RESEARCH ARTICLE

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

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Introduction

Hepatocellular carcinoma (HCC) which has high incidence and high mortality is a puzzle over the world, particularly in Asia and Africa (Schafer and Sorrell 1999). The incidence rate of hcc ranks the fifth and the mortality ranks the third in the most common cancer worldwide.It caused more than 600,000 deaths annually, which threatens human's health all the time (Bosch et al., 2004). At present, hepatoma resection is still the most useful method of treatment, but only 9% of sufferer surviving more than 5 years (Sherman 2010). Current studies show that HCC is a complex process associated with multiple factors such as HBV infection, hcc-related-genes activation and inactivation, Abnormal liver cell apoptosis (Arzumanyan et al., 2013).so the elucidation of the molecular mechanism at the gene level have become more and more important for the guidance on clinical diagnosis and treatment of hepatocellular carcinoma patient.

Recent studies in our lab have revealed many important genes in HCC disease process, such as E2F8 (Deng et al., 2010), STC1 (Wang et al., 2012) , NICE-3 (Wei et al., 2012), COTE1 (Zhang et al., 2012). Among them, SHCBP1, a novel gene, the expression level in Hepatocarcinoma tissue was significantly higher than that in non-HCC tissue. In this study, we detected the expression of SHCBP1 in HCC specimens and HCC cell lines, and analyzed the potential correlation between SHCBP1 and HCC.

As known, Src homolog and collagen homolog (Shc) is an important adaptor protein of cell surface receptors, which couples activated growth factor receptors to signaling pathways, such as EGF receptor (EGFR) (Belsches et al., 1997; Yamauchi et al., 1998), FGF receptor (FGFR) (Eswarakumar et al., 2005; Chen et al., 2012), and insulin receptor (Sasaoka and Kobayashi, 2000). The function of shc protein was proved to be active the intracellular signaling pathways, such as the Ras pathway (Hutter et al., 2000; Leahy et al., 2004; Debnath 2010). Shc gene can be transcribed and translated three isoforms with distinct molecular weights (46, 52 and 66 kDa) and specific interactions, the three isoforma have common PTB, CH1, and SH2 domains. p66Shc has a unique extra CH2 region in the amino-terminal portion (Pelicci et al., 1992). Based on the present literature, p66Shc restrains activation of Ras pathway (Debnath, 2010), p52Shc and p46Shc play a role in coupling activated receptor tyrosine kinases to Ras via the formation of the GRB2/SOS complex, subsequently activate the MAPK signal pathway (Ravichandran et al.,

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1995). SHC SH2-domain binding protein 1 (SHCBP1), a cytoplasmic proteins which couples activated growth factor receptors to signaling pathways. The expressions of SHCBP1 mRNA level had evident individual differences among human normal tissue. The 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°C in a 5% CO_2 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'-ACATCCAAGCAAAGGGTGTC-3'; (reverse) 5'- GCACACAGTTTTCCAGCGTA'; SHCBP1-168bp: 5'-CCCTTCTGAGCAAGTCGAGG - 3' (forward); (reverse)5'- AACTGGTTCCCCACAATCCC'-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'-GAGCCUAUCAAGAUUACAUdUdU-3', antisense3'-GdUd CUCGGAUAGUUCUAAUGUA-5'); siRNA-2658 (sense 5'-GGAGGUUUGAAGCAGAAA UdGdA-3', antisense 3'-AdUdCCUCCAAACUUCGUCU UUA-5'); The non-specific siRNA not targeting any annotated human genes was used as negative control. The oligonucleotide sequences for shRNA were: ShRNA-SHCBP1-2658-Forward GATCCCCGGAGGTTTGAA GCAGAAATttcaagagaATTTCTGCTTCAAACCTCCT TTTTGGAAA;ShRNA-SHCBP1-2658-Reverse AGCTT TTCCAAAAAGGAGGTTTGAAGCAGAAATtctcttgaa ATTTCTGCTTCAAACCTCCGGG;ShRNA-SHCBP1-308-Forward GATCCCCGAGCCTATCAAGATTACA TttcaagagaATGTAATCTTGATAGGCTCTTTTTGGA AA;ShRNA-SHCBP1-308-Reverse AGCTTTTCCAAA AAGAGCCTATCAAGATTACATtctcttgaaATGTAATC TTGATAGGCTCGGG;ShRNA-SHCBP1-NC-Forward GATCCCCGGCCAAAGACTGTATACACttcaagaga GTGTATACAGTCTTTGGCCTTTTT GGAAA;ShRNA-SHCBP1-NC-Reverse AGCTTTTCCAAAAATTCTC CG AACGTGTCACGTtctcttgaa ACGTGACACG TTC GGAGAAGGG.

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% paraformalclehyde 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-shcbp1 antibody (1:500) (biotech) at 4°C 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°C 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°C 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°C 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.

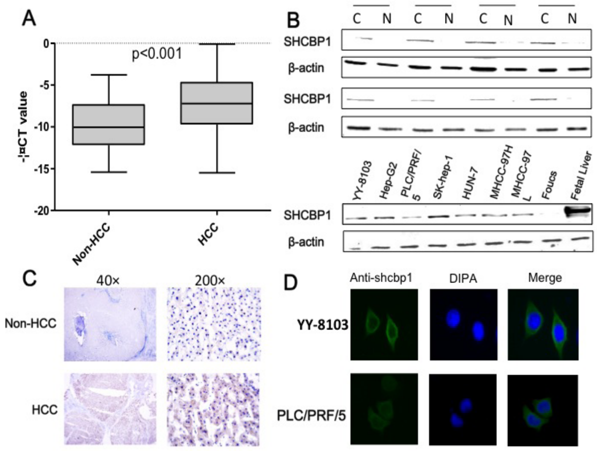

Figure 1. Expression Pattern of SHCBP1 in HCC Specimens, Cell Lines. (A) The mRNA level of SHCBP1 in 52 HCC specimens and corresponding Non-HCC livers was measured by quantitative real-time RT-PCR, β-actin as internal control. Compared with noncancerous tissues, the mRNA level of SHCBP1 was significantly 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 bolt , 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 PLC/PRF/5 cells. The nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI)

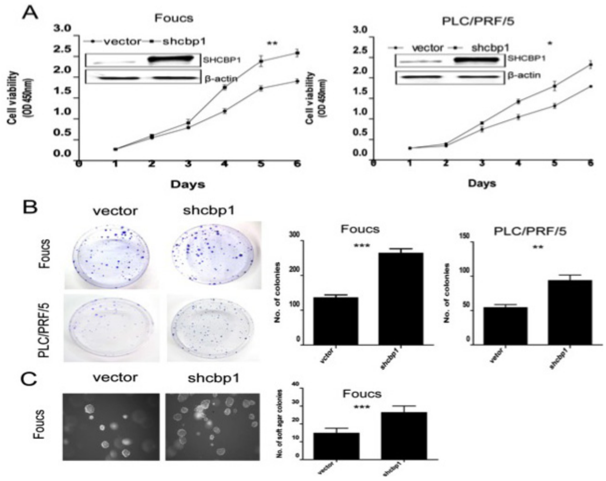

Figure 2. SHCBP1 Overexpression Promoted Cell Proliferation, Colony Formation and Soft Agar Colony Formation. (A) Ectopic SHCBP1 protein promoted the proliferation of Focus and PLC/PRF/5 cells, where SHCBP1expression was evaluated by Western blot analysis. The different symbols represent the corresponding mean values of the experiments in triplicate (mean ± SD). (B, C) Ectopic SHCBP1 enhanced the colony formation and soft agar colony formation of Focus and PLC/PRF/5 cells, as shown by representative dishes of colony formation of those transfected with pcDNA3.1-GFP-SHCBP1 and pcDNA3.1-GFP used as control. The numbers of colonies are the mean values of three independent experiments. All experiments were independently repeated at least three times. **P*<0.05, ***P*<0.01, ****P*<0.001

Cell proliferation and Colony formation assay

The proliferation ability was analyzed by cell growth curve and Colony formation assay. HCC cells (3~5 $\times 10^3$ 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 concentrationis 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 Infared 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.

Cell cycle analysis and cell synchronization

Flow cytometry was applied to analyze the effect of SHCBP1 on cell cycle. HCC cells were cultured in the medium lacking serum for 24 hours for cell cycle synchronization. cells were harvested as single cell suspensions,washed with PBS and incubated with propidium iodide (10 µg/ml) and RNase A (10 mg/ml) for 30 min at 4°C.

Statistical analysis

All experiments were repeated at least three times and each experiment was performed at least in duplicate. All the data on this research were analysed by student's t-test using GraphPad Prism 5 software. *P* value < 0.05 was considered statistically significant.

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 bolt. 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, Foucs cells (Figure 1B). In addition, we are also examined by

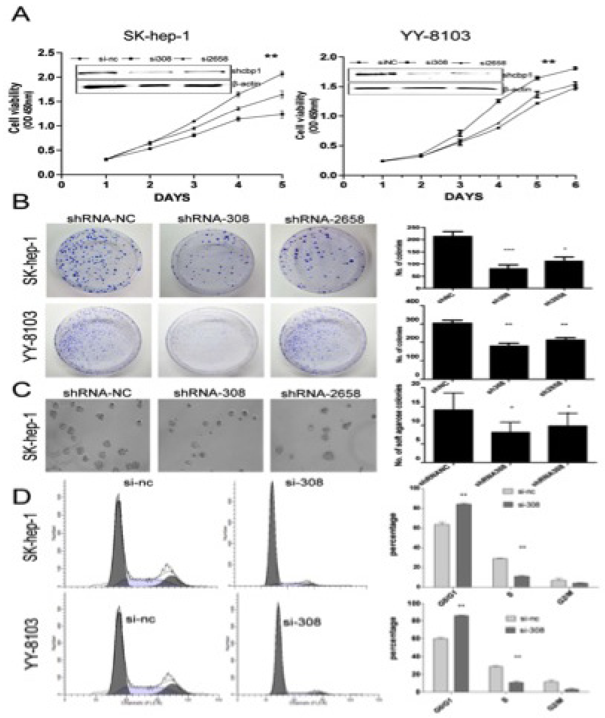

Figure 3. SHCBP1 Silencing Suppresses Cell Growth and Induces Cell Cycle Delay in HCC Cells. (A) The specific synthesized siRNA knocked down the endogenous SHCBP1 and transfected into SK-hep-1 and YY-8103 cells. The Silencing efficiencies of si-308 and si-2658 were assessed by western bolt, where si-NC was used as controls. SHCBP1 Knockdown by siRNA dramatically inhibited cell proliferation in SK-hep-1 and YY-8103 cells by using cell viability assay compared with si-NC. (B, C) Targeting SHCBP1 by pSUPER-sh308 and pSUPER-sh2658 inhibited both colony formation and soft agar colony formation in SK-hep-1 and YY-8103 cell compare with pSUPER-shNC used as a control. The numbers of colonies were counted from three independent experiments, which were stained. The mean colony number of three independent experiments (Student's t test mean ± SD) was indicated by the histograms. (D) SHCBP1 silencing by siRNA caused cell cycle significantly arrest at G0/G1 phase and inhibited the Entry of S Phase in Cell Cycle. Both in SK-hep-1 and YY-8103 cells by flow cytometry ,where si-NC was used as a control. The histograms showed that the percentages of each phase in cell cycle which was the mean values of three independent experiments by the mean and standard deviation, *$P < 0.05$ and **$P < 0.01$

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.

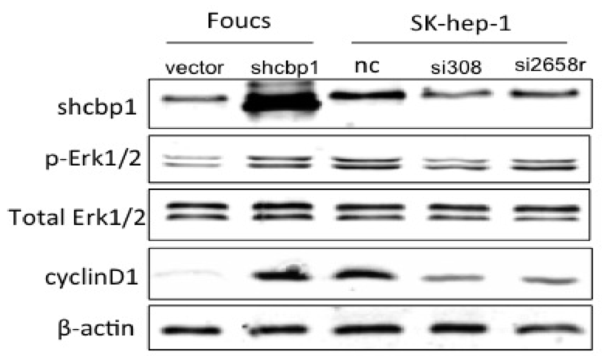

Figure 4. Western Bolt Analyses of the Expression of p-ERK1/2 and p-AKT in SHCBP1 Overexpress or Knockdown in Foucs and SK-hep-1 Cells. Note the expression of phosphorylated p-ERK1/2 and cyclin D1 expression significantly reduced in the SHCBP1 knockdown cells compared with control cells in SK-hep-1 cells. Meanwhile, overexpression of SHCBP1 significantly upregulated the protein level of cyclin D1 and phosphorylated ERK1/2 in Foucs cells. Total ERK1/2 and β-actin expression serve as internal control

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 bolt 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 (Figure3A, 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-1was 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 overexpress in Foucs 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 (Pelicci 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/RPF/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 Foucs 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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