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Original Article

RNA interference of *pax2* inhibits growth of transplanted human endometrial cancer cells in nude mice

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Abstract

The development of human endometrial carcinoma (HEC) is a complex pathologic process involves several oncogenes and tumor suppressor genes. The full-length paired-box gene 2 (*pax2*), a recently discovered oncogene, promotes cell proliferation and growth and inhibits apoptosis of HEC cells. Here, we examined the effect of *pax2* small interfering RNA (siRNA) on the growth of transplanted HEC cells in nude mice. The expression of Pax2 in 21 cases of normal endometrium and 38 cases of HEC was examined by immohistochemistry (IHC). HEC models were developed by subcutaneously transferring HEC cells into nude mice, followed by treatment with empty lentivirus vector, lentivirus vector-based *pax2* siRNA, and phosphate buffered saline, respectively. Four weeks later, tumor size was measured, tumor inhibition rate was calculated, and histological analyses were conducted after staining with hematoxylin and eosin. The expression of Pax2 and Bcl-2 was detected by Western blot; proliferating cell nuclear antigen (PCNA) was detected by IHC. Significant differences were observed in the positive rate of Pax2 between normal endometrium and HEC (14.2% vs. 60.5%, $P < 0.01$). The expression index of Pax2 in well differentiated tumors was 1.88 ± 1.68, much lower than that in tumors of moderate (3.07 ± 1.96, $P < 0.05$) or poor differentiation (5.45 ± 2.76, $P < 0.01$). Tumor necrosis increased, nuclear basophilia stain decreased, tumor growth was inhibited, and PCNA, Pax2, and Bcl-2 expression was reduced in HEC models treated with pax2 siRNA. These results indicate that Pax2 expression is related to HEC tumor biology with the increased expression of Pax2 correlated to malignancy. *pax2* siRNA down-regulates Pax2 expression and inhibits tumorigenesis of HEC in nude mice, possibly due to cell apoptosis and the inhibition of tumor proliferation induced by down-regulation of Bcl-2.

Key words RNA interference, Pax2, endometrial carcinoma, nude mice, tumor transplantation

Endometrial carcinoma, a common gynecological malignant tumor, is a group of epithelial malignant tumors with rising incidence and reducing age distribution worldwide. Statistically, the annual incidence of endometrial carcinoma is 1.5 to 2.0 per million population, second only to cervical cancer. Moreover, prognosis is poor upon metastasis or recurrence. The efficacy of conventional radiochemotherapy on endometrial cancer is unsatisfactory and the immune status of most patients is poor. In addition, the application of chemotherapy often worsens patients' physical condition. Therefore, discovering drugs with a low toxicity in an effort to improve the quality of life and patient survival is a hot issue. Molecular targeted therapy is a burgeoning field in this endeavor, supported by the understanding of molecular mechanisms and signaling pathways inherent in cancer. Several molecular targeted drugs have been applied to treat endometrial cancer and have exhibited certain effects.

It has been found that the tumorigenesis of endometrial carcinoma is related to the mutations of oncogenes and the inactivation of tumor suppressor genes. Full-length paired-box gene 2 (*pax2*), a recently discovered oncogene, is critical to promoting the

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growth of endometrial cancer cells [1]. *pax2*, an important member of the *pax* gene family, is located at chromosome 10q22.1−24.3. Pax2, a transcription factor, can indirectly regulate cell proliferation and growth, and inhibit programmed cell death and cell migration. Therefore, *pax2* is considered a proto-oncogene and is often temporarily expressed in the manner of specific spatial structure. Previous studies have demonstrated that *pax2* is closely related to some malignant tumors of the genitourinary system. It has been reported that Pax2 is expressed in renal cell cancer, prostate cancer, ovarian cancer, and other cancers, but is not expressed in normal tissues[2-4]. Wu *et al.*[5] have demonstrated that *pax2* plays a key role in endometrial cell proliferation and carcinogenesis stimulated by estrogen and tamoxifen. In addition, through genomics studies, they observed that estrogen and tamoxifen can promote cell proliferation of endometrial carcinoma *in vitro* by activating *pax2*. They also found that *pax2* is only activated by estrogen and tamoxifen in endometrial cancer cells and is not activated in normal epithelia. The difference is due to hypomethylation of the *pax2* promoter. To further study the biological effects of *pax2 in vivo*, we examined the expression of *pax2* gene in human endometrial cancer and constructed a lentiviral vector-mediated small interfering RNA (siRNA) to silence the expression of *pax2* gene in transplanted human endometrial cancer in nude mice, aiming to investigate the feasibility of targeted therapy and promote the development of new treatment drugs to improve the prevention and treatment of endometrial carcinoma.

Materials and Methods

Cell line and samples

The human endometrial cancer cell line HEC-1A was purchased from the Center for Biological Preservation, Wuhan University. Paraffin specimens were collected from 38 patients with endometrial cancer who underwent operation between October 2003 and December 2008 at the Affiliated Taihe Hospital of Hubei Medical College. No patient underwent radiotherapy or chemotherapy before operation. The patients aged 39 to 71 years (median, 57 years). According to the 2009 FIGO pathologic grading, there were 20 G1 cases, 13 G2 cases, and 5 G3 cases. Normal proliferative endometrial tissue samples obtained by diagnostic curettage were used as control.

Animals and reagents

BALB/C nude mice (female, 4 to 6 weeks old, 20 g in weight) were purchased from Experimental Animal Center of Hubei Province (Animals Certificate of Conformity: SCXK, E 2003-205) and were raised in the SPF laminar flow animal room of Hubei Medical College. Lentiviral vector (pGCL-GFP, pHelper1.0, pHelper2.0) and 293T cells were purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). Cationic liposome Lipofectamine 2000 was purchased from Gibco Company (Grand Island, NY, USA). Proliferating cell nuclear antigen (PCNA) and Bcl-2 rabbit anti-human polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Pax2 antibody was purchased from Zymed company (San Francisco, CA, USA). Universal SP immunohistochemistry kit was purchased from Beijing Zhongshan Company (Beijing, China).

Cell culture

HEC-1A cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (*V*/*V*) fetal bovine serum at 37℃ in a humidified atmosphere of 5% CO_2/95% (*V*/*V*) air. Cells were digested with 0.25% trypsin at a confluence close to 90% and subcultured in the proportion of 1:2 or 1:3.

pax2 siRNA lentiviral vector construction

Referencing siRNA design strategy, we selected sites of the *pax2* gene (NM_0000278) cDNA sequence and determined the specific sequence by BLAST. DNA oligos of *pax2* siRNA were designed and synthesized. After annealing, enzyme digestion, vector connection, and transformation into *E. coli* DH5α, it was incorporated into a pGCL expression plasmid. Then, 20 μg pGCL vector was mixed with 15 μg pHelper1.0 vector (gag/pol component) and 10 μg pHelper2.0 vector (VSVG component), packed and transfected into 293T cells.

Cell treatment and development of tumor models

At a confluence close to 90%, cells were washed with PBS twice to remove dead cells, then digested by 0.25% trypsin and resuspended in PBS. After 3 min of centrifugation at 1000 r/min at room temperature, cells were washed twice with PBS to remove serum components which can induce a non-specific immune response in nude mice. Cells were then resuspended at 6×10^6/mL in PBS. Twenty-four healthy BALB/C female mice were randomly divided into 3 groups (8 mice in each group) and were subcutaneously injected with 1 mL of 6×10^6/mL HEC-1A cells at the back near to the hind

limb. Transplanted subcutaneous tumor could be observed 5 to 7 days later. Tumor size was measured every 3 days. When tumors grew into approximately 10 mm × 5 mm in size, the mice were injected as per the following method [3]: plasmid, liposome 2000, and OPTI-MEM I were mixed by a proportion of 10 μg:30 μL: 100 μL to prepare virus solution. The mice were injected with 150 μL empty vector mixture, 150 μL *pax2* siRNA lentiviral vector mixture, and 150 μL PBS (negative control) twice, respectively, with an interval of 3 days between injections.

Immunohistochemical staining of human tissues and transplanted tumor tissues from nude mice

Specimens were prepared into 4 μm paraffin slices. Human tissue specimens were stained by Pax2 antibody; nude mice transplanted tumor specimens were stained by hematoxylin and eosin and PCNA antibody. The slices were treated with 0.3% H_2O_2 to remove endogenous peroxidase and blocked by 10% normal goat serum. Antibodies were then added sequentially. After diaminobenzidine color development, the slices were examined by light microscopy; appearing brown product in the cytoplasm was considered a positive reaction. Ten high magnification fields were observed for each slice and 100 cancer cells were counted in each field. The positive labeling index (PLI) was calculated according to the proportion of Pax2-positive cells among all cancer cells.

Examination of transplanted tumor growth inhibition in nude mice

Nude mice were killed by cervical decapitation after inoculation with HEC-1A cells for 4 weeks. Tumors were desquamated completely and weighed with an electronic balance. The maximum diameter (L) and minimum diameter (W) of tumors were measured and the volume was calculated by the following formula: V (mm^3) = ($L \times W^2$)/2. The inhibition rate of tumor growth was calculated with the following formula: tumor inhibition rate = (average tumor weight of control group − average tumor weight of injection group) / average tumor weight of control group × 100%.

Western blotting of Pax2 and Bcl-2 expression in transplanted tumors

A part of the tumor tissue injected with HEC-1A cells was used to prepare the total protein extract. An amount of 50 μg of protein extract was fractionated by SDS-PAGE; β-actin served as equal loading control. After electrophoresis, the proteins were transferred onto nitrocellulose membrane and the membrane was blocked for 2 h at room temperature. After rinsing by TBS buffer (TBST, containing 0.05% Tween20) 3 times, each time 10 min, the membrane was incubated sequentially with primary rabbit anti-Pax2 or Bcl-2 antibody (dilution, 1: 600) and HRP-conjugated goat anti-rabbit secondary antibody (dilution, 1:5000). After 2 h of incubation at 37℃, the membrane was rinsed and the image was developed by ECL.

Statistical analysis

Data were analyzed by non-parametric test and analysis of variance using SPSS11.5 software. Data are generally expressed as mean ± standard deviation. $P < 0.05$ was considered significant.

Results

Pax2 expression in normal endometrium and endometrial cancer tissues

The positive rate and expression index of Pax2 were significantly lower in normal endometrial tissues than in endometrial cancer tissues (14.2% vs. 60.5%, $P < 0.01$; 1.74 ± 1.31 vs. 6.14 ± 2.07, $P < 0.01$) (Figure 1), and were significantly lower in G1 and G2 endometrial cancer tissues than in G3 endometrial cancer tissues (50.0% and 69.2% vs. 80.0%; 3.07 ± 1.96 and 5.45 ± 2.76 vs. 1.88 ± 1.68, $P < 0.05$).

Morphologic observation of transplanted tumors in nude mice

Transplanted tumor tissues were identified as moderately differentiated adenoid carcinoma by pathologic examination. Cells were polygonal with pathologic karyokinesis and obvious nucleoli. These observations were consistent with the morphologic characteristics of endometrial carcinoma. Necrosis of transplanted tumors in the *pax2* siRNA group significantly increased compared with the empty vector group and the PBS control group; the basophilic staining of cell nuclei was much weaker in the *pax2* siRNA group than in the empty vector group and the PBS control group (Figure 2).

Paraffin immunohistochemical results of transplanted tumors in nude mice

PCNA was strongly expressed in transplanted tumor tissues, exhibited as thick and brown particles distributed in the membrane and cytoplasm of transplanted endometrial cancer cells. The positive rate and expression index of PCNA were significantly lower in *pax2* siRNA group than in the empty vector group and the PBS control group (13% vs. 55% and 61%; 2.27 ± 2.01 vs. 9.05 ± 4.11 and 10.57 ± 4.98, $P < 0.01$) (Figure 3).

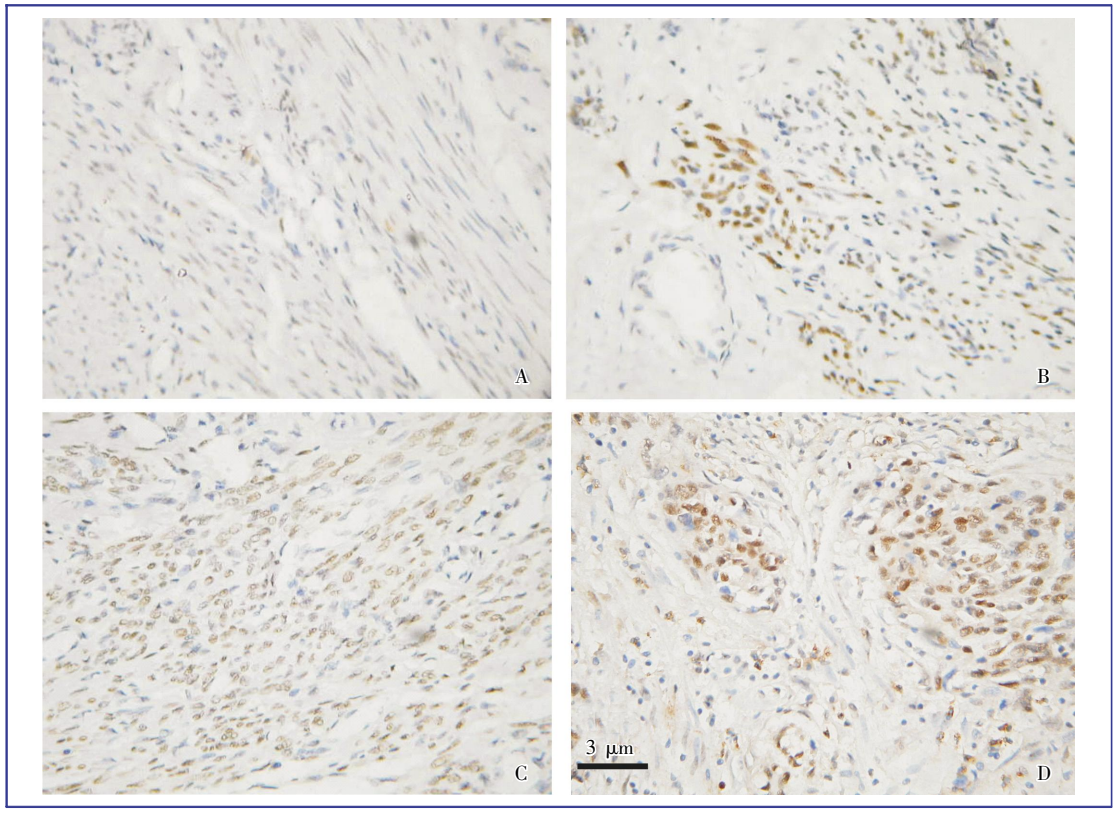

Figure 1. **Immunohistochemical SP staining of Pax2 in normal human endometrium and human endometrial carcinoma**. The endometrial tissues were stained with Pax2 antibody to show its expression. A, normal endometrium shows no Pax2 expression; B, well differentiated human endometrial adenocarcinoma shows low expression of Pax2; C, moderately differentiated human endometrial carcinoma shows high Pax2 expression; D, poorly differentiated human endometrial adenocarcinoma shows the highest Pax2 expression. Pax2 expression is related to the malignancy of endometrial cancer.

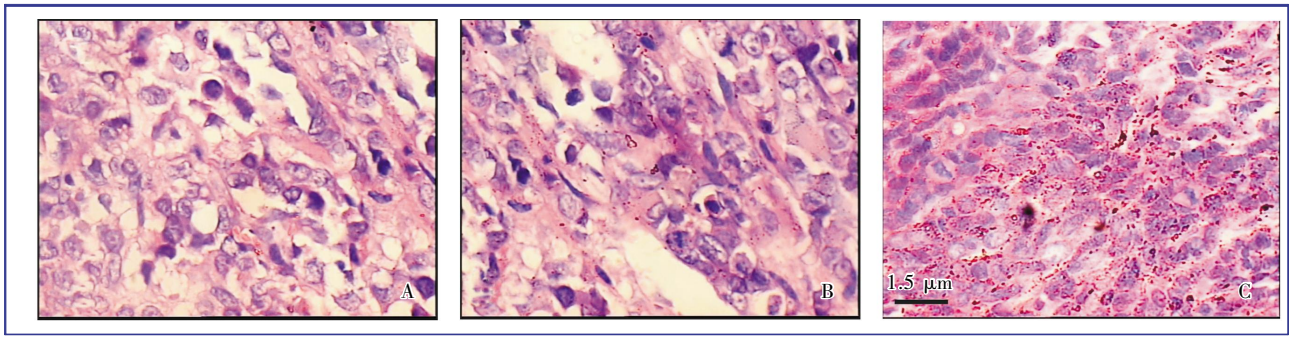

Figure 2. **HE staining of implanted human endometrial carcinoma in nude mice**. Transplanted tumors in nude mice were injected with PBS (A), empty lentivirus vector (B), and lentivirus vector-based *pax2* siRNA (C), respectively, and were stained with HE and identified as moderately differentiated endometrial carcinoma. Cells exhibit morphologic features consistent with endometrial carcinoma, such as polygonal area, distinct nucleoli and pathologic nuclear division. Tumor necrosis is greater in the *pax2* siRNA group (C) than in the empty lentivirus vector group (B) and PBS group (A), whereas nucleus basophilia dyeing was much lower than that in the other two groups.

pax2 siRNA inhibits the growth of transplanted tumors in nude mice

The tumor formation rate was 100% in nude mice with a standard subcutaneous tumor diameter of 0.3 cm. Tumor volume was almost equal among the groups before treatment ($P > 0.05$). All mice survived after the treatment. Tumor volume was sig nificantly smaller in *pax2* siRNA group than in the empty vector group and the PBS group [(741.67 ± 70.11) mm^3 vs. (1355.09 ± 100.78) mm^3 and (1503.75 ± 137.34) mm^3, $P < 0.01$],

indicating that *pax2* siRNA inhibited the growth of transplanted endometrial cancer in nude mice (Figure 4).

Tumor weight was significantly lighter in the *pax2* siRNA group than in the empty vector group and PBS group [(1.27 ± 0.04) g vs. (1.21 ± 0.05) g and (0.62 ± 0.03) g, $P < 0.01$); no significant difference in tumor weight was observed between empty vector group and PBS group ($P > 0.05$). The inhibition rate of tumor growth was 50.6% in *pax2* siRNA group and 3.7% in the empty vector group.

Western blot analysis of Pax2 and Bcl-2 expression in transplanted tumors of nude mice

Pax2 was highly expressed in transplanted tumors of the PBS group and the empty vector group, but down-regulated in the *pax2* siRNA group. Bcl-2 expression was reduced in transplanted tumors of the *pax2* siRNA group, significantly lower than in those of the PBS group and the empty vector group ($P < 0.05$) (Figure 5).

Discussion

Gene therapy, a novel treatment technology, has successfully entered phase I/II clinical trials [6,7]. The application of RNAi efficiently inhibits gene expression, showing great potential in treating viral infection, cancer, and genetic diseases[8]. *pax*, a nuclear transcription factor-paired box gene, belongs to a gene family regulating cellular development and tumor formation by initiating the inhibition of tumor epithelial cell apoptosis, inhibiting other tumor suppressor genes and cell death pathways, and stimulating cell proliferation [4,9]. Reducing Pax2 expression to inhibit tumor cell proliferation and induce

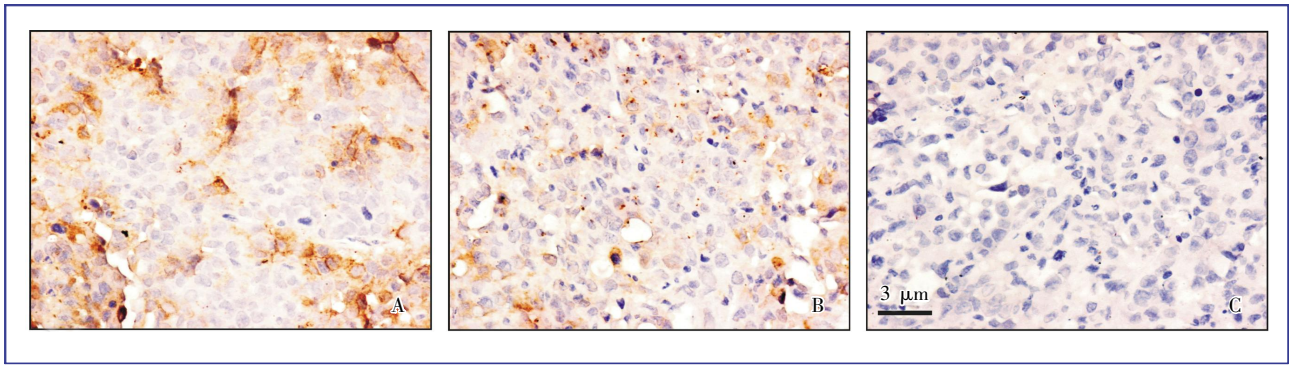

Figure 3. **PCNA protein expression in implanted human endometrial carcinoma in nude mice**. Transplanted tumors in nude mice were injected with PBS (A), empty lentivirus vector (B), and lentivirus vector-based *pax2* siRNA (C), respectively. The endometrial tissues were stained with PCNA antibody to observe its expression. Transplanted tumor tissues expressed PCNA with the positive particles submitting such features as enlargement and yellow brown tone filling the cell membrane and cytoplasm. PCNA expression is obviously reduced in *pax2* siRNA group (C) as compared with that in PBS group (A) and the empty lentivirus vector group (B).

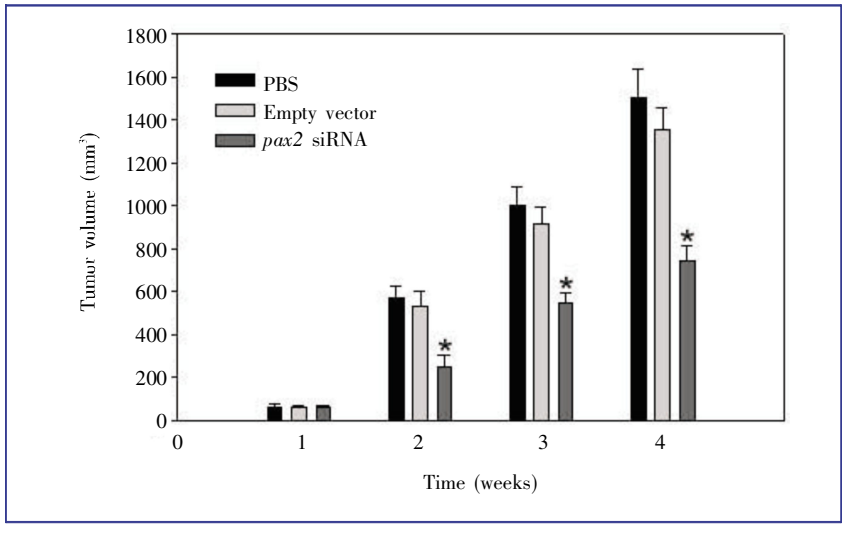

Figure 4. **Transplanted tumor growth in nude mice**. Gross tumor volume, which was equivalent before treatment, was significantly reduced in the *pax2* siRNA group after treatment, with no significant differences observed between the empty lentivirus vector and PBS groups. These results indicate that *pax2* siRNA suppresses the growth of implanted human endometrial carcinoma cells in nude mice. *$P < 0.01$, compared with the PBS and empty vector groups.

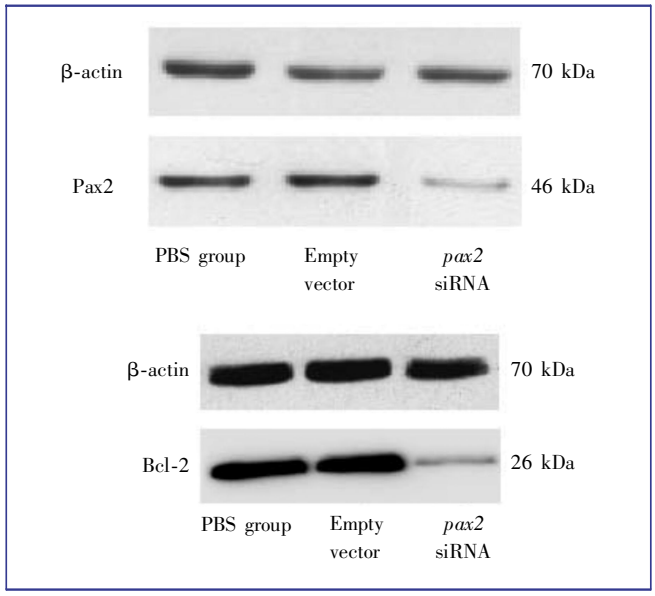

Figure 5. **Western blot analysis of Pax2 and Bcl-2 expression in transplanted tumor in nude mice**. Expression of Pax2 and Bcl-2 was high in the PBS and empty lentivirus vector groups, but was significantly down-regulated in the *pax2* siRNA group ($P < 0.05$).

tumor cell apoptosis is considered an innovative approach to treat cancer, which shows certain effect in treating renal cancer and prostate cancer [10,11]. Some researchers reported that Pax2 is a sensitive and specific immune index, predicting primary or metastatic poorly-differentiated serous ovarian cancer[12,13].

Our results showed that Pax2 was poorly expressed in normal endometrial glandular epithelial cells, whereas it was highly expressed in endometrial carcinoma epithelial cells. Furthermore, the expression index of Pax2 was closely related to histological grade of the tumor. The higher the histological grade, the higher positive rate and expression index of Pax2, indicating a high proliferative activity of cancer cells with many malignant biological behaviors such as invasion to the muscle wall, extrauterine spread and metastasis. This suggests that the expression of Pax2 may have clinical value in predicting prognosis of endometrial carcinoma. Our results are consistent with those in a previous study in which they demonstrated that *pax2* gene is closely related to the carcinogenesis and development of human endometrial carcinoma [14]. However, Rabban *et al.* [15] showed that Pax2 expression is not associated with carcinogenesis of endometrial adenocarcinoma. Therefore, the predictive and therapeutic values of Pax2 in endometrial cancer still needs further study with larger samples, survival information of patients, and further pre-clinical experiments.

Tumor transplantation models in nude mice are widely used in the study of gene therapy and play an important role during the transition of gene therapy to clinical application. To further confirm the role of *pax2* siRNA *in vivo*, we developed subcutaneous transplanted tumor models in nude mice. Literature has demonstrated the successful implementation of lentiviral vector as a gene therapy tool *in vitro* and *in vivo*[16]. In the present study, lentiviral vector was selected due to its large storage capacity, handling safety, stable genome integration, and reduced host immune response induction. Our results showed that after the injection of *pax2* siRNA carried by lentiviral vector, the growth of HEC-1A cell-transplanted tumors was clearly inhibited in nude mice. Pax2 expression in the *pax2* siRNA group was much lower than that in empty viral vector group and PBS control group. A large number of apoptotic cells within tumor tissues and spot or flake necrosis among tumor tissues were observed in the *pax2* siRNA group. Thus, the results indicated that the expression of Pax2 was reduced and tumor growth was inhibited.

Cell proliferation is closely related to the biological behavior of cancer cells. Thus, the proliferation activity of tumors is an important predictor for determining the degree of malignancy. Increased PCNA index is associated with tumor growth rate, therefore, the expression of PCNA has been used as an predictor of prognosis[17]. Li *et al.*[18] confirmed that PCNA expression can reflect the proliferation of tumor cells and the sensitivity to chemotherapy in HEC-1A and RL-952 transplanted tumors of nude mice. *Bcl-2* gene, an important inhibitor of apoptosis, is up-regulated during the tumorigenesis and development of most malignant tumors. Mitselou *et al.*[19] demonstrated that Bcl-2 expression is related to PCNA expression in normal endometrium, endometrial hyperplasia, and endometrial carcinoma. In our study, the expression of PCNA and Bcl-2 protein was examined by immunohistochemistry

and Western blot. The results showed that PCNA and Bcl-2 expression was significantly reduced in the *pax2* siRNA group as compared with empty viral vector and PBS control groups ($P < 0.05$). Reduced expression of Bcl-2 significantly slowed the proliferation of endometrial cancer cells and reduced the degree of malignancy, possibly due to increased tumor cell apoptosis.

In summary, the overexpression of Pax2 is closely related to endometrial tumorigenesis, highlighting Pax2 as an ideal target molecule in treating endometrial cancer. Lentiviral vector-based RNAi can effectively inhibit tumor gene expression and the proliferation of tumor cells *in vivo*, thereby providing new insight into the gene therapy of cancer.

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