Hypoxia-inducible factor 1α (HIF-1α) correlated with tumor growth and apoptosis in ovarian cancer

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The aims of this study were to investigate the hypoxia-inducible factor 1α (HIF-1α) protein inhibition and tumor growth by a molecular target of rapamycin inhibitor, rapamycin, in xenogeneic transplant model of ovarian cancer and to study the correlation of apoptosis with HIF-1α and vascular endothelial growth factor (VEGF) expression. Four groups of female nude mice were inoculated subcutaneous with SKOV-3 cells and treated with vehicle, rapamycin, paclitaxel, or rapamycin plus paclitaxel. The expressions of HIF-1α and VEGF and microvessel density (MVD) were assessed by immunohistochemistry. While messenger RNA (mRNA) expression of Glut1, bcl-2, and VEGF was studied by reverse transcription–polymerase chain reaction, and apoptosis of tumor cells was determined by terminal deoxynucleotidyl biotin-dUTP nick end labeling (TUNEL). The HIF-1α was expressed in epithelial ovarian cancer. There was a significant correlation between HIF-1α protein expression and VEGF or MVD. Tumor burden treated with rapamycin alone,
rapamycin plus paclitaxel, and paclitaxel alone was reduced (47.91%, 51.03%, and 31.75%, respectively) compared with controls. The expression of HIF-1α was inhibited, and apoptotic index of tumor cell increased in rapamycin and rapamycin plus paclitaxel group. HIF-1α may upregulate VEGF expression both in mRNA and protein level. There is a positive correlation between HIF-1α and MVD. Rapamycin inhibits expression of HIF-1α and suppresses ovarian tumor growth. Our data suggested that a combination of HIF-1α inhibitor and chemotherapy could provide an effective approach for inhibiting tumor growth in ovarian cancer.

KEYWORDS: apoptosis, HIF-1α, ovarian cancer, VEGF.

Epithelial ovarian carcinoma is the leading cause of gynecological cancer death(1). The poor prognosis of ovarian cancer is related to the cancer cell peritoneal dissemination. The rapid tumor cell growth and early transmission may be due to low oxygen microenvironment. Cellular adaptation to hypoxia represents an essential step in tumor progression.

One of the key factors regulating cellular hypoxia responses via transcription is hypoxia-inducible factor 1 (HIF-1)(2). HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits. The activity of HIF-1α is regulated by ubiquitin-mediated proteasomal degradation under normoxia condition. When cells are exposed to low ambient oxygen, HIF-1α is stabilized and translocated to the nucleus, where it activates angiogenesis anaerobic metabolism and tumor apoptosis(3).

Vascular endothelial growth factor (VEGF) plays an important role in tumor angiogenesis and growth(4). VEGF expression is known to increase by various stimuli, including hypoxia via upregulation of HIF-1α in human pancreatic cancer and human prostate cancer(5,6). However, the relationship between HIF-1α and VEGF is still unknown in ovarian carcinogenesis.

The molecular target of rapamycin (mTOR), which is a member of the phosphoinositide-3-kinase–related kinase family and a central modulator of cell growth, is a unique and prime strategic target for anticancer therapeutic development. The mTOR is considered as an upstream activator of HIF-1 function in cancer cells(5). In vitro study showed that rapamycin and other PI-3K/mTOR inhibitors could be for clinical use as cytotoxic agents in patients with solid tumor. The role of rapamycin and the mTOR in ovarian cancer development has not yet been defined.

Combination of rapamycin with paclitaxel was commonly used as anticancer agents in ovarian cancer. To better understand the impact these anticancer agents on the HIF-1α expression, we have investigated the HIF-1α protein expression and the correlation between HIF-1α and VEGF, MVD, bcl-2, as well as apoptotic index of tumor cell.

Materials and methods

Materials

Rapamycin, paclitaxel, and sulindac were all obtained from Sigma Chemical Co (St. Louis, MO). The human ovarian cancer cell line SKOV-3 used in this study was kindly provided by Dr Yin Yuhua, M.D. Anderson Cancer Center, Texas. Cell culture supplies, Dulbecco modified Eagle medium, were obtained from the Shanghai Shisheng Cell Biotech Ltd. (Shanghai, China).

Experimental animals

Female BALC/C nude mice (6–8 weeks of age) were purchased from and housed in the Animal Department of Shanghai Cancer Research Institute, Shanghai, China.

Methods

Cell culture

The human ovarian cancer cell line SKOV-3 was grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO2.

Animal model of ovarian cancer

The human ovarian cancer cells SKOV-3 (2 × 10⁶), 200 μL, were transplanted s.c. into the dorsal of female nude mice. Four to six weeks after inoculation, the tumors grow to 1–2 cm³. The second-passage mice with cancer were used in study. The rapamycin-only group (n = 6) was treated with rapamycin (4 mg/kg body weight [BW] i.p.) weekly, twice. The rapamycin plus paclitaxel group (n = 6) was treated with rapamycin (4 mg/kg BW i.p.) plus paclitaxel (25 mg/kg BW i.p.) weekly, twice. The paclitaxel-only group (n = 6) was treated with paclitaxel (25 mg/kg BW i.p.) weekly, twice. The remainder of the mice (n = 6) received vehicle (saline) alone as control.
Immunohistochemistry

The expressions of HIF-1α, VEGF, and smooth muscle actins (SMA) were determined immunohistochemically in paraffin-embedded specimens fixed in 4% buffered formalin. Histological slides, 5 μm in thickness, were deparaffinized in xylol and rehydrated in decreasing ethanol solutions. Slides were heated in 0.01 M citrate buffer for 16 min in a microwave oven and then cooled and washed in Tris-buffered saline (TBS) for three times. For immunohistochemical detection of HIF-1α, VEGF, and SMA, specimens were incubated overnight at 4°C with a monoclonal anti–HIF-1α antibody (clone Mab H1α67, Novus Biologicals, Littleton, CO) in a dilution of 1:80, VEGF antibody (DAKO Biologicals, Carpinteria, CA) in dilution of 1:100, SMA antibody (DAKO Biologicals) in dilution of 1:100, respectively. After specimens were washed by TBS, Envision fluid (DAKO Biologicals) was added, followed by incubation for 30 min. Bound antibodies were visualized by using 0.05% 3,3’-diaminobenzidine (DAB) plus H2O2 for 5–10 min. Then, the specimens were observed under light microscope. All sections were evaluated by a pathologist. The result was quantified with regard to the percentage of cells stained (1: less than 10%, 2: less than 10–50%, 3: less than 50–80%, 4: more than 80%) and pulsing intensity of staining (negative, –; mild, +; moderate, ++; strong, +++). Microvessel density (MVD) was assessed by immunohistochemical staining of sections with a monoclonal antibody against SMA. MVD was determined by counting all vessels at a total magnification of ×200 within an examination area. The positive control was performed on the samples of known cases. For negative control, primary antibodies were replaced by TBS.

Reverse transcription–polymerase chain reaction analyses

Fresh tissue samples were snap-frozen in liquid nitrogen. For reverse transcription–polymerase chain reaction (RT-PCR) analysis, total cellular RNA was extracted with Trizol reagent. The complementary DNA was synthesized with 1 μL of oligo(dT) primer and 5 U avian leukemia viruses (AMV) reverse transcriptase (GIBCO Carlsbad, CA). The primers for polymerase chain reaction (PCR) of human Glut1 messenger RNA (mRNA), and VEGF, bcl-2 (SIBAS Biotech Development Ltd. Company, Shanghai, China) were as follows. glyceraldehyde-3-phosphate dehydrogenase (GAPDH): upstream primer, 5’-ACCACAGTCATGCGACCAC-3’ and downstream primer, 5’-CCACACCCCTGTGCTGCTG-3’ (450 bp); VEGF: upstream primer, 5’-CTACCTCCACATGCGAGT-3’ and downstream primer, 5’-TCTCTCAATATGCTGCGCT-3’ (311 bp); bcl-2: upstream primer, 5’-GGATTGTGGCCTTCTTTGAG-3’ and downstream primer, 5’-CCAAACTGAGCAGATCTCTTC-3’ (329 bp); Glut1: upstream primer, 5’-GCAAAGTCCCTTGAGATGCTGATCC-3’ and downstream primer, 5’-GCCGACTCTCTCTCTCTCATCTCC-3’ (402 bp).

The PCR conditions for Glut1 primer sets were as follows: hot start at 94°C for 2 min; 23 amplification cycles, each at 94°C for 15 sec, 60°C for 30 sec, and 72°C for 1 min; and a final extension step at 72°C for 7 min. PCR conditions for bcl-2 and VEGF primer sets were as follows: hot start at 94°C for 2 min; 30 amplification cycles, each at 94°C for 50 sec, 60°C for 50 sec, and 72°C for 1 min; and a final extension step at 72°C for 7 min. The GAPDH mRNA was used as an internal standard. PCR products were separated on 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

TUNEL assay

Paraffin sections of ovarian cancer tissue from SKOV-3 cell–inoculated mice were used to assess apoptosis. The procedure was done as the instruction (Promega, Lyon, France). The specimens were observed under a light microscope. Nuclei that stained brown were scored as positive for apoptosis. At least three ×200 microscopic fields were scored, and the apoptotic index was calculated as the percentage of cells that were scored positive.

Statistical analysis

The results were presented as means ± SD. Data were analyzed using SPSS 10.0 for windows software. Spearman’s rank correlation was used for the VEGF and HIF-1α mRNA expressions. A correlation coefficient greater than 0.4 or less than −0.4 was considered significant. Differences were considered significant at P < 0.05.

Results

Inhibition expression of HIF-1α, VEGF, and MVD

Formalin-fixed, paraffin-embedded specimens of SKOV-3 xenografts in four groups were analyzed with immunohistochemistry for the expression of HIF-1α, VEGF, and MVD. In control group, the expression of HIF-1α was mild to strong (+ + to ++ +), and mean positive cells were 50–80% in all tumor cells. After treatment with rapamycin or rapamycin plus paclitaxel, the expression of HIF-1α decreased to negative or weak positive (− to +), and mean positive cells were less than 10% (Fig. 1). But the expression of HIF-1α has no significant difference in the group treated with paclitaxel alone and the control group.

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As shown in Figure 2, VEGF expression, which is determined by immunostaining, was related to HIF-1α. The MVD immunostaining results are shown in Figure 3A–D. The mean values of MVD in the rapamycin-alone group (12.92 ± 4.5) and the rapamycin plus paclitaxel group (22 ± 6.47) were significantly (P < 0.05) less than that of the control (30.58 ± 8.02) (Fig. 3E).

In order to understand the relationship between HIF-1α and VEGF, we studied the Glut1, a known HIF-1 target gene, and VEGF mRNA expression by RT-PCR (Fig. 4). A significant correlation between mRNA of Glut1 and mRNA of VEGF was found by Spearman’s coefficient of correlation (P < 0.01, r = 0.8264), as well as MVD showed correlation with HIF-1α (P < 0.05, r = 0.484) (Fig. 4B).

The expression of a control gene GAPDH was neither inhibited nor increased by these agents. All the experiments were repeated for three times.

Control of ovarian tumor growth

Results of treatment with vehicle, rapamycin only, paclitaxel alone, and combination of rapamycin and paclitaxel are shown in Figure 5A and Table 1.

Tumors in mice treated with vehicle grew rapidly. In other three groups, the growth of tumors was suppressed to varying degrees. Mean volume of tumor burden in the group treated with combined rapamycin and paclitaxel (0.6418 ± 0.12 g) and in group treated with rapamycin alone (0.6827 ± 0.30) was significantly (P < 0.05) less than that of control (1.3107 ± 0.38 g). Tumor burden in the rapamycin plus paclitaxel, rapamycin-alone, and paclitaxel-alone group was significantly reduced by 51.03%, 47.9%, and 31.7%, respectively, compared with the control group (P < 0.05) (Fig. 5B).

Relationship of HIF-1α with apoptosis

Cell apoptosis assed by TUNEL in the rapamycin-treated group, rapamycin plus paclitaxel–treated group, and paclitaxel-treated group was 36.33% (P < 0.01), 39.83% (P < 0.01), and 22% (P < 0.05), respectively, of the total cell population. There were no significant changes in control mice (Fig. 6).

The bcl-2 mRNA expression was also measured by RT-PCR. While the expression of HIF-1α was inhibited by rapamycin, the apoptosis rate was increased, and the mRNA expression of bcl-2 was decreased. There was very strong correlation between the apoptosis index and expression of bcl-2 mRNA (P < 0.001, r = −0.7852) (Fig. 4A, B). A significant correlation between the HIF-1α expression and apoptosis index was also found by Spearman’s correlation (P < 0.05, r = −0.519).

Discussion

Previous studies indicated that HIF-1α acts as a positive regulator of tumor growth in many solid tumors, including ovarian carcinoma. The HIF-1α–expressing cells may promote transformation and invasion of tumor. The present study investigated for the first time that HIF-1α was more expressed in transplant...
Figure 2. VEGF expression by the immunohistochemistry. A) The expression of VEGF in control group was strong, and mean positive cells were about 60%. B) After treatment with rapamycin, the VEGF staining and mean positive cells were all decreased. C) After treatment with rapamycin plus paclitaxel, the VEGF staining was negative or weak, and mean positive cells were less than 10–20% ($P < 0.05$). D) The expression of VEGF did not change in paclitaxel-alone group (original magnification A–C $\times$400; original magnification D $\times$200).

Figure 3. MVD expression by the immunohistochemistry. The immunostaining result of MVD in control, group 1, group 2, and group 3 is shown in A–D (original magnification $\times$400). E) The mean value of MVD in group 1 (rapamycin alone) (12.92 ± 4.5) and group 2 (rapamycin plus paclitaxel) (22.0 ± 6.47) was significantly less than that of the controls (30.58 ± 8.02) ($P < 0.05$), but not significantly different in group 3 (paclitaxel alone) ($P > 0.05$) (original magnification A–D $\times$400).
xenografts of human ovarian epithelial cancer SKOV-3 cells. Rapamycin could inhibit the expression of HIF-1α. Some in vitro experiments demonstrated that hypoxia increase the expression and secretion of VEGF via the upregulation of HIF-1α (10). In present study, we have shown that the expression of VEGF was regulated by HIF-1α both in mRNA and protein level. There was positive correlation between HIF-1α and MVD in ovarian cancer xenografts. HIF-1α may promote the angiogenesis through VEGF.

HIF-1α could be activated by hypoxia, and its function might be amplitude by PI-3K/AKT/mTOR signaling pathway (11). In ovarian cancer models, overexpression of an activated PI-3K/AKT/mTOR can decrease the sensitivity of anticancer agents such as paclitaxel, which promotes assembly of microtubules and causes cell death. The inhibition of signaling through PI-3K/AKT/mTOR might have therapeutic efficacy against a broad spectrum of human cancer, particularly against advanced-stage neoplasm resistant to conventional chemotherapeutic regimens (12). Previous study provided evidence that the inhibitory effect of rapamycin on HIF-1α accumulation either decreased the rate of HIF-1α synthesis or increased the rate of HIF-1α degradation in hypoxia cells (13). Other report found that increased or decreased HIF-1 activity was associated with tumor growth in mouse xenograft assay genetic manipulations (9). In our experiment, rapamycin caused a marked inhibition (47.9%, \( P < 0.05 \)) of tumor growth, while paclitaxel alone reduced tumor burden (31.75%, \( P > 0.05 \)) and rapamycin and paclitaxel combined therapy had greater effect (51.03%, \( P < 0.05 \)), without increasing the drug’s side effects. The mice treated with anticancer agents had no dry, scaly skin and survived. However, there was no significant difference between groups treated with rapamycin alone and rapamycin plus paclitaxel. It may be explained by small sample size. About 40% of ovarian cancers had abnormalities in the PI3K/mTOR pathway (14,15). Ovarian cancer might be a target of inhibitors of PI3K/mTOR (12,15,16).

Besides the role of HIF-1α in the adaptation to hypoxia, recent data demonstrated a possible role for HIF-1α in the modulation of apoptosis (17). HIF-1α was reported to influence the expression of the cell cycle regulator and apoptosis (9,15). According to previous studies, hypoxia could induce apoptosis. However, some reports have shown that hypoxia could protect cells against apoptosis induced by serum deprivation and presence of chemotherapy agents. This controversy suggested that HIF-1α could display either a proapoptotic or an antiapoptotic role according to the conditions. The correlation between cell apoptosis and HIF-1α expression was already proved in lung cancer (17,18). Cobalt chloride, a chemical inducer of HIF-1α, could inhibit the apoptotic death which is induced by tert-butyl hydroperoxide and serum deprivation of in HepG2 cells (19). The relationship between HIF-1α and bcl-2 was contradictory in previous studies. Other reports showed that HIF-1α might suppress

Figure 4. Expression of Glut1, VEGF, and bcl-2 mRNA by RT-PCR. The mRNA expression of Glut1 decreased significantly in group 1 (rapamycin alone) \( (P < 0.05) \) and group 2 (rapamycin plus paclitaxel) \( (P < 0.05) \) versus control, but not in group 3 (paclitaxel alone). VEGF also decreased in group 1 and group 2 \( (P < 0.05) \), but not in group 3. bcl-2 decreased in all treated groups \( (P < 0.05) \) versus control.

Figure 5. Tumor growth in each group. Treatments were started 1 week after inoculation. Treatment groups consist of control (vehicle alone), group 1 (rapamycin alone), group 2 (rapamycin plus paclitaxel), and group 3 (paclitaxel alone). The tumor growth in each group in shown in A. At 2 weeks after treatment, mice were sacrificed. Tumor treated with vehicle grew rapidly, and mean volume of tumor burden was 1.31 ± 0.38 g. Mean volume of tumor burden in the group treated with combined rapamycin and paclitaxel (0.64 ± 0.12 g) and rapamycin alone (0.68 ± 0.30 g) was significantly \( (P < 0.05) \) less than that of control (B).
bcl-2 expression. In present study, we observed that the apoptotic index of cancer cells increased, while mRNA expression of apoptosis inhibitor bcl-2 decreased. The expression of HIF-1α was inhibited by rapamycin in ovarian cancer xenografts. This was also supported by the observation that rapamycin promoted cell death through the inhibition of cell survival signals in a number of cell lines.

In summary, this study showed that the HIF-1α was strongly expressed in ovarian cancer. It could upregulate the expression of both protein and mRNA of VEGF, thus increasing tumor growth and angiogenesis. Rapamycin could inhibit the activity of HIF-1α, decrease tumor neoangiogenesis, and increase apoptosis. In addition, rapamycin can inhibit tumor growth in athymic mice inoculated with cells from human ovarian cancer cell line. Combination with a conventional chemotherapeutic agent, such as paclitaxel, is effective in ovarian carcinoma chemotherapy. Pharmacologic inhibition of HIF-1α activity may represent a useful therapeutic strategy, and the effect of PI3K/mTOR pathway inhibitors on HIF-1α expression may provide a basis for chemotherapeutic efficacy.

### Table 1. Effect of rapamycin and paclitaxel on tumor dimension (cm³)

<table>
<thead>
<tr>
<th>Group</th>
<th>Before treatment</th>
<th>After treatment (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>0.0607</td>
<td>0.1608</td>
</tr>
<tr>
<td>Group 1</td>
<td>0.0542</td>
<td>0.0906</td>
</tr>
<tr>
<td>Group 2</td>
<td>0.0577</td>
<td>0.0845</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.0621</td>
<td>0.1138</td>
</tr>
</tbody>
</table>

Before treatment, the tumor volume in mice in each group had no significant difference. Control: the mice treated with vehicle; group 1: the mice treated with rapamycin only; group 2: the mice treated with rapamycin plus paclitaxel; group 3: the mice treated with paclitaxel only.

### References


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