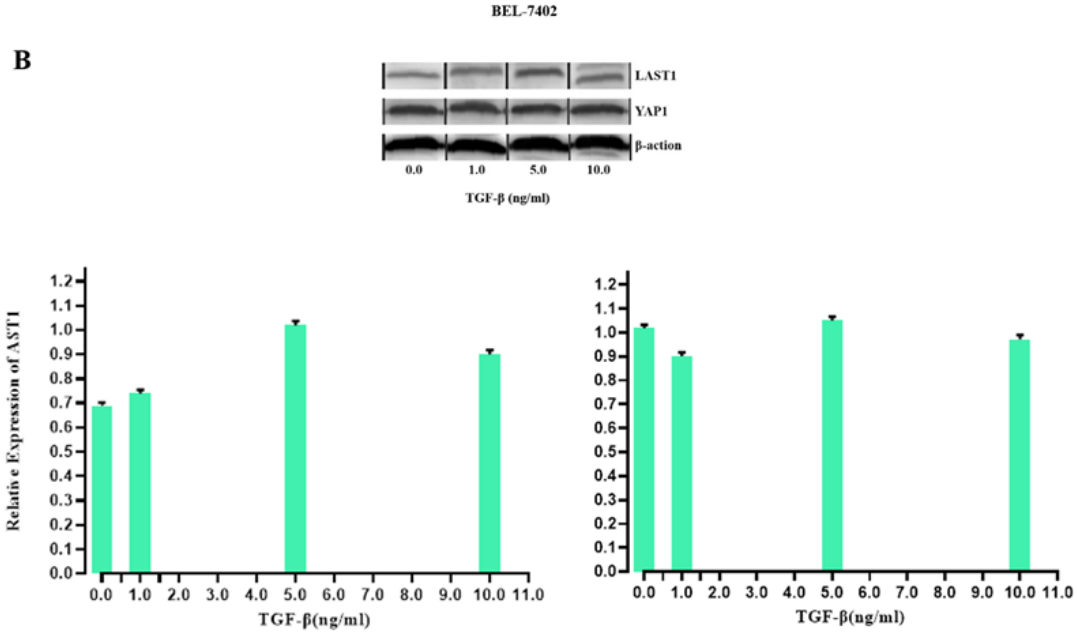


Figure 6. The application of western blotting in measuring protein levels of YAP1 and LATS1 in HCC cells after treatment for 48 hours with TGF-β in various concentrations. In both SMMC-7721 cells (A) and BEL-7402 (B), no significant association was observed in the relative expression of TGF-β and YAP1 (P>0.05).

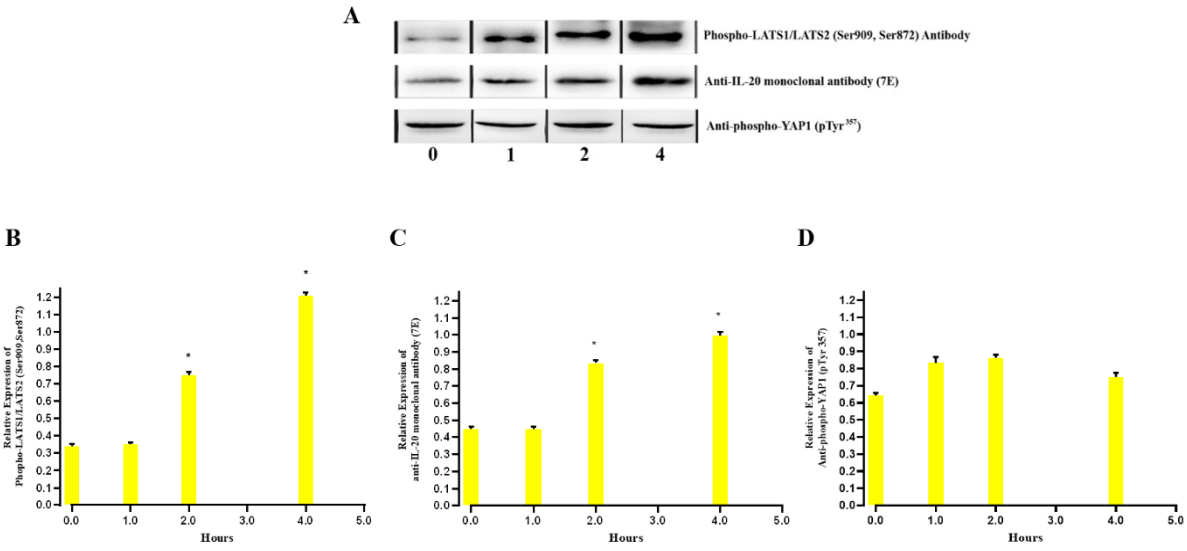


Figure 7. A) The results of western blotting in measuring protein levels of p-LATS1 (Phospho-LATS1/LATS2 (Ser909, Ser872) antibody), p-YAP1 (Anti-IL-20 monoclonal antibody (7E), and p-YAP1 (Anti-phospho-YAP1 (pTyr357)) in HCC cells after treatment with TGF-β. There were significant associations in all treatments (P<0.05). B) Levels of p-LATS1 (Phospho-LATS1/LATS2 (Ser909, Ser872) ; C) Level of p-YAP1 (Anti-IL-20 monoclonal antibody (7E) ; and D) p-YAP1 (Anti-phospho-YAP1 (pTyr357).

According to these findings, a key mechanism of TGF-β by which it suppresses the HCC cells is the regulation of the HSP through direct controlling of cellular localization and phosphorylation of YAP1. Finally, the HSP was activated by TGF-β in HCC cells. Through transfecting HCC cells by LATS1 small

interfering RNA and LATS1 genes, the current study tried to investigate the potential inhibitory effect of TGF-β on HCC cell growth by regulating the HSP to evaluate the transfection efficiency. Real-time PCR and immunoblotting assays were conducted to detect LATS1 levels after 48 hours of transfection (Figure 8). Cell treatment was then carried out with TGF-β for 48 hours. LATS1 overexpression in SMMC-7721 cells increased the phosphorylation level of YAP1 and inhibited its expression levels. On the other hand, LATS1 special siRNA caused a significant increase in YAP1 expression level and inhibited YAP1 phosphorylation (Figure 9).The results of the immunoblotting assay for examining expression levels of LATS1 YAP1, and p-YAP1 in siRNA, LATS1 siRNA, and LATS1 overexpression, all of which are transfected in SMMC-7721 after 48 hours of treatment by 5ng/mL TGF-β. Significant associations were obtained in all treatments but in the relative expression of p-YAP1 and LATS1 siRNA (P>0.05). Finally, it was concluded that TGF-β in 5ng/mL could suppress HCC cell growth by regulating the HSP.

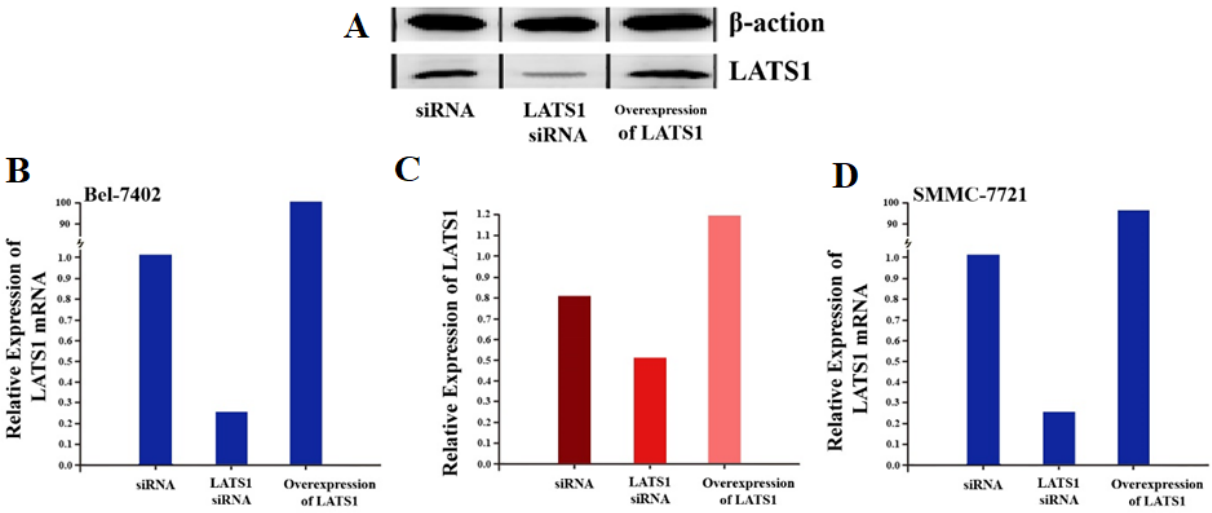


Figure 8. A and B) Protein and mRNA expression of LATS1 in SMMC-7721 and BEL-7402 cells after overexpression of LATS1 or transfection with LATS1 siRNA. In both Bel-7402 (B) and SMMC-7721 cells (D), no significant association was found between the relative expression of LATS1 siRNA and LATS1 mRNA (P>0.05). C) Relative protein expression of LATS1.

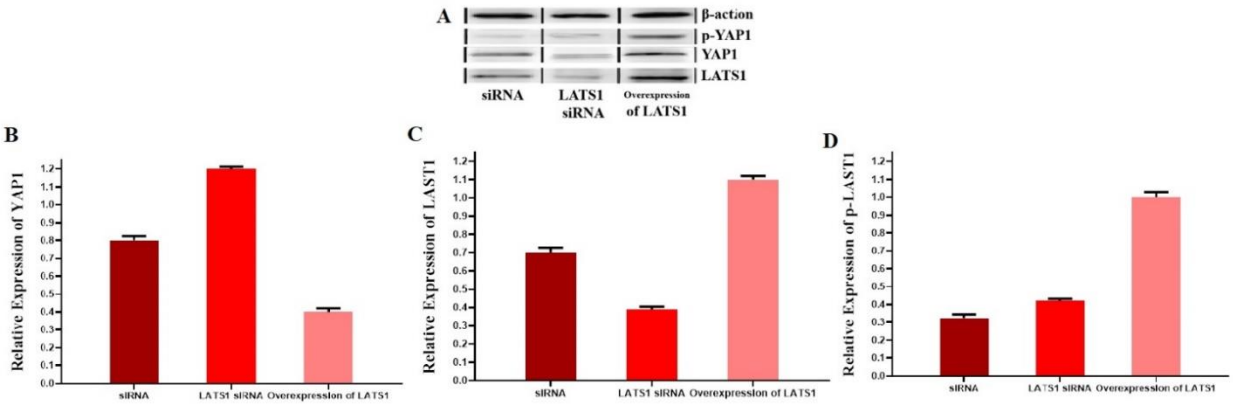


Figure 9. A) The results of the immunoblotting assay for examining expression levels of LATS1, YAP1, p-YAP1 siRNA, LATS1 siRNA, and LATS1 overexpression, all of which are transfected in SMMC-7721 after 48 hours of treatment by 5ng/mL TGF-β (C). Significant associations were observed in all treatments, except for the relative expression of p-YAP1 (B) and LATS1 (D) after siRNA treatment (P > 0.05).

Discussion

Today, it is widely accepted that the inhibitory effect of TGF-β on cell growth is through the formation of a tumor-suppressor pathway including ligands, downstream signal transducers, receptors, and their transcriptional targets (28). In advanced carcinogenic stages, this tumor-suppressor pathway is commonly lost in epithelial cells (29) as the result of transcriptional suppression or mutational inactivation of TGF-β (30). TGF-β is involved in several events in tumors including the process of epithelial-mesenchymal transition. It can also suppress the metastasis and invasion of cancerous cells (31-33).

The results of our study showed that both cytokines TBRI and TBRII inhibited HCC cell growth, increased phosphorylation of YAP1 and LATS1 time-dependently, induced nucleocytoplasmic translocation of YAP1 and upregulated the LATS1 expression at protein levels. In HCC tissues, YAP1 was highly localized and expressed in the nucleus. LATS1 overexpression, on the other hand, significantly decreased HCC cell growth, while its downregulation caused increased growth of HCC cells and YAP1 activation. Besides, HCC cell growth was inhibited by TGF-β. These results show the anti-oncogenetic role of the HSP in the suppression of HCC cell growth by TGF-β. According to our results, TGF-β suppressed the proliferative capacity and potential of HCC cells to inhibit their growth. Moreover, no significant difference was found in expression levels of Bcl-2 and Bax proteins. In a similar study, Song et al. (34) concluded that the signaling pathway of SIRT3/GSK-3β/Bax was significantly involved in HCC growth suppression and suggested this pathway as a promising therapeutic option for HCC.

Some studies reported that the HSP and TGF-β signaling pathways are independent of each other. However, some others reported the interaction between TGF-β signaling and YAP1, which is the effector protein in the HSP (35-37). According to Boopathy and Hong (37), the hippo pathway-YAP/TAZ is shown to regulate endothelial cell proliferation, migration, and survival, leading to the regulation of vascular remodeling, sprouting, and barrier formation. Most of the intracellular signaling programs simultaneously activate YAP/TAZ to regulate main events in angiogenesis (38). The result of the present study showed that, as an important protein in the Hippo signaling pathway, LATS1 can increase the expression levels induced by TGF-β. Regulation of the HSP by various signaling pathways and extracellular ligands/growth agents has been shown in several studies. Losing a key component like Salvador-1, MOB1A/MOB1B, and LATS1/2 has been shown to cause upregulation of YAP/TAZ-TEAD target gene transcription including tissue growth and cell proliferation, proving their important role in the Hippo pathway (39-42).

HCC cell transfection with siRNA vs. LATS1 led to the deletion of the LATS1 gene and the growth inhibition of HCCs was abolished by TGF-β. It is while a significant decrease occurred in HCC cell growth by upregulation of LATS1. As a result, it was found that the Hippo signaling pathway could be activated by TGF-β. AP/TAZ also participates in epithelial-mesenchymal transition, which is a key factor in cancer stemness and tumor progression. It should be noted that the tumorigenic and profibrotic roles of YAP/TAZ depend on its crosstalk with TGF-β signaling. Noguchi et al. (43) argued that nuclear YAP1 is an essential factor in maintaining and promoting tumorigenic phenotypes induced by TGF-β in either cancer or fibrosis.

Nucleocytoplasmic translocation of YAP1 hindered the growth of TGF-β-treated HCC cells. It also found that TGF-β led to the increased levels of Smad2/3 phosphorylation and expression as well as the nucleocytoplasmic translocation of Smad2/3. After knocking out of Smad2/3, however, HCC cell growth was considerably inhibited than before. Zhang et al. (44) reported reduced expression of the TGF-β receptor with HCC development both in vitro and in vivo and attributed it to the higher level of TGF-β. Although the underlying mechanism is yet to be defined, Smad2&3 may be activated when there are decreased expression and elevated levels of the TGF-β receptor. Through inhibiting the Smad2&3 pathways, the TGF-β receptor plays an inhibitory role in regulating the invasion and migration of HCCs. The results of this study showed that the TGF-β receptor is capable of being employed as a molecular target for the diagnosis and treatment of HCC cells (44). According to the results, HCC cell growth and development are inhibited by TGF-β, which targets the Hippo signaling pathway via regulating certain core proteins, including nucleocytoplasmic translocation of YAP1. Consequently, TGF-β targeted factors possess

antitumor effects that can be improved by synergistic activities if identified. A possible strategy to improve their antitumor effects may be the identification of drugs that target two molecules (45)

The Hippo signaling pathway (HSP) is involved in the suppression of HCC cell growth induced by TGF-β. A decreased level of TGF-b receptor expression is correlated with poor differentiation and portal vein invasion in HCC cells. This indicates that HCC cells are more invasive with reduced expression of the TGF- β receptor and may develop intrahepatic metastasis. The association between TGF- β signaling in HCC development is still under dispute, however, the results of our study showed a significant association between decreased expression of TGF-β receptor in HCC cells and some aggressive features including short time-to-recurrence and IM. Not only TGF-β receptor expression may serve as a biomarker for intrahepatic metastasis, but also as a predictor of HCC recurrence. Instead of promoting apoptosis, TGF-β suppresses the proliferative capacity of HCC cells to inhibit their growth. TGF-β caused nucleocytoplasmic translocation of the YAP1 and increased LATS1 expression. Both LATS1 overexpression and YAP1 nucleocytoplasmic translocation have anti-oncogenic roles in the incidence and progression of liver cancer. This study contributes to the existing insights on the inhibitory strategies for HCC progression.

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