RXF-1-dependent activation of SHP-1 inhibits STAT3 signaling in hepatocellular carcinoma cells

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Regulatory factor X-1 (RXF-1) is a transcription factor that has been linked to negative regulation of tumor progression; however, its biological function and signaling cascades are unknown. Here, we performed several studies to elucidate the roles of RXF-1 in the regulation of SHP-1 in hepatocellular carcinoma (HCC) cells. Overexpression of RXF-1 resulted in the activation of SHP-1 and repressed colony formation of HCC cells. In addition, by a mouse xenograft model, we demonstrated that RXF-1 overexpression also inhibited the tumor growth of HCC cells in vivo, suggesting that RXF-1 is of potential interest for small-molecule-targeted therapy. We also found that SC-2001, a bipyrrole molecule, induced apoptosis in HCC cells through activating RXF-1 expression. SC-2001 induced RXF-1 transcription from exon 2 is expressed in hematopoietic cells. Several major transcription factors have been found to regulate SHP-1 transcript, for example, PU.1 (16), Oct-1 (17) and regulatory factor X-1 (RXF-1) (18). RXF-1 is one of the transcription factors that can activate SHP-1 transcription in the P1 promoter region. RXF-1 overexpression in human glioblastoma cells inhibits neurosphere formation and decreases FGF-1 mRNA expression, resulting in cell growth inhibition (19). In addition, RXF-1 also exhibited an antiproliferation effect in human neuroblastoma cells through inhibiting the TGFβ2-ERK pathway (20), suggesting that RXF-1 might act as a tumor suppressor. However, the function of RXF-1 in HCC has not been fully disclosed.

In this report, we demonstrate for the first time that RXF-1 is one of the factors in determining HCC tumor cell growth. In addition, RXF-1 induction by SC-2001 activated the expression level of SHP-1 and further negatively regulated the p-STAT3 status in HCC cells. We further showed that RXF-1 may play a vital role in tumor progression using a nude mouse xenograft model. Targeting of RXF-1 by SC-2001 showed a more potent anticancer effect than sorafenib in vitro and in vivo study, suggesting a potential role for RXF-1 in HCC treatment.

Materials and methods

Cell culture and antibodies

The HuH-7 HCC cell line was obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan; JCRB0403). The PLC/PRF/5 (PLC5), SK-Hep-1 and Hep3B cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cells obtained from HSRRB or ATCC were immediately expanded and frozen down such that all cell lines could be restarted every 3 months from a frozen vial of the same batch of cells. Primary cancer cells from consenting patients were also analyzed. Study protocols were approved by Institutional Review Board of the institution, and informed consent was obtained in accordance with the Declaration of Helsinki. Human HCC samples were obtained from the patient who underwent tumor resection. The cells were isolated by mechanical mincing and digestion by collagenase. Tumor specimens were cut into small pieces of ~5 mm and then dissociated with 0.1% collagenase type IV (Sigma Chemical Co., St Louis, MO) at 37°C for 30 min. The cell suspension was filtered through a cell strainer (70 μm mesh size) and the filtrate was centrifuged at 1000 r.p.m. for 5 min. P1 cell pellet was resuspended in 5 ml of hepatocyte culture medium (XenoTech LLC, KS) and minimum essential media (Life Technologies Corp.), respectively. Approximately 1 × 10⁶ cancer cells were then seeded in plastic flasks coated with collagen (Becton-Dickinson, MA). The cultures were maintained
in a humidified atmosphere containing 5% CO\(_2\) at 37°C. A subcultivation ratio of 1:4 and 1:6 was performed while the cells reached 70–80% confluence. Under routine cultivation, Pt1 cells could secret albumin and alpha fetoprotein (AFP), this phenomenon indicated that Pt1 cells maintained the hepatic cell property in in vitro culture condition. Antibodies for immunoblotting such as p-STAT3 and STAT3 were from Cell Signaling (Danvers, MA). SHP-1, caffeine D1 and Mc1 antibodies were purchased from Abcam (Cambridge, MA), RFX-1 antibody was purchased from Novus Biologics.

**Colonies formation assay**

HCC cells were seeded in six-well plates (~1000–5000 cells per well) and subjected to the indicated treatments, with the drug being removed to terminate the treatment. Two weeks later, plates were washed in phosphate-buffered saline (PBS), fixed with 100% methanol and stained with a filtered solution of crystal violet (5% wt/vol). After washing with tap water, the colonies were counted both manually (by eye) and digitally using a ColCount™ plate reader (Oxford Optronics, Oxford, England).

**SHP-1 phosphatase activity**

A RediPlate 96 EnzChek Tyrosine Phosphatase Assay Kit (R-22067) was used for SHP-1 activity assay (Molecular Probes, Carlsbad, CA). The method was as described previously (21).

**Gene knockdown using siRNA**

Smart-pool siRNAs, including the control (D-001810-10), RFX-1, were purchased from Dharmacon (Chicago, IL). The knockdown procedure was as described previously (22). Briefly, PLC5 cells were transfected with siRNAs against the phosphatases given above or the control siRNA by the transfection reagent for 48 h and then treated with SC-2001 at the indicated concentrations. The cell extracts were analyzed by immunoblotting and colony formation assay.

**Reverse transcription PCR and quantitative PCR**

Total RNA was isolated from cells with TRIzol (Invitrogen) and cDNA was prepared from 2 μg of RNA using a First-Strand cDNA Synthesis Kit according to the manufacturer’s instructions (Amersham Biosciences, Amersham, UK). Oligonucleotide sequences were as follows: SHP-1, 5′-GCC CAG TTC ATT GAA ACC AC-3′ (sense) and 5′-GAG GGA ACC CTT GCT CTT CT-3′ (antisense); GAPDH, 5′-CGA CCA CTT TGT CAA GCT CA-3′ (antisense) and 5′-AGG GGT GTC CAT GAC AAC TG-3′ (antisense); Mc1-1, 5′-GCC ACT TGT TCT GG-3′ (sense) and 5′-CAG GGC ATG CTT CGG AAA CT-3′ (antisense). The following PCR conditions were used: denaturation at 95°C for 10 min followed by 35 cycles of 94°C for 1 min, annealing for 1 min at 57°C and elongation for 1 min at 72°C and a final elongation step at 72°C for 10 min. The amplified products were electrophoretically analyzed on a 1% agarose gel. For quantitative PCR, thermocycling was performed in a final volume of 20 μl containing 2.5 μl of cDNA sample, 200 nM of each of the primers and 6.5 μl of SYBR Green Master Mix (Roche) with Roche LightCycler 480 sequence detection system (Roche Applied Science, Foster City, CA). Expression levels of genes of interest were normalized to that of GAPDH in the same sample.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in a series of ethanol solutions with decreasing concentrations and finally in PBS. Antibiotigen retrieval was conducted in 0.01 mol/l sodium citrate buffer (pH 6.0) at 100°C for 15 min. Frozen tissues were cut into 7 μm sections and fixed in 4% paraformaldehyde for 15 min. Both paraffin-embedded tissue and cryostat sections were then treated with 3% hydrogen peroxide for 10 min, blocked with 3% bovine serum albumin (Sigma–Aldrich, MO) and 10% fetal bovine serum for 90 min and incubated with rabbit polyclonal anti-human anti-RFX-1 (1:50: antibodies (Novus Biologicals) or anti-SHP-1 antibodies (35x) (Abcam, Cambridge, UK) overnight at 4°C. All sections were counterstained with hematoxylin. All pathologic samples from the patients in this study were obtained after written informed consent had been obtained. The Research Ethics Committee of National Taiwan University Hospital approved the protocol of this study.

**Preparation of nuclear extracts and cytosolic extracts**

Nuclear extracts were prepared using NE-PER™ nuclear and cytoplasmic extraction reagent, according to the manufacturer’s instructions (Pierce Biotechnology). Briefly, 1 × 10⁶ cells were trypsinized, followed by lysing in 200 ml cytoplasmic extraction buffer I after. The lysates were vortexed for 15 s and incubated on ice for 10 min, 11 ml of cytoplasmic extraction reagent II was added. The lysates were vortexed for 5 s, incubated on ice for 1 min and vortexed for 5 s. The nuclei were pelleted at 16,000 rpm at 4°C for 5 min, and the cytoplasmic extracts were removed and collected. Nuclei were resuspended in 25 ml nuclear extraction buffer and vortexed 3 s. The nuclei were extracted on ice and vortexed for 15 s every 10 min, for a total of 40 min. The extracts were centrifuged at 16,000 rpm at 4°C for 5 min and the supernatant was collected as nuclear extract. Protein concentration was determined by BCA Protein Assay using bovine serum albumin as a standard (Pierce Biotech).

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) assay was performed, according to the protocol provided, with the EZ ChIP and EZ-Zyme Chromatin prep kit (Upstate Biotechnology, Lake Placid, NY). Briefly, after cross-linking with 17.5% paraformaldehyde, PLC5 cells were washed with PBS and lysed in lysis buffer. The DNA was fragmented to ~200–500 base pairs by the EZ-Zyme. Approximately 5 × 10⁶ cells were used per ChIP assay and the resulting DNA fragments were incubated with 2 μg RFX-1 antibodies, which were generated from rabbit or non-specific rabbit IgG (Millipore). The immunoprecipitation products were washed sequentially with low-salt immune complex wash buffer, high-salt immune complex wash buffer, LiCl immune complex wash buffer and twice with Tris-EDTA buffer. The chromatin was eluted from the agarose by incubating with elution buffer (1% sodium dodecyl sulfate, 100 mM NaHCO\(_3\)); and the DNA–protein complexes were reversely cross-linked by high-salt solution containing 200 mM NaCl at 65°C for at least 5 h. To eliminate contamination of proteins and RNAs, the mixture was treated with 10 mg RNase A at 37°C for 30 min and then treated with protease K for 2 h at 45°C. Finally, the precipitated DNA was recovered using the spin column provided in the ChIP kit and eluted with 50 μl elution buffer. The elution was conducted using Taq DNA polymerase (MyTaq). Two microliters of the precipitated DNA was used as template. The sequences of the primers used in the ChIP assay were as follows: 5′-CC TCTTGGAGGTCCTTTAGC-3′ and 5′-TGGAAAGGCAAAGGGAAATCCG-3′.

**Construction of SHP-1 promoter**

The SHP-1 promoter was amplified using human genomic DNA as the template. The promoter fragment extended from −1080 to 0 bp of the SHP-1 gene. Conditions used for the amplification of this region of the SHP-1 promoter were 5 min at 94°C, 1 min at 94°C, 1 min at 60°C and 3 min at 72°C for 35 cycles. The amplified product was electrophoresed on a 1% agarose gel. A discrete band of expected size 1 kb was isolated from the gel, digested with HindIII and XhoI and subsequently cloned into pGL3 basic vector. The deletion fragments were made by PCR amplification using different 5′ deletion primers with a 5′ XhoI restriction site and the same 3′ primer and were ligated to the pGL3 basic vector in the multiple cloning sites between HindIII and XhoI and DNA sequences were confirmed.

**Dual luciferase assay**

After transfection with firefly luciferase reporter construct and reference pCMV-tenilla luciferase plasmid for 48 h, cells were collected and lysed with passive lysis buffer. The lysate was placed into glass tube and promoter activity was analyzed by dual luciferase assay, according to the manual description. The STAT3 Reporter Kit was purchased from SA Biosciences. The activity of STAT3 promoter was determined as described above (23).

**Mutation of RFX-1-binding site on SHP-1 promoter**

The mutagenic constructs were made using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene). For this, the mutagenic primers with mutations in RFX-1 and other transcription factors sites were synthesized by the MDBio. The sequences were as follows: RFX-1: F: 5′-GGCCAGGGCTATGATGAGGCAAGAAACCGGCC-3′; R: 5′-TGACCGAGCTATGAGGCAAGAAACCGGCC-3′; AP-1: F: 5′-TTGGTG CGGCCATAGTCTGCCCCCCTAGG-3′ and R: 5′-CCATGGGAGGGAGCAGCA TATGCGGGCGACAGA-3′; AP-4: F: 5′-GGCTTGGCATGCGAATGAACT GGCGTGTTC-3′ and R: 5′-AAGACGCCATTTATGGTGGCTACAGGCG -3′ and NF-κB: 5′-AGGGGAGGAAAGAAGGCCATGATGAGGCAAGAAACCGGCC-3′ and R: 5′-TGTCCTGAGAACATGCGGACATGGTGACTGG-3′.

**Immunofluorescence**

PLC5 cells were seeded at a density of 2 × 10⁵ cells per well on glass coverslips in six-well plates 2 days prior to experimental manipulation. For immunofluorescence staining of RFX-1, cells grown on coverslips were washed twice with cold PBS, fixed with ice-cold methanol for 10 min at −20°C and permeabilized with 0.5% (vol/vol) Triton X-100 in PBS for 5 min at room temperature, followed by incubation with blocking solution for 1 h at room temperature. Coverslips were incubated with primary antibodies against human SHP-1 (Novus Biologicals) for 1 h at 37°C, washed with phosphate buffered saline with 0.2% Tween 20 and subsequently incubated with rabbit secondary antibody conjugated with rhodamine for 30 min at room temperature.
Afterwards, the coverslips were washed and mounted with fluorescent mounting medium (Dako). Fluorescence images will be taken with Zeiss LSM 510 confocal microscope scanning.

Generation of PLC5 cells with stable knockdown of RFX-1

The RFX-1-silencing stable cell line, PLC5-sh-RFX-1, was obtained by transfection of HuSH-shRNA-GFP cloning vector (pGFP-V-RS) or HuSH-sh-RFX-1-GFP cloning vector (pGFP-V-RS-sh-RFX-1) into PLC5 cells using Dharmafect 4 Transfection Reagent (Thermo Scientific). Two days posttransfection, the PLC5-sh-control (sh-vector) and the PLC5-sh-RFX-1 (sh-RFX-1) cells were selected and placed in medium containing 1.5 µg/ml puromycin for 2 weeks. Cells were routinely maintained under constant culture conditions. Control and shRNA plasmids were purchased from Origene.

Animal studies

Male NCr athymic nude mice (5–7 weeks of age) were obtained from the National Laboratory Animal Center (Taiwan, Taiwan). PLC5 vector and PLC5-OR cells were injected subcutaneously and the tumor volume was measured for 15 days. In RFX-1 knockdown experiments, when PLC5-sh-vector and PLC5-sh-RFX-1 tumors reached 100 mm3, mice received SC-2001 (10 mg/kg) orally (every other day). Controls received vehicle. Tumor measurements were converted to tumor volume (V) as follows: L x W2 x 0.52, where L and W are the length and width, respectively. Measurements were taken by the Vernier caliper. For orthotopic animal studies, mice were inoculated into the liver directly with luc2-expressed PLC5 cells. Once the tumors were visible, mice were separated into groups of three mice each. The control group was treated with vehicle alone (PBS) and the remaining two groups were treated with sorafenib (10 mg/kg/every other day) or SC-2001 (10 mg/kg/every other day). Tumor infiltration was monitored by bioluminescence imaging twice a week.

Statistical analysis

Data are expressed as mean ± SD or SE. Statistical comparisons were based on t-tests and statistical significance was defined as P < 0.05. All statistical analyses were performed using SPSS for Windows version 12.0 software (SPSS, Chicago, IL).

Results

Association between RFX-1 and SHP-1 in vitro and in vivo

STAT3 activation is often associated with progression and increased malignancy of HCC (24,25), therefore, targeting STAT3 has emerged as a novel potential anticancer therapy. Previously, we demonstrated that SHP-1 is a critical negative regulator of STAT3 and its activation is important for suppressing survival of HCC cells in vitro and in vivo (26). In addition, SHP-1 has been proved that is a major target of sorafenib (27), the only approved small molecule by FDA for HCC therapy, indicating its vital role in HCC development. However, the mechanism by which SHP-1 is modulated in HCC is far from clear. RFX-1, one of the transcription factors of SHP-1, has been shown that its induction is associated with tumor suppression in variety types of cancer (20,28,29). Since the role of RFX-1 in HCC remains unclear, we tried to further identify the function of RFX-1 in HCC cells. By transient overexpression of RFX-1, we found that ectopic expression of RFX-1 reduced PLC5 cell colony formation compared with non-transfected and vector control groups. This effect correlated with levels of SHP-1 protein and mRNA enhancement, implying that RFX-1 might play a tumor suppressor role by regulating SHP-1 (Figure 1A).

To further investigate the role of RFX-1 in tumor malignancy, we generated RFX-1 overexpression (PLC5-OR) and vector control RFX-1 (PLC5-vec) stable clones in PLC5 cells. Both of these stable clones were implanted subcutaneously into nude mice. Tumors overexpressing RFX-1 (PLC5-OR) grew slower than vector control RFX-1 (PLC5-vec) (Figure 1B, left, top). In addition, the tumor weights of PLC5-OR were lower than those seen in PLC5-vec (Figure 1B, right, top). Western blot analysis of tumor sections revealed that RFX-1 overexpression tumors had higher SHP-1 expression (Figure 1B, right, bottom) and lower p-STAT3 activity (Figure 1B, left, bottom) than vector control tumors, which correlated with the in vitro results. Furthermore, we used immunohistochemical staining to check the correlation between RFX-1 and SHP-1 in 101 clinical patient samples. We observed the high correlation between RFX-1 and SHP-1 protein expression in HCC patient tumor tissue with a R2 at 0.5156 (Figure 1C, left). Strong RFX-1 staining correlated with high levels of SHP-1 in patient samples (Figure 1C, right, top). Weak RFX-1 staining showed low levels of SHP-1 (Figure 1C, right, bottom), clinically supporting that RFX-1 positively regulates SHP-1 in HCC.

SC-2001 enhances SHP-1 promoter activity through RFX-1

Recently, several reports have shown that several natural products such as evodiamine (30), butein (31) and betulinic acid (32) dephosphorylate STAT3 through SHP-1 activation. Although these compounds increased SHP-1 mRNA and protein expression, we showed that SC-2001 exerts its antitumor effect through increasing expression of SHP-1 (26). Here, we further elucidated the mechanism of SHP-1 activation by SC-2001. A luciferase reporter driven by the SHP-1 promoter was constructed and transfected into four HCC cell lines. As shown in Figure 2A, the promoter activity of SHP-1 was enhanced by SC-2001 treatment. To further elucidate the involvement of transcription factors leading to SC-2001-induced SHP-1 activation, a series of truncated SHP-1 promoters were constructed and transfected into PLC5 cells. As shown in Figure 2B, enhancement of the SHP-1 promoter activity by SC-2001 treatment was abolished when the promoter region was truncated to ~556bp, indicating that a key element for activating the SHP-1 promoter was located between ~843 and ~556bp. Based on the literature (18), there are several transcription factors located in this region, including AP-1, AP-4, RFX-1 and NF-KB. Therefore, next, to find out which one of these transcription factors is crucial for activating SHP-1 in the presence of SC-2001, we mutated the binding sites of these transcription factors and examined the transcription activity by luciferase assay. Based on the results of luciferase assay, SC-2001 treatment-induced SHP-1 promoter activity was abolished when the RFX-1 binding site was mutated, suggesting that RFX-1 is involved in regulating the SHP-1 promoter (Figure 2C). Immunofluorescence staining was used to further confirm the movement of RFX-1 in HCC cells exposed to SC-2001. The results showed that RFX-1 translocated into the nucleus after SC-2001 treatment (Figure 2D, top). In addition, isolation of the nuclear extract showed that SC-2001 induces RFX-1 translocation into the nucleus in a time and dose-dependent manner, indicating that SC-2001 induced RFX-1 translocation and further regulated SHP-1 expression (Figure 2D, bottom). To examine the direct involvement of RFX-1 in modulating the transcription of SHP-1, we used ChIP assay to investigate whether SC-2001 enhances the interaction between RFX-1 and the SHP-1 promoter. As shown in Figure 2E, RFX-1 binding to the SHP-1 promoter was obviously increased after SC-2001 treatment. These data suggest that SC-2001-induced RFX-1 translocation can activate SHP-1 transcription by direct binding to the SHP-1 promoter.

RFX-1 is involved in SC-2001-mediated SHP-1 transcription in HCC cells and affects the antitumor effect in vivo

To further determine whether SC-2001 has potential as an anti-HCC agent through its RFX-1 activation, SK-Hep-1 and primary HCC cells were used as models to examine the effect of SC-2001 treatment on RFX-1 protein expression. SC-2001 increased RFX-1 protein expression in the time-dependent manner (Figure 3A). In addition, knockdown of RFX-1 in PLC5 and SK-Hep-1 cells resulted in significant reduction of SHP-1 mRNA and protein expressions (Figure 3B and 3C). This treatment also reversed the status of p-STAT3 even in the presence of SC-2001, implying that RFX-1 is a major target of SC-2001 (Figure 3C). To elucidate the function of SHP-1 activity by SC-2001-induced RFX-1, we performed SHP-1 activity assay. SC-2001 significantly increased SHP-1 activity, whereas silencing RFX-1 reversed the effect of SC-2001 in PLC5 and SK-Hep-1 cells (Figure 3D). We further examined the role of SC-2001-induced RFX-1 in cell growth by colony-forming assay. SC-2001 significantly decreased the PLC5 cell colony formation. Conversely, knockdown of RFX-1 reversed this inhibitory effect. In accordance with these results, overexpression of RFX-1 in PLC5 cells enhanced the
inhibitory effect of SC-2001 on colony-forming ability (Figure 3E). These results suggested that RFX-1 is involved in modulating SHP-1 expression and activity under SC-2001 treatment. We further tested SC-2001 efficacy in mice bearing sh-RFX-1 PLC5 xenograft tumors. Treatment with SC-2001 at 10 mg/kg/other day did not significantly inhibit the growth of sh-RFX-1 PLC5 tumors (Figure 3F, left) and failed to suppress p-STAT3 expression (Figure 3F, right) in these tumors compared with that in sh-vector PLC5 tumors, indicating the key role of RFX-1 in mediating the drug effects of SC-2001. Furthermore, RFX-1-depleted tumors grew more rapidly (3-fold) than sh-vector PLC5 tumors (Figure 3F, left). Also, p-STAT3 expression was relatively higher in the sh-RFX-1 PLC5 xenograft tumors than in the sh-vector PLC5 tumors (Figure 3F, right). Further, there were no apparent differences in body weight or toxicity in the drug-treated mice in comparison with the control group (Figure 3F, middle).

**SC-2001 showed a more potent anticancer effect than sorafenib in HCC cells**

In order to evaluate the potential of SC-2001 as an anti-HCC agent, we compared the anticancer effect of SC-2001 with sorafenib. As shown in Figure 4A, both sorafenib and SC-2001 decreased the viability of HCC cell lines. However, SC-2001 had a more potent inhibitory effect on cell growth than sorafenib. Moreover, DNA fragmentation assay indicated that SC-2001 exhibited a more potent effect than sorafenib in inducing cell apoptosis (Figure 4B). These two agents induce HCC cell apoptosis through various mechanisms, even though sorafenib has been shown to bind with SHP-1 protein and directly activate its
RFX-1-dependent activation of SHP-1 in HCC cells

Fig. 2. SC-2001-enhanced SHP-1 promoter activity is related to RFX-1 transcription factor binding. (A) HCC cells were transiently cotransfected with SHP-1 P1 promoter between −1080 and 0 bp and renilla control vector. To assay luciferase activity, 100 ng of P1/pGL3 reporter was cotransfected with 50 ng of renilla control vector in HCC cells, then cells were treated with SC-2001 for 6 h. Luciferase activity and renilla activity were measured after SC-2001 treatment. The graph represents the percentage of ratio of luciferase and the renilla readings of three experiments conducted in duplicate. *P < 0.05, **P < 0.01. (B) SC-2001-enhanced SHP-1 promoter activity was abolished when it was truncated to −556 bp. Luciferase activity of various deletion fragments of SHP-1 P1 promoter in PLC5 cells. PLC5 cells were transiently cotransfected with various deletion fragments of the SHP-1 P1 promoter and renilla control vector then treated with SC-2001 for 6 h. (C) SHP-1 promoter activity was abolished in SC-2001 treatment when the RFX-1-binding site was mutated. The fragments of SHP-1 promoter containing RFX-1-, AP-1-, AP-4- or NF-xB-binding sites were constructed and binding site mutagenic constructs were made. PLC5 cells were cotransfected with 100 ng of binding site promoter fragment of the aforementioned transcription factors or their mutagenic constructs and 50 ng of renilla control vector were then treated with SC-2001 for 6 h. After SC-2001 treatment, luciferase activity and renilla activity were measured. *P < 0.05. (D) Top: immunofluorescence staining, RFX-1 nuclear translocation in PLC5 cells treated with SC-2001. PLC5 cells were treated with 10 μM SC-2001 for 12 h. After the end of treatment, RFX-1 was determined by immunofluorescence staining (red). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). Representative confocal micrographs are shown. Bottom, nuclear fraction protein extraction has shown that RFX-1 translocates to the nucleus after SC-2001 treatment. RFX-1 protein levels in nuclei of PLC5 cells treated with or without SC-2001. Nuclear extract (NE) and cytosolic extract (cyto) from PLC5 cells treated with SC-2001 in dose- and time-dependent manner were prepared and subjected to western blot analysis using antibody against RFX-1. NE was prepared as described in the Materials and methods. Histone H1 was included as a loading control NE. (E) ChIP assay was performed to examine the binding of RFX-1 to the SHP-1 promoter with or without SC-2001 treatment. After SC-2001 treatment, RFX-1 binding site fragment was detected by PCR in ChIP samples precipitated with RFX-1 and rabbit IgG control antibodies in PLC5 cells.

Discussion

In this study, we investigated the roles of RFX-1 in the regulation of SHP-1 in HCC cells and uncovered possible mechanisms by which novel molecule SC-2001 inhibits HCC growth. We first demonstrated that RFX-1 acts as antitumor protein in HCC cells in vitro and in vivo. In agreement with the previous literature (18), our results suggest that RFX-1 regulates SHP-1. Ectopic expression of RFX-1 increased SHP-1 mRNA and protein expression, and silencing RFX-1 reduced SHP-1 in vitro and in vivo (Figures 1A and B and 3F). We further proved that RFX-1 is involved in SC-2001-induced SHP-1 expression in HCC cells. Importantly, we also found that RFX-1 translocates into the nucleus and binds to the SHP-1 promoter under SC-2001 treatment (Figure 2). Strikingly, in comparison with the FDA-approved target therapy for HCC treatment, sorafenib, SC-2001 had a much more potent effect on HCC cells both in vitro and in vivo (Figure 4), implying that SC-2001 may be a potential drug for HCC therapy.

SHP-1 was first found in hematopoietic cells and it functions as a regulatory protein in some biological processes in lymphocytes (33). SHP-1 has been found to negatively regulate STAT3 in various cancer cell types, implicating it as a tumor suppressor (34). Previously, we identified SHP-1 as a target of SC-2001 in inhibition of p-STAT3 expression; however, how SHP-1 was regulated by SC-2001 remained unknown. In this study, we discovered that RFX-1 is crucial for SC-2001-induced SHP-1 activity. RFX-1 is a member of a family of transcription factors that contain a highly conserved winged helix DNA-binding domain that forms homo- and heterodimers by binding to the conserved X box in the major histocompatibility complex.
class II antigen promoter region (35). Although RFX-1 has been reported to regulate some biological processes in viruses such as hepatitis B, polyoma, cytomegalovirus and Epstein–Barr (36–39), the role of RFX-1 in cancer has not been fully explored. Some reports have indicated that RFX-1 can negatively regulate cancer cell proliferation through suppressing its target genes, such as c-myc (40), proliferating cell nuclear antigen (PCNA) (41) and FGF (19). In addition, RFX-1 expression was found to be lower in human glioma tissues and glioma cell lines than in brain tissue, and transient transfection of the RFX-1 gene reduced the proliferation of glioma cells (28). RFX-1 overexpression also inhibits human neuroblastoma proliferation through the direct effect of RFX-1 on the TGF-β promoter (20). Moreover, the expression of RFX-1 was found decreased in progressive Barrett’s with dysplasia and esophageal adenocarcinoma in comparison with Barrett’s esophagus, indicating that RFX-1 might function as a negative marker of tumor progression (29). However, it remains unclear whether RFX-1 also contributes to HCC malignancy. In this study, we provided evidence that ectopic expression of RFX-1 can decrease the colony-forming ability of HCC cells (Figure 1A), and based on a xenograft model, we further showed that RFX-1-knockdown HCC cells grew faster than sh-control cells (Figure 3F). Importantly, we observed that SHP-1 expression is associated with RFX-1 level both in HCC cells and HCC patient samples (Figure 1C). These results strongly suggest that RFX-1 might be an antitumor protein in HCC by regulating SHP-1 expression.

To our knowledge, this is the first study to demonstrate that RFX-1 can function as a negative regulator of HCC cell growth. Meanwhile, SC-2001 is the first agent to be shown to activate RFX-1 expression in HCC cells. Although we have shown that SC-2001 induced RFX-1-mediated tumor inhibition, the mechanism of RFX-1 upregulation by...
SC-2001 still needs to be addressed. Our data showed that SC-2001 can mediate both translocation of RFX-1 to the nucleus (Figure 2D) and also increase its mRNA and protein (Figure 3A). Protein kinase C (PKC) is involved in the nuclear import activity of RFX-1 (42), implying a possible mechanism of RFX-1 nuclear translocation under SC-2001 treatment. In addition, in human kidney cells, RFX-1 was found to interact with Adducin, which might regulate the transcriptional activity of RFX-1 (43). Thus, we checked whether PKC or Adducin are involved in SC-2001-induced cancer cell death. However, the results showed that SC-2001 affected neither p-Adducin (s6 and s716) nor total Adducin protein. Pretreatment with PKC inhibitor did not alter the phenotype under SC-2001 treatment, suggesting that PKC or Adducin did not involve in the SC-2001-induced anticancer effect (Supplementary Figure 1, available at Carcinogenesis online). Further studies are necessary to decipher mechanisms by which SC-2001 mediates both translocation of RFX-1 to the nucleus and upregulation of RFX-1.

Sorafenib is a small molecule that inhibits the kinase activity of Raf-1 and B-Raf in addition to the VEGFRs, platelet-derived growth factor receptor b (PDGFR-b), Flt-3 and c-KIT (44–46). Importantly, sorafenib is the only FDA-approved drug for use in HCC patients. Recently, we reported that sorafenib dephosphorylates STAT3 through directly activating SHP-1 in its PTP domain (47). However, SHP-1 expression is decreased in chronic sorafenib treatment and could be one of the mechanisms that contribute to the poor clinical results for sorafenib (23). Here, we showed that SC-2001 activates SHP-1 through upregulation of transcription and exhibits a more potent anti-HCC effect than sorafenib in vitro and in vivo (Figure 4). In addition, SC-2001 alone or in combination with sorafenib might be a novel strategy to overcome sorafenib resistance in HCC cells.

In summary, this study showed that SC-2001 can activate SHP-1 through the induction of RFX-1 activity in HCC cells. We also demonstrated that RFX-1 is related to HCC tumor growth and malignancy. Most importantly, we demonstrated that SC-2001 has a much better antitumor effect on HCC cells than sorafenib. We conclude that SC-2001 might be a potential strategy for HCC therapy.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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References