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TGF-β1 promotes proliferation and invasion of hepatocellular carcinoma cell line HepG2 by activating GLI-1 signaling

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Abstract. – OBJECTIVE: To investigate the role of GLI-1 signaling pathway on TGF-β1 induced proliferation and invasion in hepatocellular carcinoma (HCC) cell line HepG2.

MATERIALS AND METHODS: Cultured HepG2 cells were treated with various concentration of TGF-β1 for 24 h and the effect of TGF-β1 on invasion ability of HepG2 was detected with transwell assay. Next, cultured HepG2 cells were treated with various concentration of TGF-β1 for 12, 24 and 48 h; then, the proliferation rate was detected by MTT. The mRNA and protein expression levels of GLI-1 were detected by RT-PCR and Western blot. Furthermore, GLI-1 siRNA was used to investigate the role of GLI-1 on TGF-β1 induced proliferation and invasion.

RESULTS: The results demonstrated that TGF-β1 could promote HepG2 cells proliferation and invasion. The mRNA and protein level of GLI-1 were upregulated by TGF-β1 treatment, whereas GLI-1 siRNA could block these processes.

CONCLUSIONS: TGF-β1 promotes HCC cell line HepG2 proliferation and invasion by activating GLI-1 signaling pathway.

Key Words:

Hepatocellular carcinoma, TGF-β1, Glioma-associated oncogene family zinc finger 1, Proliferation, Invasion.

Introduction

Hepatocellular carcinoma (HCC) , as the most common type of malignancy in China, ranks second in the incidence rate of the tumors. It is frequently seen in the adult males aged between 40 and 50 years old with a high malignancy, severely threatening the life and health of human beings[1]. In recent years, with the rapid development in medical theory and technique, great progress has been seen in the diagnosis and treatment of HCC, but the mechanisms involving the proliferation and invasion of HCC cells have not yet been elucidated. Recurrence and metastasis after resection of HCC are the major factors affecting the efficacy, which have become a hotspot in research on the pathogenesis of HCC[2,3]. Therefore, experimental studies on the proliferation, invasion and metastasis of HCC require not only the theoretical significance, but also the application value. Transforming growth factor-β1 (TGF-β1), a pleiotropic cytokine, has been reported to be involved in the proliferation and invasion of multiple malignant tumors[4,5]. It has been reported that Hedgehog signaling pathway participates in cell proliferation and development through the transposition of GLI-1 into the nucleus to interfere with the involvement of downstream target genes in the modulation of cell proliferation, apoptosis and invasion[6,7]. However, whether TGF-β1 can affect the signal of GLI-1 in HCC cells to promote its development and progression remains unknown. Thus, in this research, with HepG2 cells as subjects, we induced the proliferation and invasion of HepG2 cells using TGF-β1, aiming to investigate the changes in GLI-1 signaling pathway in proliferation and invasion of HepG2 cells induced by TGF-β1, so as to provide experimental evidence for research on the invasion and metastasis of HCC.

Materials and Methods

Materials

HepG2 cell lines provided by the Laboratory of Life Science Institute of Southern Medical University; fetal bovine serum (FBS), high-glucose Dulbecco's modified eagle medium (DMEM) medium and penicillin/streptomycin mixture were provided by HyClone (South-Logan, UT, USA); polyclonal rabbit anti-human antibodies of GLI-1 and β-actin were provided by Abclonal

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Technology Co., Ltd., (Woburn, MA, USA), and horseradish-peroxidase (HRP)-labeled goat anti-rabbit secondary antibody of IgG was provided by Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., (Beijing, China); PCR primers were designed and synthesized by Beijing Tianyi Huiyuan Biotechnology Co., Ltd., (Beijing, China); reverse transcription kit provided by TaKaRa (Dalian, China) Bioengineering Co., Ltd.; vertical electrophoresis tank and membrane transferring tank were provided by Beijing Liuyi Biotechnology Co., Ltd., (Beijing, China); RIPA lysis buffer for tissue and cell provided by Beyotime Biotechnology Institute; BCA protein quantification kit provided by Beijing Solarbio Biotechnology Co., Ltd.,(Beijing, China); GLI-1 siRNA was designed and synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China); TGF-β1 was provided by PeproTech Co., Ltd. (London, England, UK); 6-well and 96-well plates, 25 cm2 culture flask and 24-well transwell chambers (8 μm) were obtained from by Beaver (Waltham, MA, USA).

Methods

Cell Culture

HepG2 cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with penicillin-streptomycin (100 U/mL) and 10% fetal bovine serum (FBS) in an incubator at 37°C and 5% CO_2. After that, more than 90% of the area was covered by cells, which were digested for following experiments. 6 hours prior to the experiment, the medium was replaced with fresh serum-free medium, in which cell growth could be synchronized for intervention. Cells were grouped as follows: Control group, TGF-β1 group and TGF-β1 + Gant61 group.

Measurement of the Proliferation of HepG2 Cells Using MTT

HepG2 cells in logarithmic phase were digested using trypsin for preparing the single-cell suspension, and inoculated onto the 96-well plate at density of 5×103/well. Following 7 days of culture, in each well, 20 μL MTT reagent were added and well mixed, followed by incubation at 37°C for 4 to 6 hours. Then, septic pipette was used to collect the supernatant, in which 150 μL dimethylsulfoxide (DMSO) were added and well mixed by stirring for 15 min, so as to dissolve the crystal sufficiently. At 24, 48, 72 and 96 hours, we detected the absorbance at wavelength of 490 nm, and with the absorbance as the ordinate and time as abscissa, the proliferation of HepG2 cells was quantified. This experiment was carried out in triplicate.

RNA Extraction and RT-PCR

RT-PCR was used to detect the expression of GLI-1. Cells in each group were washed three times using pre-cooled phosphate buffer saline (PBS) on ice, and in each well, 1 mL TRIzol reagent was added for RNA extraction in accordance with the instructions of manufacturer. The concentration of RNA was measured through the A260/A280 ratio using the ultraviolet spectrometer for three times. After cDNA was prepared using the reverse transcription kit, we performed the PCR with the primers synthesized by Shanghai Tsingke Biological Technology Co., Ltd., (Shanghai, China): β-actin, upstream 5'-TGCCATCAACGACCCCTTCA-3', downstream 5'-TGACCTTGCCCACAGCCTTG-3'; GLI-1, upstream 5'-CCAAACTTTTCCTCCCTGAACC-3', downstream 5'-GTGATGCTGAGAAGTTTCGTTGA-3'. Real-time quantitative PCR was performed in 20 μL reaction system supplemented with 10 μL mixture, 1 μL probe primer and 2 μL cDNA as follows: denaturation at 95°C for 3 min; 94°C for 30 s; 48°C for 30 s; 72°C for 1 min; extension at 72°C for 10 min after 35 cycles of amplification. This experiment was carried out in triplicate.

Cell Transfection

GLI-1 siRNA was synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China) with the following primers: Negative control of siRNA (si-NC), upstream 5'-UUCUCCGAACGUGUCACGUTT-3', downstream 5'-ACGUGACACGUUCGGAGAATT-3'; GLI-1 siRNA, upstream 5'- GGGAGCAAAUCAGAGAAAUTT-3', downstream 5'-AUUUCUCUGAUUUGCUCCCTT-3'. In this experiment, cells in logarithmic phase were regularly digested for preparation of single-cell suspension, and then they were inoculated on the 6-well plate at density of 1.5×109/L for culture at 37°C and 5% CO_2 for 24 h. Cell transfection was performed according to the instruction of Lipofectamine 2000. After transfection, cells were incubated for 4 to 6 h, and then the transfection buffer was abandoned. After that, cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO2 for 24 h or 48 h. Transfection efficiency was measured under the fluorescent microscope.

Western Blotting Assay

After specific treatment, HepG2 cells were washed using pre-cooled PBS three times, and then placed in the RIPA lysis buffer. Lysate was collected using a cell scraper and transferred into a 1.5 mL Eppendorf (EP) tube. After 30 min, cells were centrifuged at 12000 rpm for 10 min, and with the sediment being abandoned, supernatant was preserved at -20°C. BCA method was employed to measure the concentration of protein. Then, 5×loading buffer was well mixed with proteins, and placed in 95°C water bath for denaturation. Proteins in each group were loaded on the 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for isolation as follows: 80 V for 30 min and 120 V for 80 min. Thereafter, proteins on the gel were transferred electrically onto the membrane, followed by blocking using 5% skimmed milk for 2 h. After that, proteins were probed using polyclonal rabbit anti-human antibodies of GLI-1 (1:500) and β-actin (1:3000) at 4°C overnight. Next, three washes in Tris-buffered saline and Tween-20 (TBST) on a shaker were carried out, proteins were again probed using HRP-labeled goat anti-mouse or -rabbit antibodies of IgG for 1 h at room temperature. After three washes in TBST (5 min per wash), enhanced chemiluminescence reagent was added for observing the protein bands by using imaging software. Results were scanned using the Labwork software (Lehi, UT, USA).

Determination of the Cell Invasion Ability Using Transwell

HepG2 cells in logarithmic phase were treated with trypsin, followed by blow and beat for preparation of single-cell suspension, and two washes in serum-free medium. According to the cell count result, cell density was adjusted to 4×105/mL. In the upper chamber, 200 µL suspension were added and mixed with 600 µL medium supplemented with 10% fetal bovine serum (FBS), and cells were grouped into the blank control group, TGF-β1 group and TGF-β1 + GANT61 group for incubation for 24 h. After specific treatment, chambers were taken out; the cells that failed to pass through the membrane were scraped using a cotton swab, followed by fixation in paraformaldehyde (PFA) for 30 min. PFA was totally removed, and cells were dried at room temperature and stained using crystal violet for 15 min. Next, they were washed using PBS three times, and dried at room temperature. Under the inverted microscope, cell count was calculated in five fields randomly selected at a magnification of 400×, and the average was used as the result. This experiment was carried out in triplicate.

Statistical Analysis

Using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) software and GraphPad Prism 5.1 (La Jolla, CA, USA), we performed the statistical analysis with values expressed in mean ± standard deviation. Comparisons between either two groups were carried out with t-test and one-way analysis of variance (ANOVA). $p<0.05$ suggested that the difference had statistical significance. Tukey's HSD (honestly significant difference) test was used in conjunction with an ANOVA to find means that were significantly different from each other.

Results

Effect of TGF-β1 on the Proliferation of HepG2 Cells

Interventions were performed using TGF-β1 in different concentrations of 0 ng/mL, 10 ng/mL and 20 ng/mL at 12 h, 24 h and 48 h, and MTT method was applied to measure the effect of TGF-β1 on the proliferation of HepG2 cells. Results indicated that the increase in concentration of TGF-β1 can promote the proliferation of HepG2 cells in a concentration- and time-dependent manner. Compared with the control group (0 ng/mL), TGF-β1 exerted a significant promoting effect on proliferation of HepG2 cells within 10 ng/mL ($p<0.05$). At the same time point, a high concentration of TGF-β1 produces evident promoting effect on cell proliferation, which is a dose-dependent manner. In the same concentration, with the time lapse, TGF-β1 could promote the proliferation of HepG2 cells more efficiently in a time-dependent manner. In the following experiments, we chose 20 µmol /L as the optimal concentration for curcumin intervention (Figure 1).

Effect of TGF-β1 on the Invasion Ability of HepG2 Cells

After treatment with TGF-β1 in varying concentrations of 0 ng/mL, 10 ng/mL and 20 ng/mL, we detected the effect of TGF-β1 on the invasion ability of HepG2 cells through the transwell experiment. Results showed that, in comparison with the control group (0 ng/mL), signi-

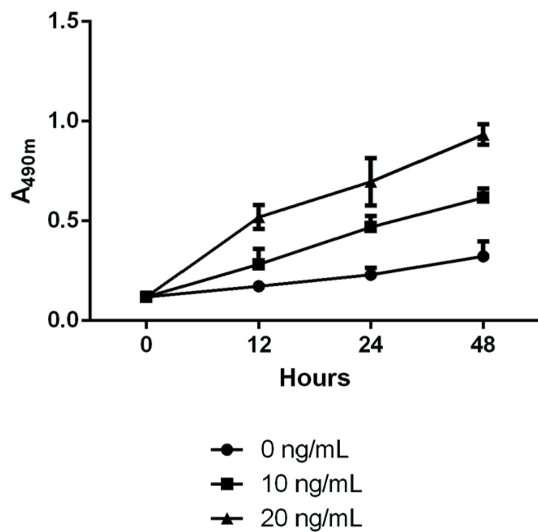

Figure 1. TGF-β1 induced HCC cell line HepG2 proliferation.

ficant enhancement was identified in the invasion ability of HepG2 cells treated with TGF-β1 in concentrations of 10 ng/mL and 20 ng/mL ($p<0.05$). The number of cells (Figure 2) passing through the membrane in the control group (0 μmol/L) was (52.6±22.3) per field, (112.3±12.6) per field in the TGF-β1 group (10 ng/mL), and (231.8±14.6) per filed in the TGF-β1 group (20 ng/mL), suggesting that TGF-β1 can significantly increase the invasion ability of HepG2 cells, and the differences had statistical significance ($p<0.05$; Figure 2).

Effect of TGF-β1 on the GLI-1 Signaling Pathway in HepG2 Cells

In HepG2 cells treated with 20 ng/mL TGF-β1 for 48 h, total RNA and proteins were extracted from cells for detection of the changes in mRNA and proteins of GLI-1 in cells through RT-PCR and Western blotting assay. In comparison with the control group, significant elevation in mRNA and protein expressions of GLI-1 occurred after TGF-β1 treatment, and the difference had statistical significance ($p<0.05$), suggesting that TGF-β1 can activate the GLI-1 signaling pathway (Figure 3).

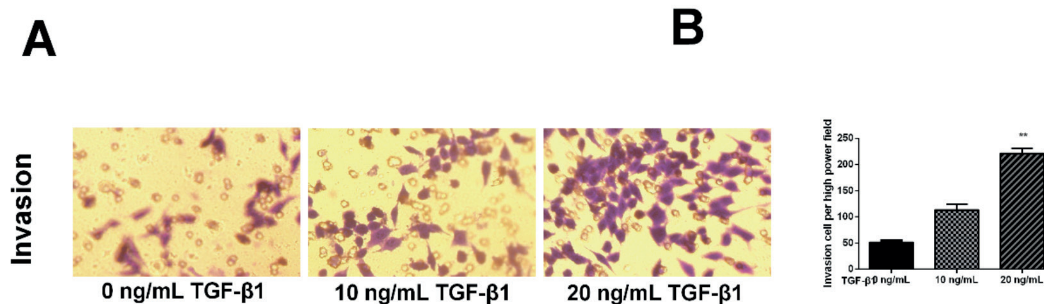

Figure 2. TGF-β1 enhances HCC cell line HepG2 invasion (crystal violet staining ×200). ***A,*** The transwell invasion assay in HepG2 cells treated with TGF-β1; ***B***, T quantitative result of transwell invasion assay in HepG2 cells.

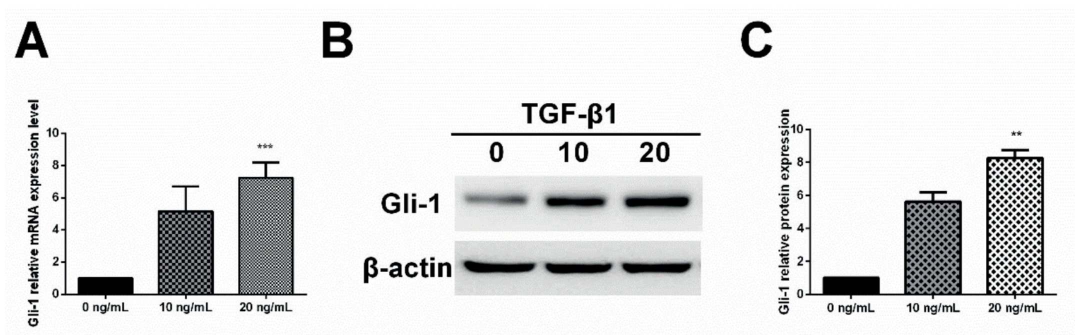

Figure 3. TGF-β1 upregulates the expression of GLI-1 in HCC cell line HepG2. ***A,*** RT-PCR for GLI-1 protein level in HCC cell line HepG2; ***B,*** Western blot assay for GLI-1 protein level in HCC cell line HepG2; ***C,*** Quantitative result of Western blot assay in HepG2 cells.

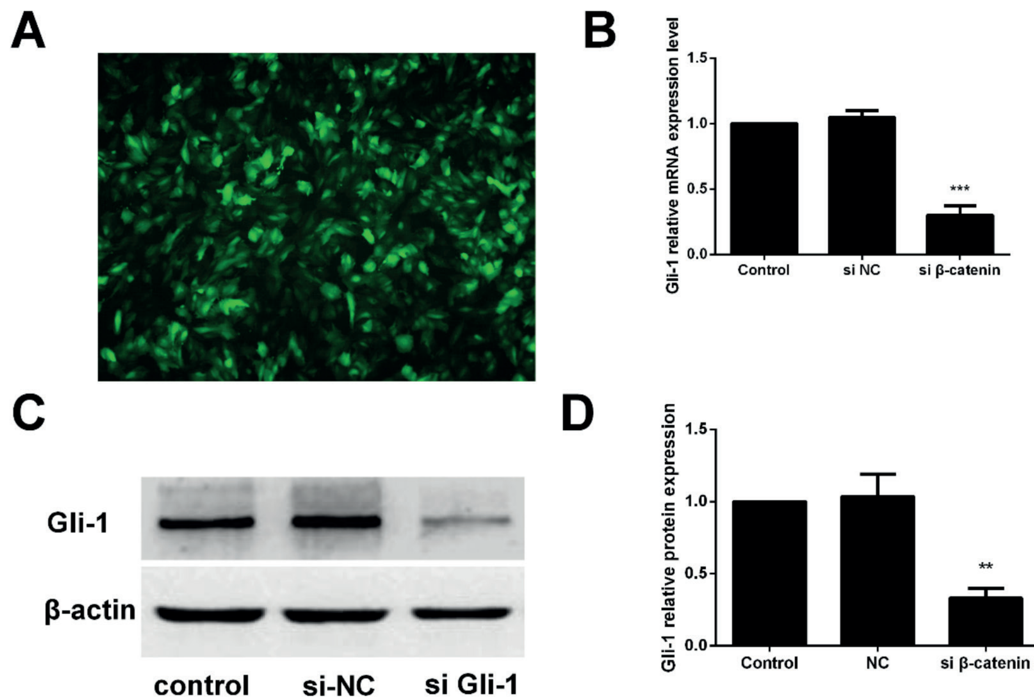

Figure 4. The effective expression of exogenous gene silence of GLI-1. ***A-B***. RT-PCR analysis of GLI-1 mRNA; ***C.*** Western blot analysis of GLI-1 protein; ***D,*** Quantification of Western blot results.

Effect of Gli-1 siRNA on the Gli-1 Signaling Pathway

48 hours after transfection with GLI-1-specific siRNA and GLI-1-NC-siRNA, we detected the mRNA and protein expressions of GLI-1 using RT-PCR and Western blotting assay. The results showed that, in comparison with the GLI-1 NC group and blank control group, mRNA and protein expressions of GLI-1 in the GLI-1 siRNA group were down-regulated significantly, and the difference had statistical significance (p<0.05; Figure 4).

Effect of Targeted Silencing of GLI-1 on TGF-β1-Mediated Proliferation of HpeG2 Cells

We detected the proliferation abilities of HpeG2 cells in the negative control group and TGF-β1 group. MTT results indicated that in comparison with the blank control group, TGF-β1 in concentration of 20 ng/mL could significantly enhance the proliferation ability with the lapse of culture time, and the difference had statistical significance (p<0.05). Cells transfected with GLI-1 siRNA exhibited a significant decrease in the proliferation ability, and the difference had statistical significance (p<0.05; Figure 5).

Effect of Targeted Antagonization of GLI-1 on TGF-β1-Mediated Invasion Ability of HpeG2 Cells

In the transwell assay, we found that after 24 h of treatment with TGF-β1 in concentration of 20

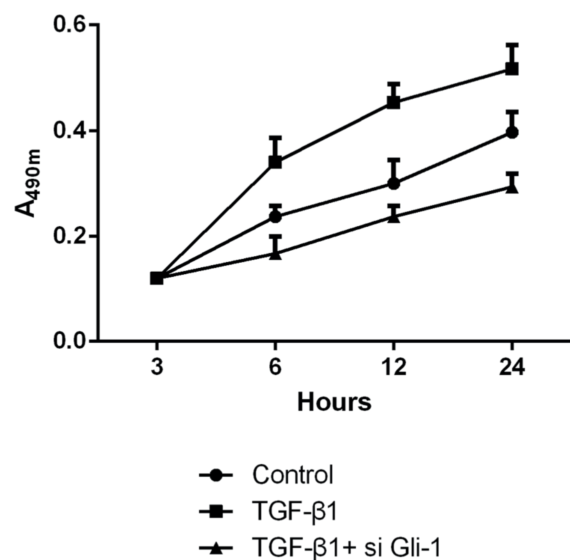

Figure 5. GLI-1 si-RNA inhibits TGF-β1 induced cell proliferation.

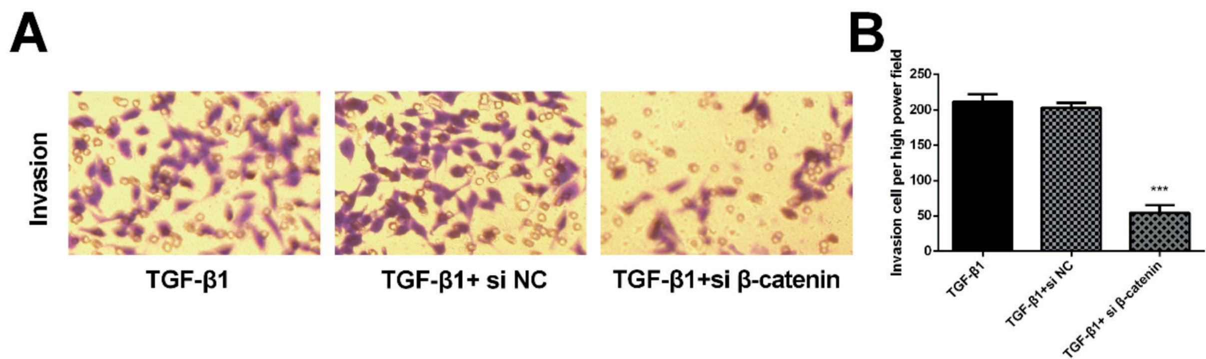

Figure 6. GLI-1 si-RNA attenuates TGF-β1 induced HepG2 cell invasion (crystal violet staining ×200). ***A-B,*** Transwell assay detect the invasion abilities of HepG2 and quantification of the results.

ng/mL, a significant increase was observed in the invasion ability of HepG2 cells in the si-NC group in comparison with the blank control group, and the difference had statistical significance ($p<0.05$). After transfection with GLI-1 siRNA, the invasion ability of cells was significantly curbed and the difference had statistical significance ($p<0.05$; Figure 6).

Discussion

HCC, a kind of malignant tumor originated from the epithelial cells or mesenchymal tissue in liver, usually manifests hidden symptoms in early stage, which are frequently ignored. In middle or advanced stage, patients suffer from symptoms like swelling pains in liver, weakness in limbs, emaciation or even progressive hepatomegaly[8,9]. Invasion and metastasis of malignant tumor are a complicated process that is regulated precisely, and tumor cells located in the primary lesion should pass through the basal membrane, then invade into the vessels or lymph-vessels, and migrate to the distant tissues or organs along the vessels, where tumor cells will be delivered outside the vessel for clone and proliferation to form new metastatic lesions[10,11]. Thus, invasion, migration and proliferation abilities of tumor cells are the major conditions guaranteeing the generation of metastatic lesions[12]. Hence, how to identify and block the proliferation and invasion of HCC cells, and the application of effective treatment method has become the key points in prophylaxis and treatment of HCC. In TGF-β superfamily, there are a variety of subtypes, in which TGF-β1, TGF-β2 and TGF-β3 are the most frequent types with highly-matched sequences and homology[13]. TGF-β1, mainly existing in the human, exerts a critical effect in the development and progression of malignant tumors[14]. Previous studies have shown that in multiple malignancies, TGF-β1 is up-regulated, and involved in the proliferation, differentiation and apoptosis in tumors[15,16]. However, in the development of malignancy, TGF-β1 has complicated but contradictory roles: on the one hand, in an early stage of tumor, TGF-β1 can be abnormally activated to inhibit the progression of tumor[17]; on the other hand, with the further development and progression of tumors, tumor cells are tolerant to the TGF-β1, resulting in a rapid growth of tumors, massive excretion of TGF-β1 and facilitating the formation of responsive tumor stroma[18]. In the development and progression of HCC, abnormally activated TGF-β1 plays a critical role, but the specific mechanism remains unknown. Hedgehog signaling pathway is involved in the proliferation, cyclin regulation, invasion and metastasis of malignant tumors[6,19]. As a critical downstream molecule in Hedgehog signaling pathway and a nuclear transcription factor, GLI-1 can activate the transcription of targeted gene in following modulating pattern: the ligand Shh, through binding with the receptor Ptch, can eradicate the inhibitory effect of Ptch on Smo, and Smo activation can further induce the transposition of GLI-1 into the nucleus to facilitate the transcription of its downstream gene, thus enhancing the invasion and infiltration of tumor cells[20]. Hence, not only GLI-1 is the key link in the Hedgehog signaling pathway, but also its up-regulation serves as the marker of the activation of this pathway. Researches

has shown that abnormal activation of GLI-1 gene has been found in a variety of malignant tumors, including ovarian cancer[21], gastric cancer[22], and liver cancer[23,24], suggesting that the activation of GLI-1 gene is closely associated with the development and progression of malignant tumors[25]. In clinical samples of human HCC, GLI-1 in the Hedgehog signaling pathway has a high positive expression rate, which is correlated with the tumor differentiation and lymphatic metastasis; thus, it serves as an indicator for evaluating the malignancy and prognosis of HCC[26,27]. Inhibiting the expression of GLI-1 can block the proliferation and apoptosis of hepatic astrocytes[28], and reduce the migration and invasion of HCC cells[29]. In addition, literature has reported that TGF-β1, through cross-talking with Hedgehog-GLI-1 signaling pathway, can regulate the phenotype trans-differentiation and collagen accumulation in renal tubular epithelial cells. Therefore, based on the previous studies, we inferred that TGF-β1 can promote the proliferation and distant invasion of HCC through inducing the expression of GLI-1 in human HCC. In the *in-vitro* experiment, we verified the effects of TGF-β1 in varying concentrations on the proliferation and invasion abilities of HepG2 cells. Results showed that after cells treated with TGF-β1 in varying concentrations of 0, 10 and 20 ng/mL for 24 h, the proliferation rates of cells were significantly accelerated in comparison with the blank control group with a significantly increased invasion ability in a concentration-dependent manner. Sequentially, we investigated how TGF-β1 induces the proliferation and invasion of HepG2 cells, and found that in comparison with the blank control group, after 48 hours of treatment using TGF-β1, mRNA and protein expressions of GLI-1 in HepG2 cells were significantly up-regulated, suggesting the potential involvement of abnormally activated GLI-1 in the TGF-β1-induced proliferation and invasion of HepG2 cells. To clarify the action mechanism of GLI-1 gene, we adopted GLI-1 siRNA to block its expression, and further detect its effects on the proliferation and invasion of cells. Results prompted that after targeted silencing, TGF-β1-induced proliferation and invasion of HepG2 cells were curbed, suggestive of the critical role of GLI-1 in this process. In light of the results above, we inferred that TGF-β1 can modulate the activation of transcription of downstream targeted genes through activating the GLI-1 in Hedgehog signaling pathway, thereby promoting the abnormal proliferation and distant invasion and metastasis of HepG2 cells, so as to facilitate the development and progression of HCC.

Conclusions

We preliminarily found that in HepG2 cell strain, TGF-β1 can activate the GLI-1, a key transcription factor in Hedgehog signaling pathway, to promote the abnormal proliferation and distant invasion of HepG2 cells, which is conducive to deepening the understanding on the pathogenesis of HCC. Thus, in-depth studies on the roles of GLI-1 gene in development, progression and modulating mechanism of HCC to block the expression of GLI-1 and inhibit the proliferation and distant invasion of HepG2 will provide new ideas and evidence for specific treatment of HCC, which has a widespread clinical significance and promising prospects in application.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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