RNAi-Mediated Knockdown of Skp2 Inhibits Human Bladder Cancer Proliferation and Invasion in T24 Cells

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Abstract

Background: Skp2 (S-phase kinase-associated protein 2) is overexpressed in many kinds of cancers, and is related to the occurrence and development of tumors. The molecular mechanism of Skp2 in the regulation of bladder cancer cell biological behavior after Skp2 expression knockdown, however, has remained unknown.

Objectives: In our present studies (experimental cytobiological studies, we used an RNAi approach to knock down Skp2 expression, and studied its impact on cell proliferation and invasion of T24 cells.

Materials and Methods: The expression of the Skp2 gene was knocked down by RNA interference (RNAi) in T24 cells. The transcription level of Skp2 was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The expression of Skp2, caspase-3, caspase-8, caspase-9, and p27 (p27⁰Kip1) were measured by western blot assay. Cell proliferation and apoptosis were detected by MTT and flow cytometry. Cell invasion analysis was performed by a matrigel transwell assay. We also detected the level of MMP2 (metalloproteinase-2) and MMP9 (metalloproteinase-9) in cell culture medium by ELISA.

Results: The levels of Skp2 mRNA in the negative control group (0.911 ± 0.071) and the blank control group (0.940 ± 0.046) was significantly higher than Skp2 RNAi group (0.220 ± 0.042) (P < 0.001). The levels of Skp2 protein in the negative control group (0.907 ± 0.049) and the blank control group (0.925 ± 0.042) was significantly higher than Skp2 RNAi group (0.220 ± 0.047) (P < 0.001). The proliferation and invasion of T24 cells were significantly inhibited in vitro upon Skp2 RNAi treatment.

Conclusions: The proliferation and invasion of human bladder cancer cells can be inhibited by RNAi-targeting Skp2. As a result, Skp2 may be a potential target for gene therapy in cases of human bladder cancer.

Keywords: Skp2, RNA, Bladder, Cancer, T24, Cell, Invasion

1. Background

Recent years, the incidence of bladder cancer has increased; it currently ranks fourth in males and eighth in females among the most common cancers in the United States (1). More than 90% of bladder cancers display urothelial carcinoma pathology (2). The main treatment for bladder cancer is surgery, although it frequently recurs after such an operation. Its 5-year survival is still very poor (3). Finding a new therapy to improve the survival rate is therefore urgent. With the development of molecular biological techniques to treat bladder cancer, gene therapy is apparently an effective adjuvant treatment for cancer. Among many genes, the Skp2 gene has been attracting researchers’ attention.

The Skp2 gene is located on 5p13; its molecular weight is about 47KD (4, 5). A number of studies have shown that Skp2 is overexpressed in many kinds of cancers and it is related to the occurrence and development of tumors (6-11). Studies have shown that Skp2 functions primarily as the substrate recognition subunit of the ubiquitin ligase complex (SCF), it can specifically recognize phosphorylated SCF, and it can promote ubiquitin degradation and cell cycle transition from phase G1 to S, resulting in excessive cell proliferation (12-14). Studies have also shown that patients with high levels of Skp2 have poor prognoses. Takanami studied the prognosis value of Skp2 mRNA in non-small cell lung cancer. Results showed that the overall survival rate of patients with high levels of Skp2 mRNA was significantly lower than that of the low-expression group. Multivariate analysis has shown that expression levels of Skp2 mRNA can be used as an independent prognostic indicator for non-small cell lung cancer patients (15). The molecular mechanism of Skp2 regulation of the biological behavior of bladder-cancer cells after Skp2 expression knockdown, however, has remained unknown. In our present study, we used an RNAi approach to knock down Skp2 expression and studied its impact on the ability of T24 cells to prolif-
erate and invade.

2. Objectives

The purpose of this study was to evaluate whether Skp2 could be a target for gene therapy. We therefore examined the following four questions in sequence: 1) Is the expression of the Skp2 gene knocked down by RNAi in T24 cells? 2) Does the down-regulation of Skp2 affect the proliferation and apoptosis of T24 cells? 3) Is the ability of T24 cells to invade affected by Skp2 RNAi treatment? 4) Is P27 activity affected by Skp2 RNAi treatment?

3. Materials and Methods

3.1. Small Interfering (SiRNA) for Skp2

RNAi plasmids of Skp2 were purchased from Wuhan Cell Marker Biotechnology Co., Ltd. (Wuhan, China). Three siRNA oligonucleotides, which were the experimental group, had the following sequences: s1: 5'-AGCCCGACAGTGAGAACAT-3'; s2: 5'-GCTTGATCGAGATGGAAT-3'; s3: 5'-GAGCTTACACCAGTTTATAGGTG-3'. The sequence 5'-ACTACCGTTGTTATAGGTG-3' was used as a negative control.

DH5α E. coli-competent cells were used for transformation according to a modified Hanahan procedure. These plasmids were purified using a QIAGEN Plasmid Maxi Kit (Qiagen, The Netherlands).

3.2. Cell Culture and Transfection

The human bladder cancer T24 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). T24 cells were cultured in RPMI-1640 medium (Life Technologies, Rockville, MD, USA), containing with 10% fetal bovine serum (Life Technologies, Rockville, MD, USA), 1% (v/v) penicillin-streptomycin solution (Life Technologies, Rockville, MD, USA) and incubated at 37°C in 5% CO2 humidified air. T24 cells were transfected with Skp2 shRNA, negative control shRNA, and empty plasmid (without any sequence) in six-well plates according to the Lipofectamine-2000 kit (Invitrogen, USA) guidance. The cells were then screened by culture in a medium containing 800 mg/L G418 (Life Technologies, Rockville, MD, USA). The stable transfected cells were harvested after 8 weeks and used for other experiments. They were divided into three groups as follows. A Skp2 RNAi group was transfected with RNA interference sequences, a blank control group was transfected with empty plasmids without any sequence, and a negative control group was transfected with negative control RNA interference sequences. All the experiments were completed at the Louhe Key Laboratory of Medical Bioengineering, Luohe, Henan, P. R. China.

3.3. Reverse Transcription-Quantitative PCR (RT-qPCR) Assay

Expressions of Skp2 in stably transfected cells were detected by RT-qPCR assay. Total RNA was isolated using Trizol reagent (Invitrogen, Grand Island, NY, USA) and quantified by spectrophotometry (Eppendorf Biopspectrometer Basic, Eppendorf, Hamburg, Germany). After isolation, 2 µg total RNA from each sample was reverse transcribed (RT) utilizing the HiFi-MMLV cDNA Kit (Beijing CoWin Biotech Co. Ltd., Beijing, China) according to the manufacturer’s protocol. The real-time PCR was used for determining relative gene expression. The primer sequence of Skp2 are as follows: 5'-CATGTGTCAGAGACCATCCCGAG-3' (sense); 5'-GAAAGCAGTCCATCAGCC-3' (antisense) (Generay Biotech Co. Ltd, Shanghai, China). RT-qPCR was performed with a SYBR® Premix Ex Taq™ (Takara, Dalian, China) according to the manufacturer’s protocol. All PCR reactions were performed in the ABI PRISM 7700 sequence detection system (Applied Biosystems, Grand Island, NY, USA). In each reaction, 1 µL cDNA, 10 µL SYBR® Premix Ex Taq (Takara, Dalian, China), and 0.4 µM forward and reverse primer in a total volume of 20 µL were used. The reaction conditions were as follows: 1 cycle of 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. The RT-qPCR for each sample was run in triplicate. β-actin was used as an internal control, and all results were analyzed using the standard 2−ΔΔCT.

3.4. Western Blot Assay

About 1 × 10⁶ stably transfected with SKP2 RNAi, negative control, and empty plasmid cells were seeded and cultured for 72 hours. Cells were collected and total proteins were extracted from T24 cells using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Shanghai, China) with 25 mM Naf, 1mM Na3VO4, and 1% protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA). Protein concentrations were quantified by spectrophotometry (Eppendorf, Hamburg, Germany). Proteins extracted from cellular lysates were separated on 10% SDS-polyacrylamide gels and then transferred to PVDF membranes (Emd Millipore, Bedford MA, USA). The PVDF membranes were blocked with 5% non-fat dry milk (Institute of Biotechnology, Shanghai, China) at room temperature for 1 h. They were then incubated with different first antibody overnight at 4°C, respectively. Rabbit monoclonal anti-human antibodies (1:1000; Caspase-3, Caspase-8, Caspase-9, Skp2, and β-actin) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The membranes were then washed three times with PBST (0.5% Tween-20, Beyotime Institute of Biotechnology, Shanghai, China) prior to incubation with goat anti-rabbit secondary antibody (1:8000, Santa Cruz Biotechnology), at
room temperature for 50 min. Immunoreactive protein bands were detected by chemiluminescence ECL buffer (Beyotime Institute of Biotechnology, Shanghai, China). Bands were analyzed by Image J software 1.48u (National Institutes of Health, Bethesda, MA, USA).

3.5. MTT Assay for the Proliferation of T24 Cells
About 5 × 10³ stable transfection cells were trypsinized, resuspended, seeded with 100 µL medium into a 96-well plate, and incubated at 37°C for the indicated times. A total of 20 µL MTT solution (5 mg/ml, Sigma, St. Louis, MO, USA) was added and incubated at 37°C for 4 hours, then 150 µL DMSO (Sigma, St. Louis, MO, USA) was added. After being shaken for 10 minutes, the 570 nm absorbance was determined by microplate reader (Thermo Multiskan MK3, Thermoelectric Electronics Co., Ltd, Shanghai, China).

3.6. Flow Cytometry Analysis for the Apoptosis of T24 Cells
The apoptosis cells were measured by flow cytometry assay. Briefly, the cells were scraped, washed twice with PBS, and centrifuged at 1000rpm. The pelleted cells were incubated with 1 × binding buffer containing FITC-annexin V (BD Biosciences, San Jose, CA) and PI (KGI Biological Development Co. Ltd. Nanjing, China) at room temperature in the dark for 15 min. Apoptotic cells were examined using flow cytometry (Novolyte Technologies, Louisiana, America). Each experiment was repeated for three times.

3.7. Transwell Invasion Assay for T24 Cells
The ability of cell invasion was measured by transwell chamber coated with Matrigel Basement Membrane Matrix (BD Biosciences, USA). Seeded 1 × 10⁵ stably transfected cells were placed in the upper compartment with 200 µL serum-free medium, while 600 µL RPMI-1640 with 10% fetal bovine serum was added to the lower chamber. The chamber was incubated at 37°C for 24 hours and then a cotton swab was used to remove the non-migratory cells in the upper chamber. The lower-chamber cells were fixed by 4% paraformaldehyde buffer (KGI Biological Development Co. Ltd. Nanjing, China) for 15min at 4°C. After that, the migrated cells were detected by 0.1% crystal violet staining.

3.8. Enzyme-Linked Immunosorbent Assay
About 1 × 10⁴ stably transfected cells were seeded in a 6-well plate for 72 hours. The concentrations of MMP-9 and MMP-2 in the cell culture supernatants were detected by MMP-2 and MMP-9 ELISA kits (R&D Systems, Minneapolis, MN USA). Briefly, the cellular supernatants were collected and centrifuged for 5 min at 500 g. A total of 100 µL supernatants, standard samples, or positive control samples were added into 96-well plates and incubated for 4h. This was followed by 100 µL enzyme-linked antibodies incubation for 0.5 hours at 4°C. After washing 9 times with washing buffer, the template was added and incubated for 0.5 hours, followed by a 2 M H₂SO₄ termination reaction. The 450 nm absorbance was determined by microplate reader. Each sample was repeated three times.

3.9. Statistical Analysis
The data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using Student’s t-test and one-way ANOVA by the software package SPSS 17.0 (SPSS Inc., Chicago, IL, USA) for Windows. In all cases, differences were considered statistically significant when P < 0.05.

4. Results
4.1. Expression of Skp2 Was Evidently Inhibited by RNAi
The positive expression rate of GFP was measured by fluorescence microscopy (Olympus, Japan) after the cells were transfected and cultured for 48 hours. Expression of Skp2 was detected by RT-qPCR and Western blot after cells were cultured for 72 hours. The results showed that the mRNA expression levels in the negative control group (0.911 ± 0.073) and the blank control group (0.940 ± 0.046) were significantly higher than that of the Skp2 RNAi group (0.185 ± 0.033) (P < 0.001). There was no significant difference between the negative control group and the blank control group (P = 0.532) (Figure 1 A). The protein expression levels of the negative control group (0.907 ± 0.049) and the blank control group (0.925 ± 0.042) were significantly higher than that of the Skp2 RNAi group (0.220 ± 0.047) (P < 0.001). There was no significant difference between the negative control group and the blank control group (P = 0.649) (Figure 1 B and D).

4.2. Effect of RNAi on the Growth of T24 Cells
The rate of cell proliferation was gradually decreased significantly in the Skp2 RNAi group (0.567 ± 0.071) after 72 hours compared to the negative control group (0.732 ± 0.029) and the blank control group (0.790 ± 0.053) (P = 0.009 and P = 0.002, respectively). There was no significant difference between the negative control group and the blank control group (P = 0.237), as shown in Figure 1C.

4.3. Effect of RNAi on the Apoptosis of T24 Cells
Cell apoptosis was significantly increased in the Skp2 RNAi group (23.133 ± 2.442) % compared to the negative control group (5.337 ± 1.002)% and the blank control group
There was no significant difference between the negative control group and the blank control group (P = 0.621), as shown in Western blots. Cell proliferation was monitored with MTT assay (C). Statistical analyses were performed using the t-test and one-way ANOVA. *(P < 0.05) indicated a significant difference compared with the control group.

4.4 Impact on the Invasion of T24 Cells by RNAi

The number of cells that passed through the membrane in the Skp2 RNAi group (22.567 ± 4.506)% was significantly lower than that of the negative control group (90.0 ± 7.0)% and blank control group (88.0 ± 9.165)% (P < 0.001). This result suggests a reduced capacity of tumor invasion in the T24 cells interference group, as shown in Figure 4.

4.5 Effect of RNAi on the Expression of Invasion-Related Proteins

We also detect the expression of invasion-associated proteins. Results showed that the proteins of MMP2 in the Skp2 RNAi group (1.567 ± 0.473) μg/L were reduced significantly compared to the negative control group (6.605 ± 0.735) μg/L and blank control group (6.189 ± 0.679) μg/L (P < 0.001); The proteins of MMP9 in the Skp2 RNAi group (0.163 ± 0.047) μg/L were reduced significantly compared to the negative control group (0.417 ± 0.10) and blank control group (0.493 ± 0.085) μg/L (P = 0.008 and P = 0.002, respectively).
5. Discussion

RNAi is a process of typical post-transcriptional regulation of gene expression (16). It has been widely used in experimental studies (17-20). Zhang Bing used the method of RNA interference to reduce the expression of Skp2 protein in GBC-SD cells. The results showed that the migration and invasion abilities of cells were gradually decreased in the interference group compared with the control group (21). Similar results were observed in gastric cancer tissues (19).

In this study, three specific shRNAs against the Skp2 gene were successfully synthesized and used for investigating the effects of Skp2 inhibition on T24 cells. RNAi of the Skp2 gene was studied on T24 cells via RT-qPCR and Western blots. Two days later, expression of Skp2 mRNA and protein were significantly decreased (P < 0.05) in the Skp2-RNAi group compared to the control groups. MTT assays showed that the proliferation of T24 cells was decreased due to RNAi. Apoptosis studies (flow cytometry) also showed that T24 cells lost its anti-apoptotic effects due to RNAi.

The expression of apoptosis proteins (caspase-3, caspase-8, and caspase-9) increased significantly due to the Skp2 RNAi treatment. This result indicated that down-regulated Skp2 could induce the apoptosis of T24 cells through the caspase signaling pathway.

Low expression of p27 is associated with highly aggressive tumors, while high levels of p27 may suppress the proliferation of cancer cells (22-24). We also observed that the level of p27 protein was up-regulated significantly after Skp2 was down-regulated by RNAi in T24 cells (Figure 3). This result indicated that down-regulated Skp2 could inhibit the proliferation of T24 cells through the P27 signaling pathway.

Many proteins are involved in the process of tumor metastasis. The digestion of the extracellular matrix by the tumor is associated with the invasion of tumors. MMP family members, which tumor cells secrete, can degrade all of the important extracellular matrix components. Studies have also shown that Skp2-RNAi downregulates MMP-2 (metalloproteinase-2) and MMP-9 (metalloproteinase-9) secretion and inhibits the invasion ability of tumors (25, 26).

The experimental results of the transwell assay showed that the invasion capability of T24 cells was reduced, evidently due to Skp2-RNAi. Further studies showed that Skp2-RNAi can down-regulate the expression of MMP-2 and MMP-9 in T24 cells. Skp2 downregulation by RNAi is therefore one of the predominant mechanisms that inhibit the malignant biological behaviors of bladder cancer by reducing the secretion of MMP-2 and MMP-9.

In conclusion, the ability of T24 cells to proliferate and invade was inhibited by Skp2-RNAi. This suggests that Skp2 may be a promising target for bladder cancer gene therapy. Tumor metastasis, however, is a complex, multifactorial and multistep process that involves various genomic mutations and signal transductions. There are many unknown mechanisms that require further study. Our study used the vector plasmid. Compared with viral vectors, it has the advantages of easy preparation and transfection. Its disadvantage, however, is low screening efficiency. The application of plasmid and viral vectors are not absolutely safe therapy for human tumors. In subsequent research,
we will try to find a carrier that can be used on human beings safely and efficiently. RNAi technology may play an important role in the treatment of human bladder cancer with the safe and effective vectors.

Acknowledgments

This work is supported (a) the Natural Science Foundation of Luohe Medical College, P.R.C. (2013-DF-002), (b) the Plan of Science and Technology of Henan Province (142102310465) and (c) Basic and Advanced Technology Research Project of Henan Province, P.R.C. 2015 (No.152300410177).

Footnote

Authors’ Contribution: Yong-Chao Ma conducted the experiment design; RT-qPCR and Western blot were performed by Song-Tao Xu; MTT and flow cytometry were performed by Jun-Ping Xing; Guo-Jian Gu and Song-Tao Xu conducted statistical analysis and manuscript writing; ELISA was performed by Song Meng. Song-Tao Xu; Jun-ping Xing; Guo-Jian Gu: Equal contributors

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