The Role of Inhibitor of DNA-binding 1 (ID-1) Protein and Angiogenesis in Serous Ovarian Cancer

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Abstract. *Background: Overexpression of Inhibitor of DNA-binding 1 protein (ID-1) is correlated with poor prognosis in some malignancies and few studies have assessed its role in ovarian cancer. This led us to investigate its association with the microvessel density (MVD) in patients with ovarian cancer. Materials and Methods: Fifty-six patients with epithelial serous ovarian cancer were selected. The early-stage group consisted of 14 patients and the advanced-stage group comprised 42 patients. ID-1 expression and MVD were evaluated by immunohistochemistry. Results and Conclusion: The histoscore for ID-1 and MVD were significantly higher in advanced-stage cancer ($p<0.05$). The MVD was significantly higher in the high ID-1 expression group compared to the low ID-1 expression group ($p<0.001$). The mean follow-up time was 52 months. The survival period in patients with high ID-1 expression was not significantly shorter than for those with low ID-1 expression ($p=0.62$). The role of ID-1 protein requires further investigation.*

Ovarian cancer is the second most common form of gynecological cancer and the deadliest considering absolute figures for women in industrialized countries (1, 2). Due to the fact that early cancer is usually asymptomatic and women with advanced-stage disease present with non-specific symptoms, the diagnosis of malignancy is delayed, which contributes to a poor survival rate despite surgery and platinum-based chemotherapy, with a median progression-free survival of 18 months (3, 4).

Prognosis depends on a large number of clinical, biological and therapeutic variables, yet their real involvement is still controversial and new prognostic markers are needed to better-stratify patients regarding subsequent therapy (5-9).

Inhibitors of DNA-binding proteins (ID;) are represented by four transcription factors, members of the basic helix-loop-helix (bHLH) family that lack the DNA-binding domain (10, 11). Their dominant negative mode of DNA-binding activity results from their capacity to form inactive heterodimers with other bHLH proteins, and thus they have an important role in cell-cycle progression and differentiation (12).

It has been shown that ID-1 has an important role in the cell cycle and its overexpression leads to an increased activity of cyclin-dependent kinase 2, inactivation of retinoblastoma protein, and other regulator proteins, inducing cell proliferation, increased DNA synthesis and invasion of tumor cells (13).

Angiogenesis has also been strongly linked with ID-1 in several articles, thus supporting its role in tumor growth and neovascularization by activation of endothelial cells (14).

ID-1 is correlated with poor prognosis in malignancies such as gastric (15), breast (16), prostate (17), and cervical (18) cancer but only few studies have assessed its role as a marker for poor prognosis in ovarian cancer.

This led us to investigate its association with MVD in patients diagnosed with serous ovarian cancer.

Materials and Methods

Informed consent was obtained from all patients and the study was approved by the Ethical Committee of the Iuliu Haţieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania (No. 414/01.11.2011).

Patients were selected by retrospectively investigating all files of cases that were registered as ovarian cancer within the Prof. Dr. Ion Chiricuţă Institute of Oncology between 2001 and 2007. Fifty-six patients diagnosed with serous ovarian cancer met the inclusion criteria (histologically-confirmed serous ovarian cancer, optimal surgery as a first-line treatment followed by platinum-based chemotherapy, regular follow-up within the Institute), with a mean age at diagnosis of 55 years, ranging from 27 to 74 years. Patients were divided into two groups, 14 with early-stage (FIGO stage I-II) and 42 with advanced-stage (FIGO stage IIIC) disease.

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None of the patients received therapy prior to radical surgery. After surgery, they were treated with a platinum-based chemotherapy and followed-up at 3-month intervals. Patients with end-stage disease, sub-optimal surgery, different histology, diagnosis of other malignancies and incomplete treatment were excluded from the present study.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue samples were assessed using tissue microarray (TMA) technology. The cores were retrieved from microscopically-representative hematoxylin-eosin-stained ovarian cancer tissues. Sections of 4 μm were mounted on silanized slides and incubated overnight at 37˚C. The NovoLink Polymer Detection System (Novocastra; Newcastle upon Tyne, UK) protocol was used. Sections were de-paraffinized in xylene, rehydrated with graded ethanol and washed. Antigen retrieval was performed by heating the slides in 10 mM citrate buffer (pH 6.0) for 20 min. After cooling at room temperature, rinsing in tris-buffered saline (TBS), endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 min and tissue sections were incubated with Protein Block (Novocastra) for 5 min to reduce non-specific binding. Each slide was then incubated with the primary rabbit polyclonal antibody ID-1 (SC-488; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) for 60 min and monoclonal mouse antibody to human CD34 (M7165; Dako, Glostrup, Denmark) for 30 min at dilutions of 1:100 and 1:350, respectively. After washing in TBS, the slides were incubated with Post Primary Block (Novocastra) for 30 min and with the secondary antibody to mouse/rabbit IgG-Poly-HRP (Novolink Polymer; Novocastra). Peroxidase activity was developed with diaminobenzidine (DAB) working solution (Novocastra). Sections were counterstained with Mayer's hematoxylin, rinsed in saturated solution of lithium carbonate, dehydrated, cleared in xylene and mounted in Faramount Mounting Medium (S3025; Dako).

As a positive control for ID-1 expression, staining was performed on normal human pancreatic tissue (19) which showed a strong cytoplasmic signal for exocrine glandular cells due to the fact that ID-1 lacks nuclear localization (18). Negative controls were performed by omitting the primary antibodies for assessment of nonspecific binding of the secondary antibody.

Evaluation of ID-1 histochemical score. ID-1-stained slides were evaluated semi-quantitatively using a scoring system which took into account both the percentage of positive cells and the intensity of staining. Slides were classified as 0 (0-10% positive cells, weak intensity), 1 (10-50% positive cells, weak/moderate intensity), 2 (>50% positive cells, weak/moderate intensity), or 3 (>50% positive cells, strong intensity).

Evaluation of MVD. The MVD was determined using slides stained with monoclonal mouse antibody to CD34 by counting all of the vessels that had a clearly defined lumen by sequentially scanning the entire TMA core area of 3.14 mm^2 at a total magnification of 400×. Clusters of positive endothelial cells were also interpreted as microvessels (20).

Statistics. Fisher's F test, Student's *t*-test, Chi-squared test and Spearman's correlation coefficient were used when appropriate. Log-rank test and the Cox proportional hazards model were used for univariate and multivariate analyses of survival rate. A test was considered significant when $p \leq 0.05$. All *p*-values given are the result of two-sided analysis.

Results

Overall, following immunohistochemistry analysis for ID-1, 17 (30%) cases scored 0 (Figure 1a), 15 (27%) scored 1 (Figure 1b), 13 (23%) scored 2 (Figure 1c) and 11 (20%) scored 3 (Figure 1d). The ID-1 histoscore was significantly higher in patients with advanced stage disease (mean ID-1 score FIGO IIIC=1.52±1.07) in comparison to those with early-stage cancer (mean ID-1 score FIGO I-II=0.71±0.95, $p<0.05$).

CD34 expression in endothelial cells was used for the assessment of MVD (Figure 2). MVD was higher in the advanced-stage group compared with the early-stage group (mean MVD of 80±34 *vs.* 58±22 respectively, $p<0.05$).

By dividing the patients into two groups based on ID-1 score (low expression: ID-1 score 0-1, high expression: ID-1 score 2-3), the value of MVD was statistically significant higher in the high ID-1 expression group compared with the low-expression group for all stages (FIGO I-IIIC) and for the advanced-stage group (FIGO IIIC) with $p<0.0001$ and $p<0.01$, respectively. The average MVD overall (FIGO I-IIIC) was 60±20 in the low ID-1 expression group and 93±38 in the high ID-1 expression group. In the advanced-stage group (FIGO IIIC), MVD was 64±19 and 95±40, respectively.

The odds ratio (OR) for high MVD (above the mean CD-34 value) are 6-times higher for those with high ID-1 values (2, 3) in comparison with low ID-1 values (0, 1; 95% confidence interval (CI)=1.86-19.26; $p<0.01$, Chi-square). The ID-1 histoscore correlated with MVD (r=0.57, 95% CI=0.36-0.72; $p<0.0001$) as shown in Figure 3.

The OR for samples with poor histological differentiation (G3) were 1.96-times higher for those with high ID-1 values (2, 3) *vs.* low ID-1 values (0,1) but not statistically significant (95% CI=0.67-5.77; $p=0.21$, Chi-square). The OR for samples with high MVD (above the mean CD-34 value) were 1.46 times higher for those with poor histological differentiation (G3) *vs.* good histological differentiation (G1, G2; 95% CI=0.50-4.24; $p=0.48$, Chi-square).

The median follow-up time was 52 months. During the study period, from a total of 56 patients 33 (59%) died and 43 (77%) patients experienced relapse. The median 5-year survival rate was 45%. In univariate and multivariate analysis (FIGO stage I-II *vs.* IIIC, histological grading 1-*2 vs.* 3, age <50 *vs.* ≥50 years) the survival interval was not significantly different in patients with high ID-1 expression from that of patients with low expression (ID-1 score 0-1 *vs.* 2-3, $p=0.62$ and $p=0.22$ respectively, log-rank test).

Discussion

Only a limited number of studies investigating the role of ID-1 in ovarian cancer have been published to date (21, 22) despite the fact that the role of neo-angiogenesis in ovarian cancer has been investigated in other studies (23, 24). ID-1 expression was

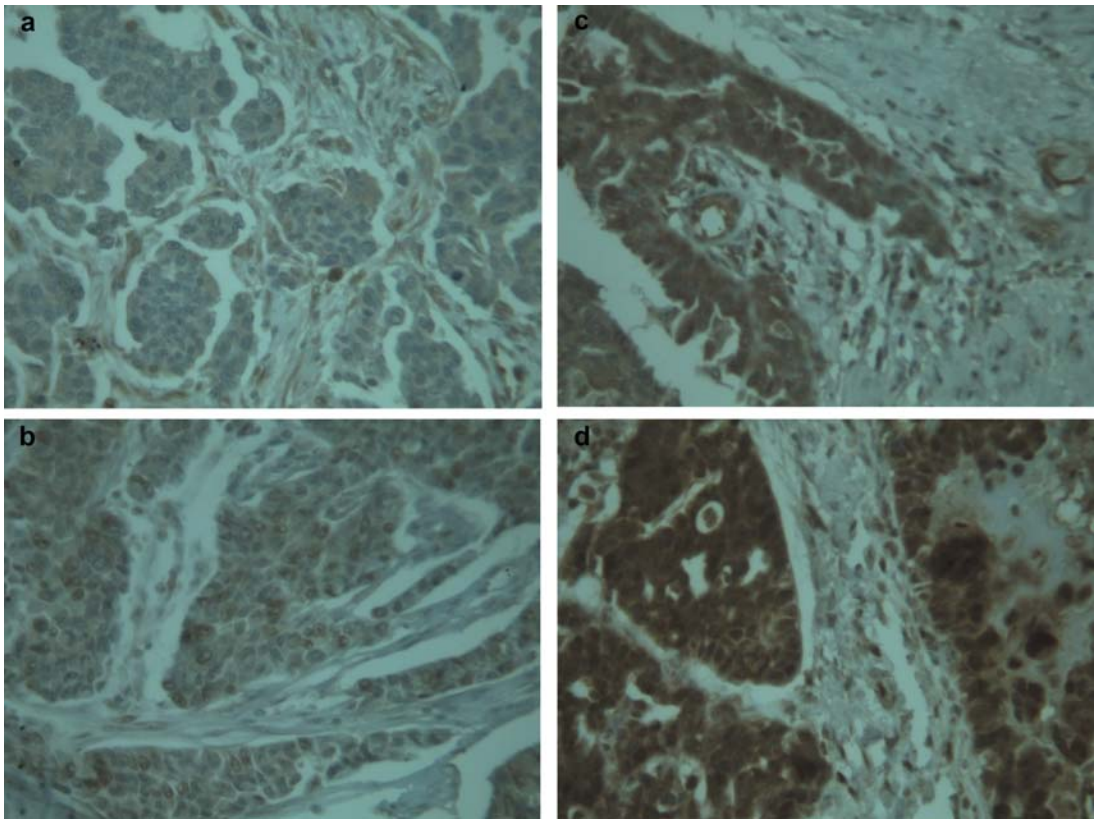

Figure 1. *Sample of ovarian cancer with histoscore 0 (a), 1 (b), 2 (c) and 3 (d) for Inhibitor of DNA-binding-1 (ID-1) expression, ×400 magnification.*

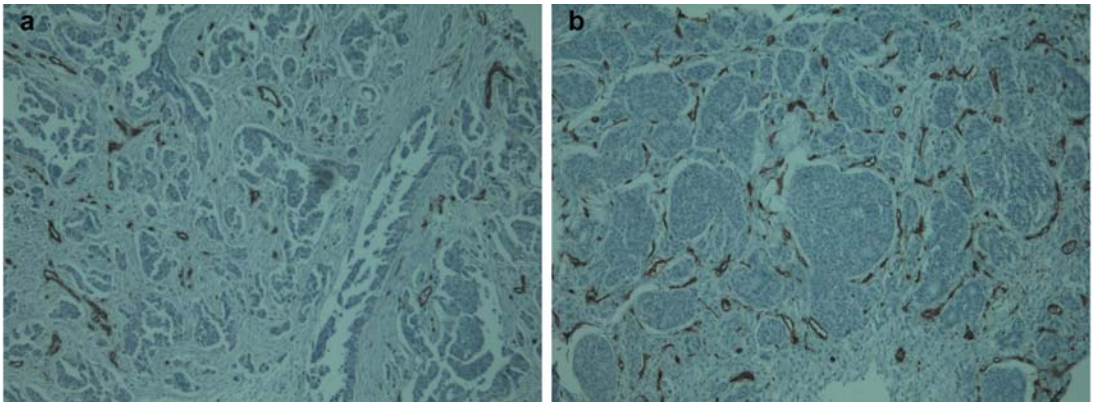

Figure 2. *Sample of ovarian cancer with low (a) and high (b) microvessel density (MVD), ×400 magnification.*

positive in a large number of tissue specimens examined in the present study. The expression of ID-1, evaluated through the histoscore, was significantly greater in patients with advanced disease in comparison to early-stage cancer. In our study ID-1, expression could not be linked to histological grading or to a longer survival rate, but tumor MVD did positively correlate with ID-1 expression and was significantly greater in the high ID-1 expression group. Thus, ID-1 might play a role in the assessment of ovarian cancer progression and neo-angiogenesis. ID-1 protein overexpression in ovarian cancer needs further detailed investigation and might represent an interesting element in anti-angiogenic therapeutic strategies.

Competing Interests

The Authors declare that they have no competing interests.

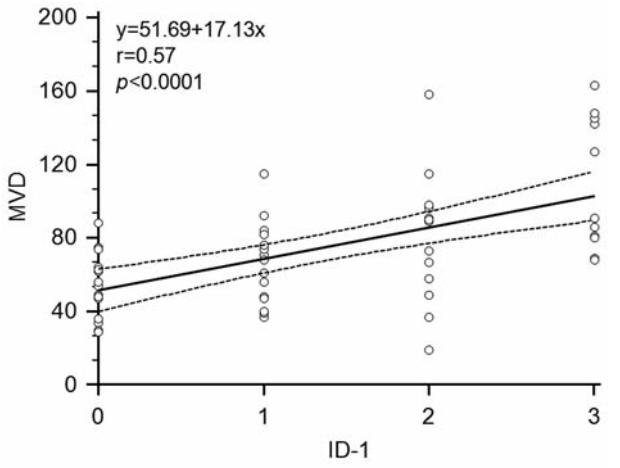

Figure 3. *Correlation of Inhibitor of DNA-binding-1 (ID-1) histoscore with microvessel density (MVD), showing regression line with 95% confidence interval curve.*

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