

Skp2-RNAi suppresses proliferation and migration of gallbladder carcinoma cells by enhancing p27 expression

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Abstract

AIM: To explore the role of S-phase kinase-associated protein-2 (Skp2) in gallbladder carcinoma and to identify whether depletion of Skp2 by Skp2-RNAi could attenuate proliferation and migration of gallbladder carcinoma.

METHODS: Skp2-RNAi was transduced into cells of the gallbladder carcinoma cell line GBC-SD, using a lentiviral vector. The effect of Skp2-RNAi on the proliferation, migration, invasion and cell cycle of GBC-SD cells was studied using *in vitro* assays for cell proliferation, colony formation, wound healing and cell cycle. The expression of Skp2 and p27 was detected by real-time polymerase chain reaction and Western immunoblotting. The effect of Skp2-RNAi on the proliferation of GBC-SD cells *in vivo* was investigated by tumorigenicity experiments in nude mice.

RESULTS: Lentivirus-mediated RNAi reduced the expression of Skp2 in cultured cells. The expression of the p27 protein increased along with the down-regulation of Skp2, although no significant difference was found in p27 mRNA expression. Flow cytometry revealed that Skp2-RNAi transfection significantly increased the

proportion of cells in the S phase and significantly decreased the proportion of cells in the G₂/M phase. No significant difference in the frequency of cells in the G₀/G₁ phase was observed. The results from the cell proliferation, colony formation and wound healing assays revealed that Skp2-RNAi transfection markedly inhibited the proliferation and migration of GBC-SD cells *in vitro*. Additionally, tumorigenicity experiments showed that suppression of Skp2 significantly decreased the weights of the tumors (0.56 ± 0.11 and 0.55 ± 0.07 g in the control and Scr-RNAi groups vs 0.37 ± 0.09 and 0.35 ± 0.08 g in the Skp2-RNAi-L and Skp2-RNAi-H groups).

CONCLUSION: The expression of Skp2 in GBC-SD cells was inhibited following Skp2-RNAi transfection. Silencing of the *Skp2* gene inhibited proliferation, migration and invasiveness of GBC-SD cells by mechanisms dependent on enhanced expression of the p27 protein.

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Key words: Gallbladder carcinoma; S-phase kinase-associated protein-2; p27; Gene therapy; Cell cycle

Core tip: The association between S-phase kinase-associated protein-2 (Skp2)/p27 and gallbladder carcinoma has rarely been reported. This study investigated the effects of Skp2-RNAi on *in vitro* and *in vivo* growth and the invasive potencies of gallbladder carcinoma cells. The authors proposed that the effects were due to the accumulation of the p27 protein following Skp2-depletion.

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INTRODUCTION

Primary gallbladder carcinoma is a common biliary malignancy. Its incidence is estimated to be approximately 1.2-10.6/100000, and this cancer accounts for almost 3% of all tumors^[1]. Unfortunately, the majority of patients with primary gallbladder carcinoma have intermediate-advanced disease at presentation due, in part, to diagnostic difficulties and a high degree of malignancy. Thus, for these patients, the prognosis is extremely poor.

The cancer suppressor gene *p27* (wherein *p27* represents the gene and *p27^(Kip1)* represents the protein) is a cyclin-dependent kinase inhibitor (CKI), which plays an important role in tumorigenesis and tumor development^[2]. Altered expression of *p27^(Kip1)* is closely associated with the prognosis in several types of human cancers^[3,4]. It has been shown that the stability of *p27^(Kip1)* can be enhanced by a specific proteasome inhibitor, which can further inhibit the growth of the tumor^[5]. Over-expression of *p27^(Kip1)* with an adenoviral vector (adenovirus-*p27*) can inhibit tumor growth and induce apoptosis^[6,7]. In addition, the expression of *p27* mRNA was determined to be constant during a normal cell cycle. The highest expression of *p27^(Kip1)* was found during the G₀/G₁ phase of the cell cycle, and the lowest expression was throughout the S and M phases^[8-10]. The expression of *p27^(Kip1)* was found to be predominantly regulated by S-phase kinase-associated protein-2 (Skp2)^[8,9].

Skp2 (wherein SKP2 represents the gene and Skp2 represents the protein) is an S-phase dependent protein kinase that was originally found by Rodriguez *et al.*^[11], constituting the F-box unit of the SCF-E3 ligase that specifically targets CKIs, such as *p21^(Cip1)*, *p27^(Kip1)*, *p57^(Kip2)* and *p130*, for degradation^[12]. Functional deletion of Skp2 leads to stabilization of CKIs, which can subsequently induce cell-cycle delay or arrest; conversely, the over-expression of Skp2 is frequently associated with a variety of human cancers^[11,13]. Nelsen *et al.*^[14] reported that cotransfection of cyclin E and Skp2 synergistically promoted cell cycle progression in cultured primary hepatocytes in the absence of mitogen or in the presence of growth inhibitors. Furthermore, transfection of hepatocytes with cyclin E and Skp2 *in vivo* promoted abundant hepatocyte replication and hyperplasia of the liver. Hence, Skp2 is thought to be closely associated with cell cycle regulation, tumor emergence, tumor development and disease prognosis.

p27^(Kip1) and Skp2 have been studied in many types of tumors^[15-19]. The determination of an association between Skp2/*p27^(Kip1)* and gallbladder carcinoma has been rarely reported^[20,21]. In the current study, we constructed a lentiviral vector of Skp2-RNAi, and explored the role of Skp2/*p27^(Kip1)* in the proliferation and metastasis of gallbladder carcinoma cells.

MATERIALS AND METHODS

Groups

The gallbladder carcinoma cell line (GBC-SD) cells

(Shanghai Cell Library, China) were divided into four groups: (1) control group: without any treatment; (2) Scr-RNAi group (Scr-RNAi group): GBC-SD cells were transfected with a negative control RNA interference sequence (TTCTCCGAACGTGTCACGT) using lentivirus vectors (SunBio, United States); (3) for the Skp2-RNAi-Low group (Skp2-RNAi-L group); and (4) the Skp2-RNAi-High group (Skp2-RNAi-H group), the cells were transfected with an RNA interference sequence of Skp2 (AGGTC-TCTGGTGTITGTAA) at a dose of 10 and 20 MOI, respectively.

Construction and identification of the RNAi lentivirus vector

The GBC-SD cells were plated and cultured in 24-well plates until cell fusion reached 40%-60%. Next, the appropriate amounts of lentivirus were added to the cells according to the different MOI values (2.5×10^4 TU/well in Skp2-RNAi-L group and 5×10^4 TU/well in Skp2-RNAi-H group). The transduction efficiency was assessed by fluorescence microscopy (Nikon, Japan) after 96 h. The cells were harvested 10 d following transduction.

The effect of the RNAi-Lentivirus on the expression of *Skp2* gene was assessed by determination of the mRNA and protein levels of Skp2 in the GBC-SD cells after infection with lentivirus for 5-7 d; real-time polymerase chain reaction (PCR) and Western immunoblotting were used for these assessments.

Cell proliferation assay

Cell proliferation ability was assessed with a methylthiazol tetrazolium (MTT) assay kit (SunBio, United States). The cells were inoculated into 96-well plates (1×10^4 cells per well). After incubation for 1, 2, 3, 4 and 5 d, 100 μ L of sterile MTT (5 mg/mL, Sigma-Aldrich Corp, United States) was added to each well. The cells were further incubated at 37 °C for 4 h, and the reaction was stopped by adding 200 μ L of dimethyl sulfoxide. After mixing for 10 min at room temperature, formazan production was determined by measurement of the optical density (OD) at 570 nm using an enzyme immunoassay analyzer (1420 multi-label counter).

Colony formation assay

Two hundred cells were prepared and plated into 35-mm culture plates for a period of 10 d. The resulting cellular clones were counted using an inverted microscope (BX45-72P15, Olympus, Japan). A cell clone was scored as positive following confirmation that the number of cells within the clone exceeded 50. The experiment was repeated 8 times.

Wound healing assay

Cells were inoculated into 6-well plates, and a 100- μ L pipette tip was used to scribe a line across the cell monolayer. The cells that moved into the interspace of the wound line were counted 24 h later using a phase contrast microscope (BX45-72P15, Olympus, Japan). This assay was

repeated 8 times.

Cell cycle assay

Cells were seeded into a 6-well plate and harvested after infection for 10 d. After two washes in pre-cooled PBS, the cells were fixed in 70% alcohol. The percentage of cells in each stage of the cell cycle was determined by staining with propidium iodide (PI, Santa Cruz, United States). The cell cycle distribution was analyzed with a FAC-Scan Flow Cytometer (BD, United States), in accordance with the manufacturer's guidelines.

RNA extraction and real-time PCR

Total RNA (2 µg) was isolated and reverse-transcribed into cDNA. The cDNA samples (2 µL) were employed for real-time PCR in a total volume of 20 µL on a GeneAmp Thermal Cycler 9700 (ABI, United States). The reactions were incubated in 96-well optical plate at 94 °C for 4 min, followed by 35 cycles of 94 °C for 10 s, 57 °C for 15 s and 72 °C for 20 s, and a final extension reaction at 86.5 °C for 5 s. Melting-curve analysis was performed from 72 °C to 99 °C at a rate of 1 °C every 5 s. The average of the triplicate data obtained for each sample was employed to calculate the relative change in gene expression after normalization to β-actin mRNA. The primer sequences were as follows: Skp2: 5'-CCTAAGCAGCTGTCCAGAC-3' (sense) and 5'-GTGTCAGTCGGCATTTGATG-3' (antisense); p27: 5'-ACCCAACAATACCACCGACC-3' (sense) and 5'-CCCGCCTAATCTGCACTGTG-3' (antisense); β-actin: 5'-CCAAGGCCAACCGCGAGAAGATGAC-3' (sense) and 5'-AGGGTACATGGTGGTGCCGC-CAGAC-3' (antisense).

Western immunoblotting

After lysis with pre-cooled lysis buffer, 40 µg of protein extracted from the cells was loaded onto 10% SDS-PAGE gels, and the resolved proteins were transferred to a PVDF membrane over a 2-h period (Bio-Rad, United States). Next, the membrane was blocked in 5% non-fat milk for 1 h at room temperature and then probed overnight at 4 °C with antibodies against Skp2 (CST, United States, 1:1000 dilution) and p27 (CST, United States, 1:1000 dilution). After three washes with TBST (tris buffered saline with 0.5% Tween-20), the membrane was incubated with the appropriate secondary antibody (anti-mouse IgG, Santa Cruz, United States, 1:1000 dilution) for 2 h at room temperature. Following a further washing with TBST, the membrane's image was developed using enhanced chemiluminescence (ECL + plus™, Amersham, United Kingdom). β-actin was employed as an internal standard, and expressions of Skp2 and p27 were determined and normalized against the level of β-actin.

Tumorigenicity experiments in nude mice

Forty male nude mice weighing 18 to 21 g, provided by Shanghai Laboratory Animal Center (Chinese Academy of Science, China), were bred under aseptic conditions;

the animals were housed in an area with a constant humidity of 60%-70% and a room temperature of 18 °C-20 °C. Animal maintenance, husbandry and experimental procedures were performed in accordance with the United States National Institute of Health Guidelines for the Use of Experimental Animals and approved by the Medical Animal Care and Use Committee of Renji Hospital (Shanghai, China). All of the mice were separated into four groups as described above: control, Scr-RNAi, Skp2-RNAi-L, and Skp2-RNAi-H groups. Lentivirus transfected cells from each group were administrated by subcutaneous injection (0.1 mL of a solution containing 1×10^4 cells/mL). The mice were examined every 4 d and were sacrificed 28 d after the initial subcutaneous injection. The tumors were resected and weighed.

Statistical analysis

All measurement data were expressed as the mean \pm SD. The association analysis among the groups was performed using one-way analysis of variance with the SPSS17.0 statistical software package. Statistical significance was defined as having a *P* value less than 0.05.

RESULTS

Validation of Skp2-RNAi-lentivirus and expression of p27

The studies showed that Skp2-RNAi transfection could significantly reduce the level of Skp2 mRNA ($P < 0.05$, Figure 1A) and protein ($P < 0.05$, Figure 1B and C) in the Skp2-RNAi-L and Skp2-RNAi-H groups, compared with the control and Scr-RNAi groups, and that these alterations were closely related to the dosage of Skp2-RNAi. In addition, the expression of p27 was also detected in GBC-SD cells after infection with RNAi-Lentivirus. Expression of p27 mRNA did not change following the down-regulation of Skp2. Densitometric analysis of the immunoblot images showed that the ratios between the p27 protein in the Scr-RNAi, Skp2-RNAi-L and Skp2-RNAi-H groups and the p27 protein in the control group were 0.99, 1.52 and 1.93, respectively ($P < 0.05$, Figure 1). This result suggests that p27 was increased at the protein level but not at the mRNA level following Skp2-RNAi transfection. Expression of cyclin D1 and E mRNA and protein was unaltered (data not shown).

Skp2-RNAi inhibited cell growth

The effect of Skp2-RNAi on cell growth was evaluated using an MTT assay kit. As shown in Figure 2, the *A* values in the Skp2-RNAi-L and Skp2-RNAi-H groups were significantly higher than the values in the control and Scr-RNAi groups (0.94 ± 0.12 and 0.87 ± 0.11 vs 0.48 ± 0.06 and 0.41 ± 0.05 , respectively, $P < 0.01$). This result suggests that cell growth was significantly inhibited along with the down-regulation of Skp2 by Skp2-RNAi.

Skp2-RNAi inhibited colony formation of GBC-SD cells

Cell colony formation was significantly decreased in the

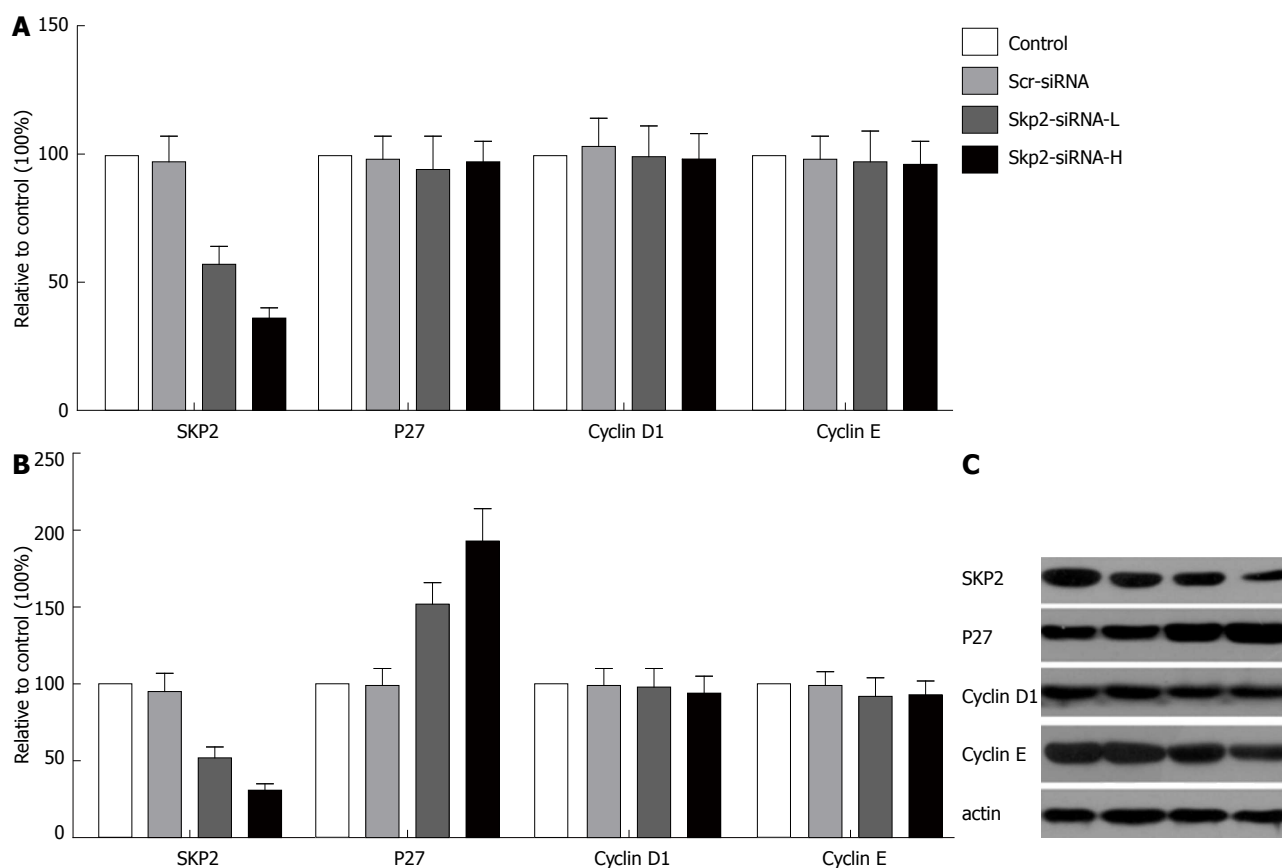


Figure 1 Detection of downstream gene and protein expression after kinase-associated protein-2-RNAi transfection. A: Expression of p27 mRNA did not change following down-regulation of S-phase kinase-associated protein-2 (Skp2); B and C: Protein expression of p27 was upregulated. Lane 1: Control; Lane 2: Scr-RNAi; Lane 3: Skp2-RNAi-L; Lane 4: Skp2-RNAi-H.

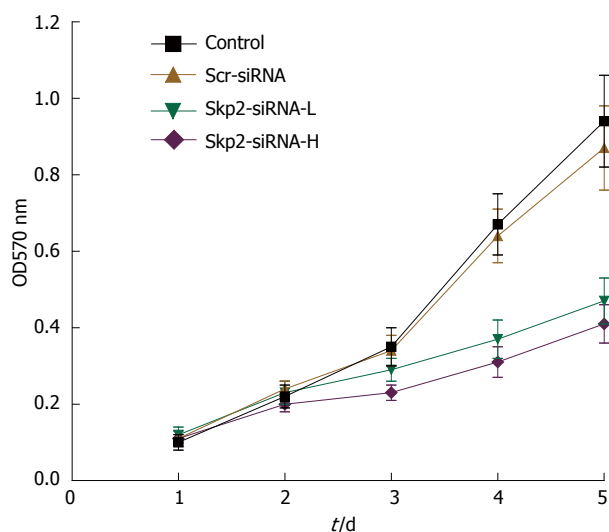


Figure 2 Proliferation curves for the gallbladder carcinoma cell line cells after the inhibition of kinase-associated protein-2 expression. Cell proliferation was significantly inhibited after down-regulation of S-phase kinase-associated protein-2 (Skp2) by Skp2-RNAi. GBC-SD: The gallbladder carcinoma cell line.

Skp2-RNAi-L and Skp2-RNAi-H groups, compared to that in the control and Scr-RNAi groups, (13.50 ± 5.90 and 7.25 ± 5.12 vs 51.25 ± 7.54 and 48.88 ± 11.93 cells

per well, respectively, $P < 0.01$, Figure 3). Colony formation of GBC-SD cells was significantly inhibited after transfection with Skp2-RNAi, and those colonies formed were closely related to the dosage of Skp2-RNAi.

Skp2-RNAi suppressed migration ability of GBC-SD cells

The migrated cells in the control, Scr-RNAi, Skp2-RNAi-L and Skp2-RNAi-H groups were found to be 111.75 ± 19.96 , 101.38 ± 14.32 , 76.50 ± 13.15 and 63.16 ± 11.00 cells per mm^2 , respectively. The migrated cells in the Skp2-RNAi-L and Skp2-RNAi-H groups were markedly decreased compared with the control and Scr-RNAi groups ($P < 0.01$, Figure 4).

Cell cycle changes

No significant difference was observed in the proportion of cells in the G_0/G_1 phase following inhibition of Skp2. However, in the Skp2-RNAi-L and Skp2-RNAi-H groups, the proportion of cells in S phase increased. Nevertheless, inhibition of Skp2 decreased the proportion of cells in the G_2/M phase as compared with the control and Scr-RNAi groups ($P < 0.05$, Figure 5).

Skp2-RNAi inhibited tumor growth in nude mice

Twenty-eight days after the mice were injected with carcinoma cells, the weights of the tumors in the control,

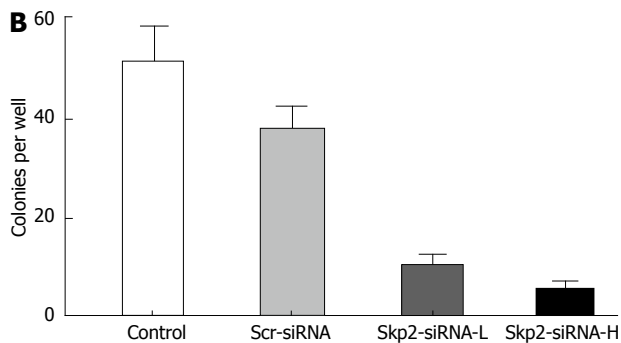
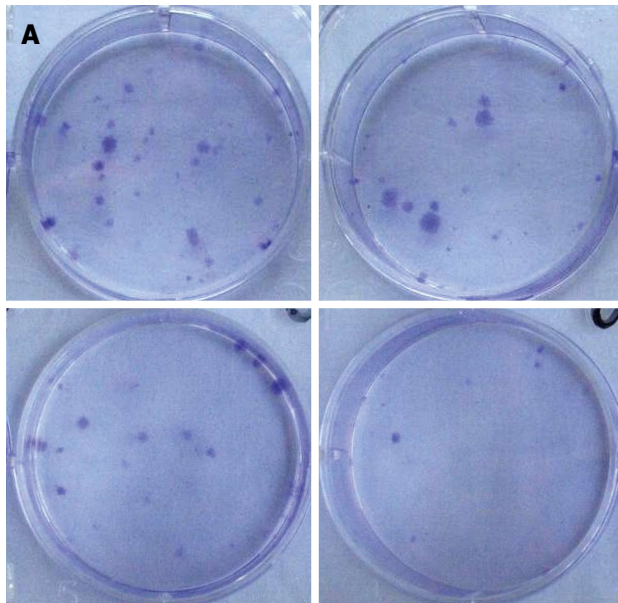


Figure 3 Colony formation assays after inhibition of kinase-associated protein-2 expression. A: Colony formation assays; B: Colony formation of the gallbladder carcinoma cell line (GBC-SD) cells was significantly inhibited after transfection with S-phase kinase-associated protein-2 (Skp2)-RNAi.

Scr-RNAi, Skp2-RNAi-L and Skp2-RNAi-H groups were 0.56 ± 0.11 , 0.55 ± 0.07 , 0.37 ± 0.09 and 0.35 ± 0.08 g, respectively. Thus, treatment with Skp2-RNAi inhibited the growth of tumors as compared with both the control and Scr-RNAi groups ($P < 0.01$, Figure 6).

DISCUSSION

Gallbladder carcinoma was first described by Clemente *et al.*^[1]. Despite advances in hepatobiliary imaging techniques, the preoperative diagnosis of this condition remains a daunting task. Furthermore, the long-term survival remains dismal, not only because of the non-specific presentation of the disease and its similarity to benign biliary tract disorders, but also because of the malignant entity. Currently, the mean survival time of advanced stage gallbladder carcinoma is approximately 6 mo, and the 5-year survival rate is less than 5%^[22]. Hence, the prognosis of gallbladder carcinoma remains poor despite improvements in surgical techniques. Moreover, the molecular mechanisms underlying the development of gallbladder carcinoma remain largely unknown.

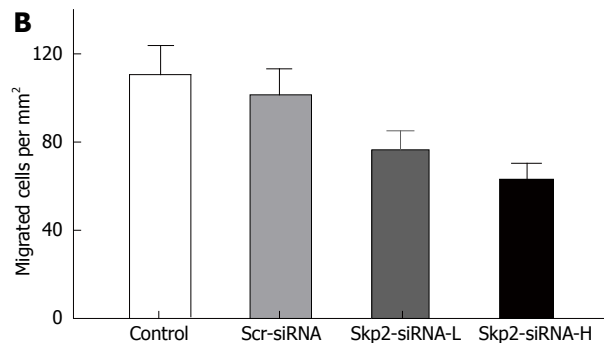
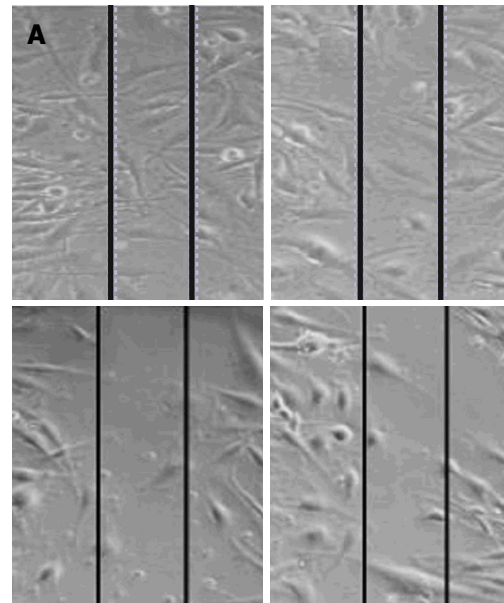


Figure 4 Results of the wound healing assay after inhibition of kinase-associated protein-2 expression. A: The wound healing assay after inhibition of kinase-associated protein-2 (Skp2) expression; B: The migrated cells in the two S-phase Skp2-RNAi groups were markedly decreased as compared with the two control groups.

Skp2 is an F-box substrate-recognition subunit of the SCF ubiquitin-protein ligase complex, which regulates progression of the cell cycle by targeting regulators such as p27^(Kip1) for ubiquitin-mediated degradation. Decreased levels of p27^(Kip1) are thought to be associated with highly aggressive tumors and related to a poor prognosis in a variety of cancers^[21,23-25].

In the current study, Skp2 expression was inhibited in GBC-SD cells by transfection of a Skp2 specific vector, namely, Skp2-RNAi. Consequently, cells in the S-phase of the cell cycle were increased, whereas cells in the G₂/M phase were decreased. No significant difference was observed in the proportion of cells present in the G₀/G₁ phase; thereby, the cell cycle was blocked in the S phase. Cell growth was significantly decreased in several *in vitro* experiments, suggesting that silencing Skp2 could markedly reduce cell proliferation and the group-dependent capability to form colonies.

In most tumors, deletion or mutation of p27 rarely occurs, and its transcription is negligibly changed. Our research found that suppression of Skp2 had no effect on the mRNA expression of p27, but it was found to upreg-

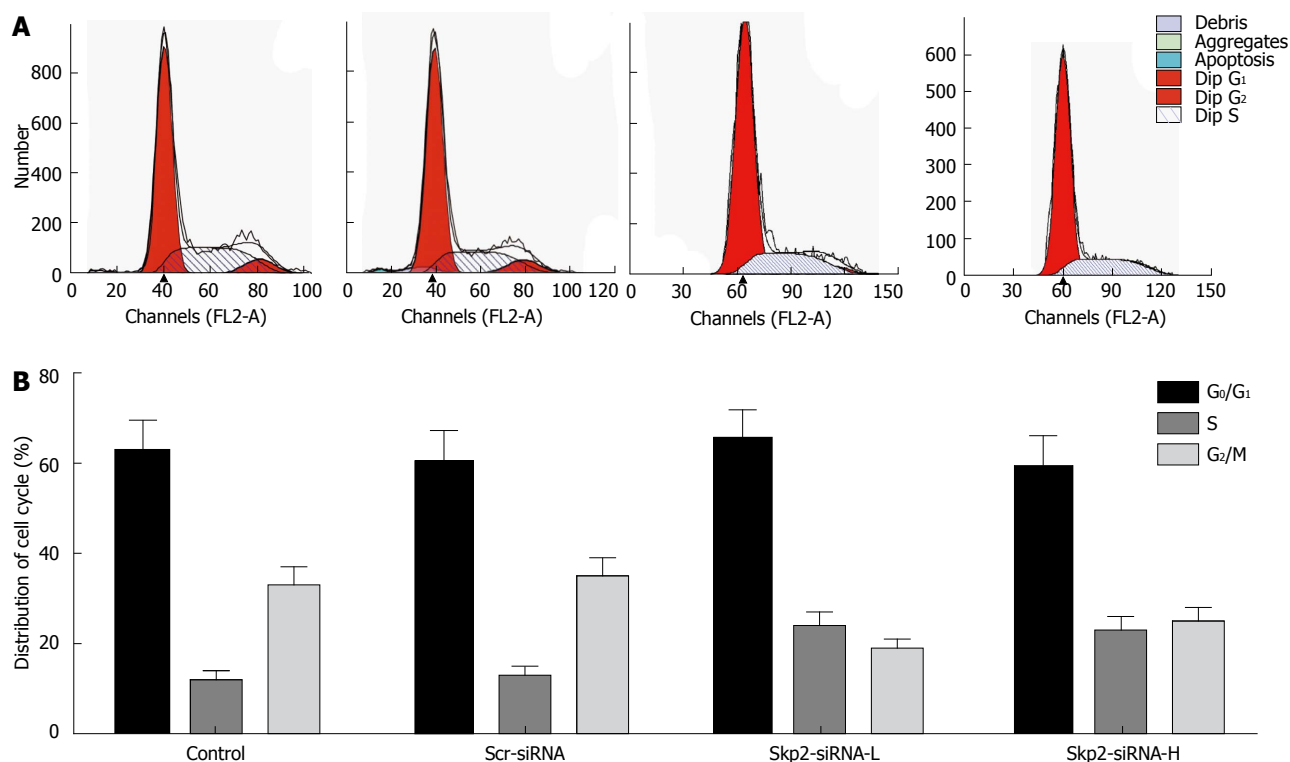


Figure 5 Proportion of cells in the cell cycle stages. A: Detection of the proportion of cells in the cell cycle stages after S-phase kinase-associated protein-2 (Skp2) expression was inhibited; B: The proportion of cells in the S phase of the cell cycle increased, and the proportion of cells in the G₂/M phase decreased in Skp2-RNAi-L and Skp2-RNAi-H groups.

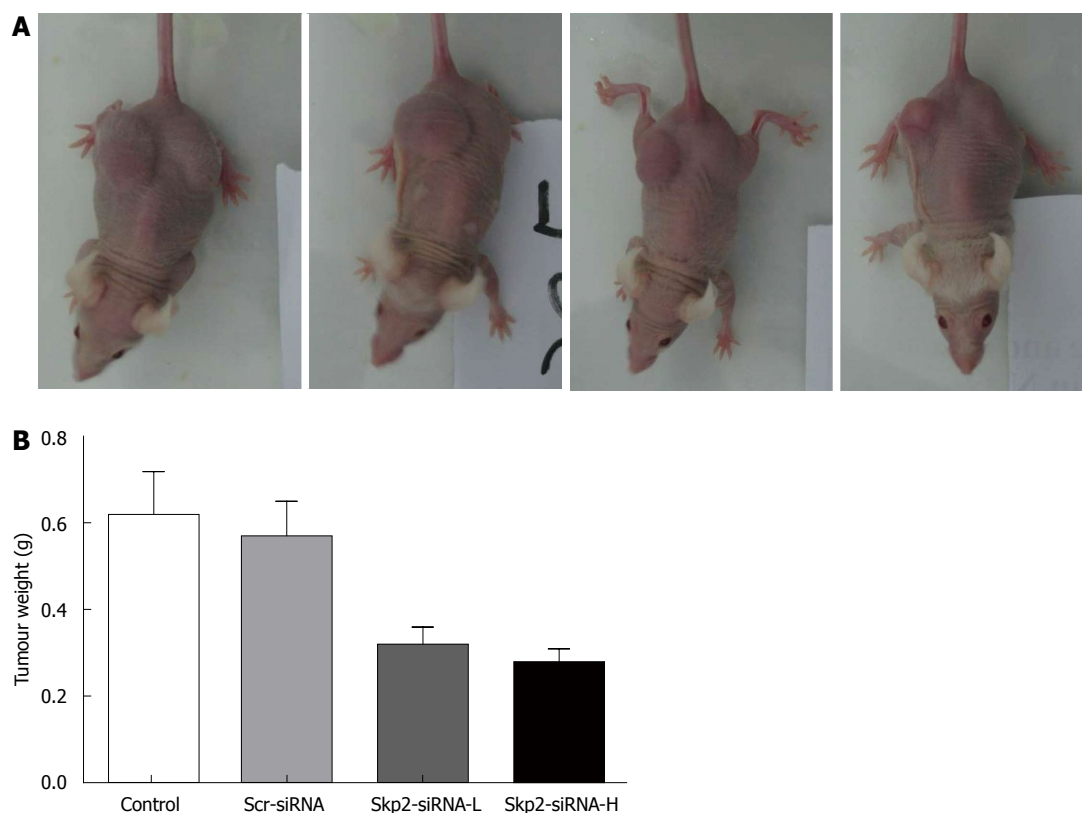


Figure 6 Tumorigenicity experiments in nude mice. A: Tumorigenicity experiments; B: Transfection with S-phase kinase-associated protein-2 (Skp2)-RNAi inhibited the growth of tumor cells.

ulate p27's protein expression. This observation suggested that regulation of gallbladder carcinoma proliferation by Skp2-siRNA is dependent on p27 protein expression, but not expression at the gene level. Nuclear polyubiquitination of p27^(Kip1) is dependent on Skp2 and phosphorylation of p27^(Kip1) at threonine 187. However, Hara *et al.*^[26] found that polyubiquitination activity was also detected in the cytoplasm of Skp2^(-/-) cells, even with a threonine 187 to alanine 187 mutant of p27^(Kip1) as the substrate. This outcome suggested that the polyubiquitination activity in the cytoplasm might contribute to an early phase of p27^(Kip1) degradation in a Skp2-independent manner.

In addition to inducing the degradation of p27^(Kip1) and promoting cellular proliferation, Skp2 also plays an important role in tumor invasiveness and metastasis. The numbers of migrated cells in the two Skp2-RNAi treated groups were found to be significantly fewer than those in the control groups. This observation suggested that Skp2-RNAi could inhibit the proliferation, migration and invasiveness of GBC-SD cells. We also studied the antitumor effect of Skp2-RNAi on nude mice in tumorigenicity experiments; the weights of the resulting tumors were decreased. This outcome suggested that treatment with Skp2-RNAi repressed the growth of metastatic tumors *in vivo*. Furthermore, the inhibition was shown to be positively associated with the dose of the lentivirus used. Hung *et al.*^[27] established Skp2-overexpressing stable transfectants in A549 human lung cancer cells and found that these stable transfectants exhibited increased migratory and invasive capabilities. Additionally, the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9 were up-regulated and neutralization of these two MMPs using antibody-mediated approaches reduced cellular invasion. These data suggest that Skp2 promoted both tumor growth and metastasis and that enhanced expression of both MMP-2 and MMP-9 may have provided a contributory mechanism.

Moreover, Skp2 and p27^(Kip1) have been shown to be useful indicators of prognosis^[28-31]. Sanada *et al.*^[21] reported that Skp2 and p27^(Kip1) were independent predictors of poor prognosis in patients with biliary tract cancers (BTCs). Discrepancies between *SKP2* DNA copy number and the level of Skp2 protein were observed, although a correlation was found between copy number and protein expression in some primary BTCs. Therefore, it is formally possible that Skp2 protein expression could be considered a more accurate prognostic marker for BTCs than *SKP2* gene copy number. Hashimoto *et al.*^[32] revealed that low levels of protein expression of p27^(Kip1) and high Skp2 were associated with aggressive tumor behavior and that both p27^(Kip1) and Skp2 could be considered useful markers in predicting the outcome of patients with intrahepatic cholangiocarcinomas. In an immunohistochemical study of 62 cases using tissue microarray, Li *et al.*^[20] confirmed that Skp2 over-expression represented the most significant independent adverse prognostic indicator in gallbladder carcinoma. Beyond the prognostic importance of Skp2/p27^(Kip1), the development of drugs targeting Skp2 may

provide novel molecular therapeutic approaches.

In summary, the results from our studies support the idea that Skp2 inhibitors and/or Skp2 regulatory sequences could provide a useful therapeutic protocol for the treatment of gallbladder carcinoma. In the future, the role of Skp2/p27^(Kip1) in gallbladder carcinoma can be expected to be gradually unveiled.

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COMMENTS

Background

Primary gallbladder carcinoma is a common biliary malignancy, with a poor prognosis. p27 and S-phase kinase-associated protein-2 (Skp2) may play an important role in tumorigenesis and tumor development, and are closely associated with prognosis.

Research frontiers

Inhibition of Skp2 or over-expression of p27^(Kip1) could inhibit tumor growth and induce apoptosis.

Innovations and breakthroughs

The authors explored the effect of Skp2-RNAi on GBC-SD cells, and found that suppression of the *Skp2* gene inhibited proliferation, migration and invasiveness of GBC-SD cells by mechanisms dependent on enhanced expression of p27 protein.

Applications

The results indicated that Skp2 inhibitors and/or Skp2 regulatory sequences such as Skp2-RNAi could provide a useful therapeutic protocol for the treatment of gallbladder carcinoma.

Peer review

The authors used several assays to explore the role of Skp2 in gallbladder carcinoma. The results indicated that Skp2-RNAi might provide a useful therapeutic protocol for the treatment of gallbladder carcinoma.

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