RNAi-mediated silencing of the Skp-2 gene causes inhibition of growth and induction of apoptosis in human renal carcinoma cells

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Abstract: Renal cancer ranks one of the most frequent causes of cancer death in the world. S-phase kinase-associated protein 2 (SKP2) is overexpressed in human tumors and has prognostic value in many cancers including renal cancer, indicating its potential as a therapeutic target. In this study, we investigated the therapeutic potential of Skp-2 in renal cancer using the technique of RNA silencing via short hairpin RNA (shRNA). Synthetic shRNA duplexes against Skp-2 were introduced to down-regulate the expression of Skp-2 in a highly malignant renal carcinoma cell line, ACHN. The results indicated that siRNA targeting of Skp-2 could lead to an efficient and specific inhibition of endogenous Skp-2 activity. Furthermore, we found that depletion of Skp-2 caused a dramatic cell cycle arrest, followed by massive apoptotic cell death, and eventually resulted in a significant decrease in growth, viability and tumor formation in renal cancer cell lines studied.

Keywords: Renal carcinoma, Skp-2, RNA silencing, cell cycle, apoptosis

Introduction

S-phase kinase-associated protein 2 (SKP2) is one of the components of the E3 ubiquitin ligase. Overexpression of SKP2 was found in various human cancers and has been shown to be associated with poor prognosis.

SKP2 genes, located at chromosome 5p13, produces a substrate-recognizing E3 ubiquitin ligase and targets p27Kip1 for degradation by the 26S proteasome [1-8]. SKP2 protein, a part of SKP1-CUL1-F-box (SCF) complexes, is considered a negative regulator of the cell-cycle inhibitor, p27Kip1, and positively regulates the G1/S transition. Frequently, SKP2 is overexpressed in various human cancers, including lung cancer, head and neck cancers, myxofibrosarcomas, and CRC [9-12]. Besides accelerating cell growth, SKP2 protein over-expression was also proven to be associated with aggressive phenotypes of promoting cell migration, invasion, and metastases in various human cancers [13, 14]. Recently, correlations of SKP2 with ErbB family-induced Akt ubiquitination, aerobic glycolysis, and tumorigenesis were demonstrated [15]. Targeting glycolysis by an SKP2 deficiency also sensitizes Her2-positive tumors to Herceptin treatment, highlighting the value of SKP2 targeting in clinical cancer therapy. Moreover, overexpression of SKP2 was also demonstrated to increase radioresistance of esophageal squamous cell carcinoma and be associated with poor survival of nasopharyngeal cancer patients treated with radiotherapy [15, 16]. These emerging evidences suggest that SKP2 is involved in resistance to chemotherapy and radiotherapy by human cancers and deserves further evaluation in rectal cancers with neoadjuvant CRT. Nevertheless, thus far, no systematic study has specifically addressed whether SKP2 has effects on CRT for rectal cancers. This is the first study to focus on the expression status, prognostic significance, and in vitro therapeutic effects of chemotherapy and radiotherapy coupled with SKP2 targeting in rectal cancers.
A study on Skp-2 mRNA levels in several renal cell carcinoma cell lines (ACHN, 768-O) was carried out. In ACHN and 768-O Skp-2 expression was found to be maximal. In order to investigate the possibility of turning Skp-2 into a novel therapeutic agent for the treatment of renal cancer, ACHN and 768-O were chosen to silence the expression of Skp-2 with the highly specific post-transcriptional suppression of RNAi. Thereafter, proliferation, cell cycle status, apoptosis, and chemosensitivity to doxorubicin were also studied. The results obtained suggest that targeting of Skp-2 may be used as a potential and specific therapeutic tool for the treatment of renal cancer.

**Materials and methods**

**Cells, plasmids and antibodies**

ACHN and 768-O were cultured in DMEM supplemented with 10% FBS and antibiotics. All cells obtained from the cell bank of the Committee on Type Culture Collection of the Chinese Academy of Science (CCTCC, Shanghai, China).
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Figure 2. Skp-2 depletion results in a significant decrease in the viability and growth of renal cancer cells. A. Skp-2 depletion reduced cell proliferation. Following shRNA transfection, cells were collected and counted at the indicated time points. The y-axis represents the average n-fold increase in cell numbers. The error bars represent the standard errors of 3 independent experiments. B. Effect of Skp-2 depletion on cell viability. Cell viability was measured using Trypan blue exclusion analysis as described in Materials and Methods. Cell viability data are expressed as the percentage of viable cells of the total number of cells. The error bars represent the standard errors of 3 independent experiments.

Generation of stable cell lines

293T cells were transfected with the pQCXIP vector encoding each gene as well as the pVPack-GP and pVPack-Ampho vectors (Stratagene, Tokyo, Japan). The culture supernatant was collected 48 h later and applied to ACHN or 768-O cells with 2 μg/ml of polybrene (Sigma). Cells were cultured for 24 h, and then 1 μg/ml of puromycin (Sigma) was added to select for infected cells. To produce ACHN cells that constitutively expressed shRNAs, oligonucleotides encoding shRNA specific for human Skp-2 (5’-GGAATATATCATCCGCCAT-3’) and luciferase (5’-CTTACGTCAAGTACTTACG-3’) were cloned into the pSIREN-RetroQ retroviral vector (Clontech). Recombinant retrovirus was produced, and infected ACHN cells were selected with 1 μg/ml puromycin for 3 days.

Quantitative real-time PCR

Total RNA was extracted from cultured cells using EZNA Total RNA Kit (OMEGA Bio-tek, USA), and cDNA was generated using PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Otsu, Japan). Quantitative real-time PCR was performed using the SYBR Premix ExTaq II (TLRNAShe Plus) (TaKaRa, Otsu, Japan) with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).

Cell cycle assay

The cell cycle was analyzed by using flow cytometry (FCM) with propidium iodide staining. Both floating and attached cells were collected by trypsin digestion and low-speed centrifugation, washed with cold PBS, and then fixed in ice-
cold 70% ethanol overnight. The fixed cells were collected by brief centrifugation and resuspended in PBS, after which the cells were treated with RNaseA and stained with propidium iodide for 1 h at room temperature, and finally analyzed by FCM.

**Apoptosis assay**

Apoptosis was assayed using the Annexin V-FITC Apoptosis Kit (keygen, China) according to the manufacturer’s instructions. Briefly, the cells were harvested and washed twice with PBS, followed by resuspension in Annexin-V binding buffer, and then FITC-conjugated Annexin V and PI were added. After incubation for 10 min at room temperature in the dark, another binding buffer was added, and the samples were immediately analyzed using FCM.

**MTS assay**

The MTS assay was used to detect the effect of plasmids on the growth of ACHN cells and to determine the 50% inhibitory concentration (IC$_{50}$) of doxorubicin. For measurement of cell growth curves, cells (1104/well) were plated into 96-well plates and allowed to grow for 4 days after transfection with pSuper-retro/Skp-2-si and pSuper-retro/GFP-si. Growth curves were plotted as optical density (490 nm) versus days after transfection.

**Immunoblotting analysis**

Cells were lysed with RIPA buffer (Beyotime, China) and boiled for 5 minutes. The protein concentration of each lysate was measured using the BCA method (Beyotime, China). Equal quantities of protein from each cell lysate were separated on SDS-polyacrylamide electrophoresis gels and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked with 5% skim milk, incubated with each primary antibody overnight at 4°C, washed with TBS-T buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween 20) and incubated with secondary antibodies. The proteins were visualized using enhanced chemiluminescence (GE Healthcare Biosciences).

**Statistical analysis**

All experiments were performed at least three times and statistical analysis was done using the SPSS13.0 package (SPSS Inc., Chicago, USA). The values were expressed as mean with SD. The ANOVA test was used whenever more than two groups were compared, and the significance level was set at $P < 0.05$. Dunnett’s post hoc test was used to analyze multiple comparisons. $P$ values of less than 0.05 ($P < 0.05$) were considered to be statistically significant.

**Results**

**Silencing of Skp-2 expression at the mRNA and protein levels**

In the first step of our study, we determined the depletion of the Skp-2 gene activity in the targeted ACHN cells. ACHN cells were transiently
transfected with pSuper-retro/Skp-2-si and pSuper-retro/GFP-si. Quantitative RT-PCR and Western blot analysis demonstrated that Skp-2 expression was significantly inhibited at both mRNA and protein levels 48 h after transfection, whereas the expression of actin was unchanged (Figure 1). The level of Skp-2 protein was efficiently reduced by at least 90% 48 h after transfection.

**Skp-2 depletion inhibits renal carcinoma cell proliferation and decrease viability**

Skp-2 depletion strongly inhibited the growth rate of ACHN, whereas control shRNA did not affect the proliferation of the cells. We also examined the viability of Skp-2 depleted cells by Trypan blue exclusion assay. As indicated in Figure 2, compared with control shRNA which showed very little effect on cell viability, Skp-2 shRNA significantly reduced cell viability.

**Skp-2 depletion induces mitotic cell cycle arrest**

Next, we analyzed the effect of Skp-2 depletion on cell cycle progression using flow cytometry. Skp-2 depletion induced an obvious increase in the number of cells at G0/G1 phase and reduction in S phase, as 77.10% of the ACHN and 768-O cells in shSkp-2 were noticed at G0/G1 phase, compared to 63.75% and 64.84% cells in the control and shCtrl, respectively (Figure 3A). There were significant differences between Bmi-1si and controls (P < 0.05). Western blot results clearly showed a reduction in the expression of cyclin D1 and an increase of p21 in sh Skp compared to the blank control and shCtrl (Figure 3B, 3C).
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Furthermore, we determined whether or not Skp-2 depletion resulted in apoptosis in esophageal cancer cells because Skp-2 depletion has been shown to induce apoptosis in certain cancer cells. Flow cytometry analysis indicated that the cells with DNA content increased dramatically at later stages after transfection, suggesting that Skp-2-depleted cells undergo apoptosis (Figure 4A). About 14-76% of Skp-2-depleted cells displayed G1 DNA 72 h after transfection, whereas only 3-5% of control cells had this phenotype.

Skp-2 silencing inhibited tumor growth in nude mice

ShCtrl and shSkp-2 cells were subcutaneously injected to the femoral area of nude mice and tumor formation was examined. Both cell lines formed 6 subcutaneous tumors of 7 injected sites. The tumor formation of shSkp-2 cells was suppressed compared with the tumor formation of shCtrl cells (Figure 5A). Mice were sacrificed 36 days after tumor cell injection and the tumor weight was determined. The average tumor weight of shSkp-2 cells was significantly reduced compared with that of shCtrl cells (Figure 5B).

Discussion

The SKP2 gene, located at chromosome 5p13, produces a substrate recognizing E3 ubiquitin ligase and targets \(^{p27}Kip1\) for degradation by the 26S proteasome [17]. SKP2 protein, part of SKP1-CUL1-F-box (SCF) complexes, is considered a negative regulator of the cell-cycle inhibitor, \(^{p27}Kip1\), and positively regulates the G1/S transition. Frequently, SKP2 is overexpressed in various human cancers, including renal cancer, head and neck cancers, myxofibrosarcomas, and CRC. Increased levels of Skp2 and reduced levels of \(^{p27}\) occur in various types of cancer, such as gastric carcinoma, prostate cancer [4], oral squamous cell carcinoma, and diffuse large B-cell lymphoma. In breast cancer, Zheng et al. reported that high level of Skp2 expression were more frequently found in ER-negative tumors and tumors with metastatic axillary lymph nodes [18]. Traub et al. found that the combined assessment of Skp2 and \(^{p27}\) expression identifies aggressive breast cancer, and high Skp2 and low \(^{p27}\) expression indicates an unfavorable clinical course [19]. All the above mentioned studies analyzed the relationship of nuclear Skp2 expression with clinicopathological characteristics, concluding that nuclear Skp2 expression predicted a poor prognosis.
This in vitro and vivo study was an effort to investigate whether Skp-2 could be exploited as a novel therapeutic target for the treatment of renal cancer. We have found that the specific shRNA-mediated depletion of Skp-2 leads to a significant decrease in cell viability, mitotic arrest followed by massive apoptosis, tumor formation in human renal cancer cell studied. These results strongly demonstrated that, apart from being of diagnostic value in esophageal cancer, inhibition of Skp-2 in renal cancer may additionally serve to be of therapeutic value.

In summary, our present study strongly indicates that the specific shRNA-mediated silencing of Skp-2 resulted in the elimination of renal cancer cells via the inactivation of p21/cyclin B1-mediated mitotic cell cycle arrest followed by massive apoptotic cell death. Therefore, Skp-2 may serve as a potential target in the treatment of human renal cancer. Consequently and conceivably, gene therapeutic approaches and/or pharmacological small molecule inhibitors aimed at Skp-2 may be developed for the management of renal cancer.

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Disclosure of conflict of interest

None.

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References


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is associated with PIK3CA mutation, low stage, and favorable outcome in 717 colorectal cancers. Cancer 2011; 117: 1399-1408.


