Up-regulation of Foxp3 inhibits cell proliferation, migration and invasion in epithelial ovarian cancer

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The transcription factor Forkhead Box P3 (Foxp3) has been shown to play important roles in the occurring of regulatory T cells (Tregs). Limited evidence indicated that it was also expressed in tissues other than thymus and spleen, while, very recently, it was identified as a suppressor gene in breast cancer. However, the precise role and molecular mechanism of the action of Foxp3 in ovarian cancer remained unclear. To elucidate the function of Foxp3, we examined the expression of Foxp3 in ovarian cancerous cells and the consequences of up-regulation of Foxp3 in epithelial ovarian cancer cell lines, respectively. By multiple cellular and molecular approaches such as gene transfection, CCK-8 assay, flow cytometry, RT-PCR, in-cell western, wound healing assay, and invasion assay, we found that Foxp3 was weakly/no expressed in ovarian cancerous cells. Up-regulation of Foxp3 inhibited cell proliferation, decreased cell migration, and reduced cell invasion. Compared with control, Foxp3 up-regulated cells showed decreased expression of Ki-67 and cyclin-dependent kinases (CDKs). Moreover, up-regulation of Foxp3 reduced the expression of matrix metalloproteinase-2 (MMP-2) and urokinase-type plasminogen activator (uPA), resulting in the inhibition of cell migration and invasion. In addition, Foxp3 up-regulation inhibited the activation of mammalian target of rapamycin (mTOR) and NF-κB signaling. These findings suggested that up-regulation of Foxp3 could be a novel approach for inhibiting ovarian cancer progression.

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1. Introduction

Epithelial ovarian cancer (EOC) is the leading killer among all gynecological malignancies and the 5-year survival rate for all ovarian cancer patients remains at less than 40% for the past 30 years [1]. This could be due to the fact that no effective methods of early diagnosis are currently available, as well as due to the lack of effective systemic therapies resulting in the high mortality of patients diagnosed with EOC. This disappointing outcome strongly suggests that there is a dire need for innovative research that will lead to a dramatic improvement in the survival of patients diagnosed with this deadly disease.

Foxp3 is a new member of the Forkhead/winged helix family of the transcription factors. It was identified during position cloning of Scurfin, a gene responsible for X-linked autoimmune diseases in mice and human (immune deregulation, polyendopathy, enteropathy, X-linked, IPEX) [2,3]. Mutation of Foxp3 gene resulted in ablation of the suppressor function of regulatory T cells, although it was still debatable whether such mutation ablated the regulatory T lineages, as was originally proposed [4,5].

Limited evidence indicated that it was also expressed in tissues other than thymus and spleen, while, very recently, Foxp3 mRNA as well as protein was detected in some tumor cell lines, albeit in variable levels, not related to the tissue of origin. That expression correlated with the expression levels of IL-10 and TGF-β1 [6]. In support of the above data, a very recent publication described the expression of Foxp3 in pancreatic carcinoma cells providing
evidence that this could be an important tumor escape mechanism [7]. Moreover, Foxp3 was noted as a transcriptional repressor of the HER2/ERBB2 oncogene, and interacted with SKP2 gene directly in breast cancer [8], while widespread deletion and somatic mutations of Foxp3 were observed in human breast cancer. In addition, germline mutation of Foxp3 resulted in a high rate of spontaneous breast cancer and increased susceptibility to carcinogens in a mouse model [9]. According to the above findings, Foxp3 was identified as a suppressor gene in breast cancer. However, it also suppressed cell growth and induced cell death in tumor cells without HER2/ERBB2 over-expression [9], which suggested that Foxp3 was involved in more other signaling pathways. Since the role and molecular mechanism of Foxp3 in ovarian cancer remained unknown, this study was scheduled to investigate whether expressions of Foxp3 transcripts and mature protein could occur in ovarian cancerous cells as well as whether it might play a suppressive role in ovarian cancer.

2. Materials and methods

2.1. Samples and immunohistochemistry

A total of seven normal ovarian epithelium and 27 malignant epithelial ovarian tumors were obtained from patients (age: 40.35 ± 9.02 years and 55 ± 14.03 years) admitted at The Hospital of Obstetrics and Gynecology of Fudan University after the approval of the local ethics committee and informed consent were obtained according to the Declaration of Helsinki from 2006 to 2007. Immunohistochemical staining of deparaffinized tumoral and normal ovarian tissues was done according to standard protocols using Foxp3 antibody (236A/E7, eBioscience, USA). The staining intensities were graded as strong, moderate, and weak, respectively, by two pathologists specialized in ovarian cancer.

2.2. Cells and cell culture

Human epithelial ovarian cancer cell lines SKOV3 and ES-2 were obtained from American Type Culture Collection. OMC685 were generously provided by Dr. Xu (M.D., Ph.D., Fudan University, Shanghai, China). All cells were cultured in RPMI1640 medium with 10% fetal bovine serum in a humidified atmosphere at 37°C. SKOV3 cells were seeded in 6-well plates and transfected with pEF-1α-Foxp3 and pEF-1α-vector, respectively, using Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) for 24 h without antibiotic selection. Then cells were cultured in medium containing 1600 μg/ml G418 (Sigma, St. Louis, MO) until all of the cells in the non-transfected control culture were killed. The antibiotic-resistant cells were pooled and passaged in medium containing 800 μg/ml G418. Cells were named as SKOV3/pEF-1α-Foxp3 and SKOV3/pEF-1α-vector, respectively, for further experiments.

2.4. Cell growth assay

Cell growth was analyzed using a WST-8 Cell Counting Kit-8 (Beyotime, Jiangsu, China). Cells (7.5 × 10^4) suspended in RPMI1640 medium (100 μl) containing 10% fetal bovine serum were seeded in 96-well plates and incubated for 2d, 4d, and 6d, respectively. CCK-8 solution (10 μl) was added to each well and the cultures were incubated at 37°C for 90 min. Absorbance at 450 nm was measured using an immunoreader. The results were plotted as means ± SD of three separate experiments having four determinations per experiment for each experimental condition.

2.5. Clone formation assay

Cells were seeded at 1.0 × 10^4 cells/well in 6-well plates and left to form clones over a period. Cultures were stained with 0.1% crystal violet and the number of clones in a 2 × 2 cm grid (on the culture plates) was scored to determine the clone-forming ability of the cells. Clones containing over 50 cells were counted.

2.6. Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry. After indicated treatments, cells were trypsinized, rinsed with PBS, fixed with 70% ethanol at 4°C overnight, and treated with RNaseA (0.02 mg/ml) in the dark at room temperature for 30 min. Cells were resuspended in 0.05 mg/ml propidium iodide and analyzed with flow cytometry (Becton Dickinson). DNA histograms were analyzed by the ModFit LT V2.0 software. For each sample, at least 10^4 events were recorded.

2.7. Western blot analysis

Two types of western blot, regular western blot and in-cell western, were conducted. Regular western blot was according to previous documents. In-cell western was a promoted method [10]. Briefly, cells were seeded in 96-well plates at 5 × 10^4 cells/well and cultured for 12 h. After being washed twice by PBS, cells were fixed with 4% paraformaldehyde for 20 min, incubated with 0.1% Triton-100 for 20 min, and then washed twice by PBS. Blocking was carried out with 5% BSA for 1 h at room temperature. Each well was incubated overnight at 4°C with target primary antibody (anti-MMP-2 1:50; anti-MMP-9 1:50; anti-uPA 1:50; anti-Ki-67 1:50; anti-PCNA 1:50; anti-CDKs 1:50; anti-NF-κB 1:50 (RnD, Minneapolis, USA), anti-AKT 1:50; anti-phospho-AKT 1:50; anti-mTOR 1:50; anti-phospho-mTOR 1:50; anti-ERK 1:50; anti-phospho-ERK 1:50 (Cell Signaling Technology, Beverly, MA)) and β-actin 1:100 as control, followed by IRDye38 conjugated anti-mouse (800 nm) or Alexa Fluor®880 conjugated anti-rabbit (700 nm) secondary antibody 1:5000 (BD PharMingen, California, USA) incubation at room temperature for 1 h in the
fluorescence was visualized, scanned and analyzed on Odyssey (LI-COR, US).

2.8. Real-time RT-PCR analysis

Total RNA was isolated with TRI-zol (Tarkara, Japan) according to the manufacturer’s protocols. RNA concentration was determined by Ultrospec3000 spectrophotometer (Pharmcia, Sweden). First strand reverse transcription kit was obtained by MBI (Fermentas, Lithuania, USA). Each reaction mixture (final volume 20 μl) contained RT template or negative control (2 μl), ddH2O, primers (0.3 μM), and SYBR Green taq-enzyme mix buffer (2 ×, 10 μl) (Takara, Japan). GAPDH was used as the control reaction. GAPDH: sense primer 5'-GAA GGT GAA GGT CGG AGTC-3' and anti-sense primer 5'-GAA GAT GGT GAT GGG ATT TC-3'. Foxp3: sense primer 5'-TCA CCT ACG CCA CGC TCA T-3' and anti-sense primer 5'-ACT CAG GTT GTG CGG ATGG-3'. Data were analyzed with ABI7000 software. Reaction products were also analyzed on 2% agarose gels, and visualized by ethidium bromide staining.

2.9. Wound healing assay

Cells were cultured and grown to 100% confluence. A clear area was then scraped in the monolayer with a 200 μl pipette tip. After being washed with serum-free RPMI1640 medium, cells were incubated at 37 °C in RPMI1640 medium containing 5% fetal bovine serum. Migration of cells into wounded areas was evaluated at the indicated times with an inverted microscope and photographed. The healing rate was quantified with measurements of the gap size during the culture. Three different areas in each assay were chosen to measure the distance of migrating cells to the origin of the wound.

2.10. Cell invasion assay

Invasion assay was performed using 24-well Transwell units with 8 μm pore size polycarbonate inserts. The polycarbonate membranes were coated with Matrigel (Becton Dickinson) and cultured at 37 °C for 1 h. Cells (1.0 × 10⁴) suspended in 200 μl of RPMI1640 medium containing 5% fetal bovine serum were seeded in the upper compartment of the Transwell unit. 800 μl of RPMI1640 medium containing 10% fetal bovine serum was added into the lower compartment as a chemoattractant. After 24 ~ 48 h incubation, cells on the upper side of the membrane were then removed, whereas the cells that migrated through the membrane to the underside were fixed and stained with 0.1% crystal violet. Cell numbers were counted in five separate fields using light microscopy at 200 × magnification. The data were expressed as the mean value of cells in five fields based on three independent experiments.

2.11. Statistical analysis

Experiments were independently done three or more times. Comparisons were made between SKOV3/pEF1α-vector and SKOV3/pEF1α-Foxp3 cells. Two-tailed Student’s t test or one-way ANOVA (SPSS software version 11.5) was used to analyze. P < 0.05 was considered to indicate statistical significance.
3. Results

3.1. Foxp3 expression in normal and cancerous ovarian tissues

We assessed Foxp3 expression in human epithelial ovarian cancer specimens (n = 27) and normal ovarian epithelium (n = 7) by immunohistochemistry. Foxp3 protein was detected in normal ovarian epithelium, whereas no or weak expression was determined in tumor cells (three histological subtypes, Fig. 1a–d). Moreover, the existence of Foxp3-positive Tregs was observed in 1/27 cancerous ovarian tissue (Fig. 1e). Real-time RT-PCR analysis confirmed the results obtained by immunohistochemistry. Analysis of transcript level showed that the expression of Foxp3 gene in 26 epithelial ovarian cancer specimens (exclusion the existence of Foxp3-positive Tregs) was lower than in normal ovarian epithelium (Tumor ΔCt = −8.089 vs. normal ΔCt = −2.239, P < 0.01) (Fig. 1f).

3.2. Foxp3 decreased cell growth and inhibited cell cycle associated proteins

Several epithelial ovarian cancer cell lines included SKOV3, OMC685, and ES-2 were taken to detect Foxp3 expression. However, the results showed Foxp3 expression only at mRNA level but devoid on protein level compared with activated T cells as positive control (Fig. 2a and b). To explore whether Foxp3 gene suppressed the growth of epithelial ovarian cancer cells, we transfected the empty vector or the vectors carrying Foxp3 cDNA into SKOV3 cells. The untransfected cells were removed by a pEF1α-vector and SKOV3/pEF1α-Foxp3 cells. Western blot analysis confirmed that Foxp3 protein expression was significantly increased in SKOV3/pEF1α-Foxp3 cells (Fig. 2b). While the empty-vector-transfected cells grew into large clones, the Foxp3-transfected cells grew slowly (Fig. 3a). CCK-8 assay demonstrated that pEF1α-Foxp3 inhibited cell proliferation compared with SKOV3/pEF1α-vector cells (Δ cell viability rate >10%, P < 0.05) (Fig. 3b). Cell cycle analysis showed that Foxp3 over-expression caused a significant accumulation of cells in the G0–G1 phase, with a concomitant decrease of cells in the G2–S phase compared with empty-vector-transfected cells (G0–G1 93.7 ± 2.5% vs. 78.4 ± 1.8%, P < 0.05) (Fig. 3c). To study cell proliferation in further detail, some proteins involved in cell cycle control were examined. In-cell western analysis detected that the levels of Ki-67 and CDKs were inhibited by Foxp3 up-regulation (Fig. 3d).

3.3. Foxp3 decreased cell migration and invasion

Wound healing assay was carried out to test cell migration. SKOV3/pEF1α-Foxp3 cells had a much slower wound-healing rate compared with SKOV3/pEF1α-vector cells (0.25 ± 0.053:1, P < 0.05) (Fig. 4a). Matrigel-Transwell assay was used to determine effects of Foxp3 on cell invasion. SKOV3/pEF1α-vector cells showed about 5.89 ± 0.97-fold penetration rate through the Matrigel-coated membrane compared with SKOV3/pEF1α-Foxp3 cells (Fig. 4b), which indicated that Foxp3 reduced the invasion ability of tumor cells. To further characterize above effects, several regulatory factors involved in cell migration and invasion were examined. The expressions of MMP-2 and uPA at protein level were decreased in SKOV3/pEF1α-Foxp3 cells (Fig. 4c).

3.4. Foxp3 inhibited mTOR and NF-κB signaling

To further explore the mechanism of Foxp3, several signaling pathways were tested. In-cell western analysis showed that Foxp3 inhibited the activation of mTOR and NF-κB, but almost no effects on PI3K/AKT and ERK1/2 signaling (Fig. 5).

4. Discussion

Foxp3 was considered as a master control gene of the function of thymically derived naturally occurring regulatory T cells [3,4]. Due to the regulatory T lineage specification by Foxp3, its tissue expression was expected in thymus, spleen and lymph nodes which had been well documented. Recent data showed that quantification of Foxp3 mRNA in tissues was represented an independent prognostic variable in terms of overall survival and progression-free survival [11]. However, those data were mostly focused on the expression of Foxp3 in Tregs. The large number of Tregs infiltrated in cancerous tissues was associated with a dismal prognosis in ovarian cancer [12]. Quantification of Foxp3 mRNA serve as a surrogate marker for the ovarian cancer tissue infiltration grade by Tregs, and immunohistochemical staining of Foxp3 on a tissue microarray corroborated Foxp3 mRNA data in terms of the identification of tumors with Foxp3-positive Tregs [11,12].

Albeit to a far lesser extent, Foxp3 expression had also been observed in other normal and cancerous tissues [6,7]. The present study extended those findings by investigating Foxp3 expression in ovarian cancerous cells. First, immunohistochemistry staining was used to detect Foxp3-positive Tregs in cancerous tissues. As a result, the...
existence of Foxp3-positive Tregs was observed only in 1/27 cancerous ovarian tissues by immunohistochemical staining, which might due to the small series of specimens. Of note, Foxp3 expression was readily detectable in ovarian epithelium from healthy women, whereas no or weak expression was determined in tumor cells. Furthermore, real-time RT-PCR analysis confirmed the results obtained by immunohistochemistry. Analysis of transcript level showed that the expression of Foxp3 gene in 26 epithelial ovarian cancer specimens (exclusion the existence of Foxp3-positive Tregs was observed only in 1/27 cancerous ovarian tissues by immunohistochemical staining, which might due to the small series of specimens. Of note, Foxp3 expression was readily detectable in ovarian epithelium from healthy women, whereas no or weak expression was determined in tumor cells. Furthermore, real-time RT-PCR analysis confirmed the results obtained by immunohistochemistry. Analysis of transcript level showed that the expression of Foxp3 gene in 26 epithelial ovarian cancer specimens (exclusion the existence of
Foxp3-positive Tregs by immunohistochemical staining) was lower than in normal ovarian epithelium. Although it was a small series of specimens, a correlation between Foxp3 expression and ovarian cancer was indicated. That Foxp3 expression in normal ovarian epithelium but weak/no in epithelial ovarian cancerous cells and cell lines might represent an example of molecular mimicry and reveal another mechanism of etiology of ovarian cancer [13].

We selected stable cell clones which constitutively expressed Foxp3 protein to detect the function and mechanism of Foxp3 in epithelial ovarian cancer cell lines. Up-regulation of Foxp3 elicited a dramatic effect on the proliferation of SKOV3 cells by CCK-8 assay and clone formation assay. To explore the mechanism of Foxp3 in cell growth inhibition, we postulated whether it was due to cell cycle arrest. Actually, Foxp3 up-regulation increased cell population in the G0–G1 phase and decreased cell progression into the DNA replication phase (G2–S phase). We also observed a reduction in the expressions of CDKs [14,15] and Ki-67 [16,17] at protein level, which were strongly correlated with cell cycle distribution phenotype and growth suppression.

mTOR was involved in numerous regulatory functions of cell biology [18]. Inhibition of mTOR by everolimus promoted cisplatin-induced apoptosis [19], delayed tumor onset and progression in a transgenic mouse model of ovarian cancer [20], and enhanced effects of chemotherapy in advanced renal cell carcinoma by a double-blind, random, placebo-controlled phase III trial [21]. Interestingly, Foxp3 over-expression inhibited the activation of mTOR in SKOV3 cells, which was similar to the effect of rapamycin as documented [22,23]. But almost no effects on the activation of PI3K/AKT and ERK1/2 signaling, suggesting that Foxp3 might induce cell cycle arrest and decrease cell proliferation through down-regulating mTOR signaling.

Peritoneal metastasis was a big problem in ovarian cancer. When we observed that the up-regulation of Foxp3 inhibited cell growth, we further examined the effects of Foxp3 up-regulation on the migration and invasion of SKOV3 cells. We found that up-regulation of Foxp3 inhibited the invasion rate of SKOV3 cells through the Matrigel, and reduced the migration rate in wound healing assay. It was known that MMPs were critically involved in the processes of tumor cell invasion and metastasis [24,25], and MMP-9 and MMP-2 were directly associated with metastatic processes in ovarian cancer [26,27]. Here, we found that up-regulation of Foxp3 inhibited MMP-2 expression at protein level, suggesting that the potential anti-metastatic activities of Foxp3 could be interpreted partly through the down-regulation of MMP-2. In addition, up-regulation of Foxp3 inhibited the activity of NF-κB, which confirmed that Foxp3 might inhibit cell migration and invasion partly through the inhibition of NF-κB and its target genes MMPs [28–30]. However, further in detail studies were needed to ascertain the precise molecular regulation of Foxp3 and NF-κB and their crosstalk in elucidating the role of Foxp3 in cell growth, invasion, and migration in animal models and human ovarian cancer.

Another important molecule involved in cell invasion and metastasis was uPA and its receptor. It was reported that cancer cells with high levels of uPA or its receptor would tend to invade surrounding tissues and subsequently migrate to blood vessels, thereby developing cancer cell metastasis [31,32]. In the present study, we found that up-regulation of Foxp3 decreased the expression of uPA, which suggested Foxp3 could potentiate the anti-metastasis activities partly through the down-regulation of uPA.

In summary, our study herein presented experimental evidences that supported the role of Foxp3 up-regulation in inhibiting cell proliferation, migration and invasion in epithelial ovarian cancer.
as anti-tumor and anti-metastatic mechanisms in ovarian cancer. From these data, we could expect that up-regulation of Foxp3 might potentially be an effective therapeutic approach for the inhibition of cell growth, migration, and invasion of ovarian cancer.

Conflicts of interest

None declared.

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