

Insulin-like growth factor-1 induces epithelial-mesenchymal transition in hepatocellular carcinoma by activating survivin

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Received December 21, 2017; Accepted May 31, 2018

DOI: 10.3892/or.2018.6516

Abstract. Insulin-like growth factor-1 (IGF-1), a small polypeptide hormone similar to insulin in protein structures, has been identified as an activator of epithelial-mesenchymal transition (EMT) pathways in several types of cancers. As a member of the inhibitor of apoptosis protein (IAP) family, survivin is implicated in the EMT of some cancers. However, the role of survivin on IGF-1-mediated EMT of hepatocellular carcinoma (HCC) has not been clarified. In the present study, we demonstrated that survivin was involved in the EMT process induced by IGF-1 in HCC cell line SMMC7721. With administration of different concentrations of IGF-1, survivin mRNA and protein expression were significantly increased and stimulated EMT in the tested cell line, while the increased invasive and migratory abilities of HCC cells and activation of the EMT process induced by IGF-1 were reversed after silencing of survivin expression by transfecting small interfering RNA. This was further confirmed by the observation of morphological changes, the decrease of invasive and migratory abilities and the downregulation of EMT markers, N-cadherin, vimentin and Snail, and the upregulation of E-cadherin. In conclusion, survivin may play a vital role in the IGF-1 signaling pathway by mediating EMT in HCC through the upregulation of the expression of EMT markers, and the knockdown of survivin expression may suppress the metastasis of HCC, which may provide new insights for the molecular therapy of HCC patients in clinical treatment.

Introduction

Liver cancer is the fifth most common cancer in men and the seventh in women. Hepatocellular carcinoma (HCC), which

accounts for >85% of primary liver cancers, has a poor prognosis with 5-year overall survival rates of <12% (1,2). Since its poor prognosis has been reported to be closely associated with HCC recurrence and metastasis, it is essential to determine the possible underlying mechanisms which mediate tumor invasion and metastasis. EMT has been demonstrated to be involved in the progression of various cancers, including liver, prostate and breast cancer (3-5), and functions as a main step toward tumor metastasis. To date, increasing studies have been directed at uncovering the possible signaling pathways in EMT of HCC.

IGF-1 has been demonstrated to be upregulated in many different tumor cell lines compared with normal cells and involved in tumorigenesis and progression, which is mediated through the activation of multiple signal transduction pathways, including the JNK, MAPK, PI3K/Akt pathways (6). IGF-1 was found to elevate the expression of transmembrane glycoprotein MUC1 in MCF-7 cells for the initiation of EMT in a PI3K/Akt signaling pathway-dependent manner (7). Furthermore, it was reported that IGF-1 could promote the growth and metastasis of HCC cell lines via the upregulation of cathepsin B expression (8). As a member of the inhibitor of apoptosis protein (IAP) family, survivin is overexpressed in some tumor specimens, including HCC, while it is nearly negative in normal tissue (9). In human sacral chondrosarcoma, SDF-1/CXCR4 signaling could upregulate the expression of survivin via the MEK/ERK and PI3K/AKT pathways, leading to cell cycle and EMT occurrence (10). Overexpression of survivin in HCC cells was revealed to suppress the ability of migration via upregulation of glucose-regulated protein 78 (GRP78) and reduce the EMT marker, vimentin (11). Previous studies have revealed that high levels of survivin exhibited anti-apoptotic and pro-metastatic potential in cancer cell lines but not in normal cells (12).

Although both IGF-1 and survivin could mediate metastasis in cancer cells, the mechanisms by which they co-regulate metastasis have not been uncovered. In the present study, we used various molecular and cellular methods to investigate the existence and significance of the relationship between the IGF-1 and survivin proteins. Our data elicited a new mechanism in which IGF-1 induced EMT through regulation of survivin and a downstream pathway, and this can be targeted to treat HCC patients.

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Key words: insulin-like growth factor 1, epithelial-mesenchymal transition, survivin, hepatocellular carcinoma

Materials and methods

Antibodies and reagents. Monoclonal rabbit antibodies against survivin (1:5,000; cat. no. 2808), Akt (1:2,000; cat. no. 2920), p-Akt (1:5,000; cat. no. 96115), Snail (1:500; cat. no. 3879), vimentin (1:1,000; cat. no. 5741), E-cadherin (1:1,000; cat. no. 14472), N-cadherin (1:1,000; cat. no. 13116) and GAPDH (1:5,000; cat. no. 5174) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). IGF-1 was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). DAPI was purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Carlsbad, CA, USA).

Cell culture. Human HCC SMMC7721 cells were cultured in RPMI-1640 medium (Hyclone Laboratories; GE Healthcare, Chicago, IL, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO₂.

Transfection of survivin siRNA. SMMC7721 cells were seeded in a 6-well plate and adjusted to a density of 5x10⁵ cells/well and incubated at 37°C in a CO₂ incubator until the cells reached 60–80% confluence. Survivin siRNA or control siRNA was provided by Shanghai GenePharma Co., Ltd., (Shanghai, China). The survivin or control siRNAs were subjected to Opti-MEM with Lipofectamine 2000 (Invitrogen) for transfection. Following a 6-h incubation, the medium was replaced with fresh DMEM (10% FBS) and cells were collected for further experiments after 72 h of culture.

Wound healing assay. HCC cell migration was examined by a wound-healing assay. SMMC7721 cells were cultured to a confluent monolayer in a 6-well plate. A scratch (wound) was introduced in the confluent cell layer using a pipette tip. The cells were washed three times with PBS to remove detached cells. The cells were then incubated with different doses of IGF-1 for 24 h, and images of a defined wound spot were captured with a phase-contrast microscope (Olympus Corp., Tokyo, Japan) at 0 and 24 h. The width of the wound was assessed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The distance of the wound was calculated as: Distance of the wound = distance at 0 h - distance at 24 h.

Transwell filter cell migration and invasion assays. Boyden chambers containing polycarbonate filters with 8-µm pore size (Costar Group, Bodenheim, Germany) were employed. Cells were seeded at a density of 5x10⁵ cells/ml. To initiate the migration assay, cells (5x10⁴) in 10 µl of DMEM without FBS were added to the upper chamber, and the lower chamber was filled with 600 µl of DMEM with 10% fetal calf serum (FCS). For the invasion assay, Matrigel was introduced in DMEM (4:3) in the inner chamber. IGF-1 was used as an inducer of cell migration and the cells were allowed to migrate for 12 h at 37°C. Cells on the filter were first stained with crystal violet, and the cells that remained on the upper surface of the filter were removed using a cotton swab. The cells that had migrated onto the lower surface of the filter were examined using a microscope (Olympus Corp.) after mounting them onto a slide. A total of six random fields (magnification, x100) per filter were

photographed. Experiments were carried out in triplicate with consistent results.

RNA extraction and quantitative real-time PCR. RNA extraction was prepared using TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. Total RNA (1 µg) was used to synthesize the first strand of cDNA using Bestar qPCR RT kit (DBI Bioscience, Ludwigshafen, Germany). The mRNA expression was evaluated by real-time qPCR on the Stratagene Mx3000P real-time PCR platform (Agilent Technologies, Santa Clara, CA, USA) with SYBR-Green PCR core reagents. The PCR reaction system contained 10 µl of Bestar[®] SYBR Green qPCR Master Mix, 1.0 µl of forward and reverse primers (10 µM), 1 µl of cDNA template, and 8 µl of ddH₂O. GAPDH was applied as a reference. The following primers were synthesized and applied: survivin forward, 5'-TCAAGGACCACCGCATCT-3' and reverse, 5'-CGCACTTTCTCCGCAGTT-3'; Snail forward, 5'-TCC TTCGTCCTTCTCCTCTAC-3' and reverse, 5'-TGTGGCTTC GGATGTGC-3'; E-cadherin forward, 5'-CCGATCTTCAAT CCCACC-3' and reverse, 5'-CCCACGCCAAAGTCCTC-3'; vimentin forward, 5'-CGCCAGATGCGTGAAAT-3' and reverse, 5'-CACGAAGGTGACGAGCC-3'; N-cadherin forward, 5'-GGA TCAAAGCCTGGAACAT-3' and reverse, 5'-CTTGGAGCC TGAGACACGA-3'; GAPDH forward, 5'-TGTTTCGTCATGG GTGTGAAC-3' and reverse, 5'-TGTTTCGTCATGGGTGTG AAC-3'. The reaction procedure was initiated with denaturation at 94°C for 2 min and followed by 40 repeated cycles (denaturation at 94°C for 20 sec, annealing at 58°C for 20 sec and extension at 72°C for 20 sec). The Ct-value for each sample was calculated with the $\Delta\Delta C_q$ -method, and the results were expressed as 2^{- $\Delta\Delta C_q$} to analyze the fold change (13).

Western blotting. HCC cells were lysed with lysis buffer containing 135 mM NaCl, 20 mM Tris (pH 7.5), 25 mM β-glycerophosphate, 2 mM EDTA, 2 mM sodium pyrophosphate, 2 mM DTT, 10% glycerol, 1% Triton X-100, 10 mM NaF, 1 mM sodium orthovanadate and 1 mM PMSF supplemented with a complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) at 4°C. Lysates were centrifuged (15,000 x g) at 4°C for 15 min. Equal amounts of the soluble protein were denatured in SDS, resolved on 12% SDS-polyacrylamide gel, and transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Bedford, MA, USA), and incubated with blocking buffer (5% non-fat dry milk in TBST) overnight at 4°C. Immunoblotting was performed with a primary antibody (1:1,000) followed by the appropriate horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:15,000; Cell Signaling Technology, Inc., Danvers, MA, USA). Immunodetection was performed with an enhanced chemiluminescence system (ECL; Pierce Biotechnology, Rockford, IL, USA) using hydrogen peroxide and luminol as a substrate.

Immunofluorescence staining. Briefly, after experimental treatment, SMMC7721 cells were incubated with a primary antibody at 4°C overnight. After being washed three times with PBS, the cells were incubated with Alexa Fluor 488-labeled secondary goat anti-rabbit antibody (1:10,000; cat. no. 4412; Cell Signaling Technology, Inc.) for 1 h at 37°C. The cells were then stained

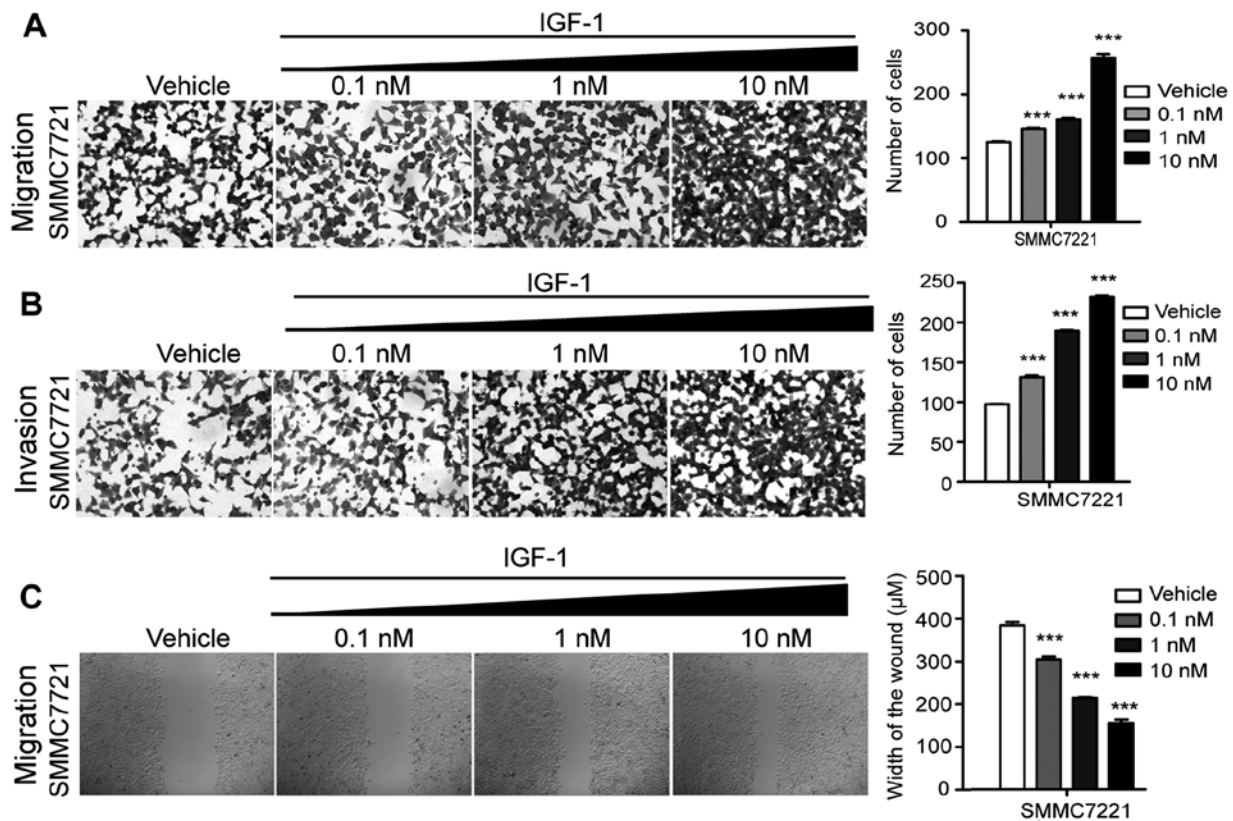


Figure 1. IGF-1 enhances the invasive and migratory abilities of HCC cells. (A) The impact of IGF-1 on migratory ability in a Transwell filter assay. (B) The impact of IGF-1 on invasive ability in a Transwell filter assay. (C) The impact of IGF-1 on migration ability in a wound healing assay. ***P<0.001 compared to the control. IGF-1, insulin-like growth factor-1.

with DAPI to visualize cell nuclei and observed under a confocal microscope (Nikon A1+; Nikon Corp., Tokyo, Japan).

Statistical analysis. GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze the experimental data, all the results were presented as the mean ± standard deviation (SD) from three independent experiments. The statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Dunnett's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

IGF-1 enhances the invasive and migratory abilities of HCC cells. To determine the effect of IGF-1 on the invasive and migratory abilities of HCC cells, different concentrations of IGF-1, including 0.1, 1 and 10 nM, were added to perform the wound healing assay and Transwell filter cell migration and invasion assays (Fig. 1). Statistical analysis revealed that in both the invasive and migratory assays the number of cells were significantly increased with the increasing doses of IGF-1, in comparison with the vehicle group (Fig. 1A and B). In addition, the distance of the wound healing was markedly decreased in the IGF-1 treatment groups in a dose-dependent manner (Fig. 1C). These results demonstrated that cellular invasive and migratory abilities were highly promoted by IGF-1, indicating that IGF-1 may act as an epigenetic activator of invasion and migration of HCC cells.

IGF-1 activates EMT in HCC cells. Losing cohesive ability and epithelial phenotype and acquiring spindle or fiber-like shapes are classic morphological changes in the process of EMT (14,15). Similar morphological changes as in EMT were observed in HCC cells after treatment with IGF-1 (Fig. 2A), hence it was speculated that IGF-1 enhances the invasive and migratory potential via activation of the EMT-related pathway. To confirm this hypothesis, we compared the mRNA and protein expression patterns of EMT-associated genes of IGF-1-treated cells to that of control cells at different concentrations. As shown in Fig. 2B and C, the expression levels of vimentin, Snail and N-cadherin were notably increased while E-cadherin was significantly decreased at both transcription levels and protein levels in a dose-dependent manner, indicating that EMT was activated in SMMC7721 cells after treatment with IGF-1.

Effect of IGF-1 on survivin expression. It was reported that survivin could be mediated by IGF-1/mTOR signaling (4) and that survivin participated in the activation of EMT in colon cancer cells (16). It could be speculated from these facts that IGF-1 may activate EMT by mediating survivin expression. Thus, we assessed the ability of IGF-1 to affect the expression level of survivin using RT-qPCR, western blot analysis and immunofluorescence staining. With the increase in the concentration of IGF-1 from 0.1 to 10 nM, the expression pattern of survivin significantly increased at both the mRNA and protein levels (P<0.01, P<0.001) (Fig. 3A and B). A similar tendency was observed in the immunofluorescence staining

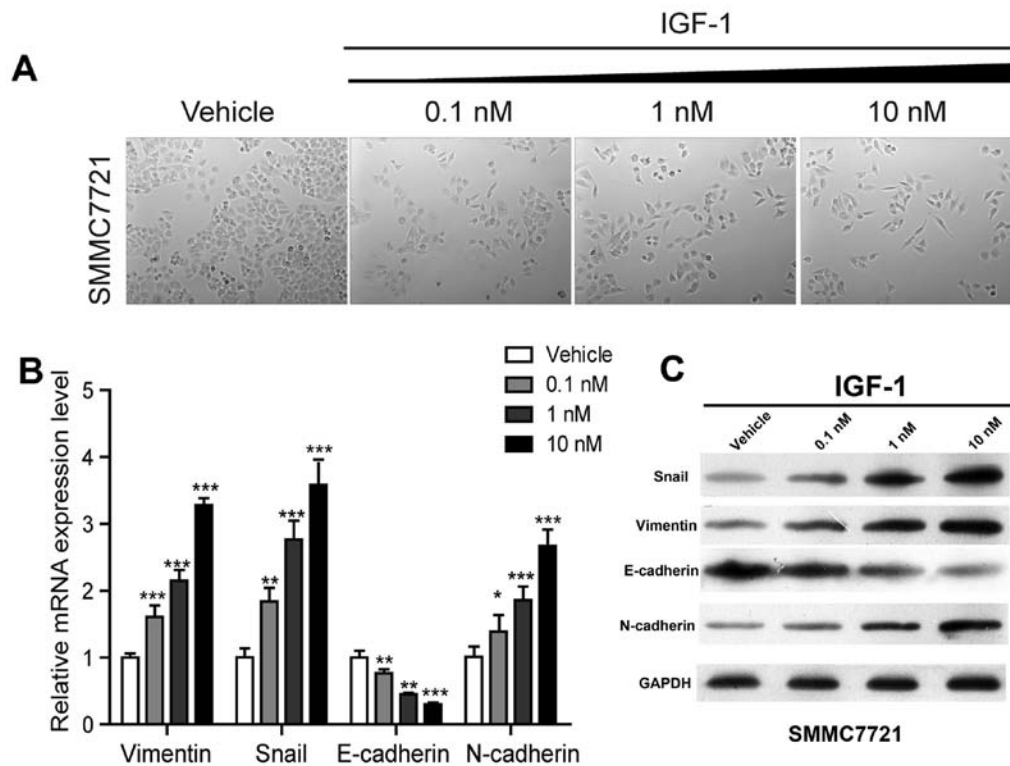


Figure 2. IGF-1 induces EMT by modulating the expression of EMT-associated genes. (A) Morphological change of HCC cells after treatment with IGF-1. (B) Relative mRNA expression of EMT-associated genes using RT-PCR after IGF-1 treatment. (C) Relative protein expression of EMT-associated genes using western blotting after IGF-1 treatment. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the control. IGF-1, insulin-like growth factor-1; EMT, epithelial-mesenchymal transition.

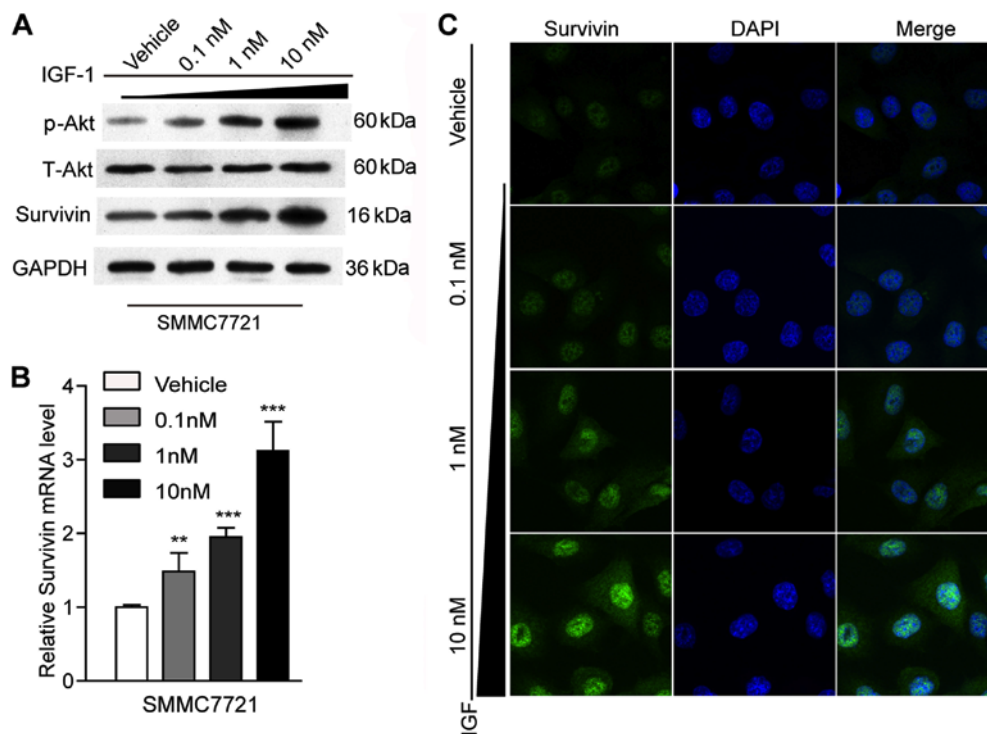


Figure 3. The expression patterns of survivin in HCC treatment with different concentrations of IGF-1. The expression of survivin was assessed using (A) western blot analysis and (B) RT-PCR. (C) Immunofluorescence staining for survivin in HCC treatment with different concentrations of IGF-1. ** $P < 0.01$ and *** $P < 0.001$ compared to the control. IGF-1, insulin-like growth factor-1; HCC, hepatocellular carcinoma.

results (Fig. 3C). These results implied that survivin may be involved in the IGF-1-induced EMT process.

In addition, it is well-known that IGF-1 participates in the activation of different signal transduction pathways, including

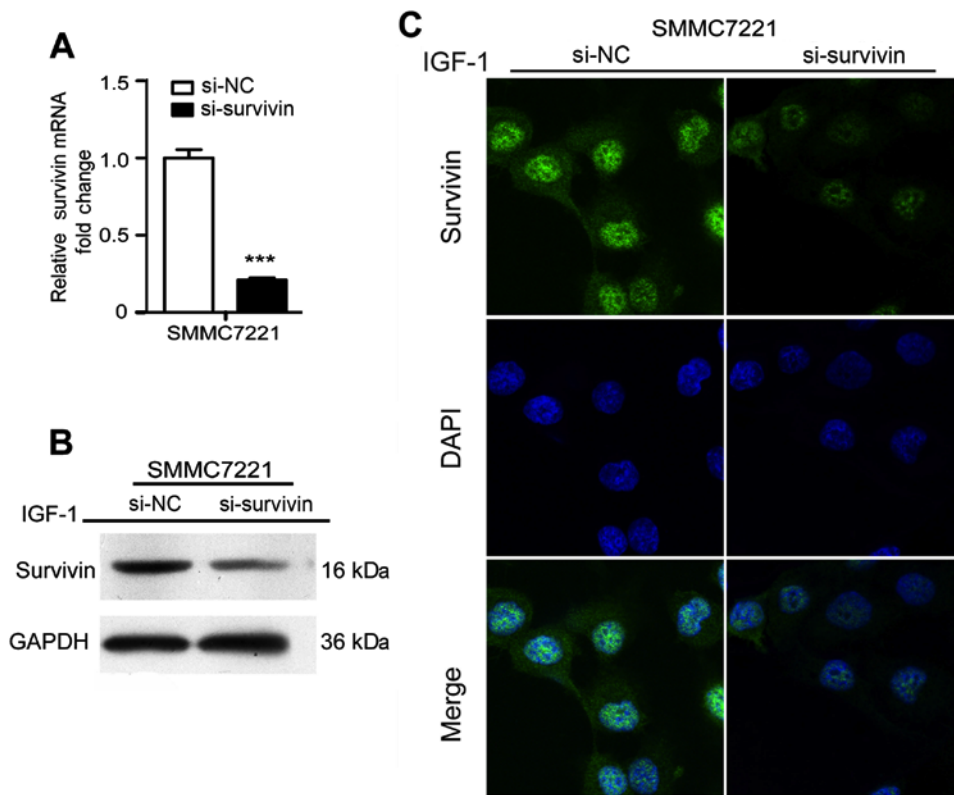


Figure 4. The effect of survivin on the EMT process. (A) Knockdown proficiency of survivin in HCC assessed by RT-PCR. *** $P < 0.001$ compared to the control. (B) Knockdown proficiency of survivin in HCC assessed by immunofluorescence staining. (C) Morphological change of HCC cells after silencing of survivin and treatment with IGF-1. HCC, hepatocellular carcinoma; IGF-1, insulin-like growth factor-1.

the PI3K/AKT/mTOR pathway (17,18). In the present study, our western blot results also revealed that AKT was activated and the p-AKT expression levels were significantly elevated in the IGF-1-treated groups in comparison with the vehicle group ($P < 0.05$) (Fig. 3A).

Survivin knockdown eradicates IGF-1-induced EMT in HCC cells. To further confirm the effect of survivin on the IGF-1-induced EMT process, survivin siRNA was transfected to silence survivin expression promoted by IGF-1 at a concentration of 10 nM and the results revealed that survivin expression at the mRNA and protein levels were significantly reduced ($P < 0.001$) (Fig. 4). After silencing survivin expression in HCC cell lines, there was a significant decrease in cell invasion and migration ($P < 0.001$) (Fig. 5E and F). Furthermore, EMT events were evaluated by observing cell morphological changes and assessed the expression levels of EMT markers in mRNA and protein. As shown in Fig. 5A-D, there was a changeover in EMT marker expression and cell phenotype in the survivin-siRNA cells compared with the control-siRNA cells. These results indicated that silencing of survivin recovered the EMT process of HCC induced by IGF-1.

Discussion

EMT is a reversible process in which epithelial cells lose their cell polarity and cell-cell adhesion and acquire mesenchymal features. During carcinogenesis, EMT enables tumor cells to become invasive via downregulation of epithelial-specific markers including E-cadherin, thyroid transcription

factor-1 (TTF-1) and ZEB and upregulation of mesenchymal markers including vimentin, α -SMA, N-cadherin and transcription factor Snail (19-26). EMT is found to be a major event in tumor metastasis by changing the cohesive ability of cells and enhancing the invasive and migratory potential (27) and different biomarkers mediating the EMT process have been identified. In addition, various tumor-associated growth factors have been identified to be involved into EMT, such as VEGF, EGF and TGF- β (28).

The important effect and involvement of IGF-1 in metastasis have been well elucidated. IGF-1 affects cell invasion by suppression of PTEN phosphorylation and interaction with the PI3K/PTEN/Akt/NF- κ B signaling pathway in pancreatic cancer (29). In addition, IGF-1, together with latent TGF- β can activate metalloproteinase activity (MMP) and then result in EMT in MCF-7 breast cancer cells (30). IGF-1 was found to elevate the expression of transmembrane glycoprotein MUC1 in MCF-7 cells for the initiation of EMT in a PI3K/Akt signaling pathway-dependent manner (7). IGF-1 was also reported to activate PI3K/AKT/mTOR signaling to increase the expression of survivin and control the expression of EMT biomarkers in the development of gastric, prostate and colon cancer cells (4,16,31). However, the relationship between IGF-1 and survivin in HCC was unclear. In the present study, we also found that AKT was activated with the upregulation of survivin in response to the treatment with IGF-1. In line with previous research, the downregulation of E-cadherin and the upregulation of N-cadherin, snail and vimentin induced by IGF-1 in HCC were positively associated with invasion and migration in a dose-dependent manner. On the basis of these findings,

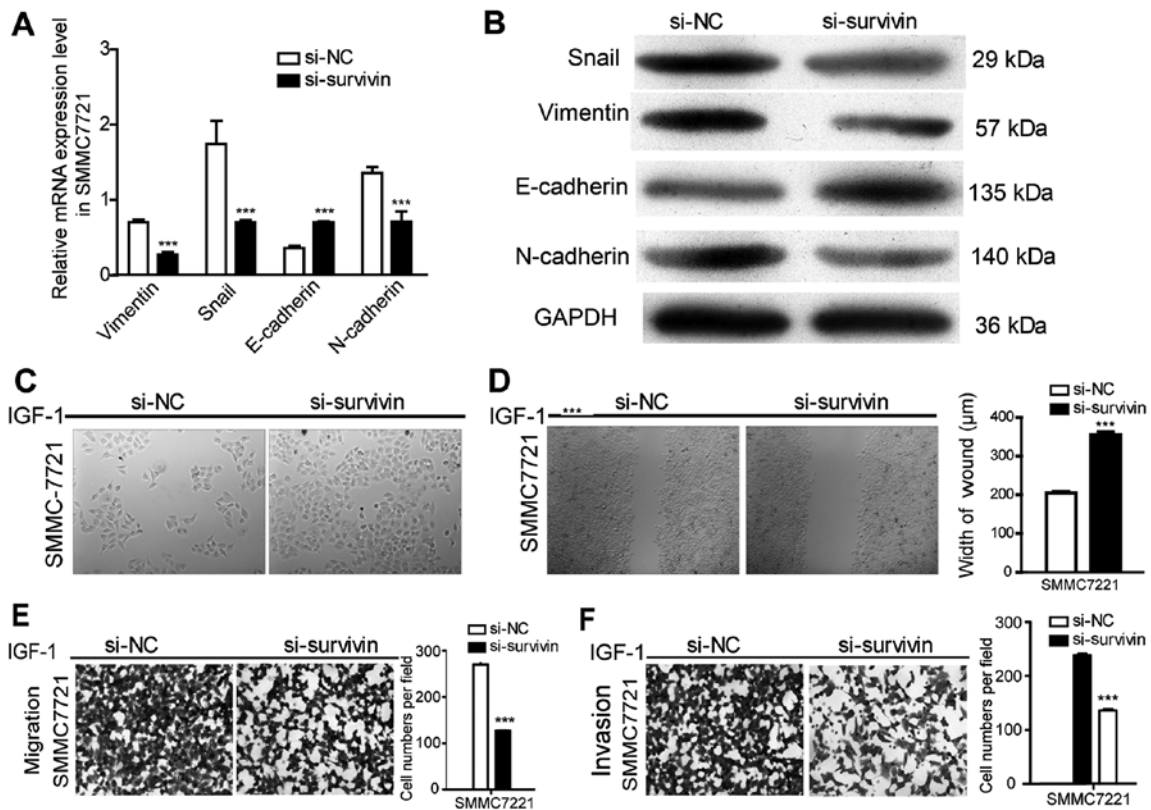


Figure 5. Knockdown of survivin inhibits the IGF-1-induced EMT process. (A) Relative mRNA expression of EMT-associated genes using RT-PCR after silencing of survivin and treatment with IGF-1. *** $P < 0.001$ compared to the control (B) Relative protein expression of EMT-associated genes using western blot analysis after silencing of survivin and treatment with IGF-1. (C) Cell morphological changes after silencing of survivin and treatment with IGF-1. (D) The impact of survivin on migration ability in a wound healing assay. *** $P < 0.001$ compared to the control. (E) The impact of survivin on migratory ability in a Transwell filter assay. *** $P < 0.001$ compared to the control. (F) The impact of survivin on invasive ability in a Transwell filter assay. *** $P < 0.001$ compared to the control. IGF-1, insulin-like growth factor-1; EMT, epithelial-mesenchymal transition.

our study suggests that IGF-1 may induce EMT involving E-cadherin, N-cadherin, Snail and vimentin dysregulation, thus facilitating the invasion and metastasis of HCC.

Survivin protein is overexpressed in most human tumors and promotes tumor cell proliferation and viability (4,32). As a nodal protein, survivin interfaced with multiple signals involved in mitosis and apoptosis and functionally integrated proliferation, cell death and cellular homeostasis. Exploring strategies to lower the expression level of survivin has been viewed as effective cancer therapy. Usually, positive correlation between IGF and survivin can be observed in tumor cells. As a target of IGF-1, survivin protein translation can be driven by IGF-1 signaling in prostate cancer cells. Binding of IGF-1 to its receptor activates downstream kinases, mammalian target of rapamycin (mTOR) and p70S6 protein kinase (p70S6K), which modulates survivin mRNA translation to increase the apoptotic threshold (4). In the present study, we focused on the role IGF-1/survivin in metastasis of HCC, which has not been reported to date. However, target-therapeutic functions have been found in other cancers. Sato *et al* also indicated that IGF-1 can induce expression of survivin in renal cell carcinoma (32). Furthermore, we demonstrated that survivin depletion could recover the cohesive ability and epithelial phenotype of HCC cells lost with IGF-1 treatment. Collectively, these data confirmed that survivin participated in the IGF-1-mediated EMT process in HCC. To the best of our knowledge, this is the first study to elucidate the IGF-1/survivin cascade in HCC metastasis *in vitro*.

In conclusion, our results displayed indirect evidence that IGF-1 had an effect on metastasis of HCC by mediating the EMT process and activating survivin. AKT was also activated with the upregulation of survivin in response to treatment with IGF-1. These data provided a new insight for the molecular therapy of HCC patients in clinical treatment.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Key Research and Development Plan in Shandong Province (2015GGH318017).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

FL, YS, HZ, HG, XZ and HC conceived and designed the study. FL, YS, BL, JL and HL performed the experiments. FL, HZ and HC wrote the manuscript. HG and XZ reviewed and edited the manuscript. All authors read and approved the

manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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