

Role of inhibitor of DNA binding-1 protein is related to angiogenesis in the tumor advancement of uterine endometrial cancers

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Abstract. The inhibitor of DNA binding (ID)-1 protein, an inhibitor of basic helix-loop-helix transcription factors, has been found to be involved in multiple cellular functions. In the present study, ID-1 histoscores and mRNA levels were both significantly ($p<0.05$) increased in uterine endometrial cancers according to clinical stage, histological grade and depth of myometrial invasion. Furthermore, the 60-month survival rate of the 25 patients with high ID-1 was poor (52%), while that of the other 25 patients with low ID-1 was significantly higher (80%) ($p<0.05$). ID-1 histoscores and mRNA levels significantly ($p<0.0001$) correlated with microvessel counts in uterine endometrial cancers. Therefore, ID-1 acts on tumor advancement via angiogenic activity and can be considered a candidate prognostic indicator in uterine endometrial cancers.

Introduction

Inhibitor of DNA binding (ID)-1 protein is a member of the helix-loop-helix (HLH) protein family which is expressed in actively proliferating cells. ID-1 regulates gene transcription by heterodimerization with the basic HLH transcription factors, and therefore inhibits them from DNA binding and transactivation of their target genes (1). ID-1 functions mainly as a regulator in cellular differentiation of muscle cells (1). ID-1 regulates cellular senescence (2,3), induces cancer cell growth (4,5), promotes cell survival as an oncogene (6) and invasion of cancer cells (7,8), and up-regulates matrix metalloproteinase (MMP), which is necessary for the breakdown of the basement membrane and extracellular matrix that restrains tumor growth and metastasis. Expression of the 120-kDa MMP protein is strongly associated with the

increase in motility and invasiveness of mammary cells (7). Moreover, screening of breast cancer cell lines revealed that expression of ID-1 as well as the 120-kDa MMP protein was directly correlated with the invasiveness of these cell lines (7), suggesting that ID-1 regulates MMP protein as well as invasion. Furthermore, down-regulating ID-1 expression through antisense or small interfering RNA (siRNA) treatment suppressed the invasion and metastatic ability of breast cancer cells (9,10). ID-1 is frequently overexpressed in cancers of the breast (11), prostate (12), thyroid (13), pancreas (14) and esophagus (15). In addition, ID-1 is associated with tumor stage and poor prognosis in prostate (16), breast (17), ovarian (18) and uterine cervical cancers (19).

In a previous study, ID-1 overexpression was associated with invasive behavior in uterine endometrial cancer (20). However, the molecular mechanism for this overexpression is not clear. This prompted us to study the manner of expression of ID proteins in uterine endometrial cancer according to clinical backgrounds with angiogenic activity in tumors.

Materials and methods

Patients and tissues. Prior informed consent for the present study was obtained from all patients and approval was given by the Research Committee for Human Subjects, Gifu University School of Medicine. Fifty patients ranging from 34 to 79 years of age with endometrioid adenocarcinomas of the uterine endometrium [stage I, 20 cases; stage II, 17 cases; stage III, 13 cases; well-differentiated adenocarcinoma (G1), 22 cases; moderately differentiated adenocarcinoma (G2), 11 cases; poorly differentiated adenocarcinoma (G3), 17 cases] underwent hysterectomy at the Department of Obstetrics and Gynecology, Gifu University School of Medicine, between March 1996 and September 2004. Patient prognosis was analyzed in relation to the 60-month survival rate. None of the patients had received any pre-operative therapy before the uterine endometrial cancer tissue was taken in surgery. A part of each tissue of uterine endometrial cancers was snap-frozen in liquid nitrogen and stored at -80°C to determine ID-1, ID-2 and ID-3 mRNA levels, and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin wax. The clinical stage of uterine endometrial cancers was

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determined by the International Federation of Obstetrics and Gynecology (FIGO) classification (21).

Immunohistochemistry. Sections (4- μ m) of formalin-fixed paraffin-embedded tissue samples from uterine endometrial cancers were cut with a microtome and dried overnight at 37°C on a silanized slide (Dako, Carpinteria, CA, USA). The protocol of the Universal Dako Labelled Streptavidin-Biotin kit (Dako, USA) was followed for each sample. Samples were deparaffinized in xylene at room temperature for 30 min, rehydrated with graded ethanol and washed in phosphate-buffered saline (PBS). The samples were then placed in 10 mM citrate buffer (pH 6.0) and boiled in a microwave for 10 min for epitope retrieval. Endogenous peroxidase activity was quenched by incubating tissue sections in 3% H₂O₂ for 10 min. The primary antibodies, rabbit anti-human ID-1 (SC-734; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse CD34 (Dako, Glostrup, Denmark) and rabbit anti-factor VIII-related antigen (Zymed, San Francisco, CA, USA) were used overnight at 4°C at dilutions of 1:50, 1:40 and 1:2, respectively. The slides were washed, and biotinylated secondary antibody (Dako, USA) was applied for 30 min after rinsing in PBS, after which streptavidin-conjugated horseradish peroxidase (Dako, USA) was added for 30 min. Slides were then washed and treated with the chromogen 3,3'-diaminobenzidine (Dako, USA) for 5 min, rinsed in PBS and counterstained with Mayer's haematoxylin, dehydrated in graded ethanols, cleared in xylene and cover-slipped with a mounting medium (Entellan New; Merck, Darmstadt, Germany). For confirmation of the specificity for the ID-1 antigen, we also used another ID-1 (SC-488) rabbit polyclonal antibody (Santa Cruz Biotechnology Inc.), and we observed the exact identification of staining for ID-1 expression in tumor cells as ID-1 (SC-734) antibody. For the negative controls, the primary antibodies of ID-1, CD34 and factor VIII-related antigen were omitted, and the corresponding preimmune animal serums (rabbit, mouse and rabbit, respectively) (Dako, USA) were used instead.

Assessment of histochemical score (histoscore). All sections for immunohistochemical staining for ID-1 were evaluated in a semiquantitative fashion according to the method described by McCarty *et al* (22), which considers both the intensity and the percentage of cells stained in each of five intensity categories. Intensities were classified as 0 (no staining), 1 (weak staining), 2 (distinct staining), 3 (strong staining) and 4 (very strong staining). For each stained section, a value-designated histoscore was obtained by application of the following algorithm: $\text{histoscore} = \sum(i+1) \times P_i$, where i and P_i represent intensity and percentage of cells that stain at each intensity, respectively, and corresponding histoscores were calculated separately. Results were assigned to four groups according to their overall scores: weak, <160; distinct, 161-220; strong, 221-280; very strong, >280.

Assessment of microvessel density (MVD). MVD was assessed with microvessel counts (MVCs) in sequential tissue sections stained with mouse CD34 and rabbit factor VIII-related antigen antibodies. Blood vessels with a clearly defined lumen or a well-defined linear vessel shape, but not single endothelial

cells, were taken into account for microvessel counting (23). Fives areas of highest vascular density were chosen, and microvessel counting was performed at x200 magnification by two investigators. MVCs were determined as the mean of the vessel counts obtained from these fields (24).

Preparation of standard template for real-time reverse transcription-polymerase chain reaction (RT-PCR). The internal standard template for real-time PCR was produced by PCR amplification using the primers of the ID-1 gene, 418-782 in the cDNA (ID-1-TS, 5'-TTGGAGCTGAAGCTCGGAA-3' and ID-1-TAS, 5'-TCTCTGGTGACTAGTAGGT-3'); ID-2 gene, 907-1253 in the cDNA (ID-2-TS, 5'-CTAAGCAGACTTTGCCTTT-3' and ID-2-TAS, 5'-CTGAAATAAAGCAGGCAATC-3'); ID-3 gene, 686-1009 in the cDNA (ID-3-TS, 5'-GAAGTGTCTCTCCAACGA-3' and ID-3-TAS, 5'-CACGCTCTGAAAAGACCT-3'). The DNA template was purified using a GeneClean II kit (Qbiogene, Irvine, CA, USA). The copy numbers of the standard template were determined to quantitate the ID-1, -2 and -3 mRNA level in samples for real-time RT-PCR.

Real-time RT-PCR to amplify ID-1, -2 and -3 mRNAs. Total RNA was extracted with the acid guanidinium thiocyanate-phenol-chloroform method (25). Total RNA (3 μ g) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (MMLV-RT, 200 U/ μ l; Invitrogen, Carlsbad, CA, USA) and the following reagents: 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂, 0.1 M dithiothreitol, 10 mM deoxynucleotide (deoxyadenosine, deoxythymidine, deoxyguanosine and deoxycytidine) tri-phosphates (dNTPs) mixture and random hexamers (Invitrogen) at 37°C for 1 h. The reaction mixture was heated for 5 min at 94°C to inactivate MMLV-RTase.

The real-time PCR reaction was performed with a Takara Premix Ex Taq (Perfect Real Time) R-PCR kit (Takara, Otsu, Japan), using a smart cycler system (Cepheid, Sunnyvale, CA, USA). The reaction solution (25 μ l) contained Takara Premix Ex Taq (2X), SYBR Green I (1:1000 dilution; CambrexBio Science, Rockland Inc., Rockland, ME, USA) and 20 μ M of the primers of the ID-1 gene, 545-675 in the cDNA (ID-1-S, 5'-ACGATCGCATCTTGTGTC-3' and ID-1-AS, 5'-CTTGTTCTCCCTCAGATCC-3'); ID-2 gene, 907-1026 in the cDNA (ID-2-S, 5'-CTAAGCAGACTTTGCCTTT-3' and ID-2-AS, 5'-CATTCAGTAGGCTTGTGTC-3'); ID-3 gene, 709-873 in the cDNA (ID-3-S, 5'-AAGGAGCTTTTGCCACTGA-3' and ID-3-AS, 5'-CCAGGAAGGGATTGTTGTA-3') with the transcribed total RNA from the tissue and a serially diluted standard template. The real-time PCR reactions were initially denatured by heating at 95°C for 30 sec, followed by 40 cycles consisting of denaturation at 94°C for 10 sec, annealing at 55°C for 5 sec and extension at 72°C for 20 sec. A strong linear relationship between the threshold cycle and the log concentration of the starting DNA copy number was always shown (correlation coefficient >0.99). Quantitative analysis was performed to determine the copy number of each sample.

Statistical analysis. ID-1, -2 and -3 mRNA levels were determined from three parts taken from each tumor, and each sample was analyzed in triplicate. ID-1 histoscores and mRNA

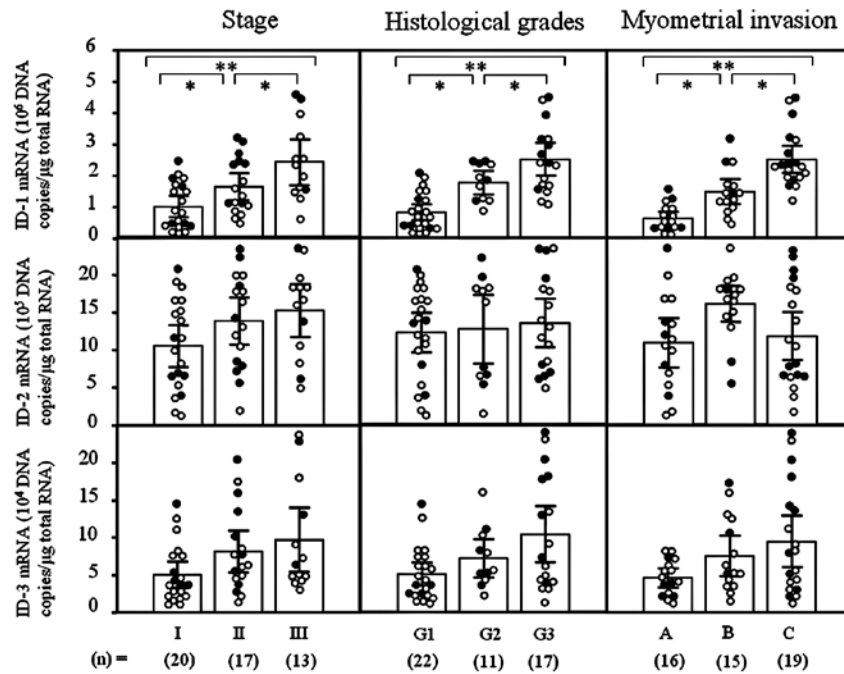


Figure 1. ID-1, -2 and -3 mRNA levels in uterine endometrial cancers classified according to clinical stage, histological grade and depth of myometrial invasion. Assessment was carried out according to FIGO classification. Each level is the mean \pm SD of nine determinations. G1, well-differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma. (A) Tumor limited to endometrium; (B) invasion to less than half the myometrium; (C) invasion to more than half the myometrium. Alive and deceased cases are numbered in open circles and closed circles, respectively. * $p < 0.05$; ** $p < 0.001$.

levels were compared using the Student's t-test. The 60-month survival rate was calculated according to the Kaplan-Meier method and analyzed with the log-rank test. The correlations between ID-1 histoscores and mRNA levels with MVCs were performed with bivariate Pearson's correlation. Differences were considered significant at $p < 0.05$.

Results

ID-1 mRNA levels significantly increased with increasing clinical stages ($I < II < III$; $p < 0.05$), histological grades ($G1 < G2 < G3$; $p < 0.05$) and depth of myometrial invasion ($A < B < C$; $p < 0.05$) of the uterine endometrial cancers (Fig. 1). However, there was no significant difference in ID-2 or -3 mRNA levels according to clinical stage, histological grade or depth of myometrial invasion in uterine endometrial cancers, as shown in Fig. 1. These results prompted us to concentrate our investigation on ID-1 in uterine endometrial cancers.

ID-1 staining was diffusely located in the cancer cells (Fig. 2; a representative case of well-differentiated endometrioid adenocarcinoma of the uterine endometrium). Since 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 signal instead (8,26). ID-1 diffuse cytoplasmic staining from moderate to strong intensity was noted in most cases whereas nuclear staining was observed only occasionally.

The ID-1 histoscore in cancer cells was significantly ($p < 0.001$) correlated with corresponding mRNA levels in each tissue, as shown in Fig. 3. Moreover, ID-1 histoscores significantly ($I < II < III$; $p < 0.05$) increased with increasing clinical stages, histological grades ($G1 < G2 < G3$; $p < 0.05$) and



Figure 2. Immunohistochemical staining for ID-1 with negative control in uterine endometrial cancers. A representative case of well-differentiated endometrioid adenocarcinoma of the uterine endometrium. Rabbit anti-human ID-1 (SC-734; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used at a dilution of 1:50 as the primary antibody (original magnification $\times 400$).

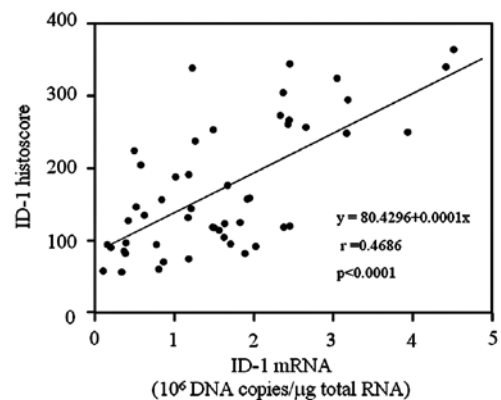


Figure 3. Correlation between ID-1 histoscores in cancer cells and mRNA (10^6 DNA copies/ μ g total RNA) levels in uterine endometrial cancers. ID-1 histoscores and mRNA levels were determined by immunohistochemistry and real-time RT-PCR, respectively. Each level is the mean \pm SD of nine determinations.

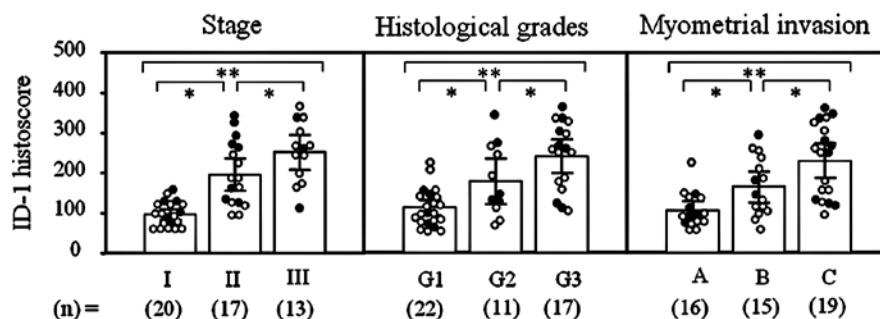


Figure 4. ID-1 histoscores in uterine endometrial cancers classified according to clinical stage, histological grade and depth of myometrial invasion. Assessment was carried out according to FIGO classification. Each level is the mean \pm SD of nine determinations. G1, well-differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma. (A) Tumor limited to endometrium; (B) invasion to less than half the myometrium; (C) invasion to more than half the myometrium. Alive and deceased cases are numbered in open circles and closed circles, respectively. * $p<0.05$; ** $p<0.001$.

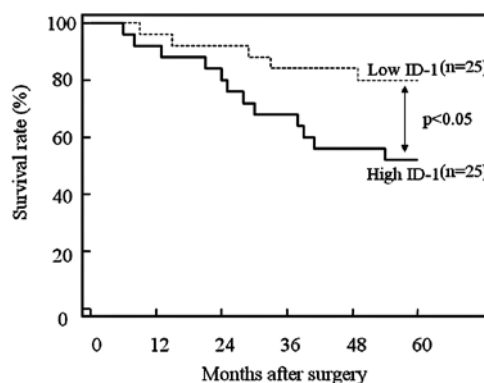


Figure 5. Survival rate after surgery for uterine endometrial cancer patients. Patient prognosis was analyzed with a 60-month survival rate. High ID-1, cases with high histoscores and mRNA levels (>150 histoscore and $>1.5 \times 10^6$ copies/ μg total RNA, respectively). Low ID-1, cases with low histoscores and mRNA levels (<150 histoscore and $<1.5 \times 10^6$ copies/ μg total RNA, respectively).

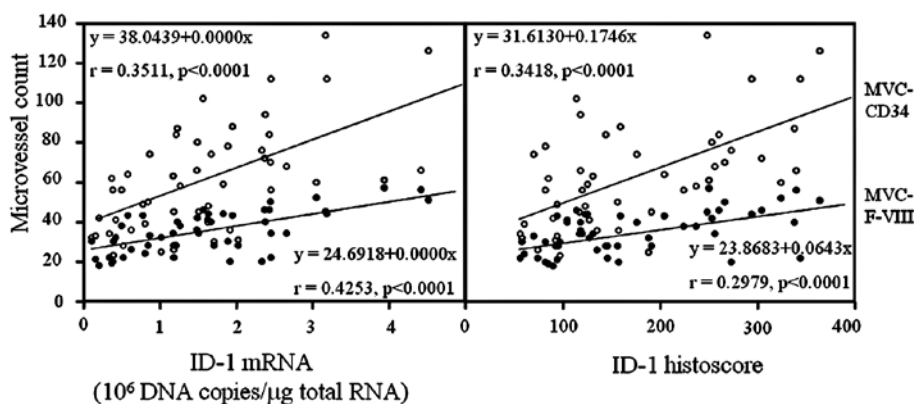


Figure 6. Correlation of ID-1 histoscores in cancer cells and mRNA levels in uterine endometrial cancers with microvessel counts (MVCs). White circles, MVC-CD34 by immunohistochemical staining for CD34; black circles, MVC-F-VIII by immunohistochemical staining for factor VIII-related antigen. Each level is the mean \pm SD of nine determinations.

depth of myometrial invasion ($A<B<C$; $p<0.05$) of the uterine endometrial cancers (Fig. 4), as did ID-1 mRNA.

Furthermore, the 50 patients who underwent hysterectomy were divided into two equal groups based on ID-1 histoscores and mRNA levels, with the midpoint being a histoscore of 150 and mRNA of 1.5×10^6 copies/ μg total RNA, respectively. The two groups, determined independently by the ID-1 histoscores

and mRNA levels, consisted of exactly the same patients. The prognosis of the 25 patients with high ID-1 (>150 histoscore and $>1.5 \times 10^6$ copies/ μg total RNA) in the uterine endometrial cancers was poor (52%), while the 60-month survival rate of the other 25 patients with low ID-1 (<150 histoscore and $<1.5 \times 10^6$ copies/ μg total RNA) was higher (80%), as shown in Fig. 5.

ID-1 expression was negative in endothelial cells, although CD34 and factor VIII-related antigen expression was strong. ID-1 histoscores significantly correlated with MVC-CD34 (MVCs determined by immunohistochemistry for CD34; $r=0.3418$, $p<0.0001$) and MVC-F-VIII (MVCs determined by immunohistochemistry for factor VIII-related antigen; $r=0.2978$, $p<0.0001$); ID-1 mRNA levels were also correlated with MVC-CD34 ($r=0.3511$, $p<0.0001$) and MVC-F-VIII ($r=0.4253$, $p<0.0001$), as shown in Fig. 6.

Discussion

In this study, ID-1 expression significantly increased with more advanced clinical stage, histological grades and depth of myometrial invasion. In addition, the patients with high ID-1 expression had a lower survival rate compared with patients with low ID-1 expression. Therefore, ID-1 contributes to tumor progression and can be recognized as a novel indicator of tumor advancement and patient prognosis in uterine endometrial cancers. In a previous study, abundant ID-1 immunoreactivity was found in endometrial carcinoma cells; ID-1 expression was associated with histological grade and invasion to the myometrium (20). However, no evidence is available on the prognostic implications of ID-1 or the relationship between ID-1 expression and angiogenesis in uterine endometrial cancer.

Studies with ID-1 knock-out mice revealed that ID-1 expression is required, not only for normal angiogenesis, but is also essential for tumor-associated angiogenesis during cancer progression (27,28). Both VEGF and its receptor were down-regulated in endothelial cells of ID-1/ID-3 double knock-out mice (27), suggesting a possible linkage between ID-1 and VEGF functioning. Accordingly, VEGF is a downstream target of the ID-1 protein, and inactivation of ID-1 by siRNA transfection, resulted in down-regulation of both VEGF gene transactivation and protein secretion (29). As VEGF is one of the most potent tumor angiogenic factors that activates, not only endothelial cell proliferation, but also blood vessel formation, these findings indicate a role of ID-1 in the induction of tumor angiogenesis. In the present study, ID-1 expression correlated with microvessel counts in endometrial cancers. Therefore, ID-1 might contribute to angiogenesis related to the tumor progression of uterine endometrial cancers.

In summary, this study demonstrates that ID-1 overexpression plays a role in tumor advancement via angiogenesis and that ID-1 can be used as a prognostic indicator in uterine endometrial cancers. Moreover, ID-1 might be a novel anticancer target molecule for anti-angiogenic drug design in cancer treatment.

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