Expression of the inhibitor of DNA-binding (ID)-1 protein as an angiogenic mediator in tumour advancement of uterine cervical cancers

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The ID protein, an inhibitor of basic helix-loop-helix (HLH) transcription factors, has been involved in multiple cellular processes. To investigate the association between tumour advancement and ID expressions of uterine cervical cancers, the levels of ID-1, ID-2 and ID-3 mRNAs were determined by real-time reverse transcription-polymerase chain reaction and the histoscore with the localisation of ID-1 was determined by immunohistochemistry and patient survival in 60 patients. ID-1 histoscores and mRNA levels both significantly (\(P<0.05\)) increased in uterine cervical cancers according to clinical stage regardless of histopathological type or lymph node metastasis. Furthermore, the 36-month survival rate of the 30 patients with high ID-1 was poor (60%), whereas that of the other 30 patients with low ID-1 was significantly higher (83%). ID-1 histoscores and mRNA levels significantly (\(P<0.0001\)) correlated with microvessel counts in uterine cervical cancers. Tumour cells show mostly diffuse to strong cytoplasmic expression of ID-1 and also very faint expression in endothelial cells. Moreover, ID-1 expression not only correlated with microvessel counts but also correlated significantly with histoscore. Therefore, ID-1 might work on tumour advancement through angiogenic activity and is considered to be a candidate for a prognostic indicator in uterine cervical cancers.

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The family of inhibitors of DNA-binding (ID) proteins consists of four members of basic helix-loop-helix (bHLH) transcription factors lacking the DNA-binding domain (Norton et al, 1998a). Therefore, they act as dominant-negative regulators of bHLH proteins by forming transcriptionally inactive Id-bHLH protein complexes (Riechmann et al, 1994; Riechmann and Sablitzky, 1995). ID proteins play key roles in the regulation of cell differentiation during neurogenesis, lymphoiesis and angiogenesis. Their functions also include the promotion of cell growth and cell cycle progression, and apoptotic induction (Lyden et al, 1999; Norton, 2000).

Regarding tumour cell proliferation, ID-1 inhibited the promoter region of the tumour suppressor gene CDKN2A/p16, supporting the role of ID-1 as a potential oncogene (Alani et al, 2001). Furthermore, ID proteins also inhibited the transformation-specific sequence (ETS) transcription factors of an avian erythroblastosis virus, E26 (Yates et al, 1999), which are able to suppress p16 expression by binding to and activating its promoter. This indicates that IDs might inhibit the p16 promoter, and thereby increase tumour cell proliferation, through interactions with ETS transcription factors directly and indirectly (Ohtani et al, 2001). Both IDs and ETS transcription factors have previously been implicated in the regulation of angiogenesis (Lyden et al, 1999; Wernert et al, 2003). The ID interactors–E protein transcription factors E12 (also known as epididymal sperm-binding protein, ELSPBP1), E47 (also known as E2A and TCF3), E2-2 (also known as TCF4) and HEB (also known as TCF12) (Yan et al, 1997), retinoblastoma protein (RB) (Lasorella et al, 2000) and ETS proteins (Ohtani et al, 2001)–are all implicated in tumorigenesis.

Recently, several reports have shown that ID-1 protein can induce cell proliferation and increase DNA synthesis, and that it can immortalise mammalian cells in corporation with other oncogenes (Norton and Atherton, 1998b; Alani et al, 1999). ID-1 overexpression has been found in several types of primary human cancers including breast, pancreatic, prostate, nasopharyngeal, melanoma and cervical cancers (Langlands et al, 2000; Lin et al, 2000; Polsky et al, 2001; Schindl et al, 2001, 2003; Wang et al, 2002; Ouyang et al, 2002a; Schoppmann et al, 2003; Lee et al, 2004; Straume and Akalen, 2005; Jang et al, 2006). IDs activate VEGF-dependent mobilisation of circulating endothelial cells and endothelial precursor cells from the bone marrow (Lyden et al, 2001). Further, ID-1 might act by transcriptional repression of thrombospondin (TSP)-1, a well-known angiogenesis inhibitor (Volpert et al, 2002). TSP-1 was more frequently expressed in leiomyoma compared with uterine smooth muscle tumour of uncertain malignant potential and leiomyosarcoma (Boedner-Adler et al, 2006).

Previous studies reported, expression of ID-1 was shown as an independent prognostic factor in cervical cancer with long-time follow-up, even more significant in early-stage disease. Over expression of ID-1 is associated with more aggressive behavior of
tumour cells in cervical cancer (Schindl et al., 2001). However, no evidence clarifying the direct association between tumour advancement and ID protein expression is available in human primary cancer so far.

To elucidate the clinical implication of IDs in uterine cervical cancers, we analysed the manner expression of ID proteins in uterine cervical cancer progression according to clinical backgrounds, their influence on the survival of the patients and the association between ID mRNA expression and neoangiogenesis, assessed by assessment of microvessel density (MVD).

MATERIALS AND METHODS

Patients and tissues

Prior informed consent for the following studies was obtained from all patients and approval was given by the Research Committee for Human Subjects, Gifu University School of Medicine. Sixty patients ranging from 35 to 79 years of age with uterine cervical cancers (stage I, 21 cases; stage II, 21 cases; and stage III, 18 cases; squamous cell carcinoma (SCC), 42 cases and adenocarcinoma (AD), 18 cases underwent surgery) at the Department of Obstetrics and Gynecology, Gifu University School of Medicine, between September 1998 and August 2004. Patient prognosis was analysed in relation to a 36-month survival rate. None of the patients had received any pre-operative therapy before the uterine cervical cancer tissue was taken in surgery. A part of each tissue of uterine cervical 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 cervical cancers was determined by International Federation of Obstetrics and Gynecology (FIGO) classification (International Federation of Obstetrics and Gynecology (FIGO) News, 1989).

Immunohistochemistry

Sections (4 μm) of formalin-fixed paraffin-embedded tissue samples from uterine cervical cancers were cut with a microtome and dried overnight at 37°C on a silanised-slide (Dako, Carpinteria, CA, USA). The protocol of universal Dako-labelled Streptavidin – Biotin kit (Dako, Carpinteria, CA, USA) was followed for each sample. Samples were deparaffinised in xylene at room temperature for 30 min, rehydrated with graded ethanol and washed in phosphate-buffered saline (PBS). The slides 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, Carpinteria, CA, USA) was applied for 30 min after rinsing in PBS, after which streptavidin-conjugated horseradish peroxidase (Dako, Carpinteria, CA, USA) was added for 30 min. Slides were then washed and treated with the chromogen 3,3′– diaminobenzidine (Dako, Carpinteria, CA, USA) for 5 min, then 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 ID-1 antigen, we also used another ID-1 (SC-488) rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and we have observed the exact identified intensity and localisation of staining for ID-1 expression in tumour 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, Carpinteria, CA, USA) were used instead.

Assessment of histochemical score (histoscore)

All sections of immunohistochemical staining for ID-1 were evaluated in a semiquantitative fashion according to the method described by Maccarty et al. (1985), which considers both the intensity and 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: histoscore = ∑(i + 1) × Pi, where i and Pi 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–. Immunohistochemistry results were analysed by two pathologists.

Assessment of microvessel density

The 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 (Giatromanolaki et al., 2001). Fives areas of highest vascular density were chosen and microvessel counting was performed at × 200 magnification by two investigators. The MVCs were determined as the mean of the vessel counts obtained from these fields (Maeda et al., 1996).

Preparation of standard template for real-time reverse transcription – polymerase chain reaction (RT – PCR)

Internal standard template for real-time PCR was produced by PCR amplification using the primers of ID-1 gene, 418–782 in the cDNA (ID-1-TS: 5′-TTCGAGCTGTCGGAATGG-3′ and ID-1-TAS: 5′-CTCTGTGACTATGAGTGT-3′); ID-2 gene, 907–1253 in the cDNA (ID-2-TS: 5′-CTAACGAGCTTTGCTTCT-3′ and ID-2-TAS: 5′-CTGAAAATAGGAGGCACT-3′); ID-3 gene, 686–1009 in the cDNA (ID-3-TS: 5′-GAACCTGTCATCTCCACACGA-3′ and ID-3-TAS: 5′-GAGCCTGCTGAAAAGACCGT-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 ID-1, ID-2 and ID-3 mRNA level in samples for real-time RT – PCR.

Real-time RT – PCR to amplify ID-1, ID-2 and ID-3 mRNAs

Total RNA was extracted with the acid guanidinium thiocyanate– phenol–chloroform method (Chomczynski and Sacchi, 1987). The total RNA (3 μg) was reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (MMLV-RT, 200 U μl−1, 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.

Real-time PCR was performed with a Takara Ex Taq R-PCR kit, Version 1.0 (Takara, Otsu, Japan), using a smart cycler system.
Inhibitor of DNA-binding-1 mRNA levels significantly increased with increased clinical stages (I < II < III; \( P < 0.05 \)) of uterine cervical cancers, regardless of histopathological type and lymph node metastasis (Figure 1). However, there was no significant difference in ID-2 or ID-3 mRNA levels according to clinical stage, histopathological type or lymph node metastasis in uterine cervical cancers, as shown in Figure 1. These results prompted us to concentrate our investigation on ID-1 in uterine cervical cancers.

Inhibitor of DNA-binding-1 staining was diffusely located in the cancer cells. Although ID-1 expression in stroma cells was weak or negative, perivascular cells of intratumoral microvessels were strongly positive in all cases (Figure 2A, a representative case, 46-year-old patient, stage IIb, squamous cell carcinoma). Because ID-1 is not a transcription factor per se, it lacks the nuclear localisation signal found on many basic HLH proteins but gives a cytoplasmic signal instead (Maruyama et al, 1999; Lin et al, 2000; Schindl et al, 2001). ID-1 diffuse cytoplasmic staining was seen from moderate to strong intensity in most cases whereas nuclear staining was observed only occasionally (Figure 2B).

Inhibitor of DNA-binding-1 histoscore in cancer cells significantly (\( P < 0.001 \)) correlated with corresponding mRNA levels in each tissue, as shown in Figure 3. Although there was no significant difference in ID-1 histoscores in cancer cells according to histopathological type and lymph node metastasis, ID-1 histoscores significantly (I < II < III; \( P < 0.05 \)) increased with increased clinical stages of uterine cervical cancers (Figure 4), as did ID-1 mRNA.

Furthermore, the 60 patients who underwent surgery were arbitrarily divided into two equal groups based on ID-1 histoscores and mRNA levels, with the midpoint being a histoscore of 140 and mRNA of 1.6 \( \times 10^6 \) copies \( \mu g^{-1} \) total RNA in uterine cervical cancers was poor (60%), whereas the 36-month survival rate of the other 30 patients with low ID-1 (< 140 histoscore and < 1.6 \( \times 10^6 \) copies \( \mu g^{-1} \) total RNA) was higher (83%), as shown in Figure 5.

Inhibitor of DNA-binding-1 expression was very weak in endothelial cells although Factor VIII-related antigen and CD34

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**Figure 1** Inhibitor of DNA-binding-1, ID-2 and ID-3 mRNA levels in uterine cervical cancers classified according to clinical stage, histopathological type and lymph node metastasis. Clinical staging of uterine cervical cancers was done according to FIGO. Each level is the mean ± s.d. of nine determinations. SCC, squamous cell carcinoma; AD, adenocarcinoma. *\( P < 0.05 \); **\( P < 0.001 \).
expression were strong. In tissue sections, weak ID-1 expression was associated with low MVD whereas strong ID-1 expression was associated with abundant microvessels. The ID-1 histoscores in cancer cells significantly correlated with MVD-CD34 (MVCs determined by immunohistochemistry for CD34; \( r = 0.833, P < 0.0001 \)) and MVD-F-VIII (MVCs determined by immunohistochemistry for factor VIII-related antigen; \( r = 0.742, P < 0.0001 \)); and ID-1 mRNA levels also correlated with MVD-CD34 (\( r = 0.892, P < 0.0001 \)) and MVD-F-VIII (\( r = 0.768, P < 0.0001 \)), as shown in Figure 6.

**DISCUSSION**

This study is, to our knowledge, the first to reveal that ID-1 expression in the cancer cells increased with tumour advancement of uterine cervical cancers. Also, the patients with high ID-1 expression had a lower survival rate compared with patients with low ID-1 expression. Negative prognostic impacts of increased ID-1 expression have been shown in breast cancers (Schoppmann et al., 2003), pancreatic cancers (Lee et al., 2004), melanoma (Straume and Akslen, 2005), cervical cancers (Schindl et al., 2001), and ovarian carcinomas (Schindl et al., 2003). This is in line with our finding. Therefore, ID-1 is recognised as a novel indicator of tumour advancement and patient prognosis in uterine cervical cancer.

Previously, no correlation between expression of ID proteins and angiogenesis, assessed by MVD was observed in cervical cancers (Schindl et al., 2001). But contrary to their finding, present study showed that mostly diffuse to strong cytoplasmic expression of ID-1 in tumour cells and also very weak ID-1 expression in endothelial cells. Moreover, ID-1 expression not only correlated with microvessel counts but also correlated significantly with histoscore. These data indicate that ID-1 overexpression is found to be closely related to tumour angiogenesis, a higher density of intratumoral vessel and poor survival in cervical cancer. Therefore, ID-1 is a candidate for angiogenic mediator as the clinical relevance of angiogenesis assessed by MVD.

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**Figure 2** (A). Immunohistochemical staining for positive and negative ID-1 staining in squamous cell carcinoma of uterine cervical cancers. (B). Immunohistochemical staining for ID-1 representing cases from each stage in uterine cervical cancers. Weak staining (score 111) stage I; distinct (score 167) stage II; strong (score 264) stage III and very strong (score 392) stage IV, showing strong cytoplasmic expression in tumour cells. Overall scores: weak, < 160; distinct, 161 <, > 220; strong, 221 <, > 280; very strong, 280 <. (original magnification ×400). 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 ×200).
Angiogenesis is essential for development, growth and advancement of solid tumours (Folkman, 1985). The angiogenic factors vascular endothelial growth factor (VEGF) (Fujimoto et al, 1999a) and thymidine phosphorylase identified with platelet-derived endothelial cell growth factor (Fujimoto et al, 1999b, c, 2000) and interleukin (IL-8) along with the angiogenic transcriptional factor ETS-1 (Fujimoto, 1999) work on angiogenesis in uterine cancers.

Inhibitor of DNA-binding-1 is a regulator of basic HLH-mediated transcription (Norton et al, 1998a) and causes cells to pass a mitogen-restricted point in late G1 phase (Le Jossic et al, 1994). Therefore, ID-1 has been suggested as responsible for some of the changes in gene expression that lead to increased growth and invasion of tumour cells (Lyden et al, 1999). Moreover, the ectopic expression of ID-1 increases the proliferation, migration, invasion and metastasis of breast cancer cells (Desprez et al, 1998; Lin et al, 2000). Although ID-1 has been shown to interact with various cell cycle regulators (Alani et al, 2001; Ohtani et al, 2001), ID-1 seems to be an essential factor in the promotion of G1/S cell cycle transition in certain cancers by inactivating of p16 and increasing of CDK4 activity/RB (Polsky et al, 2001; Ouyang et al, 2002b; Singh et al, 2002). However, additional studies are required to determine the mechanism of interaction between ID-1 and various cell cycle regulators.

Recently, TSP-1, an endogenous inhibitor of neovascularisation has been identified as a target of ID-1 that is negatively regulated by ID-1 in the regulation of angiogenesis (Volpert et al, 2002). Thrombospondin-1 suppresses angiogenesis by inhibiting endothelial cell proliferation and inducing endothelial cell apoptosis (Zhang et al, 2003). For example, in the ID-1+/− mice, transcription of TSP-1 is increased, which is associated with inhibition of bFGF and VEGF-induced angiogenesis. This phenotype can be reversed by inactivation of TSP-1 function (Volpert et al, 2002). In addition, microvessel density count was negatively
associated with the intensity of TSP-1 expression in uterine cervical cancer (Wu et al., 2004). An inverse relation between ID-1 and TSP-1 expression supports the significant role of ID-1 in the regulation of this complex multigene family (Straume and Akslen, 2005). These previous studies suggested that due to ID-1 suppression, TSP-1 expression might be lost with tumour advancement, resulting in drastic tumour angiogenesis that accelerates tumour advancement. These provide evidence that clarifies the association between ID-1 overexpression and tumour angiogenesis in human primary cancer.

Inhibitor of DNA-binding-1 expression was high in two androgen-independent prostate cancer cell lines, DU145 and PC3, but its expression is undetectable in the androgen-dependent cell line, LNCaP, under serum-free conditions (Ling et al., 2003). As prostate cancer progression and aggressiveness are judged by its response to androgen, these results support the role of ID-1 expression in tumour progression. Furthermore, ID-1 expression was higher in highly aggressive breast cancer cell lines such as MDA-MB321 and MDA-MB-435 compared with the less aggressive cell lines such as MCF7 and T47D. Ectopic ID-1 expression in T47D cells led to increased cell growth, invasion ability and reduced sensitivity to sex hormones (Lin et al., 2000). In addition, stable transfection of breast cancer cells with the antisense ID-1 vector also reduces lung metastasis after their inoculation into nude mice (Fong et al., 2003). These results support the role of ID-1 in metastasis of breast cancer and also suggest inactivation of ID-1 as a therapeutic strategy in the treatment of metastatic breast cancer. Therefore, ID-1 protein may be an important new target molecule for antiangiogenic drug design in cancer treatment. It is strongly suggested that transcriptional suppression of TSP-1 by ID-1 may play an important part in promoting angiogenesis in the uterine cervical cancers.

To conclude, this study suggests that ID-1 promotes tumour advancement of uterine cervical cancer through angiogenesis, leading to poor prognosis, and provides a novel therapeutic approach in uterine cervical cancers.

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