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Effects of RNA interference silencing SOX9 on proliferation, apoptosis and tumorigenicity of renal cell carcinoma 786-O cells in vitro

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ABSTRACT

Objective: To investigate the effect of RNA interference silencing of *SOX9* (sex determining region Y-box 9) on the proliferation, apoptosis and tumorigenicity of renal cell carcinoma 786-O cells in vitro. Methods *SOX9*-specific small interfering RNA (small interfering RNA) , siRNA) sequence was the experimental silencing group, and the non-specific siRNA sequence was the control group and were transfected into 786-O cells respectively. The proliferation of 786-O cells was detected by CCK-8 assay and plate clone formation assay, and Hoechst 33342 cell staining was used to detect the proliferation of 786-O cells. Apoptosis of 786-O cells. The effect of RNA interference silencing *SOX9* on tumorigenic ability of animals was detected by tumorigenic assay in nude mice. The expression of proliferating antigen Ki-67 protein was detected by immunohistochemistry. Compared with the control group, the OD value of the *SOX9* silencing group was significantly lower, and the number of cell colonies was significantly lower than that of the control group, and the difference was statistically significant (P<0.05). Statistical significance (P<0.05). The results of animal tumorigenesis experiments showed that the volume and weight of the tumor in the *SOX9* silencing group were significantly lower than those in the control group, and the difference was statistically significant (P<0.05). Immunohistochemical staining showed that the siRNA silencing group The proportion of Ki-67 positive cells was significantly lower than that in the control group, and the difference was statistically significant (P<0.05). Conclusion RNA interference silencing of *SOX9* can significantly inhibit the proliferation of renal cell carcinoma 786-O cells, promote their apoptosis, and inhibit renal cell carcinoma 786-O cells. Cancer cell proliferation in nude mice.

Keywords: Small interfering RNA; SOX9; 786-O cells; tumorigenesis in nude mice

1. Introduction

Renal cell carcinoma is one of the most common malignant tumors in the urinary system after bladder tumors, accounting for about 85% of renal malignant tumors [1]. Renal cancer is not sensitive to traditional drugs and radiation therapy, and the prognosis is relatively poor, so It is particularly important to find new treatment methods. *SOX9* (sex determining region Y-box 9) belongs to the SRY (sex determination region of Y chromosome) related gene family. So far, more than 20 SOX gene families have been found, and the common feature is that each gene Both contain a conserved HMG-box DNA binding domain[2]. Studies have shown that abnormal expression of *SOX9* gene is associated with the progression of various tumors such as colorectal cancer, lung cancer and pancreatic cancer[3-5]. Currently, interference with *SOX9* gene expression The effect on the proliferation, apoptosis and tumorigenesis of nude mice of renal cancer cells remains to be further studied. In this study, small interfering RNA (siRNA) technology was used to silence and down-regulate the expression of *SOX9* gene in renal cell carcinoma 786-O cells. Its effects on the proliferation, apoptosis and tumorigenesis of 786-O cells in nude mice provide a certain reference for the clinical treatment of renal cell carcinoma.

2. Materials and methods

2.1. Materials

Renal cell carcinoma 786 □ O cell line was purchased from ATCC (American Type Culture Collection) The cell culture medium is RPIM □ 1640 (hyclone company of the United States), in which 10% fetal bovine serum, penicillin 100 IU / ml and streptomycin 100 IU / ml are added The experimental animals used are BALB / C female nude mice, aged 4 ~ 6 weeks, purchased from Beijing weitonglihua Experimental Animal Technology Co., Ltd

2.2. Main instruments and reagents

Multiskan FC microplate reader was purchased from Thermo company of the United States, and CX41 fluorescence microscope was purchased from Olympus company of Japan Hoechst 33342 apoptosis kit was purchased from Beijing Lianke Biotechnology Co., Ltd., CCK □ 8 kit was purchased from Japan Dongren chemical company, and immunohistochemical antibody Ki □ 67 was purchased from santacruz company of the United States

2.3. Experiments and methods

siRNA cell transfection and qPCR

The effective sequence of *SOX9* siRNA is: upstream: 5'-AG-CAAGTCCGCGAGCCAGTAC-3', downstream: 5'-GGT-GTGCCTTCTGTGCTGCAC-3'; control sequence: upstream: 5'-UCCACUGTCACUGGUCCGATT-3', downstream: 5'-CGUGACAGUGCCGAGAAT □3′. The specific transfection method is as follows: when the cell growth confluence is 70%-80%, according to the operating instructions, respectively, INTERFERINGTM transfection reagent (Poly-plus Transfection, France) and *SOX9*siRNA or negative control sequence (50nmol/L) After 48 h, the cells were collected for subsequent functional detection. The transfected cells were taken and the total RNA was extracted by Trizol method. According to the instructions of the cDNA

reverse transcriptase kit (Beijing Dakewei Biotechnology Co., Ltd.), 2 μg of Total RNA was reverse transcribed into cDNA, which was used as a template for amplification. *SOX9* sequence: upstream: 5′-CAGAAGTACTGGGAAAGTCGT-3′ , downstream: 5'-CC-GGTACTTGTAGTTGGGGTAGT-3'. Internal reference GAP-DH sequence: upstream: 5'-AGCCTCAAGATCATCAG-CAAT-3', downstream: 5'-TGTGGTCATGAGTCCTTC-CACG-3'. The total volume of the reaction system was 20 μL, including Template cd-NA was 1.5 μL, upstream and downstream primers were 1 μL, SYBR mixture was 10 μL, and sterile distilled water was 6.5 μL. Reaction conditions: pre-denaturation at 95 °C for 3 min, denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, extension at 70 °C for 30 s, A total of 30 cycles. GAPDH was used as an internal reference, and data analysis was performed by the $2^{-\Delta\Delta Ct}$ method. Primers were synthesized by Shanghai Sangon Bioengineering Co., Ltd.

detection of cell activity by CCK □ 8 method

The 786-O cells in each group were made into cell suspensions, seeded in 96-well plates at a density of 3 × 104 cells/well, and incubated in a cell incubator for 0, 24, 48, and 72 h, respectively. Serum cell culture medium was mixed with 10 μL of CCK-8 reagent, added to each well, and incubated in a cell incubator at 37°C for 1 h. Finally, the 96-well plate was placed under a microplate reader and excited at 450 nm. The optical density (OD) value was measured. Each experiment was repeated 3 times, with 3 replicate wells in each group.

Hoechst 33342 cell fluorescence staining

48 h after transfection, 786-O cells in each group were prepared into a cell suspension with a concentration of 1 × 105 cells/mL. 2 mL of cell suspension was added to a 6-well plate (a clean glass slide was placed at the bottom). , put the 6-well plate into the cell culture incubator. After 48 h, the slides covered with cells were washed three times with PBS, and then fixed with 4% paraformaldehyde for 1 h. The slides were washed three times with PBS again. The slides were immersed in a staining jar filled with Hoechst33342 staining solution for 10 min. Under a fluorescence microscope (×100), 5 fields of view were randomly selected for counting. Viable cells showed diffuse and uniform fluorescence, and densely stained granules were seen in the nucleus or cytoplasm of apoptotic cells. Bulk fluorescence, 3 or more DNA fluorescent fragments are considered as apoptotic cells [6].

tumorigenesis experiment in nude mice

After siRNA transfection, 2×10^6 cells in each group were resuspended in 100 μL PBS, and inoculated subcutaneously into the axilla of the right forelimb of nude mice. Every 5 days, the length (L) and width (W) of the tumor were measured with a vernier caliper. The formula for calculating tumor volume is $LW^2/2$ (mm^3). Initially, there were 11 nude mice in each group. On the 7th day, one nude mouse in the *SOX9* silencing group died due to infection at the injection site, and one nude mouse in the control group remained alive from start to finish. Tumors grew. Nude mice were sacrificed on the 20th day, and the tumors were

carefully isolated and weighed. The tumor tissues were used for subsequent immunohistochemical experiments.

immunohistochemical staining

Fresh tumor tissues were isolated from nude mice and fixed with 4% paraformaldehyde solution immediately Then it is dehydrated by alcohol gradient, xylene is transparent, embedded in paraffin, and cut into about 4 thick slices with slicer μ M tissue slice Dewaxing to hydration after treatment with xylene and gradient alcohol Ki-67 antibody was added dropwise and incubated overnight, and the secondary antibody was incubated for 1H Diaminobenzidine (DAB) was used for color development, and neutral resin was sealed after hematoxylin staining Under the ordinary microscope, randomly take five 40 times high-definition fields to count the total number of cells and the number of Ki-67 positive cells. If the nucleus is brown, it is regarded as Ki-67 positive Proportion of Ki-67 positive cells = number of Ki-67 positive cells / total number of cells × 100%°.

plate clone formation experiment

The 786-O cells transfected with siRNA were made into single cell suspension and inoculated into ordinary 6-well plates at the density of 1000 / well Place the six well plate in the cell incubator and replace the fresh medium every two days After 14 days, the cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet Ordinary microscope（ × 100) randomly take 5 visual fields and count the number of cell colonies greater than 100 cells The experiment was repeated for 3 times, with 3 holes in each group

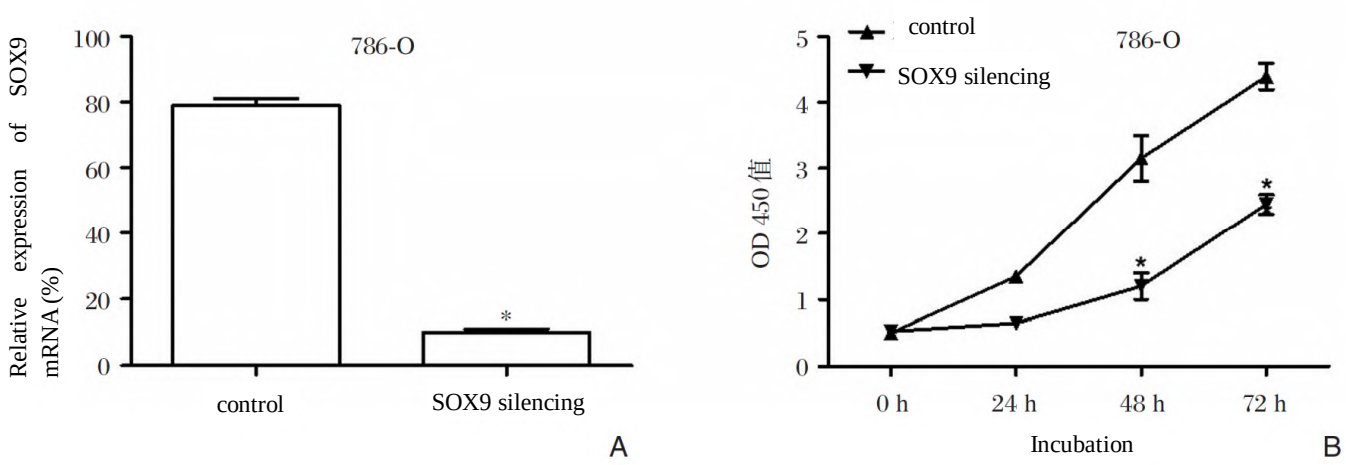
2.4. Data statistical processing

All data processing adopts SPSS 13.0 software The expression form of measurement data is mean ± standard deviation ($x \pm s$). The comparison between the two groups adopts "student" test, and $P < 0.05$ indicates that the difference is statistically significant

3. Results

3.1. Silencing the expression of *SOX9* gene affects the activity of 786-O cells

48 hours after transfection, compared with the control group, the expression level of *SOX9* mRNA in the silencing group decreased significantly, and the interference efficiency was 83.8% (Fig. 1a) After 48 h and 72 h of simultaneous transfection, the cell activity of *SOX9* silencing group was significantly lower than that of the control group ($P < 0.05$) (Fig. 1b)



A: The relative expression of *SOX9* mRNA in cells of each group; B: absorbance value of cells in each group at different time points* P < 0.05 means the difference is statistically significant compared with the control group

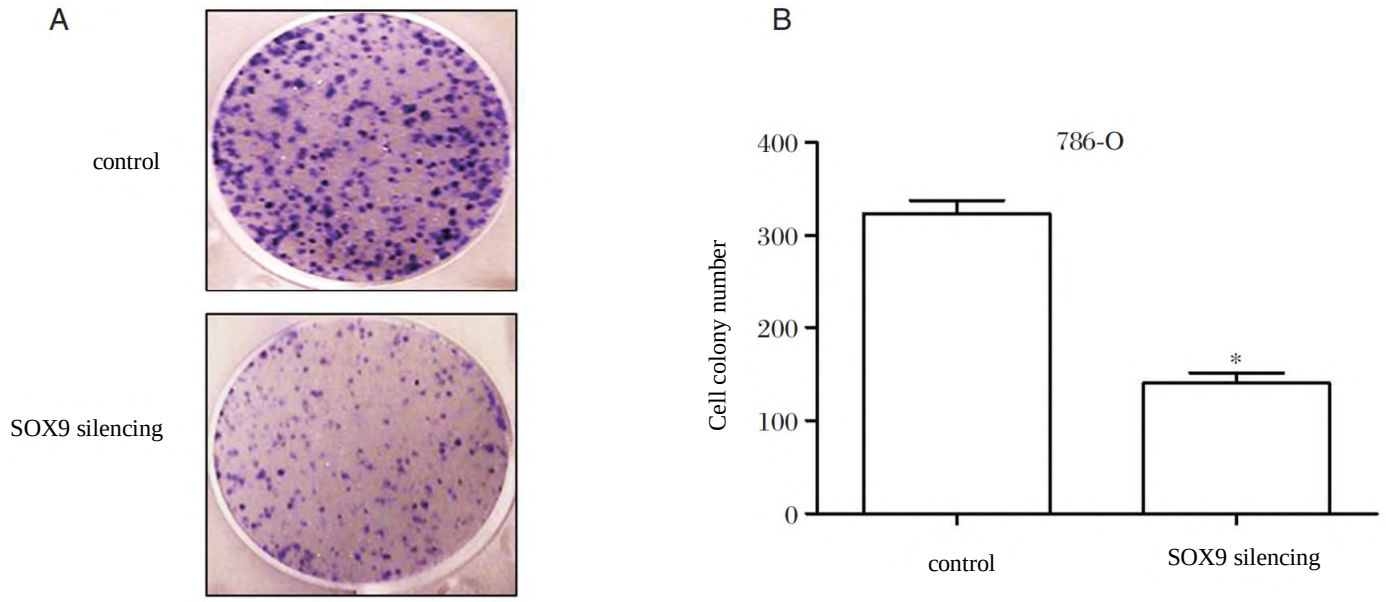
Figure 1. Effects of *SOX9* siRNA on cell viability of 786-O cells

3.2. Silencing the expression of *SOX9* gene inhibits the proliferation of 786-O cells and promotes apoptosis

The results of plate clone formation experiment showed that after renal cell carcinoma 786-O cells were transfected with *SOX9* siRNA, the number of cell colonies in *SOX9* silencing group was (140.0 ± 11.52), which was significantly lower than that in control group (323.3 ± 14.51) (f < 0.05) (Fig. 2a and Fig. 2b) The fluorescence staining results of Hoechst 33342 cells showed that the proportion of apoptosis in *SOX9* silencing group was (27.7 ± 3.25)%, which was significantly higher than that in control group (6.0 ± 0.94)% (f < 0.05) (Fig. 3a and Fig. 3b)

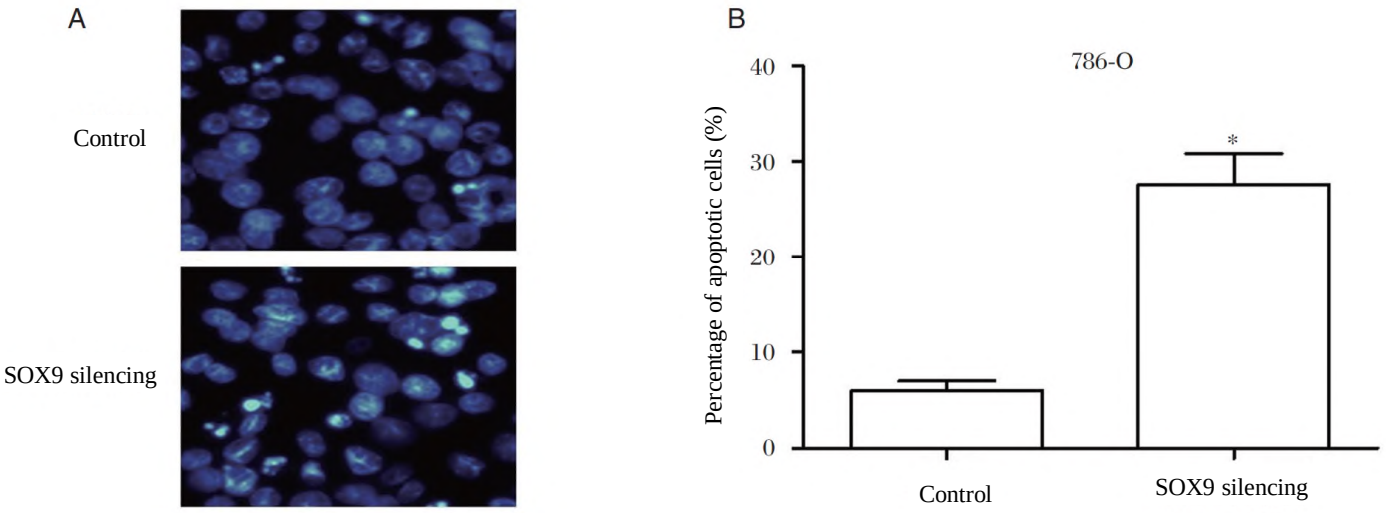
3.3. Silencing the expression of *SOX9* gene inhibits the tumorigenicity of 786-O cells in nude mice

786-O cells transfected with *SOX9* siRNA and their control cells were inoculated into BALB / C nude mice to observe their tumorigenicity At the end of the experiment on the 20th day, the average tumor volume of *SOX9* silencing group was (315.44 ± 63.73) mm^3, which was significantly lower than that of the control group (595.2 ± 50.52) mm^3 (FIG. 4A) Meanwhile, the average tumor weight of *SOX9* silencing group was (0.43 ± 0.23) g, which was significantly lower than that of the control group (0.89 ± 0.16) g (P < 0.05) (Fig. 4b) The immunohistochemical staining results of Ki □ 67 are shown in Figure 5. The proportion of Ki □ 67 positive cells in Sox silencing group was (13.3 ± 2.66)%, which was significantly lower than that in tumor tissues of control group (56.3 ± 3.21)% (P < 0.05)



A: Experimental map of cell plate clone formation in each group; B: Statistical chart of experimental results of cell plate clone formation in each group* P < 0.05 means the difference is statistically significant compared with the control group

Figure 2. Effects of *SOX9* siRNA on 786□O cells proliferation



A: Hoechst33342 staining and immunofluorescence staining of cells in each group（ × 100); B: Statistical chart of hoechst33342 staining and immunofluorescence staining results of cells in each group* P < 0.05 means the difference is statistically significant compared with the control group

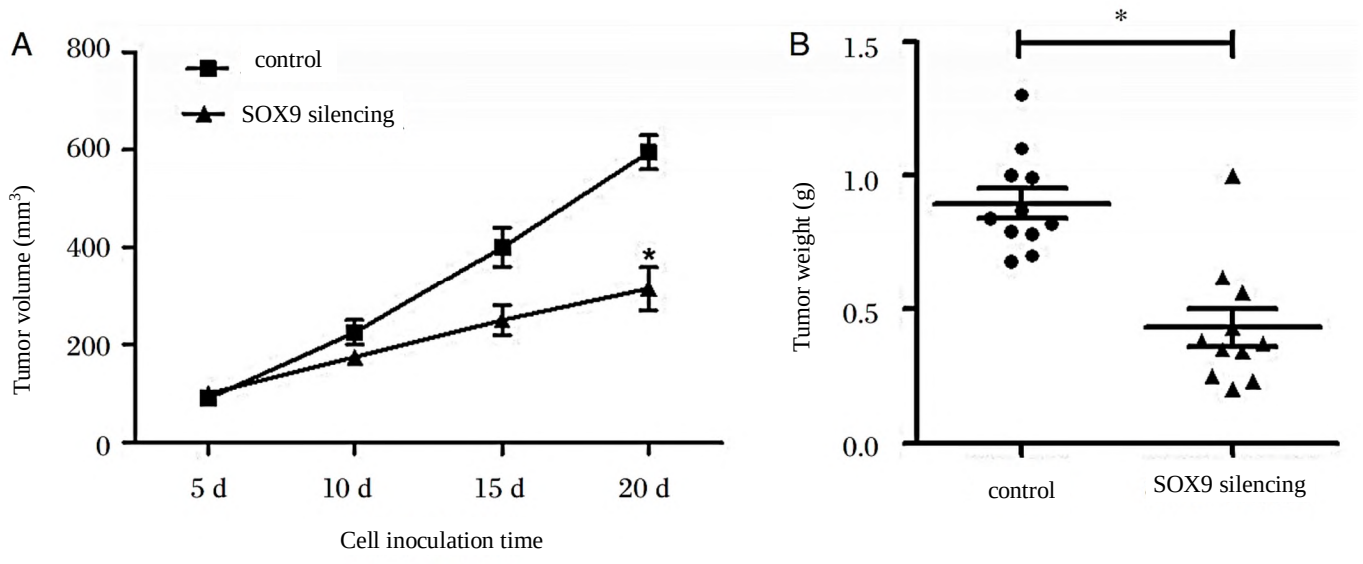
Figure 3. Effects of *SOX9* siRNA on 786□O cells apoptosis

4. Discussion

Renal cell carcinoma is a common malignant tumor of urinary system. Surgical resection combined with molecular targeted drugs is the main treatment However, the occurrence and development of renal cell carcinoma is a process involving multiple factors and genes. Some patients

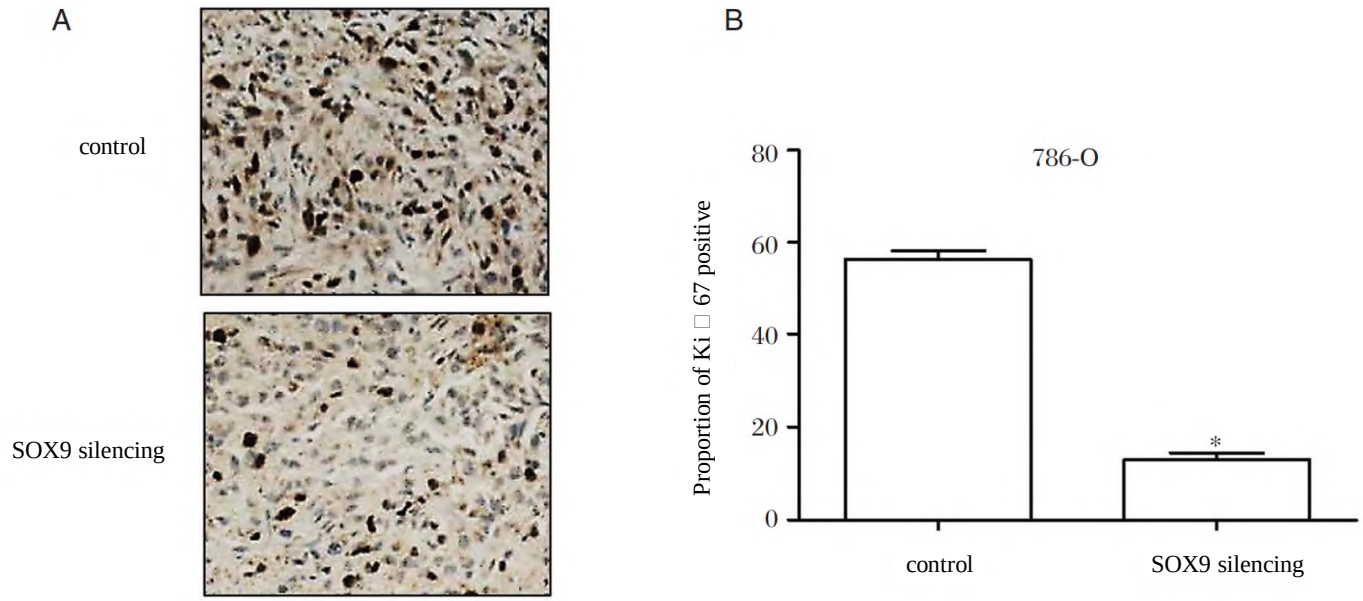
with renal cell carcinoma are not sensitive to the existing molecular targeted drug therapy Therefore, finding new molecular targets is of great significance for the treatment of renal cell carcinoma

Sox gene superfamily participates in many important physiological processes of human body, such as determining gender, regulating embryonic nervous system development, participating in cartilage formation, lens development, hematopoiesis and so on [7 □ 8] In recent years, relevant studies have shown that Sox gene family is closely related to human tumors. For example, *SOX2* gene inhibits the proliferation and metastasis of gastric cancer cells by regulating the expression of PTEN [9] The expression of *SOX6* gene in liver cancer tissue is significantly reduced, and the prognosis of patients with low expression of *SOX6* gene is poor [10] The low expression of *SOX7* in patients with oral squamous cell carcinoma is closely related to lymph node metastasis and tumor high-grade stage. Inhibiting the expression of *SOX7* can reduce the proliferation and invasion of oral squamous cell carcinoma cells [11] Abnormal expression of *SOX9* gene can lead to the occurrence of [3] in colorectal cancer, and is abundant in lung cancer and pancreatic cancer tissues. It is involved in regulating the metastasis of [4 5] in the corresponding tumor cells, and is also closely related to the prognosis of breast cancer. Enhanced *SOX9* expression can promote the metastasis of glioma cells [13], while silencing *SOX9* gene expression can inhibit the proliferation of cervical cancer cells [14] In addition, *SOX9* plays the role of tumor suppressor gene in prostate cancer. Low expression of *SOX9* gene is closely related to the recurrence and metastasis of ERG positive prostate cancer [15] It is reported that *SOX9* is highly expressed in corresponding renal cell carcinoma tissues, which is closely related to its prognosis [16] However, the effect of *SOX9* gene expression on the function of renal cell carcinoma cells has not been reported



A: The tumor volume of each group at different time points B: The weight of tumor in each group at different time points* P < 0.05 means the difference is statistically significant compared with the control group

Figure 4. Effects of *SOX9* siRNA on 786□O cells tumorigenesis in nude mice



A: Immunohistochemical staining of Ki □ 67 protein in cells of each group（ × 40); B: Statistical chart of positive proportion of Ki □ 67 protein by immunohistochemical staining in each group* P < 0.05 means the difference is statistically significant compared with the control group

Figure 5. Effects of *SOX9* siRNA on Ki□67 protein expression in 786□O cells

In order to explore the effect of *SOX9* on the proliferation of renal cell carcinoma 786 □ o cells, CCK □ 8 was used to detect the effect of siRNA interference silencing *SOX9* gene expression on cell activity The experimental results showed that the activity of 786 □ o cells decreased significantly after silencing *SOX9* expression At the same time, the corresponding plate clone formation experiment and Hoechst 33342 cell fluorescence staining results further showed that interference silencing *SOX9* could significantly inhibit the proliferation of 786 □ o cells and promote the increase of apoptosis Early studies have shown that knockout of *SOX9* gene in vitro can significantly inhibit the proliferation of thyroid cancer, rectal cancer and gastric cancer [3,17 □ 18] and promote the apoptosis of notochord neuroma cells [19]; In addition, knocking out the expression of *SOX9* gene in osteosarcoma cell line can not only inhibit cell proliferation, but also promote its apoptosis [20] The results of this study are basically consistent with the results reported in the above literature

In addition, in order to further understand the effect of silencing *SOX9* gene on the proliferation of renal cell carcinoma cells in vivo, the cells were inoculated into nude mice to observe the changes of tumor volume and weight The results showed that the tumor volume and weight of *SOX9* silencing group were lower than those of the control group Ki □ 67 is a protein related to cell cycle. Its function

is closely related to mitosis and is indispensable in cell proliferation. Its high expression reflects strong cell proliferation ability. It is a commonly used cell proliferation antigen [21] The data showed that after silencing *SOX9* gene, the expression of proliferation antigen ki □ 67 was significantly inhibited It is suggested that silencing *SOX9* gene can also inhibit the proliferation of renal cell carcinoma 786 □ o cells in vivo, which is consistent with the experimental results in vitro

Based on the above research results, interfering with and silencing the expression of *SOX9* can significantly inhibit the proliferation of renal cell carcinoma 786 □ o cells and promote the apoptosis of the cells At the same time, it is proved that targeted inhibition of *SOX9* gene expression in vitro and in vivo can effectively kill renal cell carcinoma related cell lines, and further provide reference for clinical treatment of renal cell carcinoma

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