Observational Study

Smoc2 potentiates proliferation of hepatocellular carcinoma cells via promotion of cell cycle progression

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AIM
To determine the influence of Smoc2 on hepatocellular carcinoma (HCC) cell proliferation and to find a possible new therapeutic target for preventing HCC progression.

METHODS
We detected expression of Smoc2 in HCC tissues and corresponding non-tumor liver (CNL) tissues using PCR, western blot, and immunohistochemistry methods. Subsequently, we down-regulated and up-regulated Smoc2 expression using siRNA and lentivirus transfection assay, respectively. Then, we identified the effect of Smoc2 on cell proliferation and cell cycle using CCK-8 and flow cytometry, respectively. The common cell growth signaling influenced by Smoc2 was detected by western blot assay.

RESULTS
The expression of Smoc2 was significantly higher in HCC tissues compared with CNL tissues. Overexpression of Smoc2 promoted HCC cell proliferation and cell cycle progression. Down-regulation of Smoc2 led to inhibition of cell proliferation and cell cycle progression. Smoc2 had positive effect on ERK and AKT signaling.
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CONCLUSION
Smoc2 promotes the proliferation of HCC cells through accelerating cell cycle progression and might act as an anti-cancer therapeutic target in the future.

Key words: Smoc2; Hepatocellular carcinoma; Cell cycle; Proliferation

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Core tip: In our study, we confirmed that Smoc2 was up-regulated in hepatocellular carcinoma (HCC) tissues and played an important role in regulating liver cancer cell proliferation. Besides, we verified that Smoc2 participated in promoting HCC cell proliferation mainly through regulation of cell cycle progression. We have not investigated the promotive role of Smoc2 in regulating cell proliferation, whether it is through cell cycle regulation only or involves regulation of cell apoptosis as well. Moreover, the exact mechanism of how Smoc2 regulates cell cycle remains unclear. The core contents of our study included Smoc2 promotion of HCC cell proliferation via accelerating cell cycle progression.

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INTRODUCTION
Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with high mortality rate and low early diagnostic rate[1]. HBV infection, alcohol abuse, aflatoxin exposure and HCV infection are identified as major causes of HCC. The current therapies available for HCC include surgery, interventional therapy, radio frequency therapy, radiotherapy, biological target therapy and so on[2]. All of these treatments have certain curative effects, but have inherent limitations and adverse effects, especially for HCC patients at the advanced stage[3]. Thus, it is urgent to find new treatment target for the sake of enhancing curative effect and reducing adverse effects, especially in advanced HCC patients.

Apart from the common etiologies of HCC listed above, certain oncogenes, cytokines, neurotransmitters, chemokines, extracellular secretory proteins and tumor microenvironment are thought to play important roles in origin and progression of HCC[4]. Therefore, oncogenes and tumor microenvironment, which facilitate HCC progression, can be chosen as therapeutic targets for HCC treatment[5]. The secreted protein acidic and rich in cysteine (SPARC; alternative names: osteonectin; ON or basement membrane-40; BM-40) family is recognized as extracellular matrix proteins[6]. A differential expression of SPARC in tumor tissue and its surrounding stroma compared to normal tissues has been reported for many different types of cancer[7]. And, SPARC was found to be up-regulated in several solid tumors and to facilitate tumor metastasis[8].

Secreted modular calcium-binding protein-2 (Smoc2) is a novel member of the SPARC family[9]. Previous study confirmed that Smoc2 could promote cell cycle progression of human umbilical vein endothelial cells by inducing the expression of transcripts required for cell cycle[10]. Other studies have shown that Smoc2 is necessary for DNA synthesis in the cell cycle and is likely to impact cell growth in vitro and in vivo[11]. However, to the best of our knowledge, no study has been conducted on the role of Smoc2 in HCC.

Thus, the present study was designed to investigate the effect of Smoc2 on proliferation of HCC cells and the impact on cell cycle. Our study showed that Smoc2 promoted proliferation of HCC cells and accelerated cell cycle progression. The results suggested that Smoc2 might act as an anti-cancer therapeutic target for HCC treatment.

MATERIALS AND METHODS

Clinical samples
A total of 20 pairs of HCC tissues and corresponding non-tumor liver (CNL) tissues were obtained from the Liver Surgery Department of the corresponding hospital. All the human liver tissues were obtained with informed consent and the study was approved by the Ethical Committee of the corresponding hospital.

Immunohistochemistry
The paraffin-embedded human liver tissues were incised into 4-5 µm thickness slices and then dewaxed using xylene and ethanol, in a stepwise manner. After dewaxing, the slices were rehydrated for subsequent staining. For immunohistochemistry (IHC) staining, the slices were treated with hydrogen peroxide and boiled for 15 min in citrate solution for antigen retrieval. When the slices had cooled naturally to room temperature, we added goat serum for blocking of unrelated antigens. Afterwards, the slices were incubated with Smoc2 antibody (Abcam) at 4 ℃ overnight. The following day, the slices were washed three times with phosphate buffer and incubated with horseradish peroxidase-labelled secondary antibody for 1 h at room temperature. The slices were then developed using DAB substrate liquid (Thermo) and dehydrated by ethanol, in a stepwise manner, and finally sealed with neutral balsam.

Western blotting assay
The tissue protein was isolated from human liver tissue using T-PER Tissue Protein Extraction Reagent
(Pierce Biotechnology) according to protocols provided by the manufacturer. The cell protein was lysed using lysis buffer that contained Tris-HCl, NaCl, Triton-X 100, MgCl2, PMSF and so on. The cell lysate, which was used for cell signaling detection, was obtained by IP cell lysis solution (Beyotime Biotechnology). All the proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane under constant current condition. Then, the membrane was blocked using 5% bovine serum albumin at room temperature for 1 h. The nitrocellulose membrane was then incubated using antibodies for Smoc2 (Abcam), extracellular regulated protein kinase (ERK; Cell Signaling Technology, CST), phospho-ERK (CST), AKT (CST), phospho-AKT (CST), Src (CST), phospho-Src (CST), FAK (CST), phospho-FAK (CST) and GAPDH (Sigma) at 4 °C overnight. The next day, the nitrocellulose membrane was incubated with the fluorescence-conjugated secondary antibodies at room temperature for 1 h. All the fluorescence signals were captured and saved by the Odyssey imaging system (LI-COR). The images of western blots were analyzed using ImageJ software for gray value calculation.

**Immunofluorescence staining**
For cell staining, SMMC-7721 cells transfected with Smoc2 vector using lentivirus method were seeded on rounded slides in 24-well plates and incubated at 37 °C and a 5% CO2 atmosphere overnight. The next day, cells were fixed using paraformaldehyde and washed three times with phosphate buffer before staining. Then, the slides were incubated with Smoc2 antibody (Abcam) for 75 min at room temperature. After that, the slides were washed three times using phosphate buffer and incubated with Alexa Fluor 488nm-conjugated secondary antibody. The nuclei were stained using DAPI (Sigma) and the immunofluorescence stain images were record using fluorescence microscope (Carl Zeiss).

**Real-time quantitative PCR assay**
Total RNA was isolated from frozen liver tissues using the Trizol Reagent (Takara). The isolated RNA was then immediately applied to the reverse transcription reaction. Primers used for quantitative PCR reaction were: forward primer, 5’-GCTCACGTTCTTGAGAGTCG-3’; and reverse primer, 5’-TGTAGCTGTGACACTGGACC-3’. The PCR reaction conditions were 30 s at 94 °C, 1 min at 57 °C and 1 min and 30 s at 72 °C for 35 cycles. The AmpliTag polymerase and related reagents were supplied by Takara.

**Lentivirus transfection assay**
The full-length cDNA cloning vector was purchased from GeneCopoeia and used for amplification template. The Smoc2 full-length cDNA was amplified with NheI and BamHI restriction sites using KOD-PLUS Neo polymerase. Then, the PCR-amplified cDNA was cloned into the pcDNA3.1 vector using restriction enzymes and DNA ligase. The recombined vector was packed with VSV, REV and GAG vectors and transfected into 293T cells using the Lipofectamine 2000 Reagent (Invitrogen) to obtain virus supernatent. The virus supernatent was added into SMMC7721 cells and Huh7 cells with polybrene. The transfected SMMC7721 cells and Huh7 cells were cultured under puromycin condition and verified by western blot assay.

**Small interfering (si)RNA interference assay**
MHCC-97H cells and HCC-LM3 cells were transfected with siRNA duplexes against Smoc2 or with control RNA duplex oligonucleotides using Lipofectamine 2000, following the manufacturer’s instructions. The Smoc2 interference target sequence was TTAAGAGGTTCCTGCGAAA.

**CCK-8 cell proliferation assay**
MHCC-97H and HCC-LM3 cells transfected with Smoc2-SiRNA were seeded into 96-well plates at density of 3000 cells per 100 µL and cultured for 5 d in vitro. In addition, the SMMC-7721 and HCC-LM3 cells transfected with Smoc2 lentivirus were seeded into 96-well plates at density of 2000 cells per 100 µL and cultured for 5 d at 37 °C and in 5% CO2 atmosphere. The CCK-8 reagent was added to each well at 10 µL volume and reacted for 1 h in light-free condition, at 37 °C, and in 5% CO2 atmosphere. Then, the absorbance value was detected using a microplate reader. The absorbance value at 450 nm was recorded.

**Cell cycle assay**
The cells were seeded into 6-well plates and collected at logarithmic growth phase. The cells were cultured in serum-free condition overnight before collection. Liver cancer cells were collected and washed three times using phosphate buffer. Then, the cells were centrifuged at 1000 r/min for 5 min and resuspended in 1 mL phosphate buffer. Next, the cells were added to 9 mL 70% cold ethanol, slowly and carefully. The fixed cells were then stored at -20 °C for at least 24 h. For cell cycle assay, the fixed cells were centrifuged at 1000 r/min for 10 min and washed three times using cool phosphate buffer. Then, we added 0.5 mL RNase (50 µg/mL) to each tube and incubated for 20 min at 37 °C. Lastly, we added propidium iodide at 50 µg/mL to each tube and stained on ice for 30 min in light-free condition. The stained cells were detected using flow cytometry (BD Biosciences).

**Statistical analysis**
We used SPSS 16.0 software to analyze the statistical significance of differences in our study. Statistic differences were calculated using two-tailed Student’s t-test. P < 0.05 was considered statistically significant and P < 0.01 was considered very statistically significant.
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RESULTS

Smoc2 was up-regulated in HCC tissues compared with CNL tissues

The expression of Smoc2 was significantly up-regulated in HCC tissues, compared to CNL tissues, as evidenced by IHC (Figure 1A). IHC results showed that expression of Smoc2 was mainly located in the cytoplasm of HCC cells and the extracellular lesion of liver tissues. Western blot assay showed that protein expression of Smoc2 was higher in fresh HCC tissues than in CNL tissues. 

Figure 1  Smoc2 was up-regulated in hepatocellular carcinoma tissues compared with corresponding non-tumor liver tissues. A: Representative images of immunohistochemistry (IHC) staining assay; IHC images show that expression of Smoc2 was higher in hepatocellular carcinoma (HCC) tissues compared with corresponding non-tumor liver (CNL) tissues; B: Western blot assay show the expression of Smoc2 was higher in fresh HCC tissues than in CNL tissues; C: Quantitative real-time PCR assay showed that the relative expression of Smoc2 was higher in fresh HCC tissues than in CNL tissues. *P < 0.05.
The expression level of Smoc2 was significantly higher in human HCC tissues, compared to CNL tissues (Figure 1B). The real-time quantitative PCR result indicated that mRNA expression level of Smoc2 in HCC tissues was remarkably higher than in CNL tissues. All the results above revealed that expression of Smoc2 was up-regulated in HCC tissues, compared to CNL tissues, at both protein and mRNA levels (Figure 1C).

**Silencing Smoc2 by siRNA transfection and overexpressing Smoc2 by lentivirus transfection assay**

We carried out siRNA transfection for silencing of Smoc2 in MHCC-97H and HCC-LM3 cells, and verified the silencing effect using western blot assay (Figure 2A). We induced overexpression of Smoc2 in SMMC-7721 and Huh7 cells using the lentivirus transfection method and identified the overexpressing effect using western blot assay (Figure 2B). The immunofluorescence stain results showed that expression of Smoc2 induced by lentivirus transfection can be found in cytoplasm of SMMC-7721 cells (Figure 2C).

**Silencing of Smoc2 inhibited HCC cell proliferation and overexpression of Smoc2 promoted HCC cell proliferation in vitro**

CCK-8 assay directly reflects cell viability and can be used for evaluating cell proliferation. We collected MHCC-97H and HCC-LM3 cells transfected with Smoc2-siRNA at 48 h and detected the cell viability for 5 d in vitro using CCK-8 assay. The results showed that the proliferation capacity of MHCC-97H and HCC-LM3 cells transfected with Smoc2-siRNA were significantly worse than that in the control group (Figure 3A). Besides, the proliferative capacity in Smoc2 overexpressing SMMC-7721 and Huh7 cells was remarkably stronger than in control groups in vitro (Figure 3B). These results suggested that Smoc2 plays an important role in promoting HCC cells proliferation in vitro.
Overexpression of Smoc2 accelerated cell cycle progression by increasing the proportion of S phase cells

Cell cycle directly reflects cell proliferation capacity and is composed of G0/G1 phase, S phase and G2/M phase. The cell proliferation capacity can be reflected by proliferation index (PI) and S phase cell fraction (SPF). PI equates to the percentage of sum of S phase and G2/M phase cells in total cell cycle cells. SPF equates to the percentage of S phase cells in total cell cycle (G0/G1 + S + G2/M). Our results showed that both PI and SPF values in overexpressing Smoc2 cells were higher than those in the control group, which indicated the promotive role of Smoc2 in cell cycle progression and cell proliferation (Figure 4A and B; Figure 5A and B).

Smoc2 potentiated ERK, AKT and FAK signaling in HCC cells

The ERKs consist of ERK1 and ERK2, having molecular weights of 44 kDa and 42 kDa respectively. Phosphorylation of ERK allows translocation from the cytoplasm to the nucleus, and mediates transcriptional activation of Elk-1, ATF, NF-κB, Ap-1, c-fos and c-Jun. Activation of ERK signaling participates in regulating cell proliferation and differentiation, construction of the cell skeleton, and the processes of cell apoptosis and cell canceration. Our results showed that phosphorylation of ERK was up-regulated in Smoc2 overexpressed cells and down-regulated in Smoc2 siRNA interfered cells (Figure 6).

Signaling by AKT, also known as protein kinase B, plays an important role in regulating cell survival and apoptosis. Our results indicated that phosphorylation of AKT was up-regulated in Smoc2 overexpressed cells and down-regulated in Smoc2 siRNA interfered cells (Figure 6).

FAK, also known as focal adhesion kinase, and its activation is related to cell proliferation and cell cycle. Inhibition of FAK signaling can cause decrease of S phase cell ratio in cell cycle. Thus, inhibition of FAK signaling lead to inhibition of cell proliferation and enhancement of cell apoptosis. Our results illustrated that down-regulation of Smoc2 by siRNA transfection caused inhibition of FAK phosphorylation (Figure 6).

DISCUSSION

As one of the most frequent human cancers worldwide,
the incidence and progression of HCC are affected by oncogenes and tumor microenvironment\cite{16,17}. Cytokines, chemokines, pH value, hypoxia, fibroblast and extracellular matrix proteins are important components of the tumor microenvironment\cite{18-21}. The tumor microenvironment plays an important role in regulating tumor progression\cite{22,23}. It has been reported that many extracellular matrix proteins exert significant influence on tumor progression. Thus, we focused on finding a new secretory protein which can cause tumor development.

The Smoc2 gene, encoding a protein belonging to the SPARC family and identified as an extracellular matrix protein, is up-regulated during embryogenesis and wound healing\cite{24}. The Smoc2 gene encoded protein promotes extracellular matrix assembly and potentiates endothelial cell proliferation and migration\cite{25}, and has pro-angiogenic activity\cite{26}. It was reported that the Smoc2 gene is indispensable for VEGF-induced vascular endothelial cells mitogenesis.

**Figure 4 SMMC-7721 cells.** A: Fluorescence-activated cell sorting assay for cell cycle detection. All the SMMC-7721 cells were stained by propidium iodide, which can be detected at 488 nm; B: The overexpression of Smoc2 caused increase of S stage cell proportion in SMMC-7721 cells, which indicated promotion of cell cycle progression.
and tube formation[27,28]. Due to its promotive effect on angiogenesis, the Smoc2 gene may represent a useful target for inhibition of tumor progress.

The cell cycle consists of G0/G1 phase, S phase and G2/M phase in mammalian cells[29,30]. The cell cycle is mainly regulated by various kinds of growth factors, such as platelet-derived growth factor[31,32]. Cyclin D1 expression is induced by mitogens and is crucial for G1 progression[33]. Previous results showed that Smoc2 plays an important role in promoting G1/S progression through participating in the maintenance of cyclin D1 expression[34-36]. In other words, cyclin D1 can be considered as an effector of Smoc2-induced DNA synthesis[37,38].

It was also demonstrated that Smoc2 was necessary for DNA synthesis in response to PDGF and other growth factors during the cell cycle[39-41]. Our study demonstrated that Smoc2 had a promotive effect on HCC cell G1/S phase progression, as well as on cell proliferation. The S phase cell ratio was significantly elevated in Smoc2 overexpressing HCC cells, and this result was in accordance with those from a previous
study using human umbilical vein endothelial cells transduced with Ad-Smoc2[42,43].

Compared with Swiss 3T3 cells transfected with Sicontrol, the cells transfected with SiSmoc2 oligonucleotide duplexes showed no significant change in phospho-MAPK and phospho-AKT levels[44-47]. Unlike the results in Swiss 3T3 cells, our results indicated that Smoc2 had promotive influence on MAPK/ERK and AKT signaling pathways in HCC cells. MAPK/ERK and AKT are identified as PDGF β receptor effector kinases and positively regulate the expression of cyclin D1[48]. Therefore, we speculated that Smoc2 could potentiate PDGF-induced mitogenesis and the subsequent cell proliferation. Nevertheless, another study based on Swiss 3T3 cells showed that the promotion of Smoc2 in DNA synthesis was independent of PDGF-binding activity but dependent on integrin-activated protein kinase[49].

Previous studies revealed that Smoc2 promotes growth factor-induced DNA synthesis and differs from SPARC, which was identified as an anti-mitogenic factor. It was reported that SPARC could bind various kinds of mitogens (such as VEGF, PDGF, FGF and so on) but inhibit the signaling mediated by these growth factors. Unlike SPARC, Smoc2 could facilitate growth factor-induced DNA synthesis[50]. The exact mechanism of how Smoc2 promotes growth factor-induced DNA synthesis in HCC cells remains unclear and needs to be studied further.

Our research focused on the proliferation promotion effect of Smoc2 in HCC cells. However, Smoc2 also has influence on cell migration. It has been reported that extracellular matrix protein Smoc2 can promote keratinocyte migration in vitro. Accordingly, the role of Smoc2 on HCC cell migration and invasion need to be further studied. The influences of Smoc2 on HCC cell biological behaviors may be extensive.

In conclusion, our results demonstrated that Smoc2 is an important regulator of cell mitogenesis, and plays a promotive role in growth factor signaling and cell cycle progression in HCC. Based on our study, Smoc2 might represent a promising therapeutic target for HCC treatment.

**Figure 6** Phosphorylation of ERK up-regulated in Smoc2 overexpressing cells and down-regulated in cells with Smoc2 small interfering (si)RNA. Western blot assay showed the change of phospho-ERK, Src, FAK and AKT expression by Smoc2 interference or overexpression.
Su JR et al. Smoc2 potentiates HCC progression in HCC tissues and corresponding non-tumor (CNL) tissues using PCR, western blot, immunohistochemistry methods and down-regulation and up-regulation of Smoc2 expression using small interfering RNA and lentivirus transfection assay respectively. The effect of Smoc2 on cell proliferation and cell cycle was identified using CCK-8 and flow cytometry respectively. The common cell growth signaling influenced by Smoc2 was detected. The authors found that Smoc2 promotes the proliferation of HCC cells through accelerating cell cycle progression and might act as an anti-cancer therapeutic target in the future. Overall, this study is well designed and the manuscript is well written.

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