SCYL1 binding protein 1 promotes the ubiquitin-dependent degradation of Pirh2 and has tumor-suppressive function in the development of hepatocellular carcinoma

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Pirh2 is a Ring-H2 domain containing E3 ubiquitin ligase that targets several important tumor suppressor genes for proteasomal degradation. Overexpression of Pirh2 is frequently detected in many clinical tumor tissues including hepatocellular carcinoma (HCC). However, the molecular mechanism of Pirh2 activation in tumorigenesis still remains poorly understood. In this study, we find a Pirh2-binding protein, SCYL1 binding protein 1 (SCYL1BP1), that can promote the ubiquitin-dependent degradation of Pirh2. SCYL1BP1 colocalized with Pirh2 in the cytoplasm and prevented its localization to the nucleus. Ectopic expression of SCYL1BP1 increased the expression of p53 and further inhibited the G1/S transition of HCC cell lines. Conversely, knock down of SCYL1BP1 restored the expression of Pirh2 and inhibited p53 at protein level. Functional assays found that reintroduction of SCYL1BP1 into HCC cell lines significantly inhibited cell proliferation, foci formation, colony formation in soft agar and tumor formation in nude mice, suggesting the strong tumor-suppressive function of SCYL1BP1 in HCC progression. Furthermore, SCYL1BP1 was found to be frequently downregulated in HCC clinical specimens compared to their paired non-tumor tissues by immunohistochemical staining. Taken together, our data suggested that the interaction of SCYL1BP1/Pirh2 could accelerate Pirh2 degradation through an ubiquitin-dependent pathway, SCYL1BP1 may function as an important tumor suppressor gene in HCC development.

Introduction

Pirh2 was first cloned as androgen receptor N-terminal domain-interacting protein with Ring-H2 (C3H2C3) finger domain from a prostate carcinoma cDNA library (1). Later, it was characterized as both a p53- inducible protein and an E3 ligase to promote p53 degradation independent of Mdm2. Pirh2 was involved in an autoregulatory feedback loop that tightly regulates the activity of p53 (2). Given its important role in regulating the stability of p53, the role of Pirh2 in tumorigenesis was under intensive investigation in recent years. Several lines of evidences supported that Pirh2 may function as a novel oncoprotein for its role in promoting tumorigenesis in mouse models (3,4). Pirh2 is not stable and can be degraded by proteosomal destruction. Several molecules were found to affect the stability of Pirh2. Tat-interactive protein of 60kDa (TIP60) was found to stabilize Pirh2 level by sequestering Pirh2 to nucleus (5). Ploemorphic Adenoma Gene Like 2 (PLAGL2) can bind with Pirh2 dimers and prevent its degradation (6). Besides, Pirh2 can be phosphorylated by Calmodulin-dependent kinase II and its E3 ligase activity to p53 is inhibited by the phosphorylation (4). In contrast to its fast degradation in normal cellular processes, Pirh2 was found to be stabilized and upregulated in tumor tissues including breast cancer (7,8), lung cancer (9), head and neck cancer (10) and liver cancer (11). However, the molecular mechanism that leads to the stabilization of Pirh2 in tumor tissue is still poorly understood.

SCYL1BP1 was identified as an interacting protein with SCYL1 (12), which also binds to CHD1L, a well-characterized oncogene in hepatocellular carcinoma (HCC) tumorigenesis (13–15). SCYL1BP1 can be downregulated by overexpressing CHD1L in HCC cell lines (Unpublished data), suggesting the potential tumor-suppressive function of SCYL1BP1 in HCC development. Based on yeast two-hybrid screening, SCYL1BP1 was found to interact with MDM2 and Pirh2 (12,16). Since MDM2 and Pirh2 are two major E3 ubiquitin ligase of p53 (2,17), SCYL1BP1 might play an important role in regulating p53 homeostasis. Recent study found that SCYL1BP1 can promote the degradation of MDM2 and further stabilize p53 (18). In the present study, we find that SCYL1BP1 could promote Pirh2 degradation through ubiquitin-dependent pathway. Knock down of SCYL1BP1 restored the expression of Pirh2 at protein level. Overexpression of SCYL1BP1 stabilized the endogenous p53 in a dose dependent manner and further inhibited the G1/S transition in HCC cell lines. Functional studies confirmed the tumor-inhibitory effect of SCYL1BP1 both in vitro and in vivo. Importantly, SCYL1BP1 was found to be downregulated in more than 50% of HCC clinical cases. These findings suggested that SCYL1BP1 is a novel regulator of Pirh2 stability and may function as an important tumor suppressor gene in HCC development.

Materials and methods

Cell lines and culture condition

The HCC cell lines (QGY-7703, PLC-8024, Hep3B) and an immortalized normal human liver cell line, LO2, were obtained from the Institute of Virology, Chinese Academy of Medical Sciences, Beijing, China. The cells were maintained in high-glucose DMEM (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco BRL). The cells were incubated at 37°C in a humidified chamber containing 5% CO2.

Western blot and antibodies

The western blot analysis was performed as described previously (13). The signals were quantified by ImageJ software (available at http://rsb.info.nih.gov/ij) and defined as the ratio of target protein to β-actin, and data are presented as mean ± SD (n = 3). Mouse anti-SCYL1BP1 antibody was purchased from Abnova Corporation (Taipei, Taiwan). Mouse anti-p53 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Pirh2 antibody was purchased from Bethyl Laboratories (Montgomery, TX). Mouse anti-β-actin was purchased from Abcam (Cambridge, MA).

Cell cycle analysis by flow cytometry

At the indicated incubation time, cells were detached, washed with PBS, stained with Propidium Iodide (Sigma–Aldrich, St Louis, MO) and immediately analyzed using a FACS flow cytometer (Becton Dickinson & Co., San Jose, CA). The relative portion of cells in each phase of cell cycle was analyzed using Modfit software (Verity Software House, Inc., Topsham, ME).

Abbreviations: CHX, cycloheximide; Co-IP, co-immunoprecipitation; CUL-ATR, Committee on the Use of Live Animals in Teaching and Research; HA-Ub, HA-tagged ubiquitin; HCC, hepatocellular carcinoma; IgG, immunoglobulin G; IHC, immunohistochemical; PLAGL2, Pleomorphic Adenoma Gene Like 2; qPCR, quantitative real-time PCR; RNAi, RNA interference; SCYL1BP1, SCYL1 binding protein 1; TIP60, Tat-interactive protein of 60 kDa; Vec-LO2, vector-transfected LO2; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide. PBS, phosphate-buffered saline; RIPA, radio immunoprecipitation assay.

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In vivo ubiquitination assay

Cells were transfected with plasmids expressing Pirh2, V5-SCYL1BP1 and HA-tagged ubiquitin (HA-Ub) (Sigma–Aldrich) either alone or in combination. For inhibition of proteasome-mediated protein degradation, the cells were treated with 20 µM proteasome inhibitor MG132 (Calbiochem, La Jolla, CA) 6h before harvest. Twenty-four hours after transfection, cells from each plate were harvested and lysed in RIPA buffer (Invitrogen, Carlsbad, CA). Cell lysates (40 µl) were directly denatured with protein loading buffer at 95°C for 10min and analyzed by western blotting analysis with anti-Pirh2, anti-V5, anti-β-actin or anti-HA antibody. The rest lysates were immunoprecipitated with 5 µg of anti-Pirh2 antibody at 4°C for 2h. Immunocomplexes were then precipitated using 100 µl of protein G-agarose provided by the immunoprecipitation kit (Roche). After an extensive washing with washing buffer, the beads were boiled in 50 µl of loading buffer and analyzed by western blot analysis with anti-Pirh2 or anti-HA antibody. Rabbit immunoglobulin G (IgG) (Santa Cruz) was used as a negative control. For analysis of Pirh2 stability, cells were cultured in complete DMEM medium with 50 µg/ml cycloheximide (CHX) (Calbiochem) and harvested at 0, 9, 24 and 30h.

RNA interference

A siRNA (20nM) against SCYL1BP1 (Ambion, Austin, TX) was transfected into cells in six well plates using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions.

In vitro tumorigenic assays

Cell growth rate was determined by 2,3,5-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) cell proliferation assay (Roche Diagnostics Co., Indianapolis, IN), according to manufacturer’s instructions. Briefly, 500 cells per well were plated in 96 well plates in a final volume of 100 µl. After the addition of 50 µl of XTT working solution, the absorbency was measured with a scanning multiwell spectrophotometer (Tecan Sunrise, TECAN Trading AG, Switzerland) at a test wavelength of 630 nm. Triplicate independent assays were performed. Anchorage-independent growth was assessed by colony-formation ability in soft agar as described previously (19). Briefly, 1 x 10^4 of cells were suspended in 1 ml of soft agar mixture (2 % DMEM, 20 % Fetal bovine serum and 0.7 % agarose) and then cultured in the presence or absence of the proteasome inhibitor MG132 (5 µM), and then cultured in 3 % CO₂ incubator for 2-3 weeks. Colonies (~10 cells) were counted under the microscope in 10 fields per well. Three independent experiments were performed in duplicates.

Tumor xenograft mouse model

About 2 x 10^5 SCYL1BP1-7703/8024 cells or control Vec-7703/8024 cells were injected subcutaneously into the right and left hind legs of 4-week-old nude mice (n = 6 mice), respectively. Tumor formation in nude mice was monitored over a 5-week period and the tumor volume was measured weekly and calculated by the formula V = 0.5 x L x W^2. All animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR), The University of Hong Kong.

Quantitative real-time PCR (qPCR)

For qPCR analysis, aliquots of double-stranded cDNA were amplified using a SYBR Green PCR Kit and an ABI PRISM 7900 Sequence Detector (Applied Biosystems, Foster City, CA). The sequences of the primers are described in (Supplementary Table S1 is available at Carcinogenesis Online). The cycling parameters were 95°C for 30 s, 55°C for 1 min and 72°C for 2 min for 45 cycles, followed by a melting curve analysis. The threshold cycle (Ct) was measured during the exponential amplification phase, and the amplification plots were analyzed using SDS 1.9.1 software (Applied Biosystems). The relative expression level (defined as ‘fold change’ ) of target gene is given by (2^-ΔΔCt) = (ΔCt treatment - ΔCt control) and normalized to the fold change detected in the corresponding control cells, which was defined as 1.0. All reactions were performed in duplicate.

Tissue microarray and immunohistochemical staining

A tissue microarray block containing 100 pairs of HCCs and their paired non-tumor surrounding liver tissues were constructed as described previously (20). These tumor samples were collected from archives of paraffin-embedded tissues obtained from the Cancer Center, Sun Yat-Sen University, Guangzhou, China. The paraffin-embedded tissue block was sectioned (5 µm thick) for immunohistochemical (IHC) staining. In brief, sections were deparaffinized and rehydrated. The endogenous peroxidase activity was blocked with 3 % hydrogen peroxide (H2O2) for 10 min. For the antigen retrieval, slides were immersed in 10 mM citrate buffer (pH 6.0) and boiled for 15 min in microwave oven. Non-specific binding was blocked with 5 % normal goat serum for 10 min. The slides were incubated with a 1:50 dilution of SCYL1BP1 antibody for 4°C overnight in a moist chamber. The slides were then sequentially incubated with biotinylated goat anti-mouse IgG (1:100 dilution, Santa Cruz Biotechnology) for 30 min at room temperature, streptavidin-peroxidase conjugate for 30 min at room temperature. Finally, the 3,3-diaminobenzidine Substrate Kit (Oako, Carpinteria, CA) was used for color development followed by Mayer’s hematoxylin counterstaining. To evaluate IHC staining of SCYL1BP1, expression of SCYL1BP1 was scored as absent expression (0), weak expression (1), moderate expression (2) and strong expression (3). In this study, we depicted the lower IHC scores of SCYL1BP1 in tumor tissues compared with normal-tumor tissues as ‘downregulation of SCYL1BP1 in HCC specimens’.

Statistical analysis

The SPSS statistical package for Windows version 16 (SPSS, Chicago, IL) was used for data analysis. For tissue microarray analysis, based on IHC scores, SCYL1BP1 protein levels in primary HCC tissues and their matched metastatic tissues were compared using Wilcoxon signed rank test. The comparison of SCYL1BP1-transfected and vector-transfected cells in anchorage-independent growth was ascertained by the independent Student’s t-test, which was also used to assess the difference of cell growth between SCYL1BP1 and vector-transfected cells. A P-value less than 0.05 was considered statistically significant.

Results

SCYL1BP1 promotes degradation of Pirh2

It has been reported that SCYL1BP1 can bind to Pirh2, which is able to induce p53 degradation by ubiquitination (2). In this study, the effect of SCYL1BP1 on Pirh2 expression was investigated by the transient transfection of SCYL1BP1 tagged with V5 (V5-SCYL1BP1) into LO2 cells. The results showed that the ectopic expression of SCYL1BP1 could dramatically decrease Pirh2 expression at protein level but not at mRNA level (Figure 1A). Similar results could be observed in QGY-7703 cells (Figure 1A). These results suggested that SCYL1BP1 might be involved in regulating Pirh2 stability.

To confirm that SCYL1BP1 can promote the degradation of Pirh2, LO2 cells were stably transfected with pcDNA3.1 vector or pcDNA3.1-SCYL1BP1, respectively. Cells were treated with CHX (protein synthesis inhibitor) to evaluate the stability of Pirh2 protein. The result showed that the degradation of Pirh2 was increased in SCYL1BP1-transfected LO2 (SCYL1BP1-LO2) cells compared with vector-transfected LO2 (Vec-LO2) cells. The half-life of Pirh2 in SCYL1BP1-LO2 cells (3.6h) was much shorter than in the Vec-LO2 cells (4.7h) (Figure 1B). The addition of MG132 could effectively restore Pirh2 expression in both Vec-LO2 and SCYL1BP1-LO2 cells by inhibiting ubiquitin–proteasome degradation (Lanes 4 and 8; Figure 1B).

SCYL1BP1 regulates Pirh2 stability through ubiquitin-dependent pathway

To further confirm the regulatory effect of SCYL1BP1 on Pirh2 stability is ubiquitin dependent. LO2 cells were transfected with Pirh2 expression plasmid and a construct encoding HA-tagged ubiquitin (HA-Ub) with or without V5-SCYL1BP1 expression construct and then cultured in the presence or absence of the proteasome inhibitor MG132. As shown in Figure 1C, the ectopic expression of SCYL1BP1 could decrease Pirh2 expression in the absence of MG132 (Lane 4), but not in the presence of MG132 (Lane 8). Furthermore, co-immunoprecipitation (Co-IP) assay showed that the overexpression of SCYL1BP1 enhanced Pirh2 ubiquitination (Lane 8; Figure 1D).

To address whether knock down of SCYL1BP1 could enhance the expression of Pirh2. Two siRNAs specifically targeting SCYL1BP1 were transfected into QGY-7703 cells, respectively. The knockdown effect was confirmed by qPCR. As shown in the results, knock down of SCYL1BP1 could not affect the mRNA level of Pirh2, but it increased the protein level of Pirh2 in QGY-7703 cells (Figure 1E).

SCYL1BP1 regulates the subcellular localization of Pirh2

Alteration of subcellular localization is an important mechanism in regulating ubiquitin-dependent protein degradation. Nuclear export regulating ubiquitin-dependent protein degradation. Nuclear export
proteasome-mediated degradation of Pirh2 in the cytoplasm (22,23). In an attempt to explain the enhanced degradation of Pirh2 upon SCYL1BP1 expression, the subcellular localization of Pirh2 was examined with or without SCYL1BP1 expression in 293T cells. DsRed-Pirh2, EGFP-SCYL1BP1 and control DsRed, EGFP vectors, were transfected into 293T cells in combination as indicated. Consistent with previous reports (22–24), SCYL1BP1 localized to the Golgi apparatus in the cytoplasm and Pirh2 showed diffused nuclear and cytoplasmic localization in EGFP-transfected cells. However, upon EGFP-SCYL1BP1 expression, Pirh2 predominantly colocalized with SCYL1BP1 in the cytoplasm and the nuclear Pirh2 significantly decreased (Figure 2). It is possible that SCYL1BP1 may interact with Pirh2 and retain Pirh2 in the cytoplasm. The relocation of Pirh2 from nucleus to the cytoplasm may account for the enhanced proteasomal degradation of Pirh2 upon SCYL1BP1 expression.

**SCYL1BP1 regulates p53 stability**

Since p53 is a major substrate that Pirh2 targets to ubiquitin degradation, we further checked whether SCYL1BP1 could affect the endogenous p53 level. LO2 cells were transiently transfected with

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**Fig. 1.** SCYL1BP1 promotes ubiquitin-dependent degradation of Pirh2. (A) Transient transfection of V5-SCYL1BP1 into LO2 and QGY-7703 cells could downregulate Pirh2 expression at protein level but not at mRNA level. β-actin or 18S was used as a loading control for western blot analysis or qPCR, respectively. (B) Pirh2 stability in SCYL1BP1-L02 and Vec-L02 cells was analyzed by treatment with CHX (50 µg/ml) for the indicated time points in the absence or presence of MG132. Quantification of protein expression was obtained by densitometry. The data are expressed as mean ± SD for three individual assays and shown in line chart. (C) After the treatment of dimethylsulfoxide or proteasome inhibitor MG132 (20 µM) for 6 h, the expression levels of V5-SCYL1BP1 and Pirh2 proteins were detected by western blot analysis in LO2 cells transfected with the indicated vectors. Under the same conditions, ubiquitin tagged with HA (HA-UB) was also examined by western blot analysis with an antibody against HA tag. HA-UB and β-actin were used as loading controls. (D) Cell lysates described in (C) were immunoprecipitated with an anti-Pirh2 antibody and were then analyzed for HA-UB and Pirh2 expression by western blot analysis. (E) Two siRNAs specifically targeting SCYL1BP1 (siBP1-1 and siBP1-2) were transfected into QGY-7703 cells respectively. Scrapped siRNA (Control si) was used as a negative control. Seventy-two hours after transfection, the expression of Pirh2 was detected by western blot. The mRNA level of SCYL1BP1 and Pirh2 was detected by qPCR. The data are expressed as mean ± SD for three individual assays and shown in bar chart.
SCYL1BP1 tagged with V5 (V5-SCYL1BP1). The results showed that the ectopic expression of SCYL1BP1 in LO2 cells could dramatically increase p53 at protein level in a dose-dependent manner (Figure 3A). Similar results were observed in QGY-7703 cells (Figure 3A). Ectopic expression of SCYL1BP1 can also significantly upregulate another Pirh2 substrate p27 (Supplementary Figure 1 is available at Carcinogenesis Online). To check whether SCYL1BP1 affects the transcription of p53, RT-PCR and quantitative real-time PCR were used to examine the mRNA level of p53 in vector-transfected QGY-7703 cells and SCYL1BP1-transfected QGY-7703 cells. As shown in Figure 3B, SCYL1BP1 could not affect the mRNA of p53, which suggests that SCYL1BP1 may regulate p53 stability at protein level. Furthermore, the endogenous p53 level was checked by western blot in Vec-LO2 cells and SCYL1BP1-LO2 cells upon CHX treatment. The half-life of p53 was much longer in SCYL1BP1-LO2 cells (24.5 min) than in Vec-LO2 cells (17 min) (Figure 3C). To confirm the effect of SCYL1BP1 on p53, the expression of SCYL1BP1 in QGY-7703 cells was silenced by RNA interference (RNAi). The result showed that silencing SCYL1BP1 expression in QGY-7703 cells could dramatically decrease p53 compared with that in scrambled siRNA-treated 7703 cells (Figure 3D). Consistently, the mRNA level of p53 was not affected (Figure 3D).

**SCYL1BP1 inhibits G1/S transition of HCC cell line**

As p53 is a vital gene that controls G1/S checkpoint in cell cycle, the effect of SCYL1BP1 on cell cycle progression was then studied. QGY-7703 cells were stably transfected with pcDNA3.1 vector or pcDNA3.1-SCYL1BP1, respectively. As shown in Figure 4A, most of SCYL1BP1-7703 and Vec-7703 cells were synchronized to G1/S checkpoint by serum starvation for 72 h. After simulating cells by adding serum for 18 h, the percentage of cells at S phase was significantly higher in Vec-7703 cells (48.23 ± 2.3%) than in SCYL1BP1-7703 cells (25.72 ± 2.8%; *P < 0.05, independent Student’s t-test), suggesting that SCYL1BP1 can hold QGY-7703 cells at G1/S checkpoint. To check whether the cell cycle arrest occurs through the p53 pathway, the experiment was repeated in a p53-null cell line Hep3B. As shown in Figure 4B, the extent that SCYL1BP1 could induce G1/S arrest in a p53-null cell line was less effective than in a wild-type p53 cell line, which suggested that SCYL1BP1 may regulate cell cycle arrest mainly through p53.

**SCYL1BP1 has tumor-suppressive function in vitro and in vivo**

To study the tumor-suppressive potentials of SCYL1BP1, SCYL1BP1 expression construct was stably transfected into HCC cell lines QGY-7703 and PLC-8024. Cell growth rate was measured by XTT proliferation assay. The results showed that SCYL1BP1 could significantly inhibit cell growth rates in SCYL1BP1 transfectants of QGY-7703 cells (BP1-7703-c1 and BP1-7703-c2) and PLC-8024 cells (BP1-8024-c3 and BP1-8024-c10) compared with their corresponding empty vector-transfected cells (*P < 0.01, independent Student’s t-test) (Figure 5A). The tumor-suppressive ability of SCYL1BP1 was further studied by soft agar assay and tumor formation in nude mice. As shown in Figure 5B, SCYL1BP1 could significantly inhibit colony formation in soft agar in SCYL1BP1-8024 transfectants compared with that in Vec-transfected cells (*P < 0.001; independent Student’s t-test). Similar results were observed in QGY-7703 cells. Tumor xenograft experiments in nude mice demonstrated that SCYL1BP1 could dramatically reduce tumorigenicity of QGY-7703 and PLC-8024 cells. Tumor formation was observed in 0/6 and 3/6 of mice injected with SCYL1BP1-7703 (the pool of BP1-7703-c1 and c2) and Vec-7703 cells, respectively (Figure 5C, left panel). For PLC8024 cells, tumor formation was found in 0/10 and 6/10 of mice injected with the SCYL1BP1-8024 (the pool of BP1-8024-c3 and c10) and Vec-8024 cells, respectively (Figure 5C, right panel).
SCYL1BP1 is frequently downregulated in HCC

To investigate the possible involvement of SCYL1BP1 in HCC development, we further examined the expression of SCYL1BP1 in clinical HCC specimens. A HCC tissue microarray containing 100 pairs of HCCs and their matched non-tumor liver tissues was studied by IHC staining with anti-SCYL1BP1 antibody. For IHC staining, the informative expression of SCYL1BP1 was detected in 88 pairs of HCC cases. The non-informative samples included lost samples, unrepresentative samples and samples with too few tumor cells. The IHC result showed that the higher frequency (43.2%) of absent SCYL1BP1 expression was detected in HCC tissues compared with non-tumor liver tissues (12.5%, Table 1). In addition, SCYL1BP1 was significantly downregulated in clinical HCC tissues compared with the paired non-tumor liver tissues and about 60.2% (53/88) of HCC cases showed the downregulation of SCYL1BP1 (\(P \leq 0.001\), Wilcoxon signed rank test, Figure 6).

Discussion

Pirh2 is a Ring-H2 domain containing E3 ubiquitin ligase, which targets several important tumor suppressor genes including p53 and p27 for proteasomal degradation (2,25). Emerging evidences supported that Pirh2 might be a novel important oncoprotein in tumorigenesis (3,4). Pirh2 was found to be overexpressed and negatively correlated with its substrate p27 in HCC clinical samples (11,26). However, the molecular mechanism that causes the stabilization of Pirh2 in HCC tissues still remains poorly understood. SCYL1BP1 was identified as an interacting protein with Pirh2 through yeast two-hybrid screening in the previous study (12). SCYL1BP1 is also an indirect interacting protein with CHD1L, and can be regulated by CHD1L in HCC cell lines (Unpublished data). Since CHD1L has diverse oncogenic roles in HCC development, this prompted us to investigate the role of SCYL1BP1 in HCC progression.

In the present study, we found SCYL1BP1 can promote the ubiquitin-dependent degradation of Pirh2 in HCC cell lines. Overexpression of SCYL1BP1 decreased the expression of Pirh2 at protein level but not at mRNA level. Protease inhibitor MG132 inhibited the degradation of Pirh2 in SCYL1BP1 overexpressing cells. In vivo ubiquitin assay further confirmed that the degradation of Pirh2 is through ubiquitin-dependent pathway. Conversely, knock down of SCYL1BP1 restored the expression of Pirh2. In an attempt to explain the mechanism of enhanced degradation of Pirh2 upon SCYL1BP1 expression, the effect of SCYL1BP1 on Pirh2 subcellular localization was examined. Previous report suggested that the subcellular localization of Pirh2 is important to its stability. Nucleus localization of Pirh2 may prevent
its proteasomal degradation in the cytoplasm (22,23). SCYL1BP1 is a golgin that localized to the Golgi apparatus and Pirh2 is diffused in both cytoplasm and nucleus. Interestingly, upon SCYL1BP1 overexpression, most of the EGFP-SCYL1BP1-transfected cells showed predominant cytoplasmic localization of Pirh2. SCYL1BP1 may affect the accessibility of Pirh2 to other SUMO E3 ligases or kinases that regulate the localization of Pirh2, or SCYL1BP1 may directly retain Pirh2 in the cytoplasm through interaction. The relocation of Pirh2 from nucleus to the cytoplasm may account for the enhanced proteasomal degradation of Pirh2. To assess whether SCYL1BP1 affects the downstream targets of Pirh2 in HCC cell lines, the endogenous p53 was checked in SCYL1BP1-transfected and vector-transfected cells. Significantly, ectopic expression of SCYL1BP1 can increase the expression of p53 at protein level in a dose dependent manner. Knock down of SCYL1BP1 decreased the endogenous p53 further confirmed that SCYL1BP1 is a key regulator of p53 stability. Recently, SCYL1BP1 was found to accelerate the self-ubiquitination of MDM2 and suppress the MDM2-mediated p53 degradation (16,18). MDM2 and Pirh2 are two important E3 ligases that may regulate the proteasomal degradation of p53 at different circumstances (27). It is possible that SCYL1BP1 may regulate the stability of p53 through both pathways. SCYL1BP1 may be a potential critical protein involved in the p53 pathway, for it can interact with and regulate the stability of both Pirh2 and MDM2. In addition to p53, another Pirh2 substrate, p27, was also upregulated by ectopic expression of SCYL1BP1 in HCC cell lines (Supplementary Figure 1 is available at Carcinogenesis Online). Since p53 and p27 are important regulators of G1/S cell cycle check point, we next examined the effect of SCYL1BP1 on cell cycle progression (28,29). As expected, cell cycle analysis found that SCYL1BP1 could arrest cell cycle at G1/S checkpoint in QGY-7703 cells. Although SCYL1BP1 can slightly inhibit the G1/S transition in p53-null cell line Hep3B cells, the extent that SCYL1BP1 could induce cell cycle arrest in a p53-null cell line was much less effective than in a wild-type p53 cell line. These data suggested that SCYL1BP1 may regulate cell cycle arrest mainly through.

**Fig. 4. SCYL1BP1 inhibits G1/S transition.** (A) Flow cytometry was used to compare DNA contents between SCYL1BP1-7703 and Vec-7703 cells. The DNA content of transfected cells before starvation (untreated), starved by withdrawal of serum for 72 h (withdraw serum), followed by the addition of serum for 18 h (add serum) were tested. After serum stimulation, the percentage of cells at S phase was significantly higher (*P < 0.05, independent Student’s t-test) in SCYL1BP1-7703 cells than that in Vec-7703 cells. The bars represent the mean ± SD of three independent experiments. (B) The same experiment was repeated in a p53-null cell line Hep3B. The bars represent the mean ± SD of three independent experiments.
the p53 pathway. To further explore the tumor-suppressive function of SCYL1BP1 in HCC development, SCYL1BP1 was transfected into two HCC cell lines QGY-7703 and PLC-8024. Functional studies found that the ectopic expression of SCYL1BP1 in QGY-7703 and PLC-8024 cells dramatically inhibited cell growth, colony formation in soft agar and tumor formation in nude mice, indicating that SCYL1BP1 had strong tumor-suppressive function during HCC progression. Interestingly, SCYL1BP1 was found to be frequently downregulated in HCC clinical specimens through IHC staining. In certain portion of HCC cases, the expression of SCYL1BP1 was absolutely lost. These further supported that the inactivation of SCYL1BP1 may be associated with the pathogenesis of HCC.

Based on these findings, we propose that SCYL1BP1 is a novel regulator of Pirh2 stability. Ectopic expression of SCYL1BP1 enhanced the ubiquitin-dependent degradation of Pirh2 and further stabilized the expression of p53. SCYL1BP1 inhibited the G1/S transition of HCC cell lines and exhibited strong tumor-suppressive function both in vitro and in vivo. The frequent downregulation of SCYL1BP1 may account for the overexpression of Pirh2 in the progression of HCC. Further characterization of the tumor-suppressive function of SCYL1BP1 may greatly facilitate our understanding of the molecular mechanism of HCC development and find new therapeutic targets for HCC treatment.

Supplementary material

Supplementary Table S1 and Figure 1 can be found at http://carcin.oxfordjournals.org/.
Fig 6. SCYL1BP1 was frequently downregulated in clinical HCC specimens. Representatives of SCYL1BP1 expression in three pairs of HCC and non-tumor liver tissues detected by IHC. Cytoplasmic expression of SCYL1BP1 (brown staining) was detected in all three non-tumor liver tissues but not in paired HCC tissues.

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