Targeting SHCBP1 Inhibits Cell Proliferation in Human Hepatocellular Carcinoma Cells

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Abstract

Src homology 2 domain containing (SHC) is a proto-oncogene which mediates cell proliferation and carcinogenesis in human carcinomas. Here, the SHC SH2-domain binding protein 1 (SHCBP1) was first established to be up-regulated in human hepatocellular carcinoma (HCC) tissues by array-base comparative genome hybridization (aCGH). Meanwhile, we examine and verify it by quantitative real-time PCR and western blot. Our current data show that SHCBP1 was up-regulated in HCC tissues. Overexpression of SHCBP1 could significantly promote HCC cell proliferation, survival and colony formation in HCC cell lines. Furthermore, knockdown of SHCBP1 induced cell cycle delay and suppressed cell proliferation. Furthermore, SHCBP1 could regulate the expression of activate extracellular signal-regulated kinase 1/2 (ERK1/2) and cyclin D1. Together, our findings indicate that SHCBP1 may contribute to human hepatocellular carcinoma by promoting cell proliferation and may serve as a molecular target of cancer therapy.

Keywords: Hepatocellular carcinoma - SHCBP1 - SHC - cell proliferation - HCC therapy
SHCBP1 mRNA and protein was selectively expressed in tissues containing proliferating cells even tumor cells, but are absent in normal, quiescent tissues and growth-arrested cells which was highly expressed in testis, but have a lower level or absent from expression in spleen, lung, heart, brain, liver and skeletal muscle (Schmandt et al., 1999). The research result proved that SHCBP1 might involve in the process of tumorigenesis and progression in cancer. The function of SHCBP1 is not yet fully clear. The present research showed that SHCBP1 may be play a role in cell growth and differentiation.

Materials and Methods

Tissue specimens and Cell lines
All HCC tissues were harvested from patients who Diagnosed and underwent hepatectomies in the department of hepatobiliary surgery of Wuxi Municipal People’s Hospital from 2010 to 2012. HCC tissues and non-cancerous adjacent tissues were cryopreserved in liquid nitrogen after resection. The content of the paper were obtained with informed consent. Fifteen liver tumor-derived cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco) at 37℃ in a 5% CO₂ incubator.

Extraction of RNA and reverse transcription
Total RNA was extracted from HCC Tissue specimens and Liver cancer cell lines using TRIZOL solution (Invitrogen). RNA was DNase treated using RNase-free DNase I (TaKaRa). Reverse transcription (RT) was performed with 2 μg total RNA treated by RNase-free DNase I (TaKaRa). First strand cDNA synthesis was carried out from 2 μg total RNA with PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa) according to manufacturer’s instructions.

Semiquantitative RT-PCR and quantitative real-time PCR
The following Primers used in Semiquantitative RT-PCR and quantitative real-time PCR follows as: SHCBP1-432bp: (forward) 5’-ACATCCAAGCAAGGGGTGTC-3’; (reverse) 5’-GCACACAGTTTTCCACCGTA’; SHCBP1-168bp: 5’-CCCTTCTGAGCAAGTCGAGG-3’(forward); (reverse) 5’-AACTGGTTCCCCACAATCC-3’.

RNA interference and construction of Plasmid
The special Small interference RNAs (siRNA) and Common utilized Negative Control (NC) siRNA were chemically synthesized (GenePharma). The open reading frame (ORF) of SHCBP1 (human, NM_024745.4) was amplified by PCR from the cDNA of Foucs cells. The expression vector of SHCBP1 (pcDNA3.1B-GFP – SHCBP1) were generated by inserting the open reading frame of SHCBP1 into The pcDNA3.1B- GFP (Chinese National Human Genome Center) vector. The special oligonucleotide fragment encoding short hairpin RNA (shRNA) for targeting of endogenous SHCBP1 was inserted into the pSUPER vector (OligoEngine). The sequences of siRNAs were listed as follows: siRNA-308 (sense 5’-GGAGCUUACAAAGAUUAACAUdUdU-3’, antisense 3’-GdUdUCGCGUAUUUCUAAUUA-5’); siRNA-2658 (sense 5’-GGAGGUUUGAAGCGAAAGA UdGdA-3’,antisense 3’-AdUdCCUACACUUCCGUCUU UA-5’); The non-specific siRNA not targeting any annotated genes was used as negative control. The oligonucleotide sequences for shRNA were: ShRNA-SHCBP1-2658-Forward GATCCCCGAGGTTGGAA GCAGAAATtcgaagaATTTTCTGCTTCAAACTCCTC TTGTTGAAA; ShRNA-ShCBP1-2658-Reverse AGCTT TTCCAAAAGGAAGGTGTTGAAGCAAGAAATtcgtaag ATTTCTGCTTCAACCTCCCCGG; ShRNA-SHCBP1-308-Forward GATCCCCGAGCCTATCAAGATTCA TtcgaagaATGTAGATTCGTTAGGCTTTTGGGA AA; ShRNA-SHCBP1-308-Reverse AGCTTTTCCAAA AAGAGCTCTAAGAAGATTCAATtcgtaag ATGTAGATTCGTTAGG; ShRNA-SHCBP1-NC-Forward GATCCCCGCCCAAGACTGTATAACCTcgaaga GTTATACAGTCTTTGCCCCTTTTGGAAA; ShRNA SHCBP1-NC-Reverse AGCTTTTCCAAAATTCTC CGAAGGTTCAGGTcgttaag ACGTGACACGC TTGAGAAGG.

Cell transfection
Cell transfection was performed by lipofectamine 2000. (Invitrogen) according to the manufacturer’s instructions. Westernblot and quantitative real-time PCR were then used to investigate the transfection efficiency.

Immunofluorescence
After grew on the cover glass, cells were fixed with 4% paraformaldehyde for 30 minutes, then were treated with 0.5% Triton X-100 for 10 minutes after that blocked with 5% BSA and stained with rabbit anti-shebp1 antibody (1:500) (biotech) at 4℃ overnight, washed three times in 0.1% PBST, followed incubation with Alexa Fluor 488-coupled (green) -anti- rabbit IgG antibody (Molecular Probes Inc) at 4℃ for 2 hours. After washing in 0.1% PBST three times again, the cell nucleus were dyed with 4’, 6-diamidino-2-phenylindole (DAPI), and then photographed by Zeiss confocal microscopy.

Immunohistochemistry study
The tumor tissues and non-cancerous adjacent tissues were fixed in 10% formalin and embedded in paraffin. Four micrometer thick sections were deparaffinized and dehydrated twice in xylene for 10 min and twice in ethanol for 2 min, and then the tumor sections were placed in 100 mM Tris-HCl (pH 6.0), 50 mM ethylenediaminetetraacetic acid (EDTA), heated at 94℃ for 15 min and washed 3 times with PBS. Treated with methanol containing 0.3% H2O2 to inhibit endogenous peroxidase, samples were stained by rabbit anti-SHCBP1 polyclonal antibody (1:100 dilution, Sigma-Aldrich) at 37℃ for 2 h, washed 3 times with PBS, LSAB2 kit/HRP System (DakoCytomation Denmark A/S, Glostrup, Denmark) and hematoxylin were used to counterstain. The photograph is measured using the Olympus microscope.
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Figure 1. Expression Pattern of SHCBP1 in HCC Specimens, Cell Lines. (A) The mRNA level of SHCBP1 in 52 HCC specimens and corresponding non-HCC livers was measured by quantitative real-time RT-PCR. β-actin was used as an internal control. Compared with noncancerous tissues, the mRNA level of SHCBP1 was significantly higher in 69.2% HCC specimens. P value was calculated by Student’s t test. *** indicates P<0.001. (B) The expression level of SHCBP1 was detected in HCC specimens and HCC cell lines, fetal livers by western blot, where β-actin was used as an internal reference. (C) Representative immunohistochemical staining on HCC samples with E2F8 antibody. (D) Immunofluorescence assay indicated that SHCBP1 protein was located on membrane in YY-8103 and hep-1, HepG2 cells, particularly in fetal liver. and there showed that SHCBP1 level was higher in YY-8103, SK-hep-1, HepG2 cells, particularly in fetal liver. The nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI)

Cell proliferation and Colony formation assay

The proliferation ability was analyzed by cell growth curve and Colony formation assay. HCC cells (3–5 x10⁴ per well) were plated into 96-well plates in 100 μl culture medium. Cell proliferation ability was measured by MTT test. HCC cells transfected with vector were cultured in medium added G418 (Life Technologies) (final concentration is 0.8 to 1 mg/mL) for 2-3 weeks. The medium was changed every 3-5 days until forming visible clones.

Western blot analysis

Total proteins were extracted from cultured cells by using the lysis buffer [25 mmol/L Tris (pH 6.8), 1% SDS, 5 mmol/L EDTA, protease inhibitor cocktail (Sigma)]; these extracts (30 μg) were subjected to electrophoresis by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto Hybrid-P polyvinylidene difluoride membrane (PVDF, Amersham Pharmacia Biotech Ltd., Buckinghamshire, UK). After blocking with blocking solution (5% nonfat milk and 0.1% Tween-20 in PBS) for 2 hours at room temperature, the membrane was incubated with antibody overnight at 4 °C. The membrane was incubated with the secondary antibody for 1h at room temperature. The Odyssey Infrared Imaging System (Li-COR) was used to record the electrophoretic bands. Rabbit anti-SHCBP1 polyclonal antibody (Sigma-Aldrich), anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), Mouse antibody cyclin D1 (Santa Cruz Biotechnology), pERK1/2 and total ERK1/2 (cell signaling technology) were used in this study.

Results

SHCBP1 has high level expression in HCC

To investigate the relative expression level of SHCBP1 in Hepatocellular Carcinoma, we detected it in 52 pairs of human HCC specimens through real-time RT-PCR and western blot. The results indicated that mRNA levels of SHCBP1 were significantly upregulated in HCC specimens compared with the paired non-HCC tissues (Figure 1A, B; P < 0.001). Moreover, we measured the protein expression level of SHCBP1 in 8 HCC cell lines and fetal liver by western blot analysis. The results showed that SHCBP1 level was higher in YY-8103, SK-hep-1, HepG2 cells, particularly in fetal liver. and there was relatively lower expression in PLC/PRF/5, Focuss cells (Figure 1B). In addition, we are also examined by
immunohistochemical staining with a specific antibody against SHCBP1 in 30 pairs of HCC samples (Figure 1C). The resulting data showed that SHCBP1 was significantly overexpressed in 19 of 30 HCC specimens as compared with the paired non-HCC tissues. Additional, SHCBP1 was located on cell membrane in YY-8103 and PLC/PRF/5 cells, as indicated by immunofluorescence assay (Figure 1D).

SHCBP1 overexpression promotes cell proliferation, colony formation and soft agar colony formation

To evaluate the function of SHCBP1 on HCC cells, the pcDNA3.1-SHCBP1 expression vector is successfully constructed. The recombinant plasmid was transfected into FOUCS and PLC/PRF/5 cells. The result showed that cellular growth, colony formation and soft agar colony formation were significantly promoted by the enforced SHCBP1 overexpression as compared with that of those transfected with pcDNA3.1B (empty Vector) (Figure 2A, B, C). These data indicated that SHCBP1 may play important role in promoting HCC cell proliferation, colony formation and soft agar colony formation.

SHCBP1 knockdown inhibits cellular proliferation, colony formation and soft agar colony formation

To investigate the effect of SHCBP1 knockdown on cellular growth, colony formation and soft agar colony formation of HCC cells. Chemically synthesized siRNAs (siNC, si308, si2658) were used for knocking down endogenous SHCBP1 in Sk-hep-1 and YY-8103 cells. siRNAs were transfected into HCC cells. Western blot was used to evaluating the efficiency of RNAi. The siRNAs RNA interference largely reduced the SHCBP1 mRNA expression level of Sk-hep-1 and YY-8103 cells compared with the control siNC. Relative to the controls siNC, SHCBP1 knockdown (si308, si2658) could significantly inhibit the ability of cell growth in Sk-hep-1 and YY-8103 (Figure 3A, B). ShRNA expression vector is more stable in cells and easier to be used for investigating the gene functions compared with Chemically synthetized siRNAs so we were constructed recombinant pSUPER-shRNA (shRNA-NC, shRNA-308, shRNA-2658) vectors. They were used to assay colony formation and soft agar colony formation. The stable cells Sk-hep-1 transfected with shRNA-NC had more and larger colonies compared with shRNA-308, shRNA-2658 plasmids (Figure 3C). Consequently, the above data showed that silencing SHCBP1 can inhibit HCC cells proliferation, colony formation and soft agar colony formation. These data implied that endogenous SHCBP1 may play important role in maintaining cellular proliferation and colony formation of HCC cells.

SHCBP1 influences cell cycle progression of HCC cells

To determine whether SHCBP1 expression could be affected cell growth, we investigated the cell cycle profile
of Focus and Sk-hep-1 HCC cells by Cell cycle analysis via flow cytometry. Serum starved HCC cells, Focus was transfected with pcDNA3.1-GFP-SHCBP1, then Sk-hep-1 was transfected with si-NC, si-308 and si-2658. As shown in Figure 3D, SHCBP1 knockdown caused an arrest in G0/G1 phase and blocked cells into S phase. This result inferred that SHCBP1 affects cell growth through involving in G1 to S transition of cell cycle progression. 5. SHCBP1 regulates cyclin D1 and pERK1/2 expression. To explore the mechanism by which SHCBP1 induces G0/G1 phase arrest, the expression level of cyclin D1 and pERK1/2 was detected by western blot. In this study, we found that the levels of p-ERK1/2, and Cyclin D proteins were decreased after SHCBP1 knockdown in Sk-hep-1 cells. In contrast, the protein level of cyclin D1 and pERK1/2 increased by SHCBP1 overexpression in Focus cells (Figure 4).

Discussion

Shc SH2-domain binding protein (SHCBP1), mapped on 16q11.2, which is a component of a novel signaling pathway downstream of Shc adaptor proteins (Chen et al., 2012). The Shc gene encodes three overlapping proteins with molecular weights of 46 kDa, 52 kDa and 66 kDa. The three proteins share a common carboxy terminal Src Homology 2 (SH2) domain (Pellicci et al., 1992). Shc SH2 domain serves an important function as a target point to tyrosine kinases receptor activation. Shc proteins can activate tyrosine kinases receptor via tyrosine phosphorylated (Tomilov et al., 2011; Tomilov et al., 2011; Sweet et al., 2012).The association of SHCBP1 with Shc via a novel phosphotyrosine independent interaction with the Shc SH2 domain. SHCBP1 was identified by its interaction with Shc, which is involved in FGF signaling (Schmandt et al. 1999). Based on the current literature, SHCBP1 expression is required for FGF signaling in neural progenitor cells. Knockdown the expression of SHCBP1 can lead to p-ERK1/2 and p-AKT significantly decreased in neural progenitor cells. The stability of SHCBP1 was necessary for exerting normal functions of some relative genes in FGF signal pathway and was required for Shc–Ras-MEK-ERK in EGF signal pathway (Schmandt et al., 1999; Chen et al., 2012). The latest Articles showed that Ser634 of SHCBP1 was phosphorylated during mitosis, SHCBP1 was diffusely localized between two separating chromosomes during early anaphase and started to accumulate during late anaphase at the central spindle and at the midbody during cytokinesis. This specific localization phenomenon was not observed in SHCBP1-siRNA Cells (Asano et al., 2013). The research findings indicated that SHCBP1 might be play an important role in encouraging G0/G1 phase into S phase in cell cycle changes. SHCBP1 was found to be required for viral RNA synthesis and might be exploited as a target of anti-viral compounds (Ito et al., 2013).

In our study, we firstly found and confirmed that the expressions of SHCBP1 mRNA level had evident individual differences among the 52 pairs of human normal tissue. Compared with the adjacent tissues the expression level of SHCBP1 in Hepatocarcinoma tissue was significantly higher than that in non-HCC tissue by quantitative real-time PCR. Overexpression of exogenous SHCBP1 could promote cell proliferation, colony formation and soft agar colony formation of Focus and PLC/RFP/5 cell lines. While, targeting SHCBP1 could inhibit the ability of proliferation in SK-hep-1 and YY-8103 cell lines. Targeting SHCBP1 caused an arrest in G0/G1 phase and blocked cells into S phase. These data indicated that SHCBP1 might be play important roles in HCC carcinogenesis and metastasis and indicated that SHCBP1 might represent as a potential novel therapeutic target for HCC. As known, Cyclin D1 is necessary to promote cell cycle progression from G1 to S phase, and sustained ERK1/2 activity is required for the continued expression of cyclin D1 in G1 phase. So we detected the phosphorylation level of ERK1/2 and Cyclin D1. We observed that the total ERK1/2 were unaffected but endogenous activity phospho-ERK1/2 and Cyclin D1 was reduced in YY-8103 cells transfected with si-SHCBP1 and was increased in Focus cells transfected with pcDNA3.1B-GFP–SHCBP1 plasmid.

In summary, we present data show that SHCBP1 play a positive role in the ability of proliferation, colony formation and soft agar colony formation in HCC cells. Meanwhile, SHCBP1 regulates cell cycle transition from G1 to S phase and affect the expression level of the protein level of cyclin D1 and pERK1/2. All the data suggest that the up-regulation of SHCBP1 contributes to HCC tumorigenesis and progression and can be a candidate molecular target for the treatment of Human Hepatocellular Carcinoma. And Further studies are required to explore the exact mechanisms.

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Han-Chuan Tao et al

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