Level of Id-1 Protein Expression Correlates with Poor Differentiation, Enhanced Malignant Potential, and More Aggressive Clinical Behavior of Epithelial Ovarian Tumors

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ABSTRACT

Purpose: Id (inhibitor of differentiation/DNA binding) -1 is involved in neoangiogenesis, it antagonizes basic helix-loop-helix proteins, inhibits differentiation, and enhances cell proliferation. Aim of this study was to investigate Id-1 protein expression in epithelial ovarian tumors and its clinical relevance in ovarian cancer.

Experimental Design: We have investigated Id-1 expression by reverse transcription-PCR and Western blotting in ovarian cancer samples. On the basis of these results, Id-1 protein expression was determined by immunohistochemistry in 101 specimens of epithelial ovarian cancer, in 40 borderline tumors, and in 20 cystadenomas. In these cases, Id-1 expression was correlated with p21 expression, microvessel density, and survival.

Results: By immunohistochemistry, detectable expression of Id-1 was found significantly more often in ovarian cancers (74.3%) than in borderline tumors (32.5%) and cystadenomas (0%; \( P < 0.0001 \), \( \chi^2 \) test). Cancer samples with poor or moderate histological differentiation (G3/G2) showed significantly stronger Id-1 expression than cancer samples with high differentiation (G1; \( P = 0.021 \), Mann-Whitney test), and no association of Id-1 with p21 expression or microvessel density was found. In cancer samples strong or moderate expression of Id-1 was a strong predictor for shorter overall survival in uni- and multivariate analysis (\( P = 0.001 \), Cox-regression).

Conclusions: The level of Id-1 protein expression correlates with the malignant potential of ovarian tumors. In cancer samples, stronger Id-1 expression is associated with poor differentiation and more aggressive behavior of tumor cells, resulting in poor clinical outcome. Consequently, Id-1 inhibition in the future might be of benefit for patients with ovarian cancer.

INTRODUCTION

Id\(^3\) proteins are HLH factors that lack a basic domain (1). Id proteins act as dominant inhibitors of basic HLH transcription factors by heterodimerization, thus inhibiting gene expression (2). Recent studies suggest that Id proteins may function as oncogenes, in addition to inhibiting G1 cell cycle arrest and differentiation (3–5). Id genes have been shown to enhance cell cycle progression, and their overexpression induces apoptosis in serum-deprived fibroblasts (6). In addition, Id proteins are considered as essential for vascularization of tumors (7).

Expression of Id proteins has been demonstrated in a variety of human tumors (2, 3, 8–11). We have demonstrated recently that overexpression of Id-1 is associated with dismal prognosis in early stage cervical cancer (8). Only little data on the expression of Id proteins in human ovarian cancer exists thus far (3), and no data on the expression and prognostic relevance of Id-1 protein in ovarian tumors are available.

Of all gynecologic cancers, ovarian malignancies represent the greatest clinical challenge, with two-thirds of patients presenting with already advanced disease, requiring major surgery, and intensive, complex additional therapies (12). Despite these diverse efforts of treatment, ovarian cancer still represents the highest mortality rate of all of the gynecologic malignancies. From 24,000 new cases annually in the United States, 13,600 patients are expected to die from their disease (13). There has been speculation about using Id proteins as possible targets for novel therapeutic agents (2, 7, 14), but for the development of new, more efficient therapeutic concepts, a better understanding of ovarian cancer with respect to the mode of Id protein expression is urgently needed.

The aim of our study was to investigate the expression of Id-1 proteins in epithelial ovarian tumors. On the basis of initial experiments, we immunohistochemically investigated the association of Id-1 protein expression and neoangiogenesis, assessed by MVD, and the expression of cyclin-dependent kinase inhibitor p21, which has been suggested to be regulated by Id-1 (15).
In addition, the prognostic influence of Id-1 expression in patients with epithelial ovarian cancer was determined.

**MATERIALS AND METHODS**

**Patients.** For immunohistochemistry, 101 consecutive formalin-fixed, paraffin-embedded cases of epithelial ovarian cancer, FIGO stages I-IV, were retrieved from our files, as well as 40 cases of ovarian LMP tumors (so-called borderline tumors), stage IA, and 20 cases of ovarian cystadenomas. The mean age of cancer patients at time of surgery was 57 ± 14.5 years. They were initially evaluated by clinical and ultrasound examination, chest X-ray, and computed tomography or magnetic resonance imaging of the abdomen. Treatment of cancer patients consisted of radical surgery with total abdominal hysterectomy, bilateral salpingo-oopherectomy, pelvic lymph node dissection, and omentectomy. All of the patients, except those with G1, stage IA, cancers were given adjuvant chemotherapy. Patients received six cycles of a platinum/Taxol combination, except those mentioned above. Response to chemotherapy was rated as follows.

- Progressive disease: defined as an increase of 50% in the size of measurable lesions for at least 4 weeks.
- Progressive disease: defined as an increase of 50% in the size of measurable lesions for at least 4 weeks.
- Stable disease: defined as a less than a partial response or progression <25% of at least 4-weeks duration.
- Progressive disease: 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.

**RT-PCR and Western Blotting.** Detection of Id-1 mRNA by RT-PCR and Id-1 protein by Western blotting was performed in six snap-frozen tissue samples of serous ovarian cancers. A cervical cancer sample with known strong expression of Id-1 was used as positive control (8). As internal positive controls, a G3PDH-specific RT-PCR was performed for Id-1 mRNA expression and Western blotting for actin for Id-1 protein expression. For RT-PCR, homogenized tissue samples were resuspended in TriReagent (Molecular Research Center, Cincinnati, OH) and RNA isolated according to the manufacturer’s instructions. Dried RNA pellets were resuspended in nuclease-free water and stored at −80°C until analysis. Total RNA was used for cDNA synthesis, and the reverse transcriptase reaction mixture was used for PCR reaction with a DNA thermal cycler (Perkin-Perkin-Elmer Corp., Shelton, CT) for 31 cycles (60 s at 94°C, 60 s at 60°C, and 60 s at 72°C). The following primers were used: G3PDH sense: 5'-ATTCCACCCATGGGAAATTTCCATG-3'; G3PDH antisense: 5'-GGGCGCATACGCCACAGTCTT-3'; Id-1 sense: 5'-AACCAGGCAAGTTGACAAAGTG-3'; and Id-1 antisense: 5'-ACCGATGCCGCTCAGGC-3'. Amplified PCR products (G3PDH, 452 bp and Id-1, 182 bp) were electrophoresed in 1.5% agarose gels and visualized by ethidium bromide staining.

For Western blotting, cells and tissue samples were homogenized, and lysed in reducing SDS sample buffer. Proteins were electrophoresed by 19% SDS-PAGE and transferred onto nitrocellulose membrane (BioRad, Richmond, CA). Membranes were incubated with a polyclonal rabbit antihuman Id-1 antibody (1:500; Santa Cruz Laboratories Inc., Santa Cruz, CA) and a polyclonal rabbit anti-human actin antibody (1:200; Sigma, St. Louis, MO). As negative control, MCF-7 breast cancer cells (obtained from American Type Culture Collection, Manassas, VA) grown under serum-free conditions were used (2). After washing, primary antibody binding was detected as described previously (16).

**Immunohistochemistry.** Immunohistochemistry was performed on paraffin-embedded specimens fixed in 4% buffered formalin. Histological sections, 4-μm in thickness, were deparaffinized in xylene. Slides were heated in 0.01 M citrate buffer for 16 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 detection of Id-1, specimens were incubated overnight at +4°C with a polyclonal rabbit antibody (Santa Cruz Biotechnology) in a dilution of 1:50 (8, 10, 11). Visualization of bound antibodies was performed by using a streptavidin-biotin-peroxidase complex (ChemMate kit; DAKO, Glostrup, Denmark). As chromogene, 3-amino-9-ethylcarbazole (BioGenex, San Ramon, CA) was used. P21 expression was investigated using a monoclonal anti-human p21 antibody (1:20; #OP64; Oncogene, Cambridge, MA; Refs. 17, 18). MVD was assessed by immunostaining of sections with a monoclonal antibody to CD34 (QBEnd/10; BioGenex; Ref. 19). Primary antibodies were incubated for 60 min at room temperature. Visualization of p21 and CD34 was performed using the ChemMate kit (DAKO) and diaminobenzidine.

As positive control for Id-1 expression, immunostaining was also performed on a sample of cervical cancer tissue with known strong Id-1 expression (8). Because Id-1 is strongly expressed in smooth muscle cells of vessels (20), they served as an internal positive control.

As positive control for p21 immunostaining, a sample of ovarian cancer with known strong expression was used. A specimen of breast cancer with known high MVD served as positive control for CD34 immunostaining. For negative control, primary antibodies were replaced by nonimmune, normal rabbit serum or by irrelevant isotype-matched mouse IgG, as appropriate.

Because Id-1 is not a transcription factor per se, it lacks the nuclear localization signal found on many basic HLH proteins but gives a cytoplasmic staining signal instead (2, 8, 11). So cytoplasmic localization of Id-1 expression and nuclear expression of p21 were determined by semiquantitatively assessing the percentage of decorated tumor cells and the staining intensity (21). The percentage of positive cells was rated as follows: 2 points, 11–50% positive cells; 3 points, 51–80% positive; and 4 points, > 80% positive cells. The staining intensity was rated adjusted to the internal positive control as follows: 1 point, weak intensity; 2 points, moderate intensity; and 3 points, strong intensity. Points for expression and percentage of positive cells were added, and specimens were attributed to four groups according to their overall score: negative, ≤10% of cells stained positive, regardless of intensity; weak expression, 2–3 points;
moderate expression, 4–5 points; and strong expression, 6–7 points.

MVD was determined according to Weidner (22). After scanning the immunostained section at low magnification (×40), the area within the tumor or directly adjacent to tumor formations with the greatest number of distinctly highlighted microvessels (“hot spot”) was selected. MVD was then determined by counting all of the vessels using an eye grid 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 endothelial lined lumen was regarded as a single countable microvessel. If 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 pathologists. The mean results of values from both observers was used for all additional calculations. If differences of >30% among observers occurred, these slides were reevaluated by both investigators using a multiheaded microscope.

Statistics. Spearman’s coefficient of correlation, χ² test, Mann-Whitney test, and Kruskal-Wallis test were used when appropriate. Bonferroni-Holm correction for multiple testing was applied for pairwise comparisons of groups.

Because influence of p21 (17, 23) and MVD (24–26) on prognosis in ovarian cancer has already been studied extensively, survival analysis was focused on expression of Id-1. OS was defined from the day of surgery until death of the patient. Death from a cause other than ovarian cancer or survival until the end of the observation period were considered as censored observations.

Survival probabilities were computed as outlined by Kaplan and Meier (27). Log-rank test and Cox proportional hazards model were used for uni- and multivariate analyses of OS, respectively. Id-1 expression (strong/moderate versus low/absent expression), patient age at time of diagnosis (≤50 versus >50 years), histological grading (WHO), residual tumor after primary surgery (0, <2 cm³, and ≥2 cm³), histological tumor type, and tumor stage (FIGO) were entered into Cox regression. For all of the tests, a P ≤ 0.05 was considered significant. All of the Ps given are results of two-sided tests.

RESULTS

RT-PCR and Western Blotting. RT-PCR revealed the presence of Id-1 mRNA in all of the ovarian tumors tested. Expression of Id-1 protein in cancer samples assessed by Western blotting showed good correlation to immunohistochromatic anti-Id-1 staining (data not shown). Nevertheless, in the one sample completely devoid of immunohistochromatic detectable Id-1 expression in tumor cells, weak expression was found at Western blotting, most probably because of Id-1 expression in smooth muscle cells of vessels (see below).

Immunohistochrometry. The majority of cases with epithelial ovarian cancer showed expression of Id-1, with 6 (5.9%) specimens showing a strong expression (Fig. 1A), 31 (30.8%) a moderate (Fig. 1B), and 38 (37.6%) a weak expression of Id-1 (Fig. 1C). No expression of Id-1 was observed in 26 samples (25.7%; Fig. 1D).

Six cases (15%) of LMP tumors showed a moderate, and 7 (17.5%) a weak staining. Twenty-seven (67.5%) samples of LMP tumors showed no expression of Id-1. Therefore, Id-1 expression was less common in LMP tumors than in invasive cancer (P < 0.0001, χ² test), and in analogy Id-1 expression was significantly weaker in LMP tumors compared with carcinomas (P < 0.0001, Mann-Whitney test). In none of the 20 specimens of cystadenoma was Id-1 expression found.

In invasive cancer, Kruskal-Wallis test revealed a significant difference in Id-1 expression between the various histological types (P = 0.038). Subsequent pairwise comparison between groups using Mann-Whitney test adjusted according to Bonferroni-Holm for multiple testing revealed that Id-1 expression was significantly lower in endometrioid (median: low expression) than in clear cell cancer samples (median: moderate expression; P = 0.045, Bonferroni-Holm corrected Mann-Whitney test; Table 1).

There was no significant difference in Id-1 levels of different FIGO stages (P = 0.145, Kruskal-Wallis test). Using the ordinal scale of FIGO stage, Spearman’s coefficient of correlation revealed a weak, but significant association with Id-1 expression (r = 0.277; P = 0.022). Cancer samples with poor or moderate histological differentiation (G3/G2) showed significantly stronger Id-1 expression than cancer samples with high differentiation (G1; P = 0.021, Mann-Whitney test).

In cancer samples, strong expression of p21 was found in 1 sample (1%), moderate in 16 cases (15.8%), and weak in 9 cases (8.9%). The majority of cancer samples (74.3%) showed no expression of p21. In LMP tumors, we observed strong expression in 1 case (2.5%; Fig. 1E), moderate expression in 12 cases (30%), in 4 cases p21 expression was weak (10%), and in 23 cases (57.5%) no expression was found. In cystadenomas, 2 cases showed moderate and low expression of p21 (5%), respectively. Kruskal-Wallis test revealed a significant difference in p21 expression between tumor types (P = 0.016). Subsequent pairwise comparison using Mann-Whitney test showed a significant difference in p21 expression between cystadenomas and LMP tumors (P = 0.01), between carcinoma and LMP (P = 0.039), but not between cystadenoma and carcinoma (P = 0.121).

There was a weak but significant correlation (Spearman’s coefficient of correlation) between p21 and Id-1 expression in LMP tumors (r = 0.337; P = 0.033) but not in cancer samples (r = −0.006; P = 0.956).

Id-1 and MVD. Mean MVD was 21.8 ± 11.6 microvessels/field in LMP samples and 24.2 ± 17.7 microvessels/field in cancer samples (Fig. 1F). There was no correlation of Id-1 expression and MVD in LMP or cancer samples (r = 0.003, P = 0.986 and r = 0.092, P = 0.372, respectively, Spearman’s coefficient of correlation).

Id-1 and Chemotherapy. There was a weak but significant correlation between Id-1 expression and diminished response to chemotherapy (r = 0.233, P = 0.019, Spearman’s coefficient of correlation).

Survival Analysis. The median follow-up time of patients was 36 months (range, 1–130 months). During the observation period, 27 patients (26.7%) developed recurrent disease, and 41 patients (40.6%) deceased from their ovarian cancer.

Strong/moderate expression of Id-1 was associated with
Fig. 1  
A, a sample of ovarian cancer with strong expression of Id-1. Immunoperoxidase, original magnification ×400. 
B, a sample of ovarian cancer with moderate Id-1 expression. Immunoperoxidase, original magnification ×400. 
C, a sample of ovarian cancer with low Id-1 expression. Note the dot-like staining signals only in a subset of cells. Immunoperoxidase, original magnification ×400. 
D, ovarian cancer with absent Id-1 expression. Note the strong staining of vascular smooth muscle cells. Immunoperoxidase, original magnification ×200. 
E, an ovarian tumor of LMP with strong expression of p21. Immunoperoxidase, original magnification ×100. 
F, a sample of ovarian cancer with high MVD, assessed by CD34-immunostaining. Immunoperoxidase, original magnification ×100.
significantly shorter OS in univariate analysis compared with low/absent expression (\(P \leq 0.0001\), log-rank test; Fig. 2). The 5-year OS rate was 69.5% in patients with low/absent Id-1 expression (median survival time 85 months), whereas it was only 24.3% in those with strong/moderate Id-1 expression (median survival time, 20.9 months).

In multivariate analysis of OS, Id-1 expression and FIGO stage remained independent prognostic factors (Table 2).

**DISCUSSION**

Few data on the expression of Id-proteins in human cancers exist thus far (2, 9–11), and data on their prognostic relevance are even more scarce (3, 8). In a previous study, we investigated the expression of Id-1, Id-2, and Id-3 in early stage cervical cancer, and only Id-1 was found to influence prognosis of patients (8). In ovarian cancer, a recent study also failed to demonstrate a significant influence of Id-3 expression on survival of patients (3). In contrast, no data on Id-1 protein expression in ovarian tumors were available.

In our present study we found expression of Id-1 mRNA in all of the tested ovarian cancer tissue samples by RT-PCR, with differences in Id-1 protein levels. Nevertheless, it has to be considered that Id-1 is also strongly expressed in vascular smooth muscle cells (20). So protein- and mRNA-based methods without preservation of morphology are of limited use for detection of Id-1 in tissue samples, because results might be influenced by Id-1 originating from vascular smooth muscle cells, as is also evident in our study. In contrast, immunohistochemistry allows exact identification of Id-1 expression in tumor cells. The presence of Id-1 protein, as detected by immunohistochemistry, was observed in the vast majority of specimens of epithelial ovarian cancer, but in a significantly smaller percentage in LMP tumors, and it was completely absent in ovarian cystadenomas. Higher levels of Id-1 expression were associated with increasing malignant potential of ovarian tumors. This is in good correlation with findings in a small series of breast tumors, where Id-1 was found expressed at lower levels in ductal carcinomas in situ compared with invasive breast cancers (2). Stronger Id-1 expression was associated significantly with poorer histological differentiation in ovarian cancer. This association of Id-1 with poor differentiation of tumor cells is in good concordance with the role of Id-1 as inhibitor of cellular differentiation (6) and, therefore, has to be considered as causal.

Id-1 is a regulator of basic HLH-mediated transcription (1) and causes cells to pass a mitogen-restricted point in late G1 phase (28). Therefore, Id-1 has been suggested as responsible for some of the changes in gene expression that lead to increased growth and invasion of tumor cells (7). A role of overexpression of Id-1 in carcinogenesis of prostatic cancer has been suggested recently (29), and it has also been shown that breast cancer cells acquire a more aggressive phenotype in vitro when Id-1 is expressed constitutively (2). Our results demonstrate that overexpression of Id-1 in human ovarian cancers is associated with a more aggressive behavior of ovarian tumor cells in vivo, with clear clinical impact.

In addition, we observed a correlation of Id-1 expression with lower response to chemotherapy in cancer samples. Future in vitro studies could investigate Id-1 overexpression and its possible association with increased chemoresistance of cancer cells.

Lyden et al. (7) found that Id proteins are required for the proliferative and invasive phenotype of endothelial cells during tumor-associated angiogenesis. Nevertheless, no correlation between neoangiogenesis, assessed by MVD, and expression of Id-1 was found in the present study, similar to our results in
cervical cancer (8). Our findings indicate that the effect of Id-1 expression on prognosis in ovarian cancer, as well as in cervical cancer (8), cannot be attributed to its proangiogenic effect alone.

Id-1 has been shown to interact with various cell cycle regulators (30, 31). Overexpression of Id-1 significantly reduced mRNA expression of the p21 gene in cell culture experiments (15). On the basis of these results, Prabhu et al. (15) suggested that the increased growth rate of Id-1-overexpressing cells might be explained by reduction of the cyclin-dependent kinase inhibitor p21. Our study revealed no such correlation in human ovarian cancer samples, suggesting that in ovarian cancers expression of p21 is also down-regulated by pathways independent from Id-1. Therefore, the aggressive clinical behavior of ovarian cancers overexpressing Id-1 cannot be exclusively explained by down-regulation of p21. Additional studies will have to elucidate the mechanisms of interaction between Id-1 and the cyclin system in detail.

It has been speculated by other authors that drugs interfering with Id-1 may target aggressive cancer cells (2, 7, 14). Our data support the thesis that inhibition of Id-1 might be beneficial for ovarian cancer patients with tumors overexpressing this protein, leading to the development of therapeutic concepts based on these results.

REFERENCES