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Original Article

Growth differentiation factor 15 (GDF15) contributes to invasion and anti-anoikis of hepatocellular cancer through TGF-β/Smad-associated signaling

Ming-Ze Ma[1*], Xiao-Hua Zhang[2*], Bin Li[2], Tie-Jun Liang[3], Chong-Mei Yang[2], Ji-Yong Liu[2]

Departments of [1]Infectious Diseases, [2]Gastroenterology, [3]Gastroenterology in Health Care Building, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong Province, China. [*]Equal contributors.

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Abstract: Hepatocellular carcinoma (HCC) is a common primary liver tumor featured by high morbidity and mortality. Hepatocellular carcinoma is also characterized by high metastatic capacity. Prognosis of HCC is still very poor partly due to few effective treatment measures available. It is an urgent matter to find new therapeutic targets for prevention of HCC metastasis. The present study demonstrates that oncogene GDF15, a member of TGF-β superfamily of cytokines, was significantly upregulated in HCC tissues compared to corresponding adjacent para-carcinomatous tissues. Furthermore, findings revealed that knockdown of GDF15 significantly inhibited HCC cell invasion but promoted cell anoikis *in vitro*. The prometastatic effects of GDF15 in HCC were performed through activating TGF-β/Smad signaling. Present findings demonstrated that GDF15 plays an important role in promoting HCC cell invasion and metastasis, perhaps acting as a therapeutic target against HCC metastasis in the future.

Keywords: GDF15, hepatocellular carcinoma, tumor invasion, anoikis

Introduction

Hepatocellular carcinoma is a common solid tumor, worldwide, especially in East Asia, with low survival rates and poor prognosis [1]. Hepatitis B and hepatitis C virus infections, Aflatoxin B1, and alcohol use are major causes of hepatocellular carcinoma (HCC). Prognosis of HCC can be remarkably improved by early diagnosis and treatment [2]. Progression of HCC can be affected by tumor cell and tumor microenvironment. The tumor microenvironment of HCC is composed by tumor-associated fibroblasts, endothelial cells, immune cells, extracellular matrix, secreted protein, ions, hypoxia, PH value, and cytokines [3]. Extracellular secreted protein plays an important role in regulating HCC proliferation, apoptosis, anoikis, and invasion. It may be chosen as a treatment target against HCC progression [4-6]. It has been reported that growth differentiation factor 15 (GDF15) protein expression in HCC was significantly higher than in corresponding adjacent paracarcinomatous tissues and normal livers [7]. GDF15, also known as macrophage inhibitory cytokine-1 or nonsteroidal anti-inflammatory drug-activated gene, belongs to TGF-β superfamily of cytokines [8]. Interestingly, serum GDF15 levels have been associated with poor prognosis of prostate, colorectal, pancreatic, lung, melanoma, and breast cancer [9-16]. MHCC97H and HCCLM3 cells have higher metastatic capacity, compared to HepG2 or SMMC7721 cells [17]. This study detected expression of GDF15 in several HCC cell lines, finding that its expression in MHCC97H and HCCLM3 cells was significantly higher than in HepG2 or SMMC7721 cells. Results suggest that GDF15 may play a role in facilitating HCC cell migration and invasion.

The present study also detected whether GDF15 could affect HCC cell anoikis. Results showed that HCC cell anoikis was promoted by GDF15 knockdown in MHCC97H and HCCLM3 cells. These data suggest that GDF15 may act as tumor promoting factor in HCC progression.

Materials and methods

Clinical sample collection

All paired HCC and corresponding adjacent paracarcinomatous tissues were obtained from

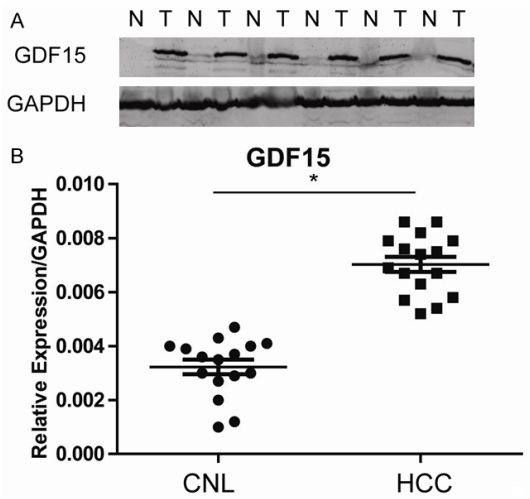

Figure 1. GDF15 expression in HCC tissues and corresponding adjacent paracarcinomatous tissues was detected by Western blot assay and qPCR assay. GDF15 expression was higher in tumor tissues than non-tumor tissues according to Western blot (A) and qPCR (B) results.

surgical samples at the Department of Liver Surgery, Shandong Provincial Hospital. Approval was obtained from the Regional Ethical Committees, Shandong Provincial Hospital, Jinan, China, for the use of clinical liver tissues.

Real-time polymerase chain reaction

Total tissue RNA was extracted from HCC tissues and corresponding noncancerous liver tissues using T-PER, tissue protein extraction reagent (Thermo Fisher Scientific). Total cell RNA was prepared using TRIzol Reagent (Takara) chloroform and isopropanol, step by step. Gene expression was measured with an Applied Biosystems 7300 Real-Time PCR System using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA) with SYBR Green ROX Mix (Thermo Scientific) and primers (Sangon Biotech, Shanghai China) at a final concentration of 0.4 μM. Primer sequences were: GDF15 Forward: TCAGAT GCTCCTGGTGTTGC; Reverse: GATCCCAGCCGCACTTCTG; GAPDH, Forward: ACCACAGTCCATGCCATCAC; Reverse: TCC ACCACCCTGTTGCTGTA.

Immunohistochemistry staining

HCC tissue histologic sections and corresponding noncancerous liver tissues sections were baked at 56°C for one hour before de-paraffinizing. All histologic sections were de-paraffinized through xylene, 50% xylene, and gradient ethanol until immersed in pure water. Histologic sections were incubated in 0.3% hydrogen peroxide solution for 30 minutes at room temperature. Antigen retrieval was performed using citric acid buffer for one hour. The histologic sections were then blocked using bovine serum albumin at room temperature for one hour. Next, histologic sections were incubated with GDF15 antibody (Anti-GDF15, purified from goat serum, polyclonal, 1/200 dilution, ab39999, Abcam, UK) at 4°C for overnight. The next day, histologic sections were washed using phosphate buffer solution three times and then incubated with HRP-coupled anti-goat secondary antibody (1:200 dilution, Dako, USA) for one hour at room temperature. Immunostaining was performed using diaminobenzidine substrate chromogen (Dako, USA) and the chromogenic reaction was carefully controlled under a microscope. Sections were immersed into hematoxylin for nuclear staining for about 2 minutes. Sections were then dehydrated using ethanol of gradient concentration and cleared with xylene. IHC staining results were judged by two experienced pathologists, independently, according to the following criterion: 0-5% positive cells scored 0; 6-35% positive cells scored 1; 36-70% positive cells scored 2; 70-100% positive cells scored 3; scores of 0 or 1 were identified as low expression; scores of 2 or 3 were identified as high expression.

Western blot

Total proteins of HCC tissues and corresponding adjacent paracarcinomatous tissue were extracted using T-Per tissue protein extraction reagent (Thermo Scientific), following manufacturer instructions. Moreover, proteins of HCC cells were extracted using IP lysis buffer (Beyotime Jiangsu Province, China). Before Western blotting, protein lysates were detected quantitatively using the BCA method. For Western blot assay, protein lysates were added into SDS-PAGE gel and electrophoresed under constant voltage condition. Proteins were then transferred from gel to nitrocellulose membranes (Millipore) using the electrophoretic transfer method under constant current condi-

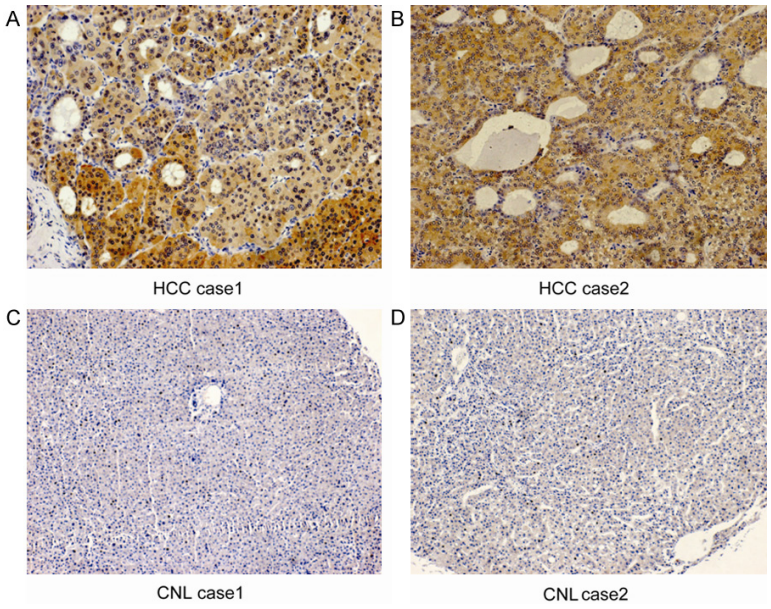

Figure 2. Immunohistochemical analysis of GDF15 expression in HCC tissues (A, B) and corresponding adjacent paracarcinomatous tissues (C, D). Original magnification, 200×.

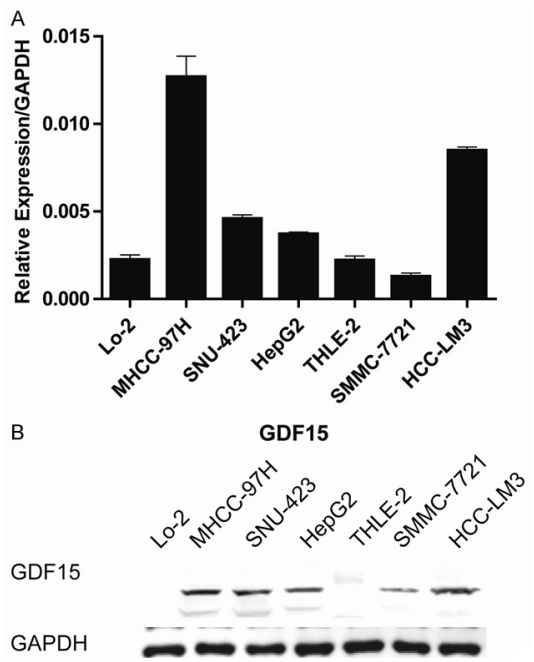

Figure 3. MicroRNA levels of GDF15 in HCCLM3, Lo-2, MHCC-97H, SNU-423, THLE-2, SMMC-7721, and HepG2 cells were detected by quantitative real-time PCR, normalized to GAPDH (A). Expression of GDF15 in hepatocellular carcinoma cell lines. Lysates from HCCLM3, Lo-2, MHCC-97H, SNU-423, THLE-2, SMMC-7721, and HepG2 cells were subjected to SDS-PAGE, followed by Western Blotting for GDF15. GAPDH was chosen as loading control (B).

tions. Next, NC membranes were blocked using 5% fat-free milk (BD) for one hour at room temperature. After blocking with milk, NC membranes were immersed into anantibodysolution(GDF15,ab39-999, Abcam, UK; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Proteintech Group, Chicago IL; Smad1/5/9, Smad1/5/9 Antibody Sampler Kit, CST; Smad1, Smad1 (D59D7) Rabbit mAb, CST; Smad2, Smad2 (D43B4) Rabbit mAb, CST; Smad3, Smad3 (C67H9) Rabbit mAb, CST; Phospho-Smad1/Smad5/Smad9, Phospho-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad9 (Ser465/467) (D5B10) Rabbit mAb, CST; Smad5, Smad5 (D4G2) Rabbit mAb, CST; Phospho-Smad2, Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) (D27F4) Rabbit mAb, CST; Phospho-Smad3, Phospho-Smad3 (Ser423/425) (C25A9) Rabbit mAb, CST) at 4°C overnight. The next day, NC membranes were gently washed using TBS (Tris Buffer Solution) three times and then incubated with fluorescent-antibodies, such as IRDye 800 anti-rabbit (LI-COR, Lincoln, NE) or IRDye 680 antimouse (LI-COR, Lincoln, NE), for one hour at room temperature. NC membranes were then gently washed using TBS supplemented with Tween-20 three times away from light. Finally, fluorescence signals were detected using Odyssey infrared imaging system (LI-COR, Lincoln, NE). Grey values were quantitatively analyzed and recorded by Image J software.

GDF15-shRNA construction and cell transfection

Sequences for GDF15-shRNA were as follows: GDF15-shRNA-F: CCGGGCTCCAGACCTATGATGACTTCTCGAGAAGTCATCATAGGTCTGGAGCTTTTTG; GDF15-shRNA-R: AATTCAAAAAGCTCCAGACCTATGATGACTTCTCGAGAAGTCATCATAGGTCTGGAGC; The pLKO.1 lentivirus vector was used to construct shRNA vector and lentiviruses were generated by co-transfecting 293T

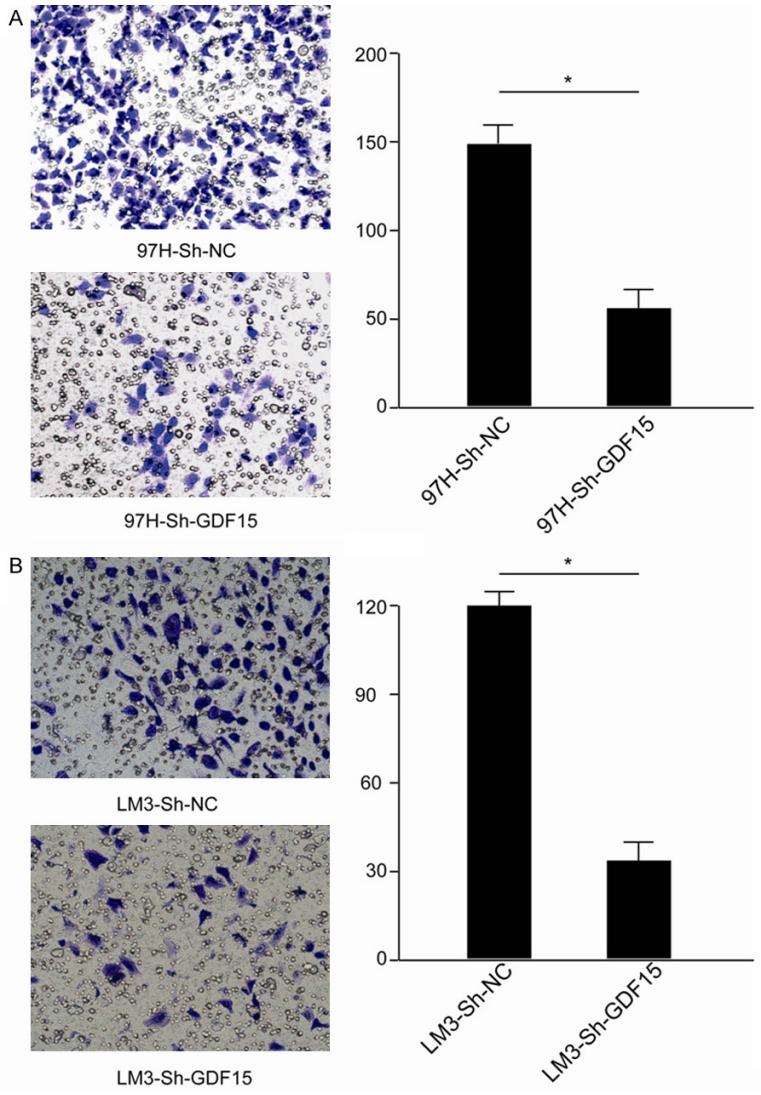

Figure 4. Silencing of GDF15 inhibits hepatocellular carcinoma cell migration *in vitro*. HCCLM3 and MHCC97H cells migrating onto the lower surface of Transwell membranes were stained with crystal violet. There were fewer migrated cells in the GDF15 shRNA groups than negative control shRNA groups.

cells using Lipofectamine 2000 (Invitrogen), according to manufacturer instructions. The virus solution generated by 293T cells was used to transfect HCC cells with the assistance of polybrene.

The full CDS sequence of GDF15 was amplified and cloned into p3xFLAG-CMV-14 (Sigma). Moreover, 10 ng/mL of recombinant human GDF15 protein (Peprotech, 120-28) was used to treat cells.

HCC cell culture

HCC cell lines were cultured *in vitro* at 37°C, 100% humidity, and 5% CO_2 condition in Dulbecco's modified Eagle medium (DMEM), supplemented with 5-10% fetal bovine serum. Cell culture medium was added with penicillin and streptomycin.

Cell migration and invasion assay

To evaluate the migration and invasion capacity of HCC cells, this study equipped 24-well plates with cell culture inserts containing 8.0 μm pore size membranes (Costar Corp., Cambridge, MA, USA). For cell migration assay, cell culture inserts were used without Matrigel added. However, Matrigel (1:5 dilution with DMEM, BD Biosciences, Bedford, MA, USA) was added into the upper chamber of cell culture inserts 30 minutes before cell invasion assay began. Briefly, cells were suspended in 100 μl of serum-free medium and placed in upper compartment chambers. The lower chamber was filled with 10% FBS as the chemoattractant. Next, added 5×10^4 cells per 100 μl into the upper chamber of cell culture inserts for migration assay and 1×10^5 cells per 100 μl into the upper chamber of cell culture inserts for cell invasion assay. Finally, residual cells were cleared on the upper surface of cell culture inserts and inserts were washed using phosphate buffer three times. Cells were then fixed on the lower surface using 4% paraformaldehyde and were stained by 0.1% crystal violet. All stained cells were counted or photographed in six fields under 100× microscope. Assays were carried out in triplicate wells and repeated three times.

Cell proliferation assay

HCC cell proliferation was measured using Cell Counting Kit-8 reagent (CCK-8, Dojindo, Japan). HCC cells were seeded into 96-well plates at density of 2000 per 100 μl and cultured at 5%

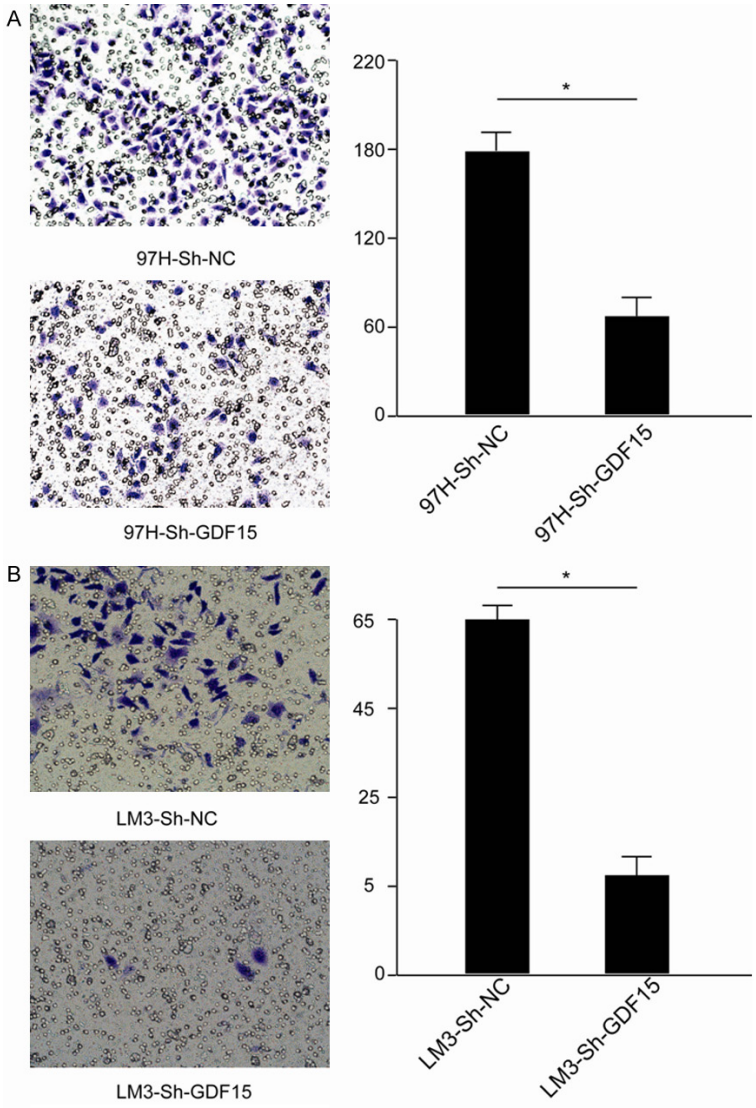

Figure 5. Silencing of GDF15 inhibits hepatocellular carcinoma cells invasion *in vitro*. HCCLM3 and MHCC97H cells invading through the Matrigel and onto the lower surface of Transwell membranes were stained with crystal violet. There were fewer invaded cells in the GDF15 shRNA groups than negative control shRNA groups.

CO_2, 100% humidity, and 37°C conditions. For the detection step, 10 μl CCK-8 reagent was added into each well and incubated for one hour. WST-8, a major ingredient of the CCK-8 reagent, can be oxidized to produce a colorimetric dye and be detected at 450 nm using a microplate reader (BIO-TEK).

Flow cytometry

Cell anoikis assay was performed using propidium iodide (PI) staining and Annexin V-FITC staining method. All cells were seeded into 6-wells plates. Plates were covered with poly-HEMA to maintain a suspended culture state for at least 48 hours. Next, suspended culture cells were collected and stained using PI and Annexin V-FITC protected from light. Finally, the anoikis rate of each group was analyzed using flow cytometry assay.

Statistical analysis

SPSS 16.0 software was used to analyze the statistical significance of differences in this study. Statistical differences were calculated using two-tailed Student's t-test. $P<0.05$ is considered statistically significant and $P<0.01$ is considered very statistically significant.

Results

GDF15 expression is elevated in HCC tissues

This study detected expression of GDF15 in HCC tissues and corresponding adjacent paracarcinomatous tissues in mRNA and protein levels. Results showed that both GDF15 mRNA and protein expression levels in HCC tissues were remarkably higher than in corresponding adjacent paracarcinomatous tissues. Results illustrated that GDF15 was upregulated in HCC tissues compared to corresponding adjacent paracarcinomatous tissues (**Figures 1**, **2**).

GDF15 is upregulated in HCC cells with high metastatic capacity

Compared to HepG2 and SMMC7721 cells, MHCC97H and HCCLM3 cells had higher metastatic capacity. GDF15 expression was detected in HCC cell lines and non-HCC cell lines (Lo2, immortal liver cell), finding that GDF15 was highly expressed in MHCC97H and HCCLM3

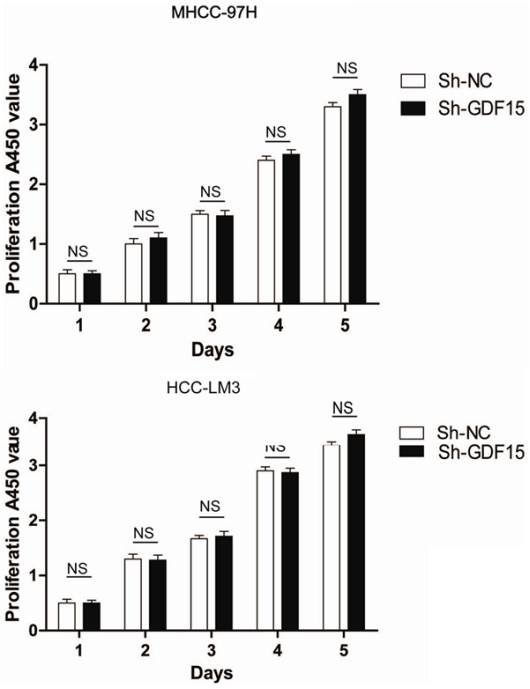

Figure 6. Effects of GDF15 knockdown on hepatocellular carcinoma cell proliferation (HCCLM3 and MHCC97H cell lines). Equal amounts of cells were seeded initially and the OD450nm value were detected by CCK-8 assay for up to five days.

cells, but downregulated in HepG2 and SMMC7721 cells. Moreover, mRNA expression levels of GDF15 in HCC cell lines were generally consistent with protein expression status. These results demonstrate that GDF15 may play a role in facilitating motility of HCC cells (**Figure 3**).

GDF15 knockdown in HCC cells inhibits cell migration and invasion in vitro

Next, cell migration and invasion assays were performed using lentivirus-mediated shRNAs assays, which targeted GDF15 expression in HCC cells. Results showed that expression of GDF15 was significantly reduced by GDF15-shRNA, in both mRNA and protein levels, in MHCC97H and HCCLM3 cells. Additionally, GDF15-shRNA remarkably inhibited migration and invasion of MHCC97H and HCCLM3 cells *in vitro* (**Figures 4**, **5**).

GDF15 knockdown in HCC cells has no influence on HCC cell proliferation in vitro

To detect the roles of GDF15 knockdown in HCC cell proliferation, CCK-8 assay was used to test cell proliferation. All GDF15-shRNA HCC cells and control HCC cells were seeded into 96-well plates in the same density and incubated for five days. Afterward, this study detected A450 values of each group every day and recorded the data for statistical comparison. Finally, it was found that knockdown of GDF15 in MHCC97H and HCCLM3 cells had no influence on proliferation of these cells (**Figure 6**).

GDF15 knockdown in HCC cells promotes HCC cell anoikis

Anoikis pertains to programmed cell death induced by cells separated from extracellular matrix. It plays and important role in tumor metastasis. PI and Annexin V staining methods were used to detect the influence of GDF15 knockdown on HCC cell anoikis. It was found that knockdown of GDF15 significantly promoted HCC cell anoikis, compared to the control group (**Figure 7**).

GDF15 promotes cell migration, invasion, and anoikis perhaps through activating TGF-β signal pathways

GDF15 is a member of the bone morphogenetic protein subfamily of the TGF-β superfamily. To identify whether GDF15 could activate TGF-β signal pathways, expression change of phospho-Smad2 and Smad3 was detected. Results showed that rhGDF15 promoted phosphorylation of Smad2 and Smad3 in MHCC-97H cells. Compared to the control group, GDF15-shRNA HCC cells showed downregulation of phospho-Smad2 and phospho-Smad3 expression but no significant changes in phospho-Smad1/5/8 expression levels. Therefore, GDF15 promoted HCC invasion and inhibited cell anoikis through activating TGF-β signal pathways (**Figure 8**).

Discussion

Hepatocellular carcinoma (HCC) has been associated with high mortality worldwide [18]. Cancer metastasis is one of the major causes of human HCC-related death. The tumor microenvironment plays an important role in affecting tumor cell invasion and metastasis [19]. GDF15 is a component of the tumor microenvironment which plays important roles in gastric cancer, epithelial ovarian cancer, and head and neck cancer progression [20]. GDF15 was first isolated from macrophages after cytokine stim-

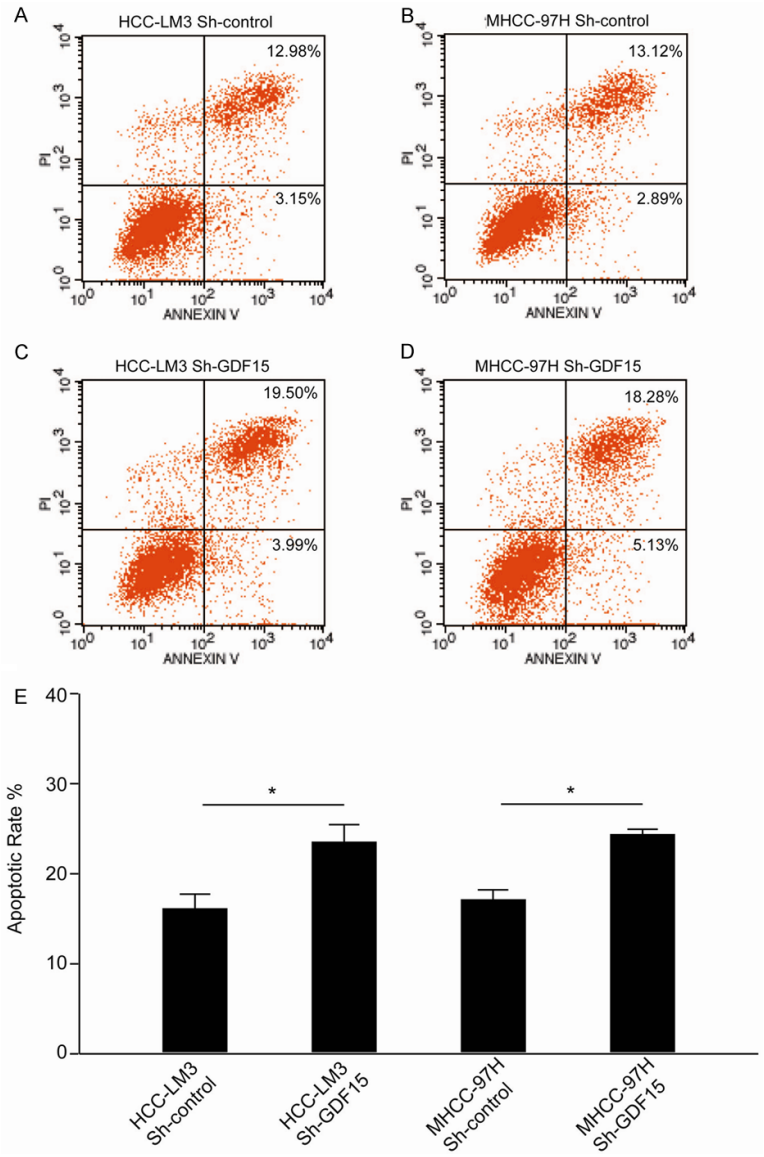

Figure 7. Effects of GDF15 knockdown on hepatocellular carcinoma cell anoikis (HCCLM3 and MHCC97H cell lines). Results showed that GDF15 knockdown promoted HCCLM3 (A, C, E) and MHCC97H (B, D, E) cell anoikis.

ulation. It has been reported that GDF15 expression can be regulated by several signal pathways, including p53/DEC1, GSK-3β, and EGR-1 pathways, playing an important role in regulating tumor progression [21].

Research by Xiuying Liu et al. revealed that GDF15 protein expression in HCC was significantly higher than that in corresponding adjacent paracarcinomatous tissues and normal livers [22]. It has been demonstrated that GDF15 overexpression in Huh7.5.1 cells resulted in increased DNA synthesis, cell proliferation, and enhanced invasiveness of HCC cells [23]. Therefore, elucidation of the biological functions of increased GDF15 in liver disease pathogenesis may promote the potential application of GDF15 in diagnosis and targeted therapy. However, a study by Zimmers TA et al. showed that genetic ablation of GDF15 in mice had no apparent effects on HCC tumor formation rate, growth rate, or invasiveness *in vivo* [24]. Present study results showed that GDF15 was upregulated in HCC tissues and promoted HCC cell invasion, but inhibited HCC cell anoikis. Anoikis is the process by which cells are triggered to die when separated from their surrounding matrix and neighboring cells [25, 26]. The metastatic capacity of tumor cells can be significantly promoted when cells gain anoikis-resistance [27, 28]. The present study showed that knockdown of GDF15 significantly improved anoikis rates of HCC cells. Results suggested that GDF15 participates in HCC cell anoikis-resistance and promotes cell metastasis. Therefore, GDF15 plays tumor-promoting roles in HCC progression.

Like other TGF-β superfamily cytokines, GDF15 is synthesized as a precursor protein [29, 30]. N-terminal propeptides originating from mature GDF15 through proteolysis can be secreted as disulfide-linked homodimer [31, 32]. The membrane receptors of GDF15 have not been identified. It has been reported GDF15 could promote human prostate cancer cells metastasis through FAK-RhoA pathways [33-35]. Moreover, it has been reported that GDF15 activates smad2 and smad3 through TGF-β I type receptors and promotes colorectal cancer metastasis [36]. The present study demonstrated that GDF15 promotes phospho-Smad2 and Smad3 expression in HCC cells. Knockdown of GDF15 resulted in downregulation of phos-

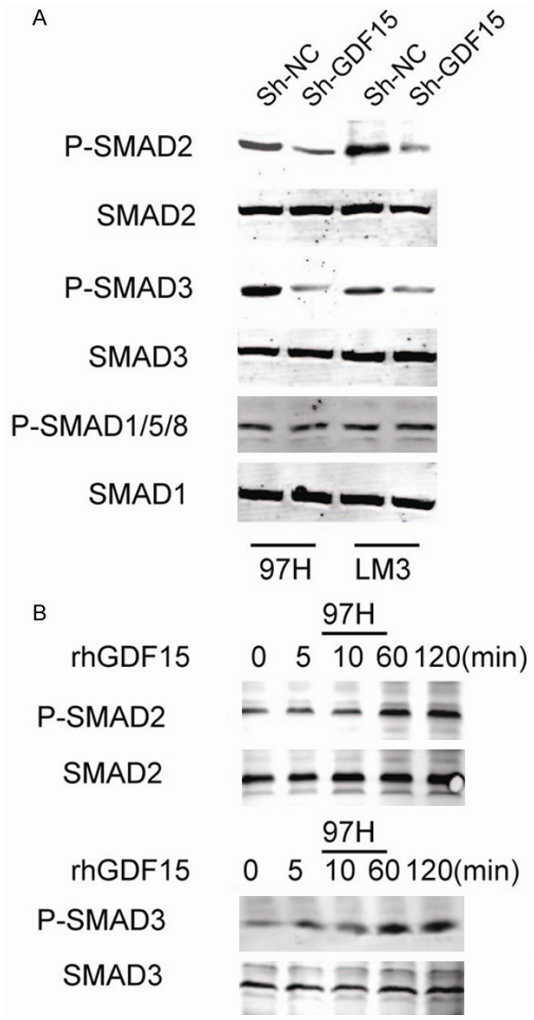

Figure 8. A. GDF15 knockdown in HCCLM3 cells and MHCC97H cells resulted in downregulation of phospho-Smad2 and phospho-Smad3 expression. B. rhGDF15 protein promoted phospho-Smad2 and phospho-Smad3 expression in HCC cells.

pho-Smad2 and Smad3 expression in HCC cells. Therefore, present results demonstrate that GDF15 promoted HCC cell invasion and metastasis through activating TGF-β/Smad2/Smad3, but not Smad1/5/8 signal pathways. Nevertheless, it has been reported that GDF15 may also have antitumorigenic and proapoptotic activities, mainly due to regulation by PI3K/AKT/GSK-3β pathways and tumor suppressors EGR-1 and p53 pathways [37-40]. Regulation of tumor initiation and metastasis is extremely complex [41]. Sometimes, candidate oncogenes like TGF ligands may play contradictory roles in tumor initiation stage and progression stage [42-46]. Present results demonstrated that GDF15 can function as a prometastatic factor in HCC progression.

In conclusion, present findings reveal that GDF15 promotes HCC cell invasion and metastasis via activating TGF-β/Smad signaling. Moreover, further research concerning how GDF15 regulates TGF-β/Smad pathways and other signaling is necessary.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Ming-Ze Ma, Department of Infectious Diseases, Shandong Provincial Hospital Affiliated to Shandong University, 324 Jing Wu Road, Jinan 250021, Shandong Province, China. Tel: +86-13793179830; +86-531-68776450; E-mail: qipengmay@163.com

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