

## Original Article

# Roles of Foxp3 in the occurrence and development of cervical cancer

Qingshuang Luo, Shulan Zhang, Heng Wei, Xiaobao Pang, Huijie Zhang

Department of Gynecology and Obstetrics, Shengjing Hospital of China Medical University, Liaoning Province, China

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**Abstract:** Objective: This study aimed to evaluate the relationship between forkhead box P3 (Foxp3) expression and clinicopathological characteristics of cervical cancer and to explore the influence of Foxp3 on the biological behaviors of cervical cancer cells. Methods: In this study, immunohistochemistry, lentivirus mediated transfection, Transwell assay; CCK-8 assay, real-time PCR and flow cytometry were employed to confirm the roles of Foxp3 in the occurrence and development of cervical cancer. Results: Foxp3 and p16<sup>INK4a</sup> were highly expressed in the cervical cancer and their expressions were related to the FIGO stage, tumor size, lymph node metastasis and serum SCC. Foxp3 had a high expression in the cervical cancer cells, tumor interstitium and metastatic lymph nodes. Foxp3 expression was positively related to p16<sup>INK4a</sup> expression in the cervical cancer. Foxp3 expression in the cervical cancer was negatively related to the prognosis: high Foxp3 expression predicted a poor prognosis. Silencing of Foxp3 was able to inhibit the proliferation and invasiveness of cervical cancer cells, promote their apoptosis, and induce the change in cell cycle. Silencing of Foxp3 also reduced the mRNA and protein expressions of p16<sup>INK4a</sup> in cervical cancer cells. Conclusion: Foxp3 is highly expressed in the cervical cancer, and able to facilitate the proliferation and invasiveness of cervical cancer, change cell cycle and inhibit their apoptosis, resulting in the occurrence, development and metastasis of cervical cancer.

**Keywords:** Cervical cancer, Foxp3, regulatory T cells, p16<sup>INK4a</sup>, cell apoptosis, human papillomavirus

## Introduction

Cervical cancer is the second common malignancies in women [1] and significantly threatens the health and life of women. In the general population, the incidence of cervical cancer shows a decreasing tendency, but the morbidity and mortality of cervical cancer are still increasing in the developing countries, and the cervical cancer patients becomes younger [2]. Cervical cancer is an immunogenic malignancy, and high-risk human papilloma virus (HPV) subtypes may cause cervical cancer through several steps: Cervical intraepithelial neoplasia (CIN), carcinoma *in situ* of cervix, invasive carcinoma of cervix and cervical cancer metastasis. Persistent infection of high-risk HPV subtypes (such as HPV16) may significantly facilitate the development of CIN (especially the CIN2 and CIN3) and has been confirmed as a major risk factor of CIN2, CIN3 and cervical cancer. For healthy subjects with HPV infection confirmed

by cervical cytology, more than 50% of them are diagnosed with transient HPV infection by re-examination at 12 months [3]. Whether HPV infection occurs after exposure to HPV and the outcome after HPV infection is closely related to the immune response of the host (especially the local immune response).

Regulatory T cells (Treg cells) are a group of mature T cells generated in the thymus following the induction of peripheral naïve T cells [4]. Treg cells are indispensable for the maintenance of non-response of host to autoantibodies and the inhibition of immune overreaction induced damage. On the other hand, over-production of Treg cells may block the protective immune response to infection and tumors. Multiple molecules of T cells are related to the specific functions of Treg cells. Forkhead/winged-helix transcription factor box P3 (Foxp3) is a member of forkhead/winged-helix family and a transcription factor specifically expressed

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**Table 1.** Clinicopathological characteristics of cervical tissues

Characteristics	Number of cases (%)
Samples	
NSCES	20 (13.5)
CIN	31 (20.9)
ICC	97 (65.5)
Differentiation	
WICC	21 (21.7)
MICC	49 (50.5)
PICC	27(27.8)
Histology	
SCC	88 (90.7)
ACC	9 (9.3)
FIGO Stage	
I	58 (59.8)
II	39 (40.2)
LN Metastasis	
Absent	77 (79.4)
Present	20 (20.6)
Tumor Size (cm)	
<4	46 (47.4)
≥4	51 (52.6)

on Treg cells. Human Foxp3 gene is mapped to Xp1 1.23 and includes 11 exons and 10 introns. Its cDNA is 1869 bp in length and it may produce a full-length mRNA and an mRNA without the 3<sup>rd</sup> exon [5]. Foxp3 plays crucial roles in the development and functions of Treg cells and especially Treg cells with high Foxp3 expression may significantly inhibit the immune response [4, 6-10]. Dysfunction of Treg cells (such as Foxp3 gene mutation) may cause severe autoimmune immune diseases, immune pathology and allergic reaction [6]. Foxp3 gene mutation may result in X-linked recessive hereditary inflammatory disease in scurfy mice and Immune dysregulation, polyendocrinopathy, enteropathy and X-linked (IPEX) in humans [4, 7]. In recent years, studies reveal that Foxp3 is not only specifically expressed in Treg cells naturally generated in the thymus, but in the cytoplasm and/or nucleus of cancer cells, and Foxp3 expression is closely related to the progression and prognosis of cancers [11-15]. For example, Foxp3 expression is aberrant in the gastric cancer [12], melanoma [13], liver cancer [14] and breast cancer [15] and closely associated with the occurrence, development and prognosis of cancers. However, the specific

mechanism underlying the role of Foxp3 in the pathogenesis of cancers is still unclear. On one hand, Foxp3 positive Treg cells may facilitate the occurrence, development and metastasis of cancers; on the other hand, abnormal Foxp3 expression may also inhibit or facilitate the occurrence and development of cancers.

p16<sup>INK4a</sup> is a cyclin-dependent kinase inhibitor and may deactivate CD4K4/CDK6 to block the phosphorylation of retinoblastoma protein (pRB), which may cause the aging of normal cells [16]. p16<sup>INK4a</sup> expression is very low in normal cells and negatively regulated by the product of pRB gene. In cervical cancer cells, high-risk HPV E7 oncogene may inactivate pRB gene and thus p16<sup>INK4a</sup> expression increases significantly in the cervical cancer [16, 17]. In the present study, the expressions of Foxp3 and p16<sup>INK4a</sup> were detected in cervical cancer, and the proliferation, apoptosis and invasiveness of cervical cancer cells were evaluated and the protein and mRNA expressions of p16<sup>INK4a</sup> were detected following Foxp3 silencing in cervical cancer cells.

### Materials and methods

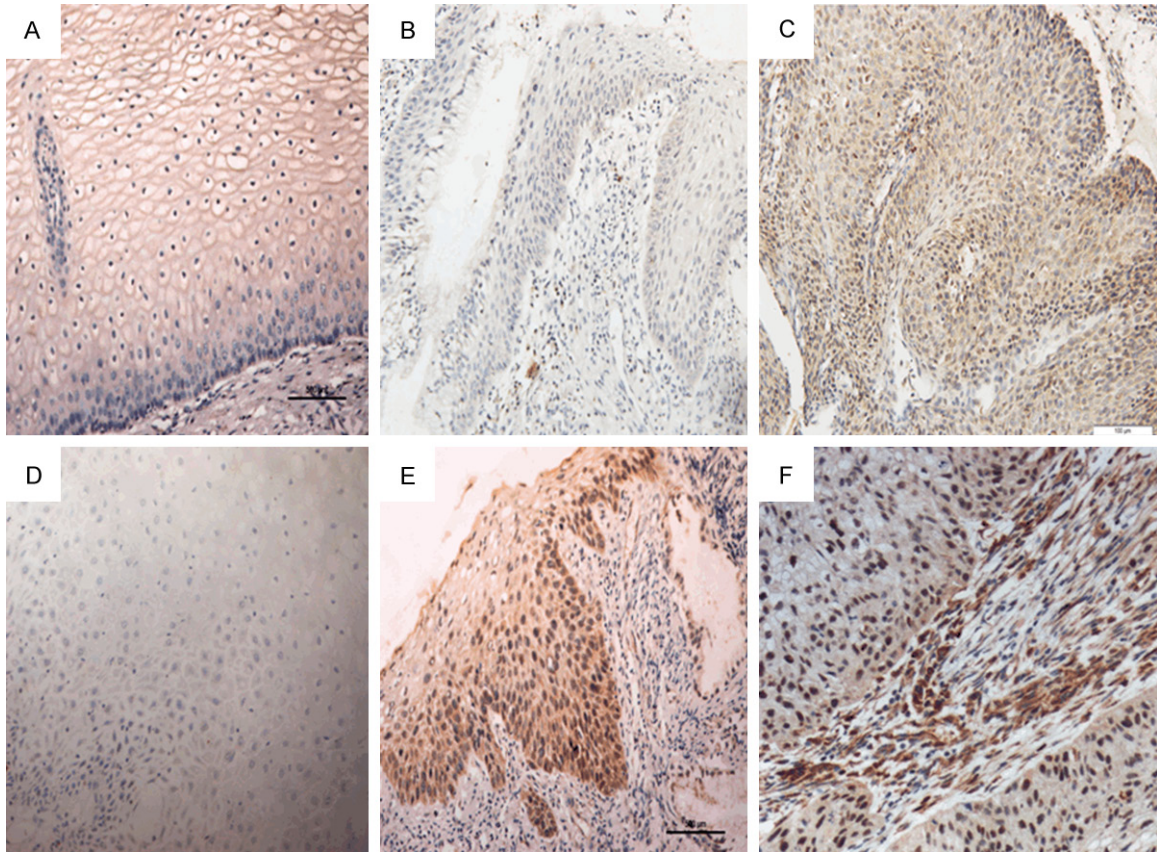
#### Subjects

A total of 148 cervical tissues were collected from inpatients and outpatients who received therapy in the Affiliated Shengjing Hospital of China Medical University between 2010 and 2011. There were 97 cervical cancer tissues, 15 metastatic lymph nodes, 5 non-metastatic lymph nodes, 31 CIN, and 20 normal cervical tissues. These patients had complete clinical information.

#### Immunohistochemistry

Neutral formalin-fixed tissues were embedded in paraffin with standard protocol. Samples were cut into 4 μm sections and mounted on glass slides. Sections were de-paraffinized with dimethylbenzene and rehydrated through a graded alcohol series, followed by blocking of the endogenous peroxidase activity by using hydrogen peroxidase (3%). The slides were then heated by microwave for antigen retrieval in citrate buffer (pH 6.0). After being cooled down in this buffer to room temperature, sections were covered with nonspecific serum, placed in a wet box and incubated at 37 °C. Primary rab-

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**Figure 1.** Expression of Foxp3 and p16<sup>INK4a</sup> in different cervical tissues. Foxp3 expression in normal cervical epithelium (A), CIN (B) and cervical cancer (C). p16<sup>INK4a</sup> expression in normal cervical epithelium (D), CIN (E) and cervical cancer (F) (200×).

**Table 2.** Foxp3 expression in normal cervical tissues, CIN and ICC

Characteristics	N	Foxp3 protein			
		-	+	++	+++
NSCES	20	17	3	0	0
CIN	31	18	10	3	0
ICC	97	2	13	45	37

bit anti-p16<sup>INK4a</sup> at 1:300 (Bioword Technology Inc., MN, USA) and mouse anti-Foxp3 at 1:50 (Abcam, USA) were respectively added on the same slides in the wet box, and then incubated at 4°C overnight. Following 3 washes in PBS, the slides were incubated at 37°C with addition of biotinylated secondary antibodies. They were again washed thrice in PBS before adding streptococcus avidin-peroxidase (Zhongshan-Golden Bridge Biotechnology Ltd., Beijing, China) and incubated at 37°C for 30 minutes. Staining was developed with DAB chromogen (Zhongshan-Golden Bridge Biotechnology Ltd., Beijing, China). They were counterstained with hematoxylin, dehydrated with ethanol gradient,

and sealed. Known positive tissue slides were used as positive controls, and PBS were used as the negative control instead of primary antibodies.

The immunostained sections were evaluated in a blinded manner by two independent researchers. The staining was evaluated as indicating expression through ten high-power fields per sample. The expression intensity was classified into four grades as follows: no staining visible was scored as 0; weak staining was scored as 1; moderate staining was scored as 2; strong staining was scored as 3. Scores for the positive cells were as follows: (score 0) means less than or equal 5% positive cells; (score 1) means 6-25% positive cells; (score 2) means 26-50% positive cells; and (score 3) means 51-75% positive cells; and (score 4) means more than or equal 76% positive cells. The scores of percentage and intensity reflect the sums of scores, with total scores of 0 indicated as (-); total scores of 1-2 as (+); total scores of 3-5 as (++); total scores of 6-7 as (+++).



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**Table 3.** Correlation between Foxp3 expression and clinicopathological characteristics of cervical cancer patients

Characteristics	N	Foxp3 protein				P-value
		-	+	++	+++	
Age (years)						
≤44	30	1	5	13	11	0.850
>44	67	1	8	32	26	
Differentiation						
WICC	21	0	5	8	8	0.189
MICC	49	0	4	25	20	
PICC	27	2	4	12	9	
Histology						
SCC	88	2	12	42	32	0.706
ACC	9	0	1	3	5	
FIGO Stage						
I	58	2	12	27	17	0.004
II	39	0	1	18	20	
LN Metastasis						
Absent	77	1	12	40	24	0.022
Present	20	1	1	5	13	
Tumor Size (cm)						
<4	46	1	11	22	12	0.014
≥4	51	1	2	23	25	
SCC (ng/ml)						
<1.5	56	1	12	32	11	<0.001
≥1.5	41	1	1	13	26	

### Cell culture

Human cervical cancer cells (Hela cells, Siha cells and Caski cells) were purchased from the Shanghai Institute of Cell of Chinese Academy of Science. Hela cells were maintained in DMEM containing 10% fetal bovine serum (FBS), and Siha cells and Caski cells were cultured in MEM containing 10% FBS. Cells were digested with 0.25% trypsin in 0.1% EDTA and then centrifuged. Cells were harvested and re-suspended, followed by incubation in an environment with 5% CO<sub>2</sub> at 37°C. Cells in logarithmic growth phase were harvested for further experiments.

### Western blot assay

Western blot assay was employed to detect Foxp3 expression, and cervical cancer cells with Foxp3 silencing were screened. At 48 h after lentivirus mediated transfection, cells were harvested and total protein was extracted. The protein concentration was determined. Total protein was subjected to 10% SDS-PAGE and transferred to PVDF membranes (Millipore,

**Table 4.** Correlation between p16<sup>INK4a</sup> expression and clinicopathological characteristics of cervical cancer patients

Characteristics	N	p16 <sup>INK4a</sup> protein				P-value
		-	+	++	+++	
NSCES	20	15	4	1	0	<0.05
CIN	31	0	11	15	5	
ICC	97	1	4	63	29	
Age (years)						
≤44	30	1	1	17	11	0.331
>44	67	0	3	46	18	
Differentiation						
WICC	21	0	0	13	8	0.504
MICC	49	0	3	34	12	
PICC	27	1	1	16	9	
Histology						
SCC	88	1	4	57	26	0.907
ACC	9	0	0	6	3	
FIGO Stage						
I	58	0	3	47	8	<0.001
II	39	1	1	16	21	
LN Metastasis						
Absent	77	0	3	55	19	0.021
Present	20	1	1	8	10	
Tumor Size (cm)						
<4	46	1	3	36	6	0.005
≥4	51	0	1	27	23	
SCC (ng/ml)						
<1.5	56	1	4	42	9	0.003
≥1.5	41	0	0	21	20	

re, Bedford, MA). After blocking with 5% non-fat dry milk in TBS, the membrane was probed with primary monoclonal antibody specific to Foxp3 (1:200, Abcam, USA), p16<sup>INK4a</sup> (1:1000, Bioword Technology Inc., MN, USA) or β-actin (1:1000, Beyotime) as an internal control for protein. Then, the membrane was incubated with secondary antibody at 1:3000, and protein bands were scanned. The optical density (OD) of bands was analyzed with the Gel-Pro-Analyzer. Each experiment was conducted three times, and data were averaged.

### Lentivirus mediated interence in siha cells

Lentivirus expressing Foxp3-siRNA was prepared in the Shanghai Genepharma Biotech Co., Ltd. Cells were divided into 3 groups: blank control group, negative control group and Foxp3 siRNA group. Cells were seeded into 6-well plates at a density of 3×10<sup>5</sup> cells/well, and then

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**Table 5.** Correlation between Foxp3 expression and p16<sup>INK4a</sup> expression in cervical cancer

Foxp3	p16 <sup>INK4a</sup>		Correlation coefficient	P
	-/+	++/+++		
-/+	16	25	0.551	<0.001
++/+++	0	87		

**Table 6.** Foxp3 expression in metastatic lymph nodes and normal lymph nodes

LN	Foxp3		P
	-/+	++/+++	
Present	4	11	<0.05
Absent	4	1	

incubated in an environment with 5% CO<sub>2</sub> at 37°C for 24 h. Cells were transfected with blank control lentivirus, negative control lentivirus and lentivirus expressing Foxp3-siRNA for 48 h, and then observed under a fluorescence microscope. Cells were harvested for the detection of mRNA and protein expressions of target genes. Western blot assay was employed to screen cervical cancer cells with high Foxp3 expression, and Foxp3 expression was silenced in these cells. After silencing of Foxp3, Western blot assay and real-time PCR were employed to detect the protein and mRNA expression of Foxp3 and p16<sup>INK4a</sup>, respectively. Each experiment was conducted three times, and data were averaged.

### Detection of cell proliferation in vitro

Cells with Foxp3 silencing were collected and seeded into five 96-well plates, followed by incubation for 24 h. Cells in 4 plates were processed for lentivirus mediated transfection, and cells in the remaining plate were processed for MTT assay (0 h). At 24 h, the medium was refreshed, and cells in 1 plate were processed for MTT assay (24 h). Cells in remaining 3 plates were maintained for 48 h, 72 h and 96 h, respectively, and then processed for MTT assay. Each experiment was conducted three times, and data were averaged.

### Detection of cell invasion in vitro

Transwell chambers were added to 24-well plates and then pre-coated with diluted Matrigel (50 µl per chamber), followed by incubation at 37°C for 2 h. The medium was

removed, and cells were washed in PBS thrice and digested in 0.25% trypsin. When the cells became round, excess trypsin was removed, and the pre-coated Transwell chambers were added to 24-well plates. After fixation in 4% paraformaldehyde at room temperature for 20 min, 0.5% crystal violet staining was performed for 5 min. After washing, cells were observed under an inverted microscope at 200×, and the migrating cells were counted. Five fields were randomly selected, and migrating cells were counted, and an average was obtained. Each experiment was conducted three times, and data were averaged.

### Detection of cell apoptosis by flow cytometry

Cells were mixed with 5 µl of Annexin V-Light and 5 µl of propidium iodide and then subjected to flow cytometry. Cells located in 4 quadrants: cells in the upper left quadrant were necrotic cells; cells in lower left quadrant were normal cells; cells in upper right quadrant were late apoptotic cells; cells in lower right quadrant were early apoptotic cells. The proportion of different cells was calculated. Each experiment was conducted three times, and data were averaged.

### Statistical analysis

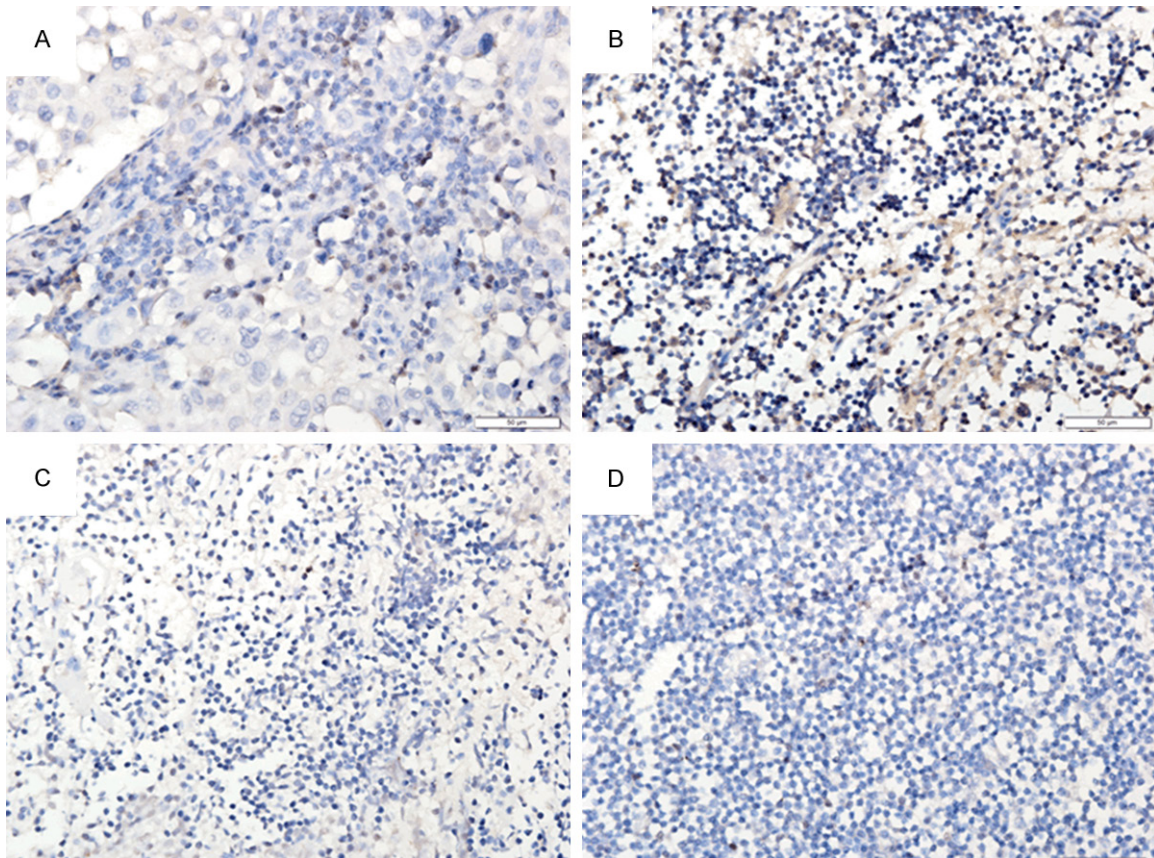
Statistical analysis was performed with SPSS version 17.0. Chi square test was employed to evaluate the relationship of clinicopathological characteristics of cervical cancer with protein expressions of Foxp3 and p16<sup>INK4a</sup>. Spearman rank correlation analysis was used to test the relationship between Foxp3 expression and p16<sup>INK4a</sup> expression. A value of  $P < 0.05$  was considered statistical analysis. Quantitative data are expressed as mean ± standard deviation and were compared with analysis of variance. Qualitative data were compared with chi square test.

## Results

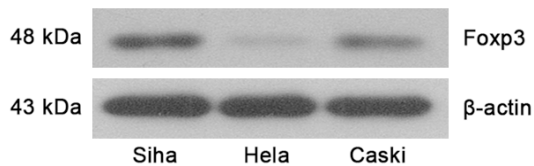
### Clinicopathological characteristics

Immunohistochemistry was done in 148 cervical tissues including 97 cervical cancer tissues, 31 CIN and 20 normal cervical tissues. All the patients had complete clinical and clinicopathological information (**Table 1**). The age of cervical cancer patients ranged from 27 years to 70 years (median: 44 years). Cervical

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**Figure 2.** Foxp3 expression of metastatic lymph nodes (A, B) and normal lymph nodes (C, D) (400×).



**Figure 3.** Foxp3 protein expression in cervical cancer cell lines.

cancer was staged according to the FIGO2009 criteria.

### *Expression of Foxp3 and p16<sup>INK4a</sup> in different cervical tissues*

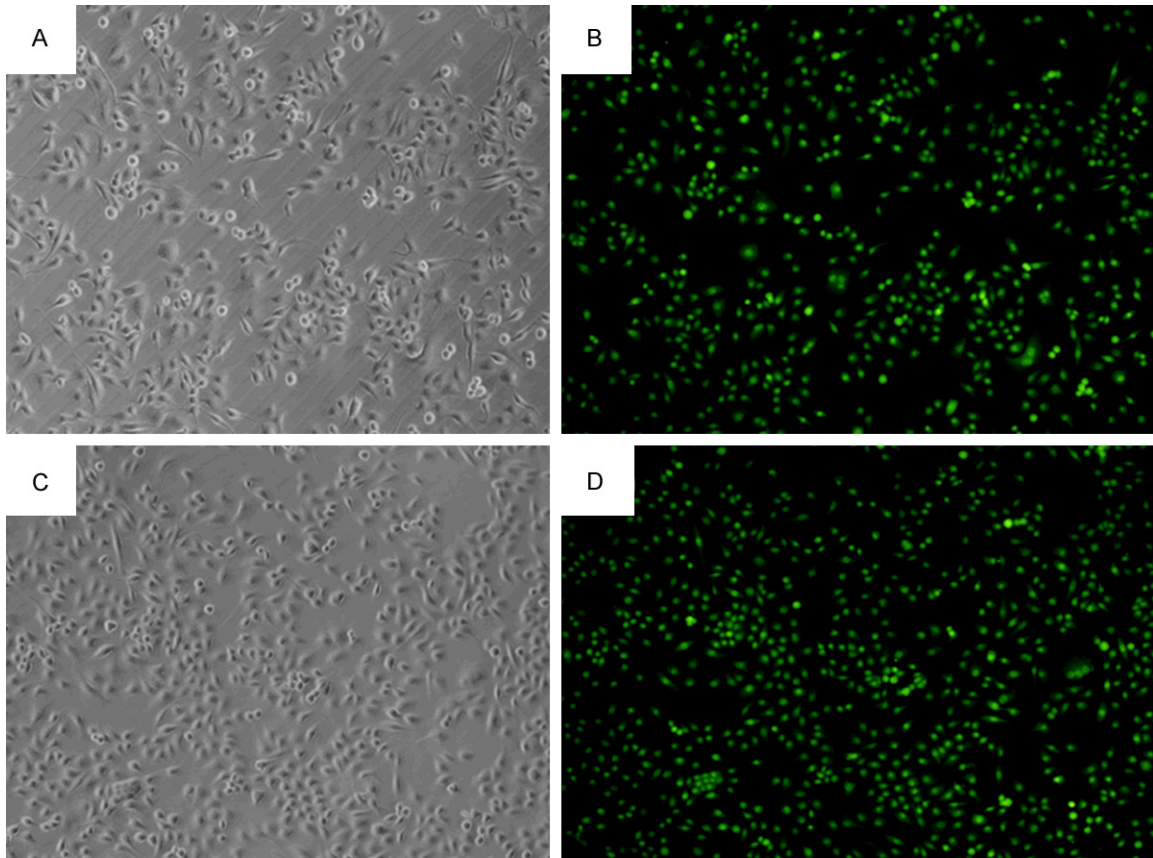
Immunohistochemistry was performed to detect the Foxp3 expression in normal cervical epithelium, CIN and cervical cancer. As shown in **Figure 1**, Foxp3 protein was not observed in normal cervical epithelium, CIN had a weak expression of Foxp3, and Foxp3 expression was observed as brown granules in the cytoplasm and nucleus of cervical cancer cells as well as in the interstitium of cervical cancer. In cervical cancer, the Foxp3 expression was sig-

nificantly higher than that in normal cervical tissues and CIN. Significant difference was also observed in the Foxp3 expression between ICC group and NSCES group ( $\chi^2=87.174$ ,  $P<0.001$ ) and between ICC group and CIN group ( $\chi^2=72.072$ ,  $P<0.001$ ), but there was no marked difference between CIN group and NSCES group ( $\chi^2=4.641$ ,  $P=0.098$ ) (**Table 2**).

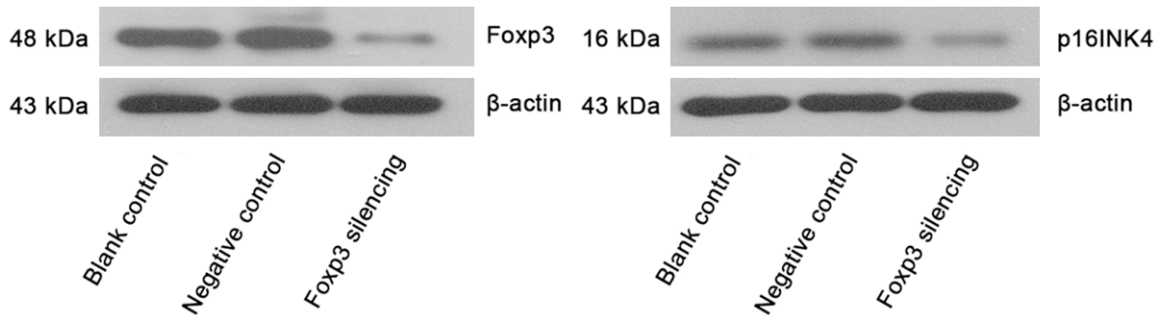
### *Correlation of Foxp3 and p16<sup>INK4a</sup> expressions with clinicopathological characteristics of cervical cancer*

The Foxp3 and p16<sup>INK4a</sup> expressions were associated with some clinicopathological characteristics (FIGO stage, lymph node metastasis, tumor size and HPV infection) of cervical cancer patients, but had no relationship with differentiation degree, pathological type and age of cervical cancer patients (**Table 3**). In cervical cancer patients, p16<sup>INK4a</sup> expression was significantly different among ICC, CIN and NSCES ( $P<0.05$ ) (**Table 4**). Moreover, in cervical cancer and CIN, Foxp3 expression was positively related to p16<sup>INK4a</sup> expression (**Table 5**).





**Figure 4.** Foxp3 expression in SiHa cells of different groups (100×). A, B: Foxp3 silencing group; C, D: Negative control group.



**Figure 5.** Expressions of Foxp3 and p16<sup>INK4a</sup> in SiHa cells of different groups (Western blot assay).

*Foxp3 expression in metastatic lymph nodes of cervical cancer patients*

Immunohistochemistry was employed to detect the Foxp3 expression in metastatic lymph nodes and normal lymph nodes (**Table 6**). As shown in **Figure 2**, Foxp3 expression was mainly found as brown granules in the nucleus of lymphocytes and the Foxp3 expression in metastatic lymph nodes was significantly higher than in normal lymph nodes ( $P < 0.05$ ).

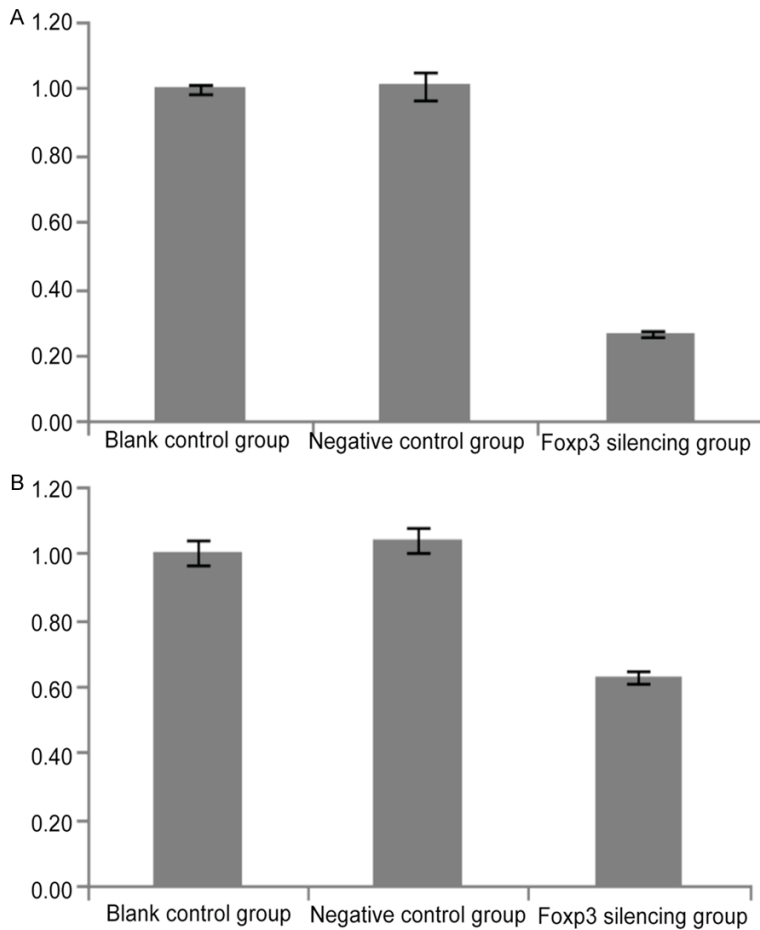
*Foxp3 expression in cervical cancer cells*

As shown in **Figure 3**, Foxp3 expression was high in SiHa cells, but low in HeLa cells.

*Foxp3 expression in SiHa cells after Foxp3 silencing*

Cells with Foxp3 silencing (GFP/LV3-Foxp3-hemo-876) were selected, and SiHa cells transfected with GFP/LV3-NC (negative control) and

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**Figure 6.** Expression of Foxp3 and p16<sup>INK4a</sup> in Siha cells of different groups (real-time PCR).

Siha cells without transfection (blank control) served as controls. Western blot assay and real-time PCR were used to detect the protein and mRNA expressions of Foxp3 and p16<sup>INK4a</sup>. **Figure 2** shows the Foxp3 expression in Siha cells following lentivirus mediated RNA interference. As shown in **Figures 3** and **4**, the mRNA and protein expressions of Foxp3 and p16<sup>INK4a</sup> in cells after Foxp3 silencing reduced significantly when compared with blank control group and negative control group ( $P < 0.05$ ) (**Figures 4-6**).

### Detection of cell proliferation in Siha cells following Foxp3 silencing by CCK-8 assay

In this study, CCK-8 assay was employed to detect the cell proliferation in Siha cells following Foxp3 silencing, Siha cells of negative control group and Siha cells of blank control group. The absorbance in difference groups is shown in **Table 7**. In Siha cells with Foxp3 silencing, the number of cells reduced significantly at 24,

48, 72 and 96 h when compared with negative control group and blank control group ( $P < 0.05$ ). This suggests that Foxp3 silencing may inhibit the proliferation of Siha cells (**Figure 7**).

### Effect of Foxp3 on the invasiveness of cervical cancer cells

Transwell chambers were employed to evaluate the invasiveness of cervical cancer cells. Results showed Siha cells after Foxp3 silencing showed a significantly reduced invasiveness as compared to blank control group and negative control group ( $45.60 \pm 5.41$  vs.  $72.40 \pm 8.20$  and  $73.60 \pm 7.89$ ,  $P < 0.001$ ) (**Table 8**; **Figures 8** and **9**).

### Effect of Foxp3 on the apoptosis of cervical cancer cells

After PI staining, cells were subjected to flow cytometry. The apoptosis rate is shown in **Table 9**. The apoptosis rate in Siha cells after Foxp3 silencing increased significantly

when compared with blank control group and negative control group (**Figure 10**).

### Effect of Foxp3 on the cell cycle of cervical cancer cells

After AnnexinV-Light staining, flow cytometry was performed to detect cells in different phases of cell cycle. As shown in **Table 10**, cells in S phase and G2 phase reduced significantly and those in G1 phase increased markedly after Foxp3 silencing when compared with blank control group and negative control group ( $P < 0.01$ ). This suggests that Foxp3 silencing reduces cells in S phase and G2 phase, but blocks cells in G1 phase (**Figure 11**).

## Discussion

Foxp3 has been regarded as a specific marker of Treg cells and plays important roles in the development and functions of Treg cells [8]. Foxp3 has three discernible domains: C<sub>2</sub>H<sub>2</sub> zinc

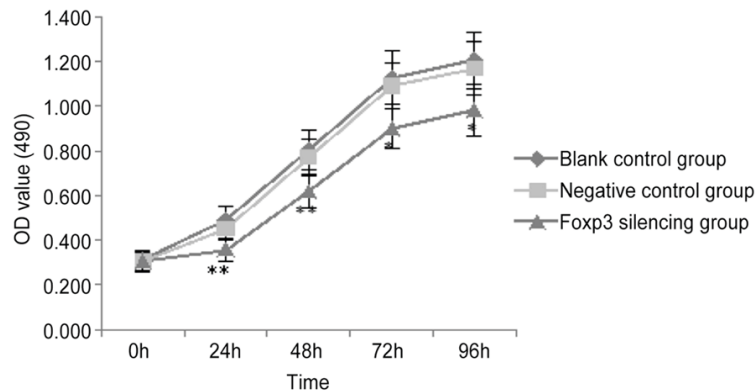


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**Table 7.** Absorbance of Siha cells in different groups at distinct time points

Group	Absorbance				
	0 h	24 h	48 h	72 h	96 h
Blank control	0.314±0.022	0.489±0.060	0.806±0.089	1.127±0.121	1.206±0.126
Negative control	0.307±0.043	0.450±0.048	0.773±0.078	1.090±0.105	1.169±0.120
Foxp3 silencing	0.305±0.046	0.355±0.053*	0.617±0.070*	0.899±0.090*	0.982±0.118*

Note: \* $P < 0.05$  vs. negative control group and blank control.



**Figure 7.** Proliferation of cells in different groups.

**Table 8.** Number of migrating Siha cells in different groups

Groups	Cell No
Blank control	73.60±7.89
Negative control	72.40±8.20
Foxp3 silencing	45.60±5.41*

Note: \* $P < 0.01$  vs. blank control group and negative control group.

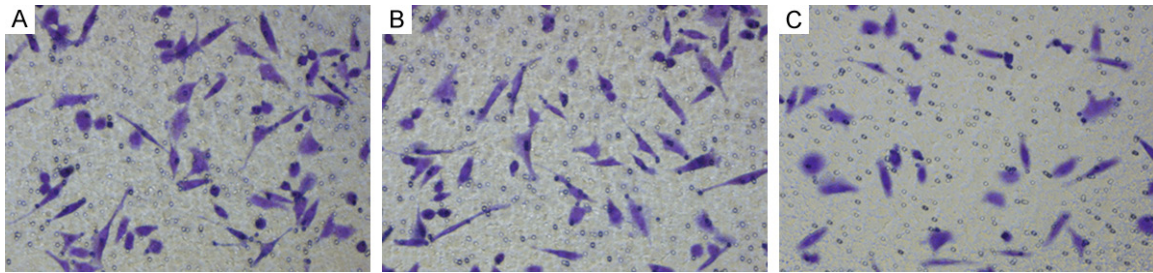
finger domain at N terminal, leucine zipper motif (LeuZip) and forkhead DNA binding domain at C terminal (FKH). The  $C_2H_2$  zinc finger domain and LeuZip are conservative in the FOX family and can bind to regulatory DNA to induce the formation of dimmers. FKH domain is a shared characteristic of FOX family and crucial for the nuclear localization and DNA binding [5]. In recent years, Foxp3 expression is found in not only T cells, but in the peripheral blood, lymph nodes and tumor microenvironment of tumor patients. The Foxp3 positive Treg cells are abundant in the liver cancer, and Foxp3 has been found to facilitate cancer metastasis and be associated with a poor prognosis of cancer patients [14]. In the local lymph nodes of ovarian cancer patients, Foxp3 over-expression was observed in Treg cells and closely related to the poor prognosis [18]. Ma et al found that Foxp3

was highly expressed in the gastric cancer cells and Foxp3 could activate the apoptosis pathway to induce the apoptosis of gastric cancer cells and inhibit the cancer progression, and high Foxp3 expression predicted a better prognosis [12, 19]. Above findings suggest that Foxp3 gene in cancer cells may serve as a tumor suppressor gene to inhibit cancer progression, or Foxp3 possesses activities similar to those of Foxp3 positive cells to suppress the immune mediated

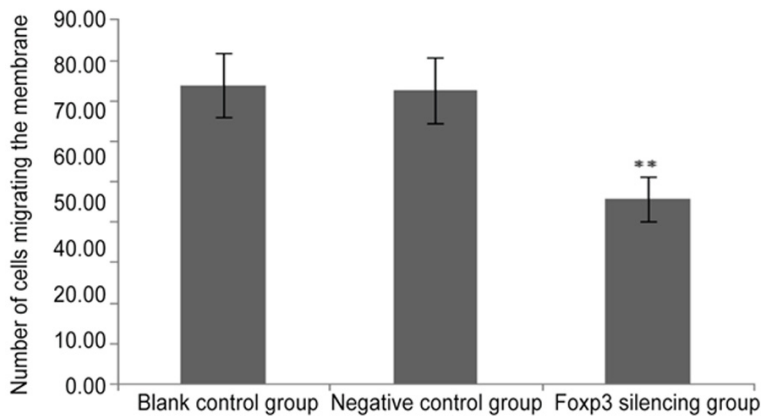
killing of cancer cells. The roles of Foxp3 in the cancer cells are still conflicting, and the specific mechanism is unclear.  $p16^{INK4a}$  is a marker of HPV infection. In tonsillar squamous cell carcinoma,  $p16^{INK4a}$  expression is related to Foxp3 expression and predicts a better prognosis [20].

Foxp3 expression increases in the gastric cancer cells, is able to inhibit the proliferation of gastric cancer cells and facilitate their apoptosis by activating apoptosis pathway and may serve as a predictor of good prognosis [12]. In melanoma SM-MEL-28 cells, no more than 1% of cells are positive for Foxp3; in cells with stable high Foxp3 expression, the proliferation is inhibited, cell colonies reduce *in vitro* and tumor growth reduces *in vivo* [13]. In melanoma cells, Foxp3 is not a key protein that is able to facilitate or inhibit the cancer progression. Some studies show that Foxp3 may inhibit the occurrence and development of cancers and its expression is positively related to the survival of cancer patients, but others reveal Foxp3 has no relationship with the occurrence and development of cancer. The exact reason for this discrepancy is still poorly understood. In studies on breast cancer, the roles of Foxp3 are conflicting: some show it is able to promote the

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**Figure 8.** Cells migrating across the membrane in different groups (200×). A: Blank control group; B: Negative control group; C: Foxp3 silencing group.



**Figure 9.** Number of cells migrating the membrane in three groups. \*\* $P < 0.01$  vs. blank control group and negative control group.

**Table 9.** Apoptosis rate in three groups

Groups	Apoptosis rate (%)
Blank control	3.68±0.13
Negative control	3.58±0.23
Foxp3 silencing	26.02±1.28*

Note: \* $P < 0.01$  vs. blank control group and negative control group.

development of breast cancer, but others reveal it inhibits the development of breast cancer. In breast cancer with HER2 over-expression, patients with Foxp3 positive cancer have improved recurrence free survival and overall survival. Foxp3 expression in breast cancer cells is an independent prognostic factor of HER2 positive breast cancer patients, and Foxp3 expression is helpful to identify high risk patients who require additional therapy after neoadjuvant chemotherapy [15].

Abnormal p16<sup>INK4a</sup> expression is closely related to HR-HPV infection. Integration of HPV DNA into DNA of the host is crucial for the stability and its maintenance of virus in the host [21].

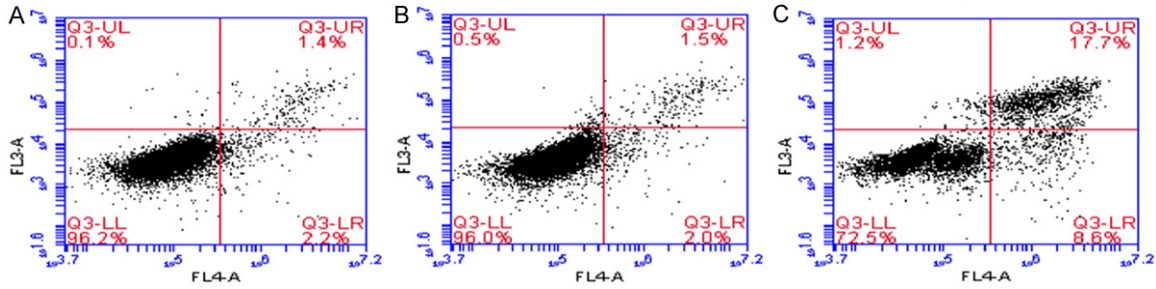
p16<sup>INK4a</sup> is a marker indicating that HPV-DNA integrates into host cells and induce the abnormal cell cycle [16, 17, 22].

In our study, the expression of Foxp3 and p16<sup>INK4a</sup> was detected in 97 cervical cancer tissues, 31 CIN and 20 normal cervical tissues. Results showed expression of Foxp3 and p16<sup>INK4a</sup> was mainly found in cervical cancer tissues, normal cervical tissues had no or weak expression of Foxp3 and P16<sup>INK4a</sup>, strong Foxp3 and P16<sup>INK4a</sup> expression was found

in lesioned cervical tissues, and CIN had intermediate Foxp3 and p16<sup>INK4a</sup> expression. Significant differences were observed in the Foxp3 and p16<sup>INK4a</sup> expression among these tissues. This indicates that Foxp3 and P16<sup>INK4a</sup> are activated in cervical cancer, and their expression shows an increasing tendency with the progression of cervical lesions. Thus, we speculate that the up-regulated Foxp3 and p16<sup>INK4a</sup> expression play an important role in the progression of normal cervical tissues into CIN or even cervical cancer. Immunohistochemistry was performed to detect the Foxp3 expression in metastatic lymph nodes of 15 patients and normal lymph nodes of 5 patients. Results showed the Foxp3 expression in metastatic lymph nodes was significantly higher than in normal lymph nodes. This implies that Foxp3 plays an important role in the lymph node metastasis of cervical cancer.

Clinical stage, pathological grade, lymph node metastasis and tumor size can be used to predict the invasiveness of cervical cancer and the prognosis of cervical cancer patients. The high-

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**Figure 10.** Normal cells, necrotic cells and cells at different stages of apoptosis (flow cytometry). A: Blank control group; B: Negative control group; C: Foxp3 silencing group.

**Table 10.** Cells in different phases of cell cycle in different groups

Groups	G1 (%)	S (%)	G2 (%)
Blank control	61.60±1.80	15.40±1.00	22.31±0.83
Negative control	62.59±1.11	14.72±0.37	21.72±0.82
Foxp3 silencing	76.29±1.09*	12.34±0.47*	11.49±0.87*

Note: \* $P < 0.01$  vs. blank control group and negative control group.

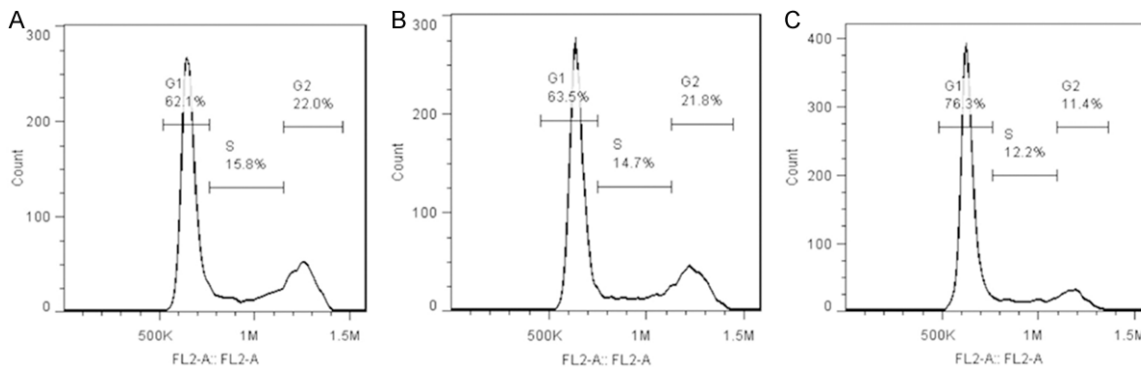
er the clinical stage and the higher the pathological grade, the more potent the invasiveness and metastasis of cervical cancer are. Under this condition, cervical cancer cells are susceptible to infiltrate the cervical interstitium and invade the blood vessels and lymphatic space, resulting in early metastasis. To investigate whether Foxp3 and p16<sup>INK4a</sup> expressions can be used to predict the malignancy and metastasis of cervical cancer, the expression of Foxp3 and p16<sup>INK4a</sup> was detected and their relationship with clinicopathological characteristics was further evaluated. Results showed Foxp3 and p16<sup>INK4a</sup> expression was closely related to the FIGO stage, tumor size, lymph node metastasis and serum SCC. The higher the FIGO stage, the higher the Foxp3 and p16<sup>INK4a</sup> expression was. This suggests that the up-regulated Foxp3 and p16<sup>INK4a</sup> expression is related to a poor prognosis of cervical cancer. However, there were no correlations of Foxp3 and p16<sup>INK4a</sup> expression with the differentiation degree, pathological type and age. On the above findings, we postulate that the up-regulated Foxp3 and p16<sup>INK4a</sup> expression play pivotal roles in the occurrence and development of cervical cancer. Moreover, Foxp3 expression was high in cervical cancer cells, cancer interstitium and metastatic lymph nodes, but the influence of Foxp3 on the behaviors of cancer cells is required to be further studied. Foxp3 plays crucial roles in the occurrence and development of cervical cancer, and

thus it is of great importance to further explore the roles of Foxp3 in cervical cancer.

The treatments of cervical cancer are multi-disciplinary and include surgery, radiotherapy and chemotherapy. Surgery and radiotherapy are traditional modalities used for the treatment of cervical cancer, but immunotherapy is an alternative for patients who are intolerable to surgery and/or radiotherapy due to poor disease condition (cachexia). In recent years, immunotherapy has been a new adjunctive therapy of cancers and a focus in studies on cancers. Immunotherapy has fewer side effects as compared to chemotherapy. Several vaccines have been developed against cervical cancer (such as E6/E7 polypeptide and gene vaccine) and used in animal studies and clinical trials. Treg cells are able to inhibit the immune response of the host against the tumor and enhance the escape of the tumor from immune clearance, suggesting that Treg cells may be a key missing part in the immunotherapy of cancers [23]. The role of Treg cells in the anti-tumor activity of immune system and Treg cells becoming a new target for the immunotherapy of cancers make Treg cells become a focus in studies on immunotherapy. In this study, the Foxp3 expression was detected in cervical cancer cell lines, lentivirus vector expressing Foxp3 siRNA was constructed to silence Foxp3 expression in Siha cells, and CCK-8 assay, Transwell assay and flow cytometry were employed to explore the roles of Foxp3 in the proliferation, apoptosis and invasion of Siha cells. Our findings will provide evidence for the new adjunctive immunotherapy of cancers. Our results showed lentivirus mediated transfection was effective to silence Foxp3 in Siha cells. Under an invert microscope, the growth



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**Figure 11.** Cells in different phases of cell cycle (flow cytometry). A: Blank control group; B: Negative control group; C: Foxp3 silencing group.

reduced and the number of viable cells also decreased following Foxp3 silencing as compared to blank control group and negative control group. CCK-8 assay showed Foxp3 silencing inhibited the proliferation of Siha cells, indicating that Foxp3 silencing affects the proliferation of Siha cells. Transwell chamber was employed to evaluate the influence of Foxp3 on the invasiveness of Siha cells. Results revealed that Foxp3 silencing significantly reduced the invasiveness of Siha cells as compared to blank control group and negative control group. This implies that Foxp3 silencing also affects the invasiveness of cervical cancer cells. Apoptosis analysis showed the apoptosis rate of Siha cells reduced dramatically after Foxp3 silencing, suggesting that Foxp3 silencing may reduce the number of apoptotic cells and also indicating that Foxp3 is involved in the proliferation of cervical cancer cells. Cell cycle is basic physiological process of cells. Our findings indicated that the proportion of cells in S phase and G2 phase reduced markedly and that of cells in G1 phase increased significantly after Foxp3 silencing, indicating that Foxp3 is able to facilitate the growth of cervical cancer cells. After silencing of Foxp3, the mRNA and protein expression of p16<sup>INK4a</sup> also decreased markedly in cervical cancer cells, further confirming that Foxp3 expression is positively related to p16<sup>INK4a</sup> expression, but the specific mechanism is required to be further elucidated. Above findings demonstrate that Foxp3 may facilitate the proliferation, reduce the apoptosis, promote the cell cycle progression and increase the cell invasion to affect the proliferation and invasion of cervical cancer cells and enhance their malignancy.

The growth, infiltration and metastasis of cancers have involvement of multiple signaling pathways and a lot of factors. More studies are required to elucidate the relationship between Foxp3 and cervical cancer, the role of Foxp3 in the occurrence and development of cervical cancer, and the specific mechanism underlying the role of Foxp3 in cervical cancer. In addition, whether the in vivo effects of Foxp3 on the cervical cancer are consistent with its in vitro effects on the cervical cancer cells and whether the cervical cancer can be treated by targeting Foxp3 are also required to be further studied.

In conclusion, the Foxp3 and p16<sup>INK4a</sup> expressions increase with the progression of cervical lesions, and Foxp3 expression is positively related to p16<sup>INK4a</sup> expression in cervical cancer. This suggests that the up-regulated expression of Foxp3 and p16<sup>INK4a</sup> may be one of mechanisms underlying the occurrence and development of cervical cancer. Foxp3 and p16<sup>INK4a</sup> expression is related to the FIGO stage, lymph node metastasis, tumor size and serum SCC-Ag. In the metastatic lymph nodes, Foxp3 expression is significantly higher than in normal lymph nodes, indicating that Foxp3 is associated with cervical cancer metastasis and may facilitate the metastasis of cervical cancer. Silencing of Foxp3 is able to significantly inhibit the proliferation and invasion of cervical cancer cells, reduce cells in S phase and G2 phase and increase the apoptosis rate of cervical cancer cells. Moreover, silencing of Foxp3 in cervical cancer cells also significantly reduces the mRNA and protein expression of p16<sup>INK4a</sup>, Foxp3 expression is positively related to p16<sup>INK4a</sup>

expression, and Foxp3 is associated with HPV infection, but the specific mechanism is still poorly understood.

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### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Shulan Zhang, Department of Gynecology and Obstetrics, Shengjing Hospital of China Medical University, Liaoning Province, China. E-mail: shulan\_zhang@yahoo.com

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