

CSR1 suppresses tumor growth and metastasis of human hepatocellular carcinoma via inhibition of HPIP

L. JIANG¹, G. HU², F.-F. CHEN¹, X.-Y. DU¹, B. LIU², C. LIU¹

¹Department of General Surgery, First Affiliated Hospital of Bengbu Medical College, Bengbu, Anhui, China

²Hubei Key Laboratory for Kidney Disease Pathogenesis and Intervention, Huangshi Central Hospital of Edong Healthcare Group, Hubei Polytechnic University School of Medicine, Huangshi, Hubei, P.R. China

L. Jiang and G. Hu contributed equally

Abstract. – OBJECTIVE: Cellular stress response 1 (CSR1) is a tumor suppressor gene that was frequently down-regulated in prostate cancer. CSR1 has critical roles in the regulation of cell apoptosis via inactivation of CPSF3 or preventing the interaction of XIAP with caspases. However, whether CSR1 plays a role in human hepatocellular carcinoma (HCC) is completely unknown.

PATIENTS AND METHODS: The expression of CSR1 in HCC clinic samples and cell lines was detected by Real-time PCR and Western blot. CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system was used to knockout CSR1 gene in HepG2 cells. The proliferation of HCC cells was measured by MTT assay. The migration and invasion abilities of HepG2 cells were determined by in vitro scratch wounding and matrigel invasion assays. Co-immunoprecipitation assay was used to determine the interaction between CSR1 and hematopoietic PBX interacting protein (HPIP).

RESULTS: The mRNA and protein levels of CSR1 were down-regulated in human HCC cell lines and clinic HCC tissues. Over-expression of CSR1 inhibited cell proliferation, migration and invasion in human HCC cell lines. Knockout of CSR1 gene by CRISPR-Cas9 in HepG2 cells achieved the opposite effects. At the molecular level, we found that CSR1 associated with HPIP and inhibited the activation PI3K/AKT pathway.

CONCLUSIONS: For the first time we demonstrated that CSR1 inhibited HCC cell proliferation, migration and invasion through inactivation of HPIP and its downstream PI3K/AKT signaling pathway and suggested CSR1 as a potential therapy target for HCC treatment.

Key Words:

Cellular stress response 1, Human hepatocellular carcinoma, CRISPR, HPIP.

Introduction

Human hepatocellular carcinoma (HCC) is the sixth most common cancer and the third cause of cancer death worldwide¹. One of the most powerful curative treatments for HCC patients is surgical resection. However, the high frequency of recurrence and metastasis following surgical resection represent the major obstacles². Therefore, it's critical to get a better understanding of the molecular mechanisms responsible for HCC carcinogenesis^{3,4}. It's also important to identify genetic or epigenetic alterations in HCC to improve the diagnosis, therapy and predicting the prognosis for HCC. CSR1, also known as scavenger receptor class A member 3 (SCARA3), is a widely expressed protein. CSR1 was originally identified as a cellular stress response protein and a macrophage scavenger receptor homolog⁵. CSR1 was significantly elevated in normal fibroblasts in response to UV radiation or hydrogen peroxide treatment and protected cells from oxidative insult⁵. Further studies demonstrate that CSR1 is down-regulated and frequently methylated in over 30% of prostate cancers and associated with a poor prognosis for prostate cancer⁶. CSR1 functions as a tumor suppressor by inhibition of mRNA mature through redistribution and hijacking cleavage and polyadenylation-specific factor 3 (CPSF3). CSR1 also inhibited X-linked inhibitor of apoptosis activity (XIAP) and promoted cell death in prostate cancer cells⁶⁻⁸. CSR1 was dysregulated in some other tumors including ovarian carcinoma and multiple myeloma^{5,9}. However, the role of CSR1 in human hepatocellular carcinoma (HCC) is completely unknown. Here, we provided evidence

to show that CSR1 was down-regulated and acted as a tumor suppressor in HCC by inhibiting HCC cell proliferation, migration and invasion through inactivation of HPIP and its downstream PI3K/AKT signaling pathway.

Patients and Methods

Human Tissue Samples

Twenty paired primary HCC cancer samples and adjacent normal tissues were collected from routine therapeutic surgery at our hospital from January 2015 to September 2016 and were obtained with informed consent. This study has been approved by the hospital Institutional Review Board.

Cell Culture

HCC cell lines Hep3B, HepG2, HCCLM3, LM3, MHCC97H, and SMCC7721 were obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CAS, Shanghai, China). All cell lines were cultured in a medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

Quantitative Real-time PCR

Total RNA was extracted from the foam cells by using an RNA kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The Real-time PCR was performed in a 30 µl reaction volume with 200 mM dNTPs, 0.3 mM forward and reverse PCR primers, 0.1 mM probe, 3.5 mM magnesium chloride, 0.01% Tween-20, 0.05% gelatin, and 0.1 U of Taq polymerase, using the following PCR program: 95°C for 10 min, then 50 cycles of 95°C for 15 s followed by 60°C for 1 min. Quantitative Real-time PCR was performed by using a SYBR Green Premix Ex Taq (TaKaRa, Dalian, China) on an Applied Biosystems 7300 Real-time PCR System (Thermo-Fisher Scientific, Waltham, MA, USA). CSR1 and beta-actin cDNAs were amplified by the primers: ATCCGAAAGCCCTGAACAAC (forward) and GGTGAGGTCCTTCAGAGAG (reverse) for CSR1, and CATCCTCACCCTGAAGTACCC (forward) and AGCCTGGATAGCAACGTACATG (reverse) for beta-actin cDNA.

CRISPR/Cas9 KO

SCARA3 CRISPR/Cas9 KO Plasmid (h): sc-409127 and SCARA3 HDR Plasmid (h): sc-

409127-HDR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HepG2 cells were co-transfected with both plasmids and selected by puromycin. The single clones were chosen and Western blot was used to verify the knockout efficiency.

Colony Formation Assay

Each 6-well plate was covered by a base layer consisting of 0.5 ml culture media (0.6% agar). Control HCCLM3 or Flag-CSR1-HCCLM3 cells were then seeded by layering about 5000 cells in 0.5 ml culture media (0.3% agar) over each base layer. Two weeks later, cells containing uniformly distributed single cell suspensions, were considered positive colony.

BrdU Assay

The incorporation of BrdU during DNA synthesis was analyzed by a cell proliferation enzyme-linked immunosorbent assay (Beyotime, Shanghai, China) following the manufacturer's protocols. Absorbance was measured at 450 nm in the Spectra Max 190 ELISA reader (Molecular Devices, Silicon Valley, CA, USA). All experiments were performed in triplicate.

CCK8 Assay

Cells were inoculated in a 96-well plate for 24 hours. CCK8 solution was added to each well of the plate. The plate was then incubated for 4 hours in an incubator. The absorbance at 450 nm was measured by using a microplate reader (Molecular Devices, Silicon Valley, CA, USA).

Migration Assay

For *in vitro* scratch wounding assays, cells were grown on 6-well plates to 100% confluence. Cells were starved in media with 0.5% serum and then scratched with sterile pipette tips to form wound. The cells were then cultured in normal media for 24 hours. The area devoid of cells was imaged immediately and 36 hours later by using Zeiss AxioVision microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

Invasion Assay

Cell invasion assays were performed in a transwell chamber with a polyethylene terephthalate filter membrane containing 8.0 µm pores on 12-well plates (Corning, NY, USA). About 5×10^4 cells per well without serum were immediately placed in the upper compartment of the plates. The lower compartment was added with medi-

um containing 10% fetal bovine serum (FBS). Cells in chambers were incubated for 36 hours. The cells remaining on the upper surface of the filter membrane were removed and the cells on the opposite surface of the filter membrane were stained with 0.1% crystal violet and fixed in 4% paraformaldehyde.

Western Blot

Clinic tissues or cultured cells were harvested and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM 2-mercaptoethanol, 2% SDS and 10% glycerol). After centrifugation at $15000 \times g$ for 15 min at 4°C, proteins in the supernatants were quantified by using BCA protein assay kit (KeyGen Biotech, Nanjing, China). Proteins were separated by 10-12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to NC membrane. Western blot assay was then performed using the following antibodies: anti-SCARA3 (CSR1), anti-HPIP, anti-E-cadherin, anti-N-cadherin (Santa Cruz, CA, USA), anti-AKT, anti-p-AKT (Cell Signaling, Boston, MA, USA) and anti-Flag M2, anti-HA (Sigma-Aldrich, St. Louis, MI, USA). Protein levels were normalized to total GAPDH, using a rabbit anti-GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical Analysis

The data shown represent the mean $\pm$ standard (SD) of three independent experiments. Significance was analyzed using Student's *t*-test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

Results

CSR1 was Down-Regulated in HCC Samples

To evaluate the expression of CSR1 in HCC cancer cells, the mRNA level of CSR1 in twenty paired primary HCC cancer samples and adjacent normal tissues was determined by Real-time quantitative PCR. We found that the mRNA expression of CSR1 was significantly decreased in HCC samples when compared with adjacent normal tissues (Figure 1A). Next, we detected the protein expression level of CSR1 among ten paired of those samples by Western blot with anti-CSR antibody. In consistent with the mRNA level, CSR1 protein was also down-regulated in HCC samples when compared with adjacent normal ones (Figure 1B). We also detected the protein levels of CSR1 in several kinds of cultured cell HCC cell lines including Hep3B, HepG2, HCCLM3, LM3, MHCC97H, and SMCC7721.

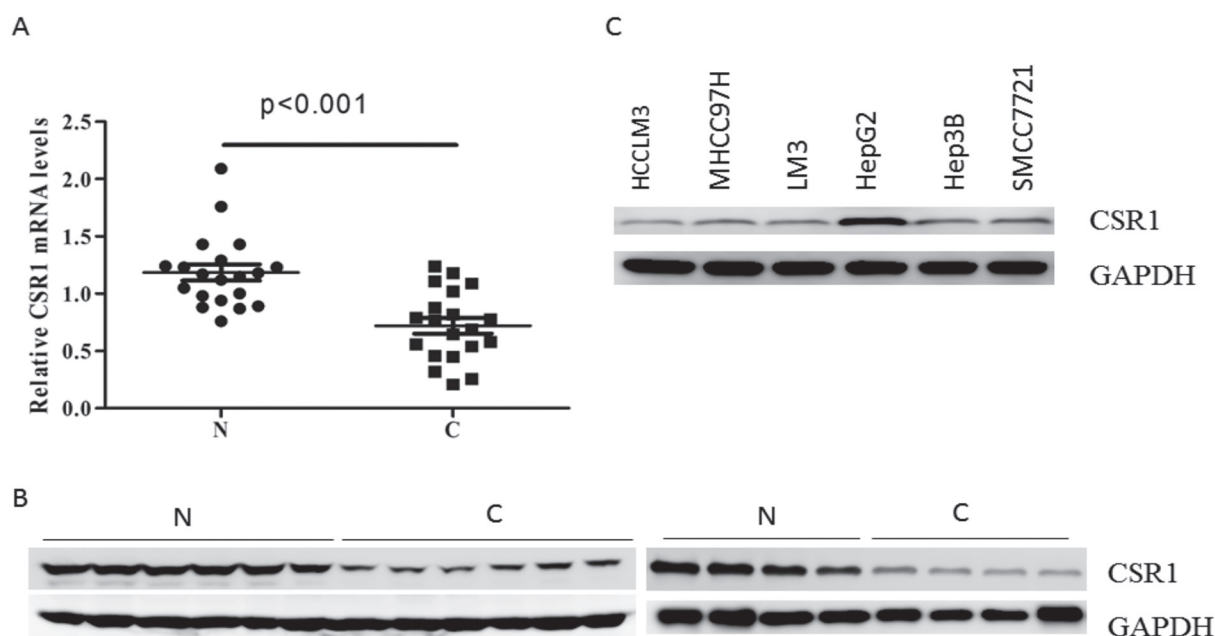


Figure 1. CSR1 was down-regulated in HCC samples. **A**, The mRNA levels of CSR1 in twenty paired primary HCC cancer samples and adjacent normal tissues were determined by Real-time quantitative PCR. N: Normal tissues; C: Cancer tissues. **B**, The protein levels of CSR1 in ten paired primary HCC cancer samples and adjacent normal tissues were determined by Western blot with indicated antibodies. **C**, The protein levels of CSR1 in HCC cell lines including Hep3B, HepG2, HCCLM3, LM3, MHCC97H, and SMCC7721 tissues were determined by Western blot with indicated antibodies.

The lowest CSR1 protein expression level was observed in high metastatic HCCLM3 cells (Figure 1C). Taken together, these data demonstrated that CSR1 was down-regulated in HCC samples and cells.

Over-Expression of CSR1 Inhibited Cell Proliferation, Migration and Invasion in Human HCC Cell Lines

To investigate the gain-of-function role of CSR1 in HCC cells, we firstly generated HCCLM3 cells stable expression of exogenous Flag tagged CSR1 by retrovirus-mediated gene expression¹⁰. The exogenous expression of Flag-CSR1 was further confirmed by Western blot with anti-Flag antibody (Figure 2A). Next, we tested whether CSR1 overexpression had a consequence on the proliferation of HCCLM3 cells. We found that HCCLM3 cells overexpressing Flag-CSR1 (Flag-CSR1-HCCLM3) exhibited

declined growth capacity when compared with vector control overexpression cells (Figure 2B). By using BrdU assay, we further confirmed this phenomenon (Figure 2C). In consistent with the growth retardant phenotype, the colony-forming ability was also decreased in Flag-CSR1-HCCLM3 cells (Figure 2D). To test whether CSR1 plays a role in cells migration and invasion of HCC cells, *in vitro* scratch wounding and matrigel invasion assays were further used. By using wound-healing assay, we found that Flag-CSR1-HCCLM3 cells had significantly slower closure of the wound area compared to control cells (Figure 2E). Moreover, flag-CSR1-HCCLM3 cells showed a lesser degree of invasion through matrigel, suggesting that CSR1 could suppress HCC cells migration and invasion *in vitro* (Figure 2F). Together, these data suggested that CSR1 inhibited cell proliferation, migration and invasion in human HCC cell lines.

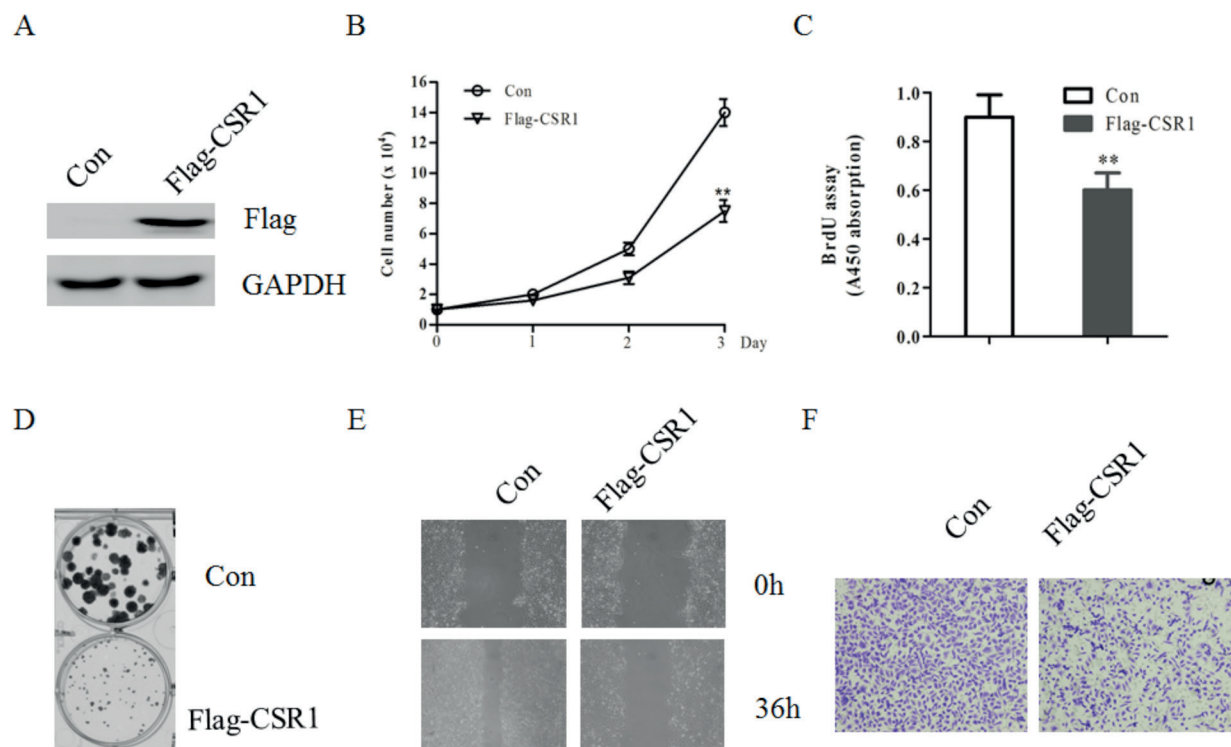


Figure 2. Over-expression of CSR1 inhibited cell proliferation, migration and invasion in human HCC cell lines. **A**, The protein level of exogenous CSR1 was analyzed by Western blot with anti-CSR1 antibody in HCCLM3 cells stably expression Flag-CSR1 or control vector (Con). **B**, The cell growth curve of in HCCLM3 cells stably expression Flag-CSR1 or control vector (Con). **C**, The proliferative potential (BrdU) assay was performed in HCCLM3 cells stably expression Flag-CSR1 or control vector (Con). **D**, The colony formation assay was determined in HCCLM3 cells stably expression Flag-CSR1 or control vector (Con). **E**, A representative image of HCCLM3 cells stably expression Flag-CSR1 or control vector (Con) at 0 and 36 hours after wound scratch in wound-healing assay. **F**, transwell chamber assay was used to analyze the cell invasion ability. The representative images of crystal violet stained HCCLM3 cells stably expression Flag-CSR1 or control vector (Con).

Knockout of CSR1 Gene by CRISPR-Cas9 in HepG2 Cells Promoted Cell Proliferation, Migration and Invasion

As HepG2 cells contain relatively higher level of CSR1 among several HCC cell lines, we then tested the loss-of-function roles of CSR1 in HepG2 cells. The CRISPR/Cas9 mediated genome editing assay had great power to knock out genes¹¹. We then used sgRNAs targeting different regions in the exon of the human CSR1 gene. HepG2 cells were co-transfected with sgRNAs and clones were selected by puromycin. Our Western blotting showed that KO3 had the most knocked out CSR1 efficiency in HepG2 cells (Figure 3A). We noticed that KO3 cells proliferated faster than the control HepG2 cells by CCK-8 assay, indicating that depleting CSR1 significantly promoted HCC cell proliferation (Figure 3B). The cell growth curve assay also showed KO3 cells grow faster than control cells (Figure 3C). We next tested the migration and invasion abilities of KO3 cells. Both scratch wounding and matrigel invasion assays demonstrated that the migration and invasive capability of HepG2

cells without CSR1 were significantly increased when compared with control cells (Figure 3 D-E). Taken together, these data demonstrated a tumor suppressor role of CSR1 in HCC cells.

CSR1 Associated with HPIP

As reported, CSR1 usually exhibited its biological function through protein-protein interaction. By searching several large protein-protein interaction databases^{12,13}, we found that PSTPIP1 (HPIP), proline-serine-threonine phosphatase interacting protein 1, was one of the binding partners of CSR1. HPIP is a scaffold protein, known to regulate the proliferation, migration and invasion in HCC¹⁴, which drove us to ask whether CSR1 interacted with HPIP in HCC cells. To this end, we tested the specific interaction between exogenous CSR1 and HPIP in HepG2 cells by using immunoprecipitation (IP) assay. Flag-CSR1 and HA-HPIP were co-transfected into HepG2 cells for 36 h; cells were lysed and subjected to IP with anti-HA antibody. As shown in Figure 4A, Flag-CSR1 was readily detected in HA-HPIP immunoprecipitate. Moreover, immunoprecipitation by Flag M2 beads

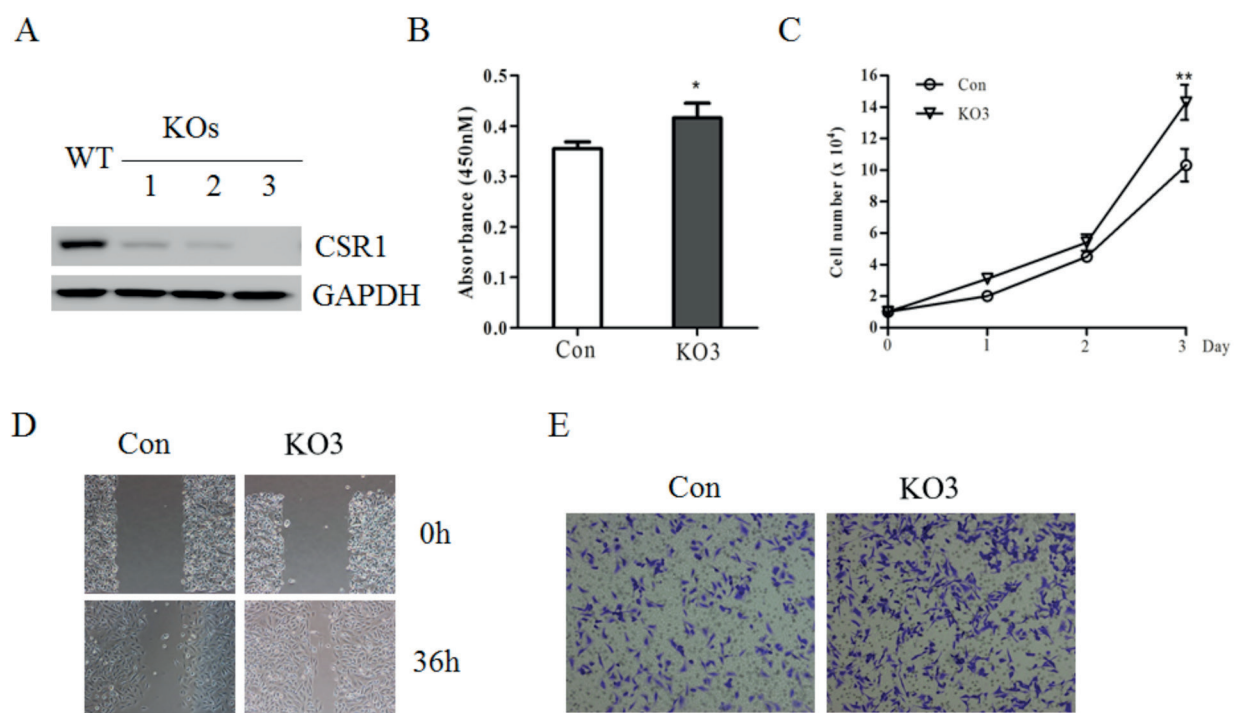


Figure 3. Knockout of CSR1 gene by CRISPR-Cas9 in HepG2 cells promoted cell proliferation, migration and invasion. **A**, protein levels of CSR1 in parental HepG2 cells and three clones of CSR1 knock out cells were detected by Western blot. **B**, the cell proliferation of HepG2 cells and CSR1 knock out cells (KO3) were tested by CCK8 assay. **C**, The cell growth curve of HepG2 cells and CSR1 knock out cells (KO3). **D**, Representative images of HepG2 cells and CSR1 knock out cells (KO3) at 0 and 36 hours after wound scratch in wound-healing assay. **E**, transwell chamber assay was used to analyze the cell invasion ability. The representative images of crystal violet stained HepG2 cells and CSR1 knock out cells.

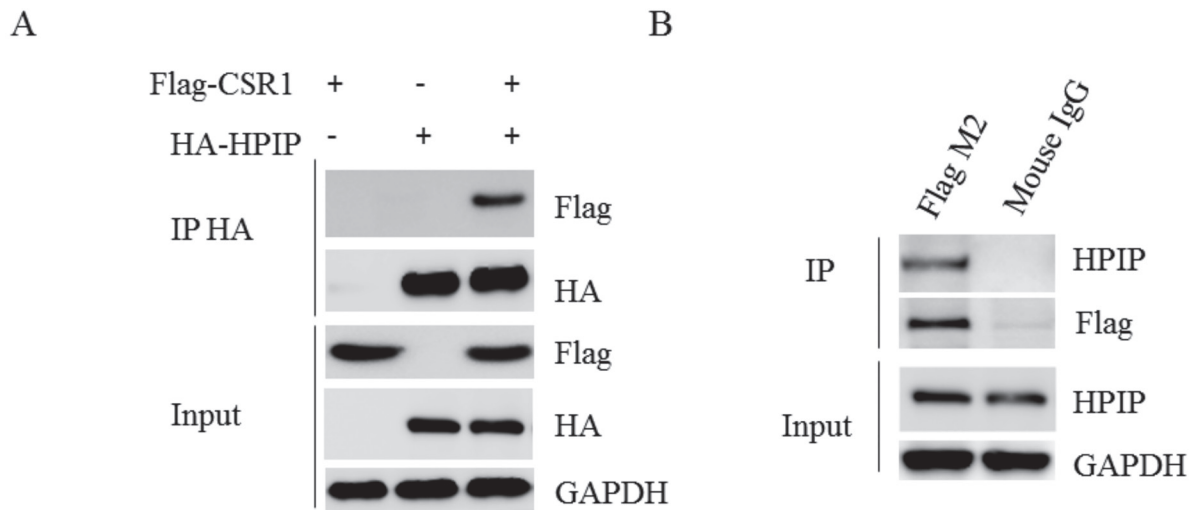


Figure 4. CSR1 associated with HPIP. **A**, hepG2 cells were co-transfected with Flag-CSR1 and HA-HPIP for 36 h, cells were lysed and subjected to IP assay with anti-HA antibody. **B**, Flag-CSR1-HCCLM3 cell lysates were subjected to IP assay with either Flag M2 beads or mouse IgG beads for 8 hours, the IP materials were subjected to Western blot with indicated antibodies.

from Flag-CSR1-HCCLM3 cell lysates followed by Western blot revealed a physical association between CSR1 and endogenous HPIP (Figure 4B). Taken together, these data suggested that CSR1 was associated with HPIP.

CSR1 Inhibited HPIP-Induced PI3K/AKT Pathway Activation in HCC Cells

Recent studies demonstrated that HPIP played a critical role in malignant tumor's infiltration and metastasis by activating several signaling pathways such as PI3K/AKT pathway¹⁵. As CSR1 function as a tumor suppressor in HCC and was interacted with HPIP, together these clues drove us to ask whether CSR1 interfering with the function of HPIP. To this end, we found that PI3K/AKT pathway was inactivated in Flag-CSR1-HCCLM3 cells, while active in HepG2 cells without CSR1 (KO3 cells), as evidenced by Western blot with phosphorylated AKT antibody (Figure 5A-B). Moreover, biomarkers for epithelial-mesenchymal transitions (EMT) were also changed according to the protein levels of CSR1 (Figure 5A-B). Thus, these data suggested that CSR1 inhibited HPIP-induced PI3K/AKT pathway activation in HCC cells.

Discussion

In the present study, we demonstrated that CSR1 had a tumor suppressor role in HCC.

Firstly, we found that the mRNA and protein levels of CSR1 were both down-regulated in HCC samples and cell line. Then, overexpression of CSR1 inhibited HCC cells proliferation, migration and invasion, while knock out of CSR1 in HepG2 cells by CRISPR/Cas9 assay archived the opposite effects. Mechanistically, we found that CSR1 was associated with HPIP and inhibited the biological function of HPIP, including HPIP-induced PI3K/AKT pathway activation. Thus, our data suggested that CSR1 interacted with and inhibited HPIP function to archive its tumor suppressor function in HCC cells. HPIP, a co-repressor for pre-B-cell leukemia homeobox 1 (PBX1), is known to act as a promoter during tumorigenesis¹⁵. HPIP is upregulated in varieties of cancers, such as astrocytoma, colorectal cancer, breast infiltrative ductal carcinoma, oral cell carcinoma, renal cell carcinoma¹⁵⁻²⁰. HPIP is also overexpressed in most of 328 liver cancer patients and regulates hepatoma cell proliferation¹⁴. HPIP promotes cell growth by inhibiting apoptosis and activation of cell cycle progression, enhances cell migration and invasion with increased EMT¹⁵. Mechanistically, HPIP increases cell proliferation and migration mainly through activation of PI3K/AKT signaling pathway¹⁵. Interestingly, we found that CSR1 interacted with HPIP and functionally inhibited HPIP in HCC cells. Previous studies demonstrated that CSR1 function as a tumor suppressor by inhibition of mRNA mature through CPSF inhibition or inhibiting XIAP to promote

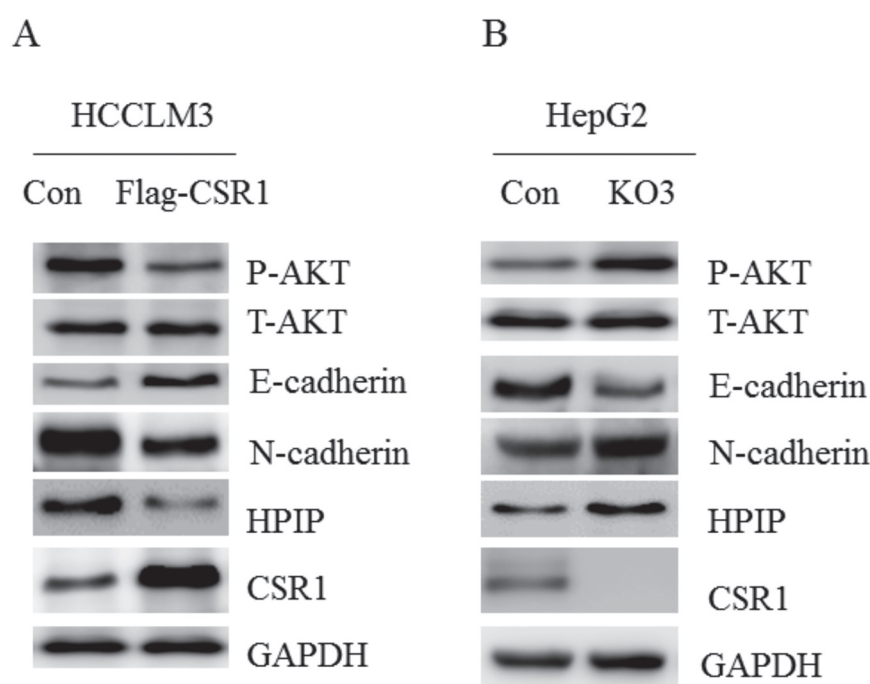


Figure 5. CSR1 inhibited HPIP-induced PI3K/AKT pathway activation in HCC cells. **A**, The lysates of HCCLM3 cells stable expressing con or Flag-CSR1 were subjected to Western blot with indicated antibodies. **B**, The lysates of control HepG2 cells or KO3 cells were subjected to Western blot with indicated antibodies.

cell death in prostate cancer cells^{7,8}. In this case, we observed the HPIP protein level could be regulated by CSR1 in HCC cells, although the changes were subtle, suggesting that a posttranscriptional modification existed. Thus, the more detail relationship between CSR1 and HPIP warrants further studies.

Conclusions

We demonstrated that CSR1 inhibited HCC cell proliferation, migration and invasion through inactivation of HPIP as well as its downstream PI3K/AKT signaling pathway. Our data suggested CSR1 might be a potential therapy target for HCC treatment.

Acknowledgements

This study is supported by grants from the Natural Science Foundation of Hubei Province (2016CFB517) and Research Foundation of Hubei Polytechnic University for Talented Scholars (16xjz01R).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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