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Advances in Brief

Overexpression of Id-1 Protein Is a Marker for Unfavorable Prognosis in Early-Stage Cervical Cancer[1]

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Abstract

Inhibitor of differentiation/DNA binding (Id) proteins are transcription factors, involved in cell cycle regulation and neoangiogenesis. Using immunohistochemistry, we investigated the prognostic influence of Id-1, Id-2, and Id-3 expression in 89 patients with cervical cancer stage pT_{1b}. In univariate and multivariate analysis, patients with strong or moderate expression of Id-1 had a significant shorter overall survival time ($P = 0.0144$, log-rank test) and disease-free survival time ($P = 0.0107$, log-rank test) compared with those with low or absent Id-1 expression. Id-1 expression is an independent prognostic marker in early-stage cervical cancer.

Introduction

The 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 G_1 cell cycle arrest and differentiation (3–5). Id genes have been shown to enhance cell cycle progression, and their overexpression can induce apoptosis in serum-deprived fibroblasts (6). In addition, Id proteins are considered essential for vascularization of tumors (7).

Cervical cancer is one of the most common cancers in women world-wide (8). Because of nation-wide screening programs in developed countries, most patients are first seen with stage 1 disease. Stage 1 cervical cancer has a favorable outcome in most patients, nevertheless, ~20–35% of patients are expected to die from their disease (9).

Expression of Id proteins has been demonstrated in a variety of human tumors (2, 3, 10–12), and there has been speculation about using them as possible targets for novel therapeutic agents (2, 7). Nevertheless, no data demonstrating a prognostic relevance of Id protein expression in human cancer exist thus far. The aim of our study was to investigate the expression of Id proteins in early-stage cervical cancer and their influence on the survival of patients. In addition, the association between Id protein expression and neoangiogenesis, assessed by MVD, was determined.

Materials and Methods

Patients and Tissues. Formalin-fixed, paraffin-embedded surgical specimens from 89 patients with invasive cervical cancer, UICC stage pT_{1b}, were examined. Diagnosis was established preoperatively by punch biopsy or cone excision, and patients were treated with radical hysterectomy and pelvic lymph node dissection. In cases with pelvic lymph node metastases or tumor invasion of the outer third of the uterine cervix, adjuvant radiation therapy was applied postoperatively. Radiation therapy consisted of brachytherapy at a total dose of 42 Gy applied intracavitarily. In patients with positive lymph nodes ($n = 29$), external beam radiation at a total dose of 50 Gy was applied.

The mean observation time was 82.1 + 42.7 months. During this observation period, 28 patients (31.5%) developed recurrent disease and deceased.

Tumors were considered bulky when they infiltrated the outer third of the cervix or had a diameter of 40 mm or more. Vascular space involvement was determined in H&E-stained sections and was considered positive if at least one tumor cell cluster was clearly visible within an endothelial lined vascular space (13).

Immunohistochemistry. Expression of Id proteins and MVD was determined immunohistochemically using paraffin-embedded specimens fixed in 4% buffered formalin. Histological sections, 4 μm in thickness, were deparaffinized in xylol, and heated in 0.01 M citrate buffer for 16 min in a microwave oven followed by incubation in methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase. Unspecific binding sides were blocked with 10% normal goat serum for 30 min.

Slides were incubated overnight at +4°C with polyclonal rabbit antibodies against Id-1, Id-2, and Id-3 (Santa Cruz Biotechnology, Santa Cruz, CA; Ref. 11) in a dilution of 1:50. Immunohistochemical detection of factor VIII-related antigen was performed on a separate slide from the same block using a polyclonal rabbit antibody (BioGenex, San Ramon, CA) according to a standard protocol (9).

Visualization of bound antibodies was performed using a Super Sensitive Kit (BioGenex), which is based on streptavidin-biotin-horseradish peroxidase complex formation, according to the manufacturer's instructions. 3-amino-9-ethylcarbazole (BioGenex) was used as chromogene. A specimen of normal human skin served as positive control for Id protein expression (11). Normal squamous epithelium in cancer samples was used as additional internal positive control (if present). Samples of breast cancer with high MVD, used already in previous studies (14, 15), served as positive controls for factor VIII-related antigen.

Whereas Id-2 and Id-3 show nuclear staining signals by immunohistochemistry, Id-1 protein lacks this typical nuclear localization signal found on many HLH proteins but gives a cytoplasmic staining signal instead (2). Therefore, cytoplasmic expression of Id-1 and nuclear expression of Id-2 and Id-3 were determined by two independent observers (P. B. and G. O.), who assessed semiquantitatively the percentage of stained tumor cells as well as staining intensity. The percentage of positive cells was rated as follows (16): 2 points, 11–50% positive tumor cells; 3 points, 51–80% positive cells; and 4 points, >81% positive cells. Staining intensity was rated 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, 3 points; moderate expression, 4–5 points; and strong expression, 6–7 points.

Determination of MVD assessed by immunostaining for factor VIII-related antigen was performed according to Weidner's method (17). In brief, after scanning the immunostained section at low magnification (×40), the area of tissue with the greatest number of distinctly decorated

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[3] The abbreviations used are: Id, inhibitor of differentiation/DNA binding; HLH, helix-loop-helix; MVD, microvessel density, UICC, International Union against Cancer; OS, overall survival; DFS, disease-free survival.

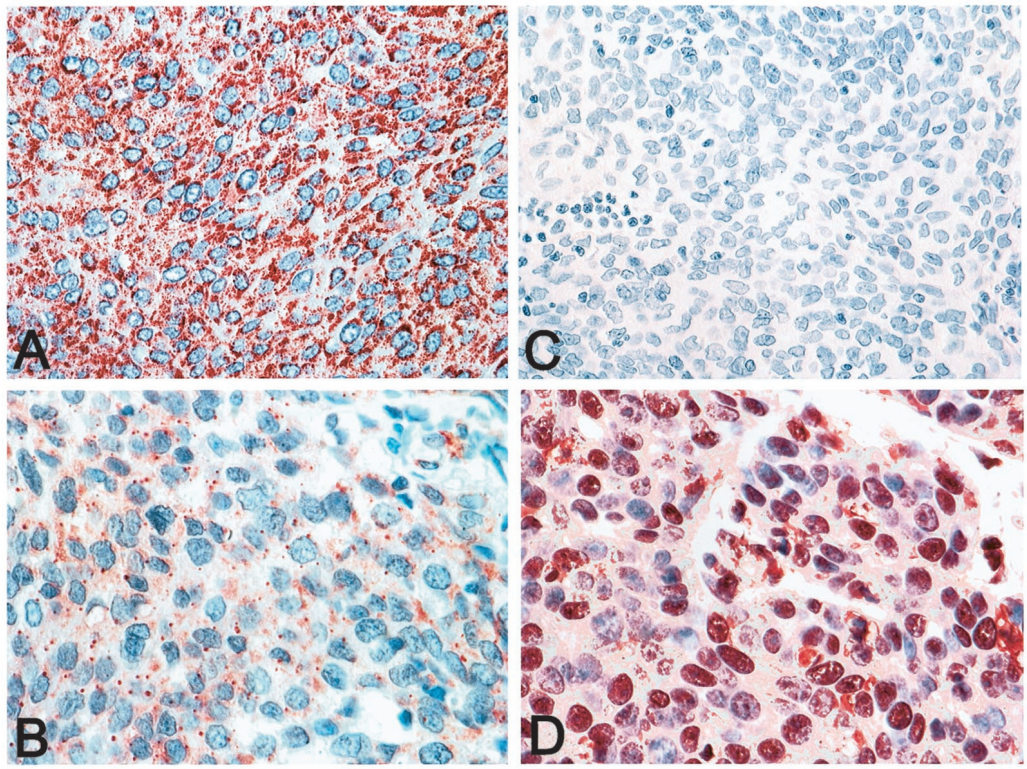

Fig. 1. *A*, a specimen of invasive cervical cancer with strong expression of Id-1. Cytoplasmic staining of strong intensity can be seen in a vast majority of cancer cells. Immunoperoxidase; ×400. *B*, a specimen of invasive cervical cancer with weak expression of Id-1. The dot-like cytoplasmic staining reaction in only a subset of cells. Immunoperoxidase; ×600. C, a specimen of invasive cervical cancer with absent expression of Id-1. Immunoperoxidase, ×400. *D*, a specimen of invasive cervical cancer with strong expression of Id-3. Nuclear staining of strong intensity can be seen in the majority of cancer cells. Immunoperoxidase, ×600.

microvessels ("hot spot") at the border of invasive cancer or inside the tumor was selected. MVD was then determined by counting all of the immunostained vessels at a total magnification of ×200 in an examination area of 0.25 mm^2. Evaluation of the staining reaction was strictly confined to the hot spots.

Negative control sections for all of the antibodies were prepared from the same tissue block. Instead of the primary antibody, a preimmune rabbit serum was applied.

Statistical Methods. Association of Id protein expression with MVD and various clinical and histopathological parameters was investigated using the Kruskal-Wallis test or Spearman's coefficient of correlation, as appropriate. OS was defined as the period from primary surgery until the death of the patient. Death from a cause other than cervical 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. Univariate analysis of OS and DFS was performed as outlined by Kaplan and Meier (18). The Cox proportional-hazards model was used for multivariate analysis. Expression of the appropriate Id protein, lymph node status, tumor size (bulky *versus* nonbulky), histological grading, and vascular invasion were entered into Cox regression. For all of the tests, a two-tailed P of ≤ 0.05 was considered significant.

Results

In normal cervical epithelium, a weak cytoplasmic expression of Id-1 of basal and parabasal cells was observed. In cancer samples, strong cytoplasmic expression of Id-1 was found in 10 cases (9.9%), moderate in 17 cases (16.8%), weak in 41 cases (40.6%), and absent in 21 cases (20.8%; Fig. 1).

Id-2 and Id-3 demonstrated moderate or strong nuclear expression in basal and parabasal cells of normal squamous epithelium. Strong Id-2 expression was observed in 1 cancer sample (1.1%), moderate in 4 cases (4.5%), and weak in 12 cases (13.5%). No expression of Id-2 was found in 72 samples (80.9%). Strong expression of Id-3 was observed in 5 cases (5%), moderate in 12 samples (11.9%), weak in 22 samples (21.8%), and no expression of Id-3 was found in 50 cases (49.5%; Fig. 1).

No association of Id-1, Id-2, or Id-3 expression with lymph node involvement or tumor size was found ($P > 0.05$, Kruskal-Wallis test). No correlation of expression of Id-1 and Id-3 was observed, but correlation of expression of Id-2 with histological grading was observed ($P = 0.008$). Nevertheless, this negative association was extremely weak (r, −0.279, Spearman's coefficient of correlation). Another weak correlation was detected between of Id-2 and Id-3 expression (r, 0.272; $P = 0.01$).

Median MVD was 20 microvessels/field (range, 8–70 microvessels/field). No correlation between expression of Id proteins and MVD was observed ($P > 0.05$, Spearman's coefficient of correlation).

At univariate survival analysis, a significant difference in OS and DFS was found between patients with no or low and those with moderate or strong expression of Id-1 [$P = 0.0144$ and $P = 0.0107$, respectively, log-rank test (Fig. 2)]. Five-years OS rate was 80.65% in patients with low or absent expression of Id-1 (median OS time, 170 months), whereas in patients with strong or moderate Id-1 expression it was 62.96% (median OS time, 96 months). Five-years DFS rate was 80.65% in patients with low or absent expression of Id-1 (median DFS time, 170 months), whereas in patients with moderate or strong Id-1 expression, it was only 47.86% (median DFS time, 66.4 months). Expression of Id-1 remained an independent prognostic factor for OS ($P = 0.016$) and DFS ($P = 0.022$) in multivariate analysis (Table 1).

No influence of Id-2 and Id-3 expression on OS ($P = 0.5633$ and $P = 0.8185$, respectively, log-rank test) or DFS ($P = 0.6329$ and $P = 0.8136$, respectively, log-rank test) was observed in univariate and multivariate analysis ($P > 0.05$, Cox regression). Nevertheless, when comparing OS and DFS between patients with absent or low *versus* patients with moderate or strong Id-3 expression, we observed a trend toward diminished prognosis of patients with absent/low expression of Id-3 (Fig. 3). Nevertheless no significance was reached in uni- and multivariate analysis ($P > 0.05$, log-rank test and Cox-regression).

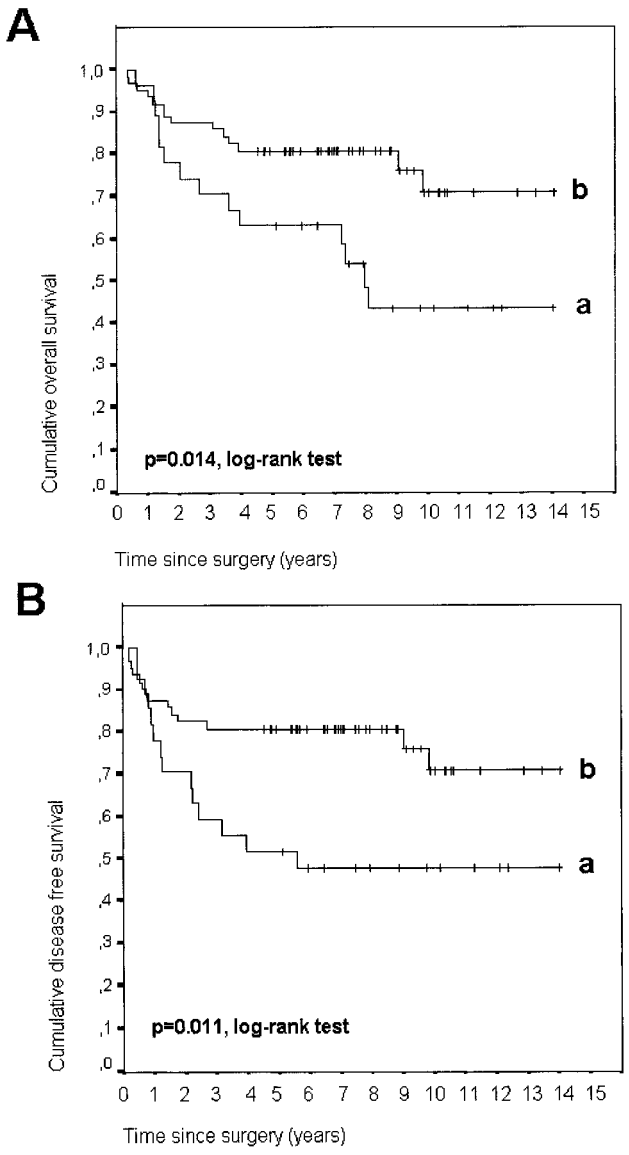

Fig. 2. *A*, cumulative OS in 89 patients with cervical cancer stage pT_{1b} with (*a*) strong or moderate expression of Id-1 and (*b*) low to absent expression of Id-1. *B*, cumulative DFS in 89 patients with cervical cancer stage pT_{1b} with (*a*) strong or moderate expression of Id-1 and (*b*) low-to-absent expression of Id-1. +, censored events.

Discussion

To our knowledge, our data presented here demonstrate for the first time a direct association of expression of an Id protein with clinical outcome in a human cancer. Expression of Id-1 was shown as an independent prognostic factor for OS and DFS in a collective of cervical cancers with long-time follow-up. The fact that this was observed in early-stage disease gives our findings even more significance.

Because Id-1 is a regulator of transcription, it may be responsible for some of the changes in gene expression that lead to the increased growth and invasion of tumor cells (7). Our results clearly indicate that overexpression of Id-1 is associated with more aggressive behavior of tumor cells in human cervical cancer. 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 proteins was found in our study, which indicated that the effect of Id-1 expression on prognosis cannot be attributed to its proangiogenetic effect alone. It has been suggested by other authors (2, 7) that drugs interfering with Id-1 may target aggressive cancer cells. Our data support the thesis that the inhibition of Id-1 might be of benefit for cancer patients, because overexpression of Id-1 significantly influences prognosis, at least in early-stage cervical cancer.

Table 1 *OS and DFS in 89 patients with cervical cancer stable* pT_{1b} (Cox regression)

	Significance *P*	95% confidence interval	Relative risk
OS			
Id-1 expression	0.016	1.2–5.62	2.6
Lymph node involvement	<0.001	2.1–13.26	5.28
Tumor size	0.088		
Grading	0.318		
Vascular invasion	0.509		
DFS			
Id-1 expression	0.022	1.14–5.35	2.47
Lymph node involvement	0.001	1.95–11.26	4.68
Tumor size	0.098		
Grading	0.645		
Vascular invasion	0.585		

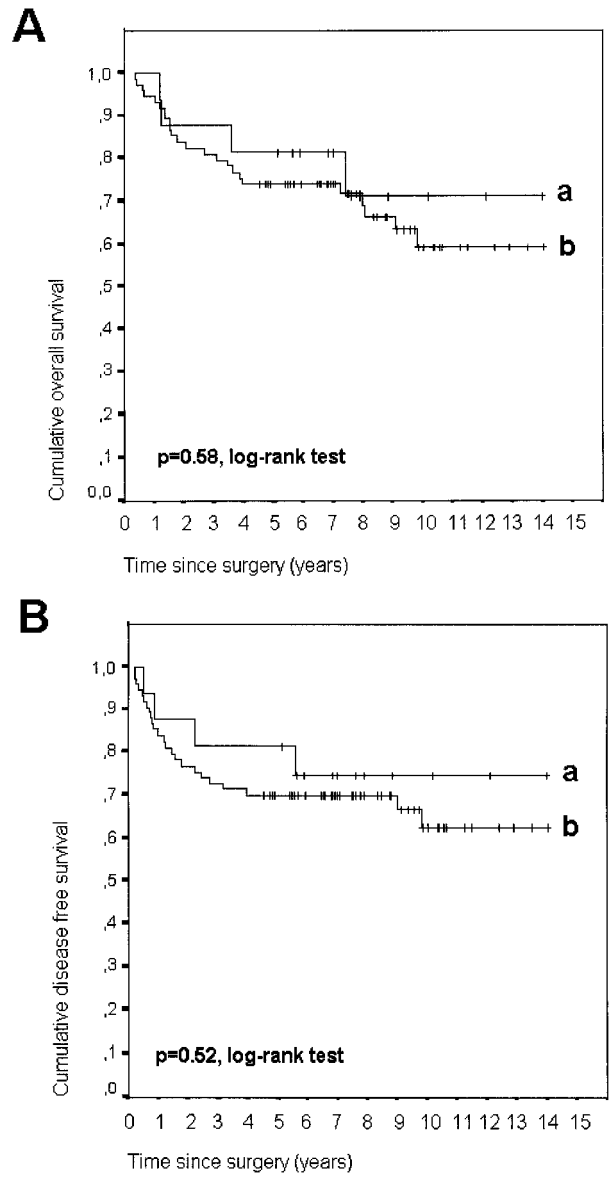

Fig. 3. *A*, cumulative OS in 89 patients with cervical cancer stage pT_{1b} with (*a*) strong or moderate expression of Id-3 and (*b*) low-to-absent expression of Id-3. *B*, cumulative DFS in 89 patients with cervical cancer stage pT_{1b} with (*a*) strong or moderate expression of Id-3 and (*b*) low-to-absent expression of Id-3. +, censored events. *P* values given are between groups.

Id-2 has been reported to alter cell cycle components normally involved in the regulatory mechanisms of cell cycle progression (*e.g.*, pRb, p16), and overexpression appears to make cancer cells refractory to the growth-inhibiting effects of various tumor-suppressor proteins (5). Our finding of a weak negative correlation between Id-2 expression and grading of tumors was also observed in a recent study of squamous cell carcinomas of the head and neck (11). In our study, the majority of cases (81%) showed no expression of Id-2, and if present, expression of Id-2 did not influence the prognosis of patients. Nevertheless, additional studies in different types of cancer in which Id-2 is more commonly overexpressed, might reveal a prognostic influence of this protein.

Although we detected a trend toward favorable prognosis in patients with strong/moderate expression of Id-3, no significance was reached, most probably because of the relatively low number of patients with moderate/strong expression of Id-3 ($n = 17$). This is in good correlation to recent findings in ovarian cancer, where there was also a trend toward diminished prognosis in patients with absent Id-3 expression, which also failed to reach significance (3). It was suggested that Id-3 might function as a tumor suppressor gene (3).

Because Id-3 is an inhibitor of transcription, overexpression of this protein might inhibit expression of various genes essential for tumor growth and progression. Additional studies are required to identify tumor-relevant genes which are influenced by Id-3 overexpression. Nevertheless, absence of Id-3 expression seems to be only a weak prognostic factor, so that studies of large collectives seem necessary to evaluate its prognostic significance.

In conclusion, we demonstrated here that overexpression of Id-1 is an independent marker for tumor progression in cervical cancer. Additional studies should investigate the question as to whether Id-1 has a similar impact on prognosis in other forms of human cancer.

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