

RESEARCH ARTICLE

SCYL1BP1 has Tumor-suppressive Functions in Human Lung Squamous Carcinoma Cells by Regulating Degradation of MDM2**Zhi-Ping Yang¹, Yong-Hong Xie², Dan-Yan Ling³, Jin-Rui Li¹, Jin Jiang¹, Yao-Hua Fan¹, Jia-Lian Zheng¹, Wan-Xin Wu^{1*}****Abstract**

SCYL1-like 1-binding protein 1 (SCYL1BP1) is a newly identified transcriptional activator domain containing protein with many unknown biological functions. Recently emerging evidence has revealed that it is a novel regulator of the p53 pathway, which is very important for the development of human cancer. However, the effects of SCYL1BP1 on human lung squamous carcinoma cell biological behavior remain poorly understood. In this study, we present evidence that SCYL1BP1 can promote the degradation of MDM2 protein and further inhibit the G1/S transition of lung squamous carcinoma cell lines. Functional assays found that reintroduction of SCYL1BP1 into lung squamous carcinoma cell lines significantly inhibited cell proliferation, migration, invasion and tumor formation in nude mice, suggesting strong tumor suppressive function of SCYL1BP1 in lung squamous carcinoma. Taken together, our data suggest that the interaction of SCYL1BP1/MDM2 could accelerate MDM2 degradation, and may function as an important tumor suppressor in lung squamous carcinomas.

Keywords: SCYL1BP1 - MDM2 - lung squamous carcinoma cells - suppressor action

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Introduction

Murine double minute 2 (MDM2) is involved in cell growth and differentiation through its interaction with other cellular proteins (Uldrijan et al., 2007; Wang et al., 2009). The MDM2 protein level is increased in a significant number of human tumors such as soft tissue sarcomas, osteosarcomas and breast tumors, underscoring its pivotal involvement in the development of human cancer (Cordon-Cardo et al., 1994; Liu et al., 2011; Ma et al., 2012; Wade et al., 2013).

MDM2 belongs to the family of RING Finger ubiquitin ligases (Jackson et al., 2000; Huang and Tindall, 2011), and the principal function of MDM2 is to mediate the ubiquitination and proteasome-dependent degradation of the p53 tumor suppressor protein and other growth regulatory proteins (Grossman et al., 1998; Manfredi, 2010; Chansaenroj et al., 2013). Accordingly, the ligase activity of MDM2 can be abolished by mutation of either one of eight cysteine and histidine residues involved in zinc coordination within the RING finger domain. Besides p53, MDM2 also ubiquitinates Numb (Yogosawa et al., 2003; Sczaniecka et al., 2012), β -arrestin binding protein (Shenoy et al., 2001; Kang et al., 2014), histone acetyltransferase Tip60 (Legube et al., 2002) as well as

MDM2 itself.

SCYL1-like 1-binding protein 1 (SCYL1BP1) is a newly identified regulator of Mdm2, which directly binds to Mdm2 and promotes Mdm2 self-ubiquitination, subsequently stabilizing p53 (Yan et al., 2010a; Yan et al., 2010b). SCYL1BP1, initially identified as a SCYL1 binding protein (Di et al., 2003; Hu et al., 2012), is an evolutionally conserved protein, existing in different organisms ranging from plants to animals. SCYL1BP1 protein is observed in many normal tissues and mainly localized in the cytoplasm, with a low level in the nucleus. However, the interaction between SCYL1BP1 and MDM2 in human lung squamous carcinoma remained unknown, and the effects of SCYL1BP1 on lung squamous carcinoma cell biological behaviors still remains poorly understood.

In the present study, we find that SCYL1BP1 could promote MDM2 degradation, which may through an ubiquitin-dependent pathway. Overexpression of SCYL1BP1 inhibited the G1/S transition in lung squamous carcinoma cell lines. Functional studies confirmed the tumor-inhibitory effect of SCYL1BP1 both in vitro and in vivo. These findings suggested that SCYL1BP1 may function as an important tumor suppressor gene in lung squamous carcinoma development.

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Materials and Methods

Reagents and cell lines

The following reagents and antibodies were used in this study: rabbit polyclonal anti-*SCYL1BP1* (Abcam), goat polyclonal anti-Mdm2 (Abcam), rabbit polyclonal anti- β -actin (Santa-Cruz, CA, USA), goat polyclonal anti-HA (Abcam). Z-Leu-Leu-Leu-al (MG132) and Cycloheximide (CHX) were purchased from Sigma Aldrich. The human lung squamous carcinoma cell lines (NCI-H226 and LTEP-S) were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, and were passaged for fewer than 6 months. NCI-H226 and LTEP-S cells were cultured in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, MD, USA) and 1xantibiotics/antimycotics at 37°C in the presence of 5% CO₂.

Plasmids, lentiviral production, and transduction

The full-length cDNA of human *SCYL1BP1* was synthesized by GeneWiz (Beijing, China). The fragment was digested by the *XhoI* and *NotI* (Fermentas, Hanover, MD, USA) incision enzymes and was cloned into the lentiviral expression vector pLVX-IRES-neo (Clontech Laboratories Inc., San Francisco, CA) (Hwang-Verslues et al., 2011; Zhou and Wang, 2011; Li et al., 2014). The resulting construct (pLVX-IRES-neo-*SCYL1BP1*) was verified by sequencing. Replication-defective VSV-G pseudotyped viral particles were packaged in the human embryonic kidney cells LentiX 293T (Clontech Laboratories Inc.) by a three plasmid transient cotransfection method (Lenti-T HT packaging mix, Clontech) using plus Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. After 48 h of cotransfection, the lentivirus-containing supernatant was collected and passed through a 0.45- μ m filter. For transduction, NCI-H226 and LTEP-S cells were infected with control lentivirus (empty vector, only lentivirus without *SCYL1BP1* was inserted) and *SCYL1BP1* lentivirus, respectively. After 48 h of transduction, the cells were stably selected with G418 (600 μ g/ml), and the drug-resistant cell populations were used for subsequent studies.

Quantitative real time PCR (qRT-PCR) analysis

TRIzol reagent (Invitrogen) was used to extract total RNA from NCI-H226-control, NCI-H226-*SCYL1BP1*, LTEP-S-control, and LTEP-S-*SCYL1BP1* cells. An aliquot of total RNA (1 μ g) was reverse-transcribed into single-strand complementary DNA using the M-MLV First Strand Kit (Invitrogen). The relative levels of RNA were detected using the ABI Prism 7000 sequence detection system (Applied Biosystems) with SYBR-Green Mix (Takara Bio, Otsu, Japan) and GAPDH as the internal control (Zheng et al., 2012). The primers used for *MDM2* were 5'-TGAACGACAAGAAAACGCAAAAT-3' and 5'-CAC TCT CCC CTG CCT GAT ACA CAG TA-3' and for GAPDH were 5'-TTG GCC AGG GGT GCT AAG-3' and 5'-AGC CAAAG GG TCA TCA TCTC-3'.

Western blotting assay

Extracts corresponding to cells were prepared by detergent lysis buffer. Sixty micrograms of total proteins was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (Millipore, Billerica, MA, USA). Antibodies for *SCYL1BP1* (Abcam), *MDM2* (Abcam), or β -actin (Santa-Cruz, CA, USA) were used. The membranes were incubated overnight at 4°C with the primary antibody diluted 1:1000 and the proteins were detected with a Phototope-horseradish peroxidase Western blot detection kit (Cell Signaling Technology, Danvers, MA, USA).

Cell cycle analysis

NCI-H226-control, NCI-H226-*SCYL1BP1*, LTEP-S-control, and LTEP-S-*SCYL1BP1* cells were plated at a concentration of 1 \times 10⁶ per 10cm dish and fixed with chilled 70% ethanol overnight at 4°C. Subsequently, we labeled single-cell suspensions with 50 μ g/ml PI (Sigma, St Louis, MO) and analyzed approximately 1 \times 10⁶ cells in 1ml by flow cytometry. Using the FlowJo Software (Treestar, Inc, Ashland, OR), the percentage of cells in the G2 phase for 10,000 cells was denoted by the 2N population phase.

Cell viability assay

NCI-H226-control, NCI-H226-*SCYL1BP1*, LTEP-S-control, and LTEP-S-*SCYL1BP1* cells were collected and then seeded in 96-well flat-bottomed plates, and 2000 cells per 100 μ l of cell suspension were used to seed in each well. After 1, 2, 4, and 6 days of cultivation, cell viability was measured by Cell Counting Kit-8 (CCK-8) system (Dojindo Laboratory, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, CCK-8 solution (10 μ l per 100 μ l of medium in each well) was added, the plates were then incubated at 37°C for 1h, and the absorbance of each well was read at 450 nm using a microplate reader (MRX; Dynex Technologies, West Sussex, United Kingdom). Six replicates for each group and the experiment were repeated at least 3 times.

Xenografts in mice

Female BALB/c nude mice, aged 4-5 weeks, were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). Mice were allowed to acclimate to local conditions for at least 1 week and maintained under a 12h dark, 12h light cycle with food and water ad libitum. NCI-H226-control, NCI-H226-*SCYL1BP1*, LTEP-S-control, and LTEP-S-*SCYL1BP1* cells were diluted to a concentration of 1 \times 10⁶/mL in physiological saline. Mice were injected subcutaneously with 0.1ml of the suspension into the back flank, each group consisted of 5 nude mice. When a tumor was palpable, tumor volume was measured by using a caliper every other day and calculated according to the following formula: $V=L \times W^2 \times 0.5$ (L, length; W, width) (Zheng et al., 2013).

Matrigel invasion assay

Cell migration and invasion ability were determined by Corning transwell insert chambers with 8 μ m pores

(Corning) and BD BioCoat Matrigel Invasion Chamber (Becton Dickinson Biosciences), respectively. About 2×10^4 (migration assay) or 2×10^5 (invasion assay) transfected cells in 200 μ l serum-free RPMI 1640 medium were seeded in the upper well and the chambers were incubated for 48h at 37°C to allow the cells to migrate to the lower well with RPMI 1640 medium plus 20% fetal bovine serum. Cells that had migrated or invaded through the membrane were fixed with methanol and stained with crystal violet (Invitrogen), imaged and counted.

Statistical analysis.

All analyses were performed in a two-sided fashion using SAS (version 9.1; SAS Institute), and $p < 0.05$ was defined as statistically significant. For comparisons, one-way analyses of variance, Fisher's exact tests, chi-squared tests, and two-tailed

Student's t-tests were performed, as appropriate. All data are presented as mean \pm SD. All experimental assays were performed in triplicated.

Results

SCYL1BP1 regulates MDM2 gene expression in human lung squamous carcinoma cells

To evaluate the effects of SCYL1BP1 on cell biological behaviors, we constructed lung squamous carcinoma cell lines with SCYL1BP1 stable overexpression and control group using lentiviruses (Figure 1A). Western blotting analysis showed that the expression of MDM2 gene was diminished when SCYL1BP1 was overexpressed (Figure 1B), but we did not observe a reduction in MDM2 mRNA levels (Figure 1C).

To determine whether SCYL1BP1 reduced MDM2 protein stability, we treated SCYL1BP1-overexpressing and control NCI-H226 cells with the protein synthesis inhibitor, cycloheximide (CHX), or the proteasome inhibitor MG-132. As shown in Figure 2A, only MG-132 abolished the degradation of MDM2 protein levels in NCI-H226 cells overexpressing SCYL1BP1, and

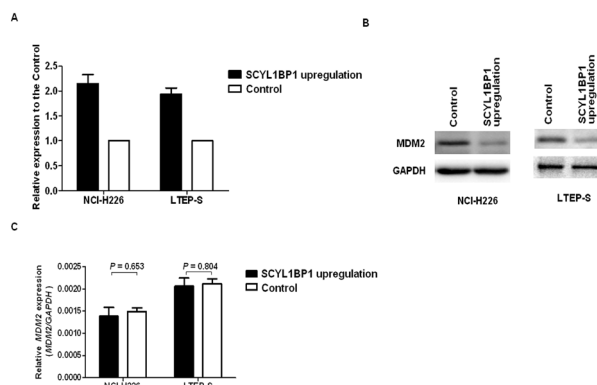


Figure 1. A) The SCYL1BP1 expression levels in lung squamous carcinoma cells after SCYL1BP1 or control lentiviruses infection. **B)** Western blot analysis for MDM2 protein expression in SCYL1BP1 stable overexpressing and control cells. **C)** The relative mRNA levels of the MDM2 in SCYL1BP1 stable overexpressing, and control cell lines, data are mean \pm SEM, normalized to GAPDH

overexpressing SCYL1BP1 promoted the degradation of MDM2 protein levels (Figure 2B). The same experiments were repeated using LTEP-S-control, and LTEP-S-SCYL1BP1 cells with similar results (Figure 2A and B).

SCYL1BP1 regulates the cell cycle in human lung squamous carcinoma cells.

We further investigated the effects of SCYL1BP1 on cell cycle using fluorescence-activated cell sorting (FACS) analysis of propidium-iodide-stained lung squamous carcinoma cells. The representative results of the cell-cycle distribution in empty vector, SCYL1BP1-transfected NCI-H226 and LTEP-S cells are shown in Figure 3A. Flow cytometry analysis revealed a statistically significant increase in the number of SCYL1BP1 overexpressed cells in the G1 phase (NCI-H226 cells: 11.08% increase, $p=0.0043$; LTEP-S cells: 8.93% increase, $p=0.0023$) accompanied by the decrease of S phase cells (NCI-H226 cells: 8.6% decrease, $p=0.0005$; LTEP-S cells: 11.48% decrease, $p=0.0007$), compared to control-transfected cells (Figure 3A).

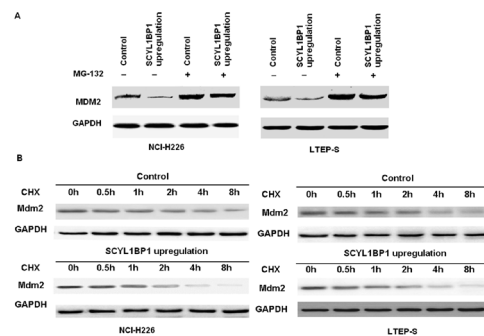


Figure 2. A and B) SCYL1BP1 stable overexpressing and control cells were incubated with the proteasome inhibitor MG-132 (5 μ M) or the protein synthesis inhibitor cycloheximide (CHX, 0.5 μ g/ μ l) for different time. The protein level of MDM2 in whole cell extracts was detected by western blotting

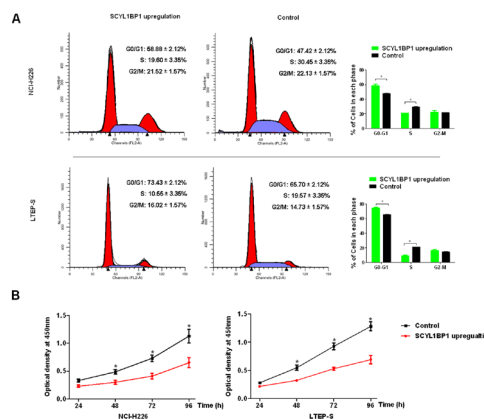


Figure 3. A) Cell cycle analysis of lung squamous carcinoma cells after transfected with SCYL1BP1 or Control lentiviruses. Results are represented as mean \pm SEM based on three independent experiments (*represents $p < 0.05$ when compared to control). **B)** SCYL1BP1 stable overexpressing and control cells were seeded in 96-well plates, and cell proliferation was assessed daily for 4 days using the Cell Counting Kit-8 (CCK-8) assay (*represents $p < 0.05$ when compared to control)

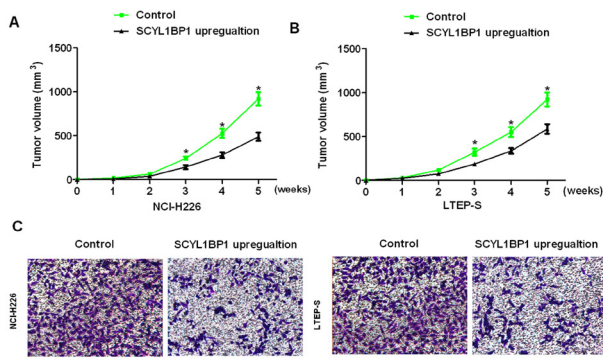


Figure 4. A) Subcutaneously implanted *SCYL1BP1* stable overexpressing and control cells xenografted tumors were established. Bars, SEM. Mean tumor volume from 6 nude mice of each group are shown (*represents $p < 0.05$ when compared to control). B) Matrigel invasion assays were performed in 24-well transwell chambers containing polycarbonate filters with 8 μ m pores coated with matrigel by using the *SCYL1BP1* stable overexpressing and control cells. Results are represented as mean \pm SEM based on three independent experiments

Effects of *SCYL1BP1* on human lung squamous carcinoma cells proliferation

Cell counting kit-8 (CCK-8) assays indicated that the cell proliferation, assessed daily for 4 days, was consistently suppressed in NCI-H226 (optical density [OD], 0.65 ± 0.09 vs. 1.13 ± 0.12 , $p = 0.011$) and LTEP-S cells (0.69 ± 0.07 vs. 1.28 ± 0.08 , $p = 0.0002$) with exogenous expression of *SCYL1BP1*, compared to the control cells on the fourth day (Figure 3B).

Effects of *SCYL1BP1* on tumor growth

To probe the effects of *SCYL1BP1* on cancer cell dynamics in vivo, *SCYL1BP1*-upregulated (NCI-H226-*SCYL1BP1* and LTEP-S-*SCYL1BP1* cells), and respective control cells were injected into the back flank of nude mice. Our results showed that the growth of tumors from *SCYL1BP1*-upregulated xenografts was significantly inhibited when compared with that of tumors formed from control xenografts: 488.2 ± 47.26 mm³ versus 919.9 ± 75.73 mm³ of NCI-H226 cells ($p = 0.0013$); 585.4 ± 54.85 mm³ versus 921.9 ± 80.04 mm³ of LTEP-S cells ($p = 0.0085$), respectively (Figure 4A and 4B).

Effects of *SCYL1BP1* on human lung squamous carcinoma cells invasion

Using transwell chambers precoated with or without Matrigel, we examined the effect of *SCYL1BP1* on cell invasion. Cells with higher expression of *SCYL1BP1* (both NCI-H226 and LTEP-S cell lines) showed significantly decreased migration and invasion when compared to the control cells (Figure 4C).

Discussion

SCYL1BP1 was initially identified as an *SCYL1*-binding protein (Di et al., 2003; Liu et al., 2012), but little is known about its biological function in human lung squamous carcinoma. Previous study showed that loss-of-function mutations of *SCYL1BP1* caused geroderma osteodysplastica (Hennies et al., 2008; Bexiga

and Simpson, 2013), which suggested that *SCYL1BP1* is an important pathogenic candidate gene. More recently, *SCYL1BP1* was identified as a new regulator of the p53 pathway (Yan et al., 2010a). So it is perhaps not surprising that *SCYL1BP1* has broader roles in physiology and pathology, such as the regulation of stress, metabolism, autophagy, tumors, and development. Here our specific aim is to investigate the potential roles of *SCYL1BP1* in the development of lung squamous carcinoma.

In the present study, we found *SCYL1BP1* can promote the degradation of MDM2 in lung squamous carcinoma cell lines. Overexpression of *SCYL1BP1* decreased the expression of MDM2 at protein level but not at mRNA level. Protease inhibitor MG132 inhibited the degradation of MDM2 in *SCYL1BP1* overexpressing cells. Recently, *SCYL1BP1* was found to accelerate the self-ubiquitination of MDM2 and suppress the MDM2-mediated p53 degradation (Momand et al., 1992; Yan et al., 2010b). Our result further confirmed this finding in lung squamous carcinoma. MDM2 is an important E3 ligase that may regulate the proteasomal degradation of p53 at different circumstances (Brooks and Gu, 2006; Sparks et al., 2013). It is possible that *SCYL1BP1* may regulate the stability of p53 through this pathway. *SCYL1BP1* may be a potential critical protein involved in the p53 pathway, for it can interact with and regulate the stability of MDM2. Since p53 is an important regulator of G1/S cell cycle check point (Martinez et al., 1991; Polyak et al., 1994; van der Laan et al., 2013), we next examined the effect of *SCYL1BP1* on cell cycle progression. As expected, cell cycle analysis found that *SCYL1BP1* could arrest cell cycle at G1/S checkpoint in NCI-H226 and LTEP-S cells. These data suggested that *SCYL1BP1* may regulate cell cycle arrest mainly through the p53 pathway. To further explore the tumor-suppressive function of *SCYL1BP1* in lung squamous carcinoma development, *SCYL1BP1* was transfected into two lung squamous carcinoma cell lines. Functional studies found that the ectopic expression of *SCYL1BP1* in NCI-H226 and LTEP-S cells dramatically inhibited cell growth, migration, invasion and tumor formation in nude mice, indicating that *SCYL1BP1* had strong tumor-suppressive function during lung squamous carcinoma progression.

Based on these findings, we propose that *SCYL1BP1* is a novel regulator of MDM2 stability in lung squamous carcinoma. Ectopic expression enhanced the degradation of MDM2 and inhibited the G1/S transition of lung squamous carcinoma cell lines and exhibited strong tumor-suppressive function both in vitro and in vivo. The frequent downregulation may account for the overexpression of MDM2 in the development of lung squamous carcinoma. Further characterization of the tumor-suppressive function of *SCYL1BP1* may greatly facilitate our understanding of the molecular mechanism of lung squamous carcinoma development and find new therapeutic targets for lung squamous carcinoma treatment.

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