Expression of Hypoxia-inducible Factor 1α in Epithelial Ovarian Tumors: Its Impact on Prognosis and on Response to Chemotherapy

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ABSTRACT

Purpose: To investigate the impact of expression of hypoxia-inducible factor (HIF)-1α on prognosis and on response to chemotherapy in epithelial ovarian tumors.

Experimental Design: Expression of HIF-1α protein was studied by immunohistochemistry in 102 specimens of epithelial ovarian cancers, in 50 borderline tumors, and in 20 cystadenomas. Results were correlated with p53, p21, and bcl-2 expression, microvessel density (MVD), apoptotic rate of tumor cells, and survival.

Results: In 68.6% of ovarian cancers and 88% of borderline tumors, expression of HIF-1α was observed. There was a significant correlation of HIF-1α protein expression and MVD (P < 0.001). HIF-1α overexpression alone and MVD showed no impact on survival of cancer patients. Furthermore, the response to platinum-based chemotherapy was independent from HIF-1α expression. Expression of HIF-1α correlated with apoptotic rate in the majority of cases, especially in low malignant potential tumors. In contrast, in cancer patients with strong expression of HIF-1α and p53 protein overexpression, not only a significantly increased MVD (P = 0.032, Mann-Whitney test) but also a significantly shorter overall survival was observed (P < 0.0001, Cox regression). The apoptotic rate was very low in these tumors.

Conclusions: HIF-1α protein overexpression alone has no impact on the prognosis of ovarian cancer. The combination of HIF-1α protein overexpression with nonfunctional p53, however, indicates a dismal prognosis.

INTRODUCTION

Angiogenesis and cellular adaptation to hypoxia represent a key step in tumor progression (1–3). This is because of the fact that the limited diffusion capacity of O2 does not allow tumor growth beyond several mm3 without neoangiogenesis (2). Also, cancer cell proliferation may outpace the rate of angiogenesis (2), and tumor cells will have to adapt to tissue hypoxia in this situation. One of the key factors regulating cellular O2 homeostasis is HIF-1α (4, 5). HIF-1α is a heterodimeric complex composed of the two bHLH-PAS (PAS is an acronym that refers to the first proteins in which this motif was identified, i.e., PER, the protein product of the Drosophila period gene; ARNT, the aryl hydrocarbon receptor nuclear translocator; and SIM, the protein product of the Drosophila single-minded gene) subunits HIF-1α and HIF-1β (6). The bHLH domain mediates dimerization and DNA binding in a large number of transcription factors. PAS is an additional dimerization motif. Whereas HIF-1β is a common subunit of multiple bHLH proteins, HIF-1α is the unique, O2-regulated subunit that determines HIF-1 activity (5).

HIF-1α may increase O2 availability or metabolic adaptation to O2 deprivation by influencing a number of genes which in part play a role in tumor progression including erythropoietin, transferrin, endothelin-1, inducible nitric oxide synthetase, heme oxygenase 1, VEGF, insulin-like growth factor-2, insulin-like growth factor binding proteins 2 and 3, and 13 different glucose transporters and glycolytic enzymes (4). Furthermore, HIF-1α protein may also influence cell cycle progression/proliferation and the rate of apoptosis, thus influencing tumor progression also independent from its regulation of VEGF expression (7). A recent study revealed that hypoxia and hypoglycemia reduce proliferation and increase apoptosis in embryonic stem cells with wild-type HIF-1α but not in cells with inactivated HIF-1α (8). This was explained by the finding that the cell cycle control genes p53, p21, and bcl-2 were found to be HIF-1α dependent, in that the levels of p53 and p21 were significantly increased, and the amount of the apoptosis inhibitor bcl-2 was reduced in stressed HIF-1α+/− but not in HIF-1α−/− cells (8).

Until now, only few data exist on the impact of HIF-1α expression on prognosis in human cancer. Immunohistochemistry revealed that HIF-1α protein may be demonstrated in a variety of human cancers, including ovarian cancer (3, 9). Our group was the first to show that expression of HIF-1α is an independent prognostic factor in cervical cancer (10).

1 The abbreviations used are: HIF, hypoxia-inducible factor; bHLH, basic helix-loop-helix; PAS, PER-ARNT-SIM; VEGF, vascular endothelial growth factor; MVD, microvessel density; FIGO, International Federation of Gynecology and Obstetrics; LMP, low malignant potential; OS, overall survival; DFS, disease-free survival.

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The aim of this study was to determine the impact of HIF-1α protein expression and its correlation with the dependent cell cycle regulators p53, p21, and bcl-2, MVD, and apoptotic rate of tumor cells on prognosis in human epithelial ovarian cancer. A better understanding of this type of cancer is urgently needed, because of all gynecological cancers, ovarian malignancies represent the greatest clinical challenge, with two-thirds of patients presenting with already advanced disease requiring major surgery and intensive and often complex additional therapies (11).

MATERIALS AND METHODS

Patients. One hundred and two consecutive cases of epithelial ovarian cancer, FIGO stages I–IV, were retrieved from our files, as well as 50 cases of ovarian LMP tumors (so-called borderline tumors), stage IA, and 20 cases of ovarian cystadenomas. The mean age of patients at the time of surgery was 57.3 ± 14.6 years. They were initially evaluated by clinical and ultrasound examination, chest X-ray, and computerized tomography or magnetic resonance imaging of the abdomen. Treatment of cancer patients consisted of radical surgery with total abdominal hysterectomy, bilateral salpingo-oophorectomy, pelvic lymph node dissection, and omentectomy. All patients, except those with grade 1, stage IA, were given adjuvant chemotherapy. Patients received six cycles of a platinum/Taxol-containing multiple drug chemotherapy, except those mentioned above. Response to chemotherapy was rated as follows.

No evidence of disease was defined as the complete disappearance of all evidence of disease for at least 4 weeks, confirmed by physical examination, computed tomographic scan, and ultrasound. Partial response was defined as a ≥50% decrease in the sum of the products of the diameters of measurable lesions for at least 4 weeks. Stable disease was defined as a steady state of response less than a partial response or progression <25% of at least 4 weeks duration. Progressive disease was defined as an increase of ≥25% in the size of the measurable lesion or the appearance of an unequivocal new lesion within 2 months after beginning of chemotherapy. Patients were followed at 3-month intervals by clinical examination and appropriate imaging studies.

Immunohistochemistry. The expression of HIF-1α, p21, p53, bcl-2, CD34, and factor VIII-related protein and the apoptotic rate of tumor cells was determined immunohistochemically in paraffin-embedded specimens fixed in 4% buffered formalin. Histological slides, 4 μm in thickness, were deparaffinized in xylol. Slides were heated in 0.01 M citrate buffer for 20 min in a microwave oven. After cooling for 20 min and washing in PBS, endogenous peroxidase was blocked with methanol containing 0.3% hydrogen peroxide for 30 min, followed by incubation with PBS containing 10% normal goat serum for 30 min. For immunohistochemical detection of HIF-1α, specimens were incubated overnight at 4°C with a monoclonal anti-HIF-1α antibody (clone MAb H1α67, NB 100-105; Novus Biologicals, Littleton, CO; Refs. 10, 12) in a dilution of 1:40, the area within the tumor or directly adjacent to tumor formations with the greatest number of distinctively highlighted microvessels (“hot spot”) was selected. MVD was then determined by counting all vessels at a total magnification of ×200 within an examination area of 0.25 mm². Determination of the staining reactions was strictly confined to the area of highest MVD (“hot spot”). Each stained lumen was counted, and the number of vessels within an examination area of 0.25 mm² was determined by counting all vessels at a total magnification of ×200 within an examination area of 0.25 mm². Determination of the staining reactions was strictly confined to the area of highest MVD (“hot spot”). Each stained lumen was counted, and the number of vessels within an examination area of 0.25 mm² was determined by counting all vessels at a total magnification of ×200 within an examination area of 0.25 mm². Determination of the staining reactions was strictly confined to the area of highest MVD (“hot spot”). Each stained lumen was counted, and the number of vessels within an examination area of 0.25 mm² was determined by counting all vessels at a total magnification of ×200 within an examination area of 0.25 mm².

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regarded as a single countable microvessel. If there was no lumen but only a single positive cell was visible, this cell was also interpreted as representing a microvessel.

Analysis of immunohistochemistry was carried out by two independent observers. The mean values of results from both observers was used for all further calculations. If differences of >30% between observers occurred, these slides were reinvestigated by both investigators using a multiheaded microscope.

**Statistics.** Spearman’s coefficient of correlation, the Mann-Whitney test, and the Kruskal-Wallis test were used as appropriate. Because the influence of p53, p21, bcl-2 (14, 22, 23), and MVD (24–26) on prognosis in ovarian cancer has already been studied extensively, survival analysis was focused on expression of HIF-1α. OS was defined from the day of surgery until death of the patient. Data on patients who had survived until the end of observation period were censored at their last follow-up visit. Death from a cause other than ovarian cancer or survival until the end of the observation period was considered a censoring event. DFS was defined from the end of primary therapy until first evidence of progression of disease, if the patient showed no evidence of disease after primary therapy.

Univariate analysis of OS and DFS was performed as outlined by Kaplan and Meier (27). The Cox proportional hazards model was used for multivariate analysis. HIF-1α expression, patient’s age at time of diagnosis, histological grading (WHO), residual tumor after primary surgery (0, <2 cm², and ≥2 cm²), and tumor stage were entered into Cox regression. For all tests, \( P \leq 0.05 \) was considered as significant. All \( P \)-values given are results of two-sided tests.

**RESULTS**

**Expression of Proteins.** The majority of cases with epithelial ovarian cancer showed expression of HIF-1α, with 26 (25.5%) specimens showing strong expression (Fig. 1A), 23 (22.5%) showing moderate expression, and 21 (20.6%) showing weak decoration by the antibody (Fig. 1B). No expression of HIF-1α was observed in 32 samples (31.4%). Expression of HIF-1α was increased in tumor cells adjacent to areas of necrosis.

When compared with invasive cancer, a higher percentage of cases of LMP showed HIF-1α expression, with 13 (26%) showing strong staining, 17 (34%) moderate staining, and 14 (28%) weak staining. Only 6 (12%) samples of LMP showed no expression of HIF-1. Nevertheless, no significant difference in HIF-1α expression between LMP tumors and invasive carcinomas was found (\( P = 0.11 \), Mann-Whitney test). In contrast, the majority of specimens with a cystadenoma [18 (80%)] were HIF-1α negative, with only 2 cases with marked epithelial proliferation showing weak and moderate expression, respectively.

There was no significant correlation of HIF-1α expression with expression of p53, p21, or bcl-2 (Table 1) in invasive cancers, LMP tumors, and cystadenomas, respectively (\( P >\)
0.05, Spearman’s coefficient of correlation). The Mann-Whitney test revealed a significant higher expression of p21 ($P = 0.003$) in p53-negative ovarian carcinomas compared with positive ones.

There was no statistical significant difference between HIF-1α and p53 expression between different histological types of carcinomas, LMP tumors, or cystadenomas (Kruskal-Wallis test). Bcl-2 expression was significantly increased in serous as compared with mucinous LMP tumors ($P = 0.004$) and cystadenomas ($P = 0.002$; Table 1). Expression of p21 was significantly higher in clear cell carcinomas compared with serous ($P = 0.001$) and endometroid ($P = 0.043$) carcinomas.

HIF-1α expression was significantly stronger in G1 invasive ovarian cancer (median: moderate expression) when compared with G3 tumors (median: low expression; $P = 0.004$, Mann-Whitney test; Table 2). HIF-1α expression of cystadenomas (median: no expression) was significantly lower than in LMP tumors and carcinomas ($P < 0.0001$, Mann-Whitney test).

HIF-1α expression correlated with apoptotic rate of tumor cells in the majority of cases, especially in LMP tumors (data not shown). In contrast, the apoptotic rate was very low in cancer samples with strong expression of HIF-1α and p53 overexpression.

**HIF-1α and MVD.** MVD, determined by immunostaining for CD34, and factor VIII-related antigen showed very strong correlation ($P < 0.001, r = 0.969$, Spearman’s coefficient of correlation). Because staining patterns of both antibodies were nearly identical, only MVD assessed by CD34 immunostaining was used for further calculations, because this marker is considered as more specific for blood vessels than factor VIII-related antigen (16). Although no increase of HIF-1α expression in tumor cells adjacent to large vessels was observed, HIF-1α-expressing cells were prominent in areas with high MVD (Fig. 1D). A significant correlation between HIF-1α expression and MVD was found by Spearman’s coefficient of correlation in invasive cancer specimens ($P < 0.001, r = 0.618$), as well as in specimens of LMP tumors ($P < 0.001, r = 0.766$; Fig. 2).

**HIF-1α and Chemotherapy.** No significant influence of HIF-1α expression on response to platinum-based chemotherapy was found in the 51 patients cancer patients with residual tumor after surgery ($P = 0.885$; Kruskal-Wallis test).

**Survival Analysis.** The mean follow-up time for patients was 28 ± 24 months (range, 1–130 months). During the obser-

### Table 1: Expression of investigated proteins in epithelial ovarian tumors ($n = 172$)

<table>
<thead>
<tr>
<th>Protein expression</th>
<th>Invasive cancer</th>
<th>Borderline</th>
<th>Cystadenoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serous ($n = 64$)</td>
<td>Mucinous ($n = 13$)</td>
<td>Endometroid ($n = 17$)</td>
</tr>
<tr>
<td>HIF-1α None</td>
<td>23</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Weak</td>
<td>14</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Moderate</td>
<td>12</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Strong</td>
<td>15</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>p53 Negative</td>
<td>32</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Positive</td>
<td>32</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>p21 None</td>
<td>53</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
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<td>6</td>
<td>3</td>
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</tr>
<tr>
<td>Moderate</td>
<td>5</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Strong</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>bcl-2 None</td>
<td>34</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Weak</td>
<td>14</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Moderate</td>
<td>14</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Strong</td>
<td>2</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Mean MVD (±SD)</td>
<td>21 ± 12</td>
<td>40 ± 30</td>
<td>27 ± 16</td>
</tr>
</tbody>
</table>

### Table 2: Median expression of HIF-1α in samples of epithelial ovarian tumors ($n = 172$)

<table>
<thead>
<tr>
<th>FIGO stage (carcinomas)</th>
<th>Median expression of HIF-1α</th>
<th>Kruskal-Wallis $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Moderate</td>
<td>0.468</td>
</tr>
<tr>
<td>Stage II</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Stage IV</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Grading</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cystadenoma</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Borderline</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Histological type, carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>Low</td>
<td>0.356</td>
</tr>
<tr>
<td>Mucinous</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Endometroid</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Histological type, borderline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>Moderate</td>
<td>0.205</td>
</tr>
<tr>
<td>Mucinous</td>
<td>Moderate</td>
<td></td>
</tr>
<tr>
<td>Histological type, cystadenoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>None</td>
<td>0.942</td>
</tr>
<tr>
<td>Mucinous</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

0.05, Spearman’s coefficient of correlation). The Mann-Whitney test revealed a significant higher expression of p21 ($P = 0.003$) in p53-negative ovarian carcinomas compared with positive ones.

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**HIF-1α and Chemotherapy.** No significant influence of HIF-1α expression on response to platinum-based chemotherapy was found in the 51 patients cancer patients with residual tumor after surgery ($P = 0.885$; Kruskal-Wallis test).

**Survival Analysis.** The mean follow-up time for patients was 28 ± 24 months (range, 1–130 months). During the obser-
vation period, 26 patients developed recurrent disease (16 showed locoregional recurrence, 8 showed distant metastases, and 2 showed local recurrence and distant metastases), and 42 patients (41.2%) died from their ovarian cancer. No influence of HIF-1α expression on OS was detected in univariate (P = 0.3959, log-rank test) and multivariate (P = 0.182, Cox regression) analyses was found (Fig. 3A). Patient’s age, tumor stage, residual tumor after primary surgery, and histological grading showed significant influence on OS in univariate analysis (Table 3). No influence of HIF-1α expression on DFS in the patients, who showed complete remission after primary therapy, was seen in univariate (P = 0.6848) and multivariate (P = 0.353) analyses. Survival analysis with regard to MVD assessed by immunostaining for CD34 (and factor VIII-related antigen) revealed no significant influence on prognosis (data not shown).

In 11 patients (10.8%), strong expression of HIF-1α and expression of p53 was observed (1 each in stages IA, IIA, and IIIB; 2 in stage IIIB; 5 stage in stage IIIIC; and 1 in stage IV). These patients showed significant shorter OS compared with the other patients (P < 0.0001, log-rank test; Fig. 3B). Mean survival time of these patients was 251 days, compared with 2129 days in all other patients. Median MVD was significantly higher in these patients compared with the other ones (31 versus 19; P = 0.032, Mann-Whitney test). In multivariate analysis, the combination of strong HIF-1α expression and p53 expression remained an independent prognostic factor (P < 0.0001), as well as tumor stage and residual tumor after primary surgery (Table 3). Expression of p53 alone was not a prognostic factor (P = 0.0974, log-rank test). Down-regulation of p21 and/or up-regulation of bcl-2 in combination with overexpression of HIF-1α protein did not influence OS or DFS of patients with ovarian cancer (P > 0.05, log-rank test).

**DISCUSSION**

This is, to our best knowledge, the first study determining the influence of HIF-1α expression on the prognosis of human ovarian carcinomas. Overexpression of HIF-1α protein, as demonstrated by immunohistochemistry, was observed in 68.6% of specimens of epithelial ovarian cancer. Determination of HIF-1α overexpression alone was of no prognostic value. In addition, response to platinum-based chemotherapy seems to be independent from expression of HIF-1α in the primary tumor.

Necrotic areas, most probably induced by hypoxia (28), were more often observed in samples of poorly differentiated ovarian cancers as compared with well-differentiated carcinomas. Although in particular tumor cells directly adjacent to necrotic tissue showed strong expression of HIF-1α, well-differentiated ovarian cancers showed an overall stronger expression of HIF-1α protein than poorly differentiated tumors. These data suggest that strong expression of HIF-1α in well-differentiated ovarian cancers cannot be attributed to hypoxia alone, thus corroborating findings in prostatic cancer (29) and hemangioblastomas (12). We show here for the first time that strong expression of HIF-1α in combination with
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nonfunctional p53 protein indicates a dismal prognosis in ovarian cancer. This combination seems to indicate a subgroup (~10%) of patients with epithelial ovarian cancer with significantly worse prognosis, affecting ~2400 patients annually in the United States alone (30). The explanation for this phenomenon might be the dual function of HIF-1α in early cancerogenesis which on the one hand stimulates tumor progression, and on the other hand supports apoptosis of tumor cells. A correlation between HIF-1α expression and apoptotic rate of tumor cells has already been observed in other types of cancers (8, 31). In our study, HIF-1α expression correlated well with apoptotic rate in the majority of ovarian tumors, but this proapoptotic function seems to get lost in cancers with nonfunctional p53. Hypoxic cancer cells with low apoptotic index have been reported to be highly aggressive (32).

HIF-1α is considered to support tumor growth through induction of angiogenesis via e.g., transactivation of the VEGF gene. On the other hand, HIF-1α was reported to associate with p53 protein, thus increasing the stability of p53 (33). In this situation, cells have a higher susceptibility to succumb because of hypoxia through p53-induced apoptosis, thus inhibiting tumor growth. The fact that HIF-1α may support hypoxia-mediated apoptosis via stabilization of p53 is supported by two findings: (a) loss of wild type p53 is associated with a marked reduction in hypoxia-mediated apoptosis (34); and (b) HIF-1α⁻/⁻ embryonal stem cells show no induction of p53 protein or apoptosis in response to O₂ and glucose deprivation (8). Therefore, the combination of p53 protein dysfunction, e.g., through somatic mutation, and HIF-1α overexpression seems to be necessary to allow HIF-1α to sufficiently stimulate tumor progression in early cancerogenesis by mediating angiogenesis and by inducing adaptive intracellular responses to hypoxia without supporting proapoptotic mechanisms. Neoangiogenesis was also significantly increased in tumors with this combination. This hypothesis explains why in cervical cancer, where p53 is inactivated by the human papillomavirus E6 protein in virtually all cases, strong expression of HIF-1α is associated with an unfavorable prognosis (10).

Interestingly enough, p53 which is up-regulated during hypoxia (35) negatively influences HIF-1α-stimulated transcription (36). Also, p53 appears to influence HIF-1α translation, as suggested by the finding in p53-deficient mouse fibroblasts which showed higher levels of HIF-1α expression in response to hypoxia, despite unchanged HIF-1α mRNA levels (37). Furthermore, by immunostaining of 75 various human cancers, Zhong et al. (3) showed a positive correlation of HIF-1α protein and p53 protein expression. In our study, however, no such association was found.

HIF-1α was reported to influence the expression of the cell cycle regulator p21 and the apoptosis inhibitor bcl-2, thus contributing also via these proteins to regulation of proliferation and apoptosis (8). In contrast to the findings with p53, down-regulation of p21 and/or up-regulation of bcl-2 in combination with overexpression of HIF-1α protein did not influence the prognosis of patients with ovarian cancer. Furthermore, the level of these proteins did not seem to be intercorrelated.

Zagzag et al. (12) demonstrated a correlation between HIF-1α overexpression and induction of angiogenesis in human brain tumors by semiquantitative assessment of the formation of blood vessels. In our study, we demonstrated for the first time a strong correlation between HIF-1α expression and increased MVD (38). This finding can be explained by the notion that angiogenesis was promoted by HIF-1α-induced expression of VEGF (8, 39, 40). It is well known that high MVD is associated with poor prognosis in a variety of human cancers (41), e.g., carcinoma of the uterine cervix (42), the breast (43, 44), the vulva (45), and a variety of other malignancies (46). In contrast to these tumors, the impact of increased MVD on prognosis of ovarian cancer is still a matter of debate, and results of various studies are contradictory (24–26, 47). In several studies including our own (24), MVD was not an independent prognostic factor. This might explain, in part, why HIF-1α protein overexpression alone did not influence the prognosis of ovarian cancer in this study.

In conclusion, HIF-1α protein overexpression alone seems not to influence the prognosis of patients with ovarian cancer, most likely because of the fact that neoangiogenesis as revealed by assessment of MVD appears to be of minor prognostic value in this type of tumor. Nevertheless, in combination with overexpression of p53 protein, however, HIF-1α protein overexpression indicates a dismal prognosis.

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REFERENCES


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