Vascular endothelial growth factor, matrix metalloproteinases, and cyclooxygenase-2 influence prognosis of uterine cervical cancer in young women

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Abstract. Recent changes in the lifestyle of young women have led to an increase in the rate of uterine cervical cancer. We investigated the clinicopathological characteristics of uterine cervical cancer in young women, and examined the expression of vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs) and cyclooxygenase-2 (COX-2). Tumor samples from 439 patients with uterine cervical cancer, who were initially treated at Osaka City University Medical School Hospital, Japan between 1995 and 2004, were stained immunohistochemically. The patients were classified into two groups according to age at onset: group Y included women aged ≤35 years, and group O included women aged ≥36 years. Group Y had more cases of squamous cell carcinoma, while group O had more advanced cases ($P<0.05$). Advanced cases (beyond stage Ib2) had a significantly worse prognosis in group Y than in group O ($P<0.05$). There were no differences between the two groups in the expressions of VEGF, MMP-2 and COX-2. However, in advanced cases (beyond stage Ib2), the expression of VEGF, MMP-2 and COX-2 was significantly greater in group Y than in group O ($P<0.05$). The above findings suggest that the expression of VEGF, MMPs and COX-2 is related to a worse prognosis for advanced uterine cervical cancer in young women.

Introduction

During the 1970s, uterine cervical cancer accounted for >90% of all uterine cancer in Japan, but since 1980, its prevalence has been declining. However, the incidence of uterine cervical cancer is increasing for young women in their 20s and 30s, despite the fact that the average age of the Japanese population is increasing (1-3). Commonly, uterine cervical cancer advances faster in young women than in older women. However, to the best of our knowledge, there have been no studies documenting this trend.

Various processes are involved with cancer invasion and metastasis, and they can be roughly divided into the following: i) an increase in the size of the primary lesion induces interstitial fusion, vascularization and lymph duct formation; ii) cancer cells detach from the primary lesion and produce proteases that destroy the surrounding matrix and basal membrane, and invade vessels; iii) cancer cells migrate inside the vessels and then adhere to endovascular cells of a target organ, which is then invaded; and iv) cancer cells multiply and form a metastatic lesion. Previous studies have documented that various proteins are involved in these processes.

The vascular endothelial growth factor (VEGF) family is a group of specific angiogenic agents that act to increase vessel permeability, as well as endothelial cell growth, proliferation and differentiation (4,5). Currently, at least six members of the VEGF family have been identified (6-12). Recent studies have shown that higher VEGF expression is correlated with poor prognosis in many human malignancies (16-23), including uterine cervical cancer (21-23).

Matrix metalloproteinases (MMPs) are thought to play a central role in stromal invasion, given their ability to penetrate the basement membrane and degrade the underlying extracellular matrix (ECM) components (24,25). At least 20 different MMP family members have been identified. Elevated expression of MMP-2 and MMP-9 has been reported in several types of human cancer (26-29), including uterine cervical carcinoma (30,31).

It is well known that cyclooxygenase (COX)-2, a key enzyme involved in the conversion of arachidonic acid to prostaglandins, plays a significant role in carcinogenesis, in addition to its well-established role in inflammatory reactions (32,33). Several studies have shown that COX-2 is up-regulated in many human malignancies (34-36), including uterine cervical cancer (37,38).

Thus, our hypothesis is that these proteins are related to the differences in the clinical course between young and older patients with uterine cervical cancer. Therefore, we

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Table I. Patient characteristics.

	Group Y	Group O
No. of cases	61	378
Age[a] (years)	31.1±3.2 (25-35)	57.9±13.2 (36-89)
Pathological types		
Squamous cell carcinoma	55	306
Adenocarcinoma	4	51
Adenosquamous cell carcinoma	1	7
Others	1	14
FIGO stage		
I	47	161
II	5	78
III	6	102
IV	3	37

[a]Mean ± SD.

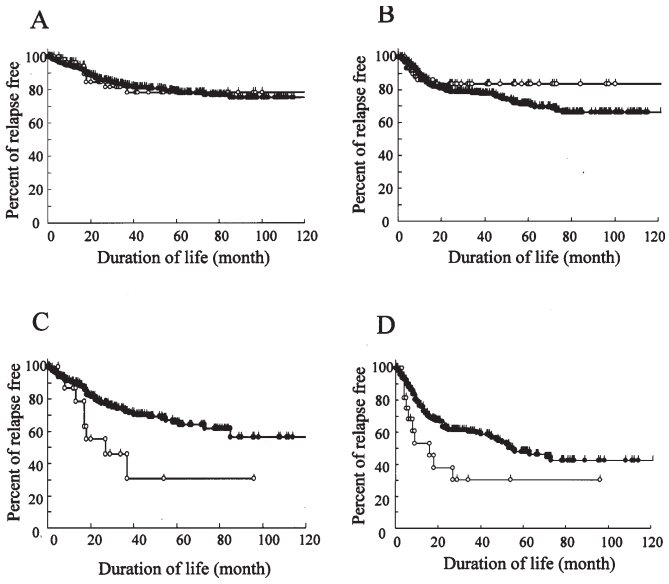

Figure 1. Survival in group Y (open circles) and group O (closed circles). There was no significant difference between the two groups (A and B). In the advanced cases beyond stage Ib2, group Y patients had significantly ($P<0.05$) worse overall and disease-free survival (C and D). Kaplan-Meier and Generalized Wilcoxon calibration.

investigated the prognosis of uterine cervical cancer in young and older patients over the past 10 years. Expression of VEGF, MMP-2, MMP-7, MMP-9 and COX-2 was determined immuno-histochemically and compared between young and older patients.

Materials and methods

Patients and samples. We reviewed 439 cases of uterine cervical cancer that were initially treated at Osaka City University Medical School Hospital, Japan between 1995 and 2004 (Table I). Tumor samples were obtained from punch biopsies or from primary surgery. The patients were classified into two groups according to age at onset: group Y included women who were ≤35 years of age, and group O included women who were ≥36 years of age. The groups were compared with respect to pathological type, clinical stage, overall survival and disease-free survival. Based on the International Federation of Gynecology and Obstetrics (FIGO) criteria, the 61 group Y cases were classified into stage I (n=47), stage II (n=5), stage III (n=6) and stage IV (n=3), and the 378 group O cases were classified into stage I (n=161), stage II (n=78), stage III (n=102) and stage IV (n=37). Clinicopathologically, the 61 group Y cases were classified into squamous cell carcinoma (n=55), adenocarcinoma (n=4), adenosquamous cell carcinoma (n=1) and others (n=1), and the 378 group O cases were classified into squamous cell carcinoma (n=306), adenocarcinoma (n=51), adenosquamous cell carcinoma (n=7) and others (n=14). Written informed consent was obtained from all patients prior to immunohistochemical examination.

Immunohistochemical analysis. Expression of MMP-2, MMP-7, MMP-9, VEGF and COX-2 was investigated in paraffin-embedded sections using the avidin-biotin-peroxidase complex method. The paraffin sections (5 μm thick) were de-paraffinized and immersed in 3% hydrogen peroxidase in methanol to block endogenous peroxidase activity. Next, an antigen retrieval procedure was performed by immersing the slides in 10 mM citrate buffer (pH 6.0) and heating in an autoclave at 121˚C for 10 min. After washing in PBS, the tissue sections were preblocked using 10% normal goat serum for 15 min. The protocol for the Dako LSAB 2 peroxidase kit (Dako, Kyoto, Japan) was followed. The sections were incubated overnight with the primary antibodies in a humidity chamber at 4˚C. The primary antibodies used for this study were monoclonal mouse anti-human MMP-2, MMP-7, MMP-9 (Fuji Chemical Industries, Toyama, Japan), VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) and COX-2 (IBL, Gunnma, Japan). The working dilutions for each primary antibody were 1:100 for anti-MMP-2 and MMP-9, 1:50 for anti-MMP-7, 1:100 for VEGF, and 1:10 for COX-2. Sections were rinsed with PBS for 15 min and incubated for 1 h with the secondary antibody (biotinylated goat anti-mouse and rabbit immunoglobulin G secondary antibody; Dako). The sections were then incubated with streptavidin-peroxidase complex, and 3,3'-diaminobenzidine was used as a chromogen. The sections were counterstained with Mayer's hematoxylin. The specificity of the immunohistochemical reactions was checked by omitting the primary antibody.

The quantitative analysis of MMP-2, MMP-7, MMP-9, VEGF and COX-2 expression was based on the scoring method of Sinicrope *et al* (39). The mean percentage of positive tumor cells was determined in five areas at x400 magnification and assigned to one of the following categories: 0, <5%; 1, 5-25%; 2, 25-50%; 3, 50-75%; or 4, >75%. The intensity of immunostaining was scored as follows: 1+, weak; 2+, moderate; or 3+, intense. For each specimen, the percentage of positive tumor cells was multiplied by the staining intensity to produce a weighted score.

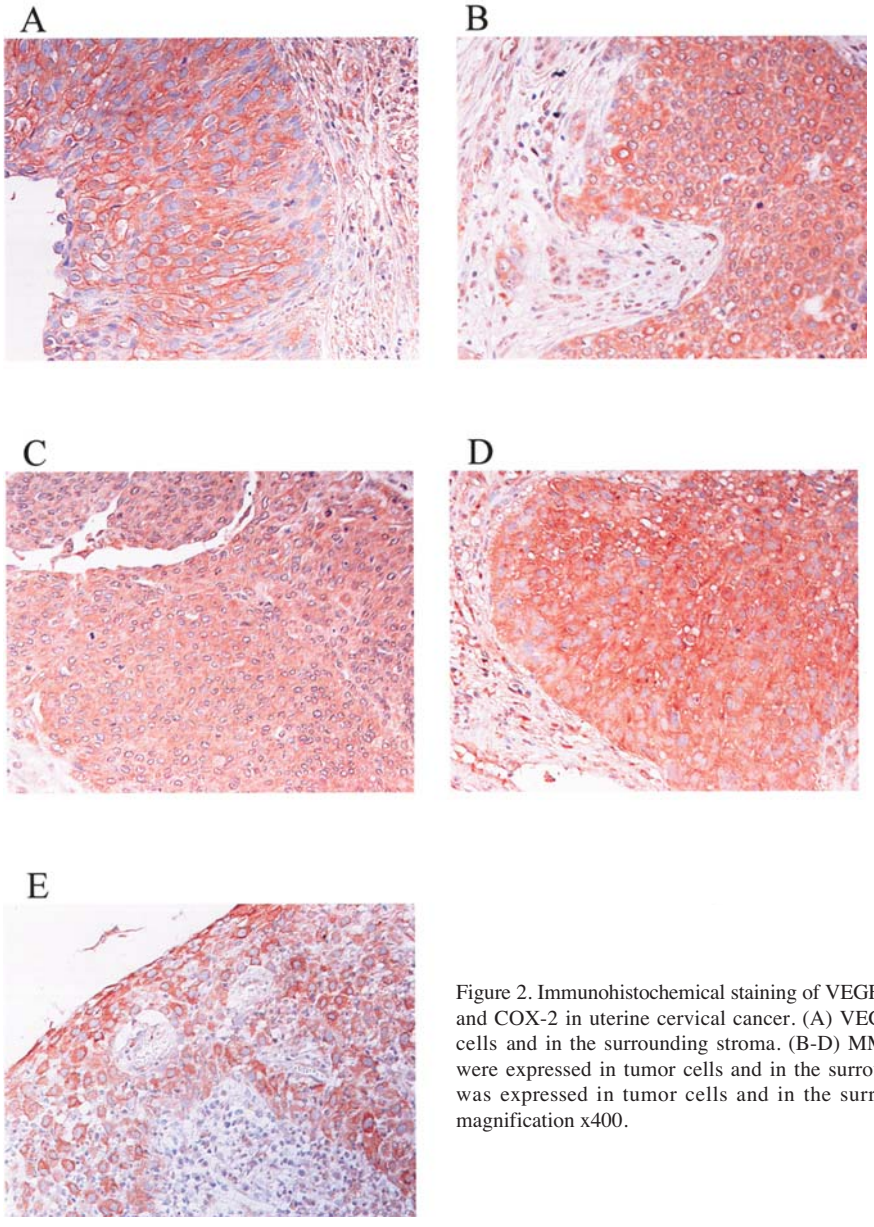

Figure 2. Immunohistochemical staining of VEGF, MMP-2, MMP-7, MMP-9 and COX-2 in uterine cervical cancer. (A) VEGF was expressed in tumor cells and in the surrounding stroma. (B-D) MMP-2, MMP-7 and MMP-9 were expressed in tumor cells and in the surrounding stroma. (E) COX-2 was expressed in tumor cells and in the surrounding stroma. Original magnification x400.

Statistics. For the prognostic analyses, Kaplan-Meier and Generalized Wilcoxon calibration were used. StatView 5.0 (Abacus Concepts, Berkley, CA, USA) was used for data analysis. Statistical significance was set at the 0.05 level. Continuous variables are expressed as the mean ± standard deviation (SD) and mean ± standard error (SE), as shown in the figures. The weighted scores were compared using the Mann-Whitney U test. Student's t-test was used to compare the number of invading cells between groups.

Results

Patient characteristics. The number of cases in group Y has been increasing over the 10 years from 1995 to 2004 (data not shown). The mean age of group Y was 31.1±3.2 years (range, 25-35 years) and that of group O was 57.9±13.2 years (range, 36-89 years). There were significantly more cases of squamous cell carcinoma in group Y than in group O ($P<0.05$) (Table I). There were significantly more advanced stages in group O than in group Y ($P<0.05$) (Table I).

Survival. There was no significant difference between group Y and group O in overall and disease-free survival (Fig. 1A and B). However, group Y patients had a significantly worse overall and disease-free survival in the advanced cases beyond stage Ib2 (Fig. 1C and D).

Expression of VEGF. VEGF was expressed in the cytoplasm of tumor cells and in the surrounding stroma (Fig. 2A). Overall, there was no significant difference in VEGF expression between group Y and group O (Fig. 3A). However, in the advanced cases beyond stage Ib2, VEGF expression was significantly greater in group Y than in group O (Fig. 3B).

Expression of MMPs. MMP-2, MMP-7 and MMP-9 were expressed in the cytoplasm of tumor cells and in the surrounding stroma (Fig. 2B and D). Overall, there was no significant difference in MMP-2 expression between group Y and group O (Fig. 4A). However, in the advanced cases beyond stage Ib2, MMP-2 expression was significantly greater in group Y than in group O (Fig. 4B). MMP-7

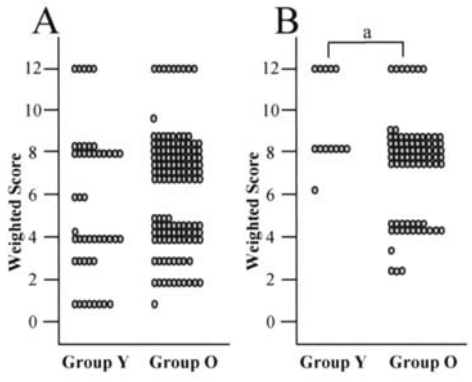

Figure 3. Weighted score of VEGF in uterine cervical cancer for all cases (A) and in the advanced cases beyond Stage Ib2 (B). (a) P<0.05 (Mann-Whitney U test).

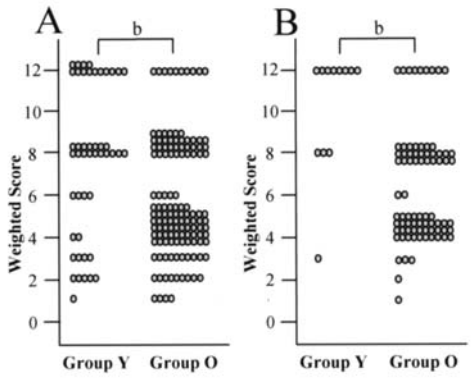

Figure 6. Weighted score of MMP-9 in uterine cervical cancer for all cases (A) and in the advanced cases beyond stage Ib2 (B). (b) P<0.01 (Mann-Whitney U test).

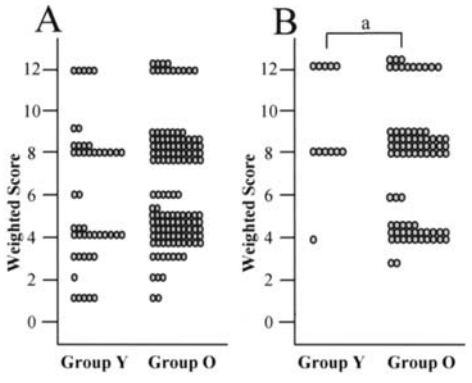

Figure 4. Weighted score of MMP-2 in uterine cervical cancer for all cases (A) and in the advanced cases beyond stage Ib2 (B). (a) P<0.05 (Mann-Whitney U test).

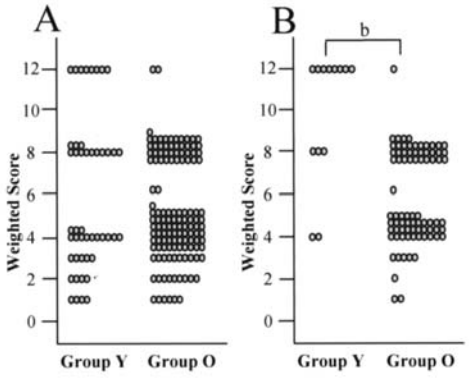

Figure 7. Weighted score of COX-2 in uterine cervical cancer for all cases (A) and in the advanced cases beyond stage Ib2 (B). (b) P<0.01 (Mann-Whitney U test).

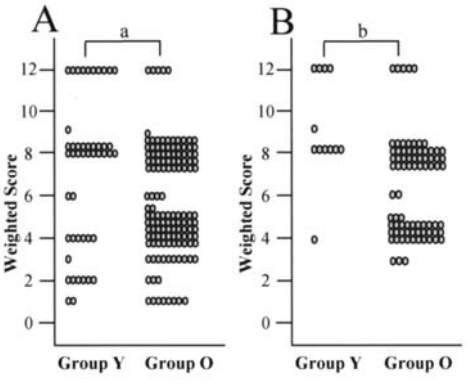

Figure 5. Weighted score of MMP-7 in uterine cervical cancer for all cases (A) and in the advanced cases beyond stage Ib2 (B). (a) P<0.05; (b) P<0.01 (Mann-Whitney U test).

expression was significantly greater in group Y than in group O, both overall and in the advanced cases beyond stage Ib2 (Fig. 5). However, in the advanced cases beyond stage Ib2, MMP-7 expression was much greater in group Y than in group O (Fig. 5B). MMP-9 expression was significantly greater in group Y than in group O, both overall and in the advanced cases beyond stage Ib2 (Fig. 6).

Expression of COX-2. COX-2 was expressed in the cytoplasm of tumor cells and in the surrounding stroma (Fig. 2E). Overall, there was no significant difference in COX-2 expression between group Y and group O (Fig. 7A). However, in the advanced cases beyond stage Ib2, COX-2 expression was significantly greater in group Y than in group O (Fig. 7B).

Discussion

While squamous cell carcinoma accounts for most uterine cancers, adenocarcinoma and adenosquamous cell carcinoma account for ~15%. Compared to squamous cell carcinoma, lymph node metastasis is likely to occur early in adenocarcinoma, which has a worse prognosis because it is often unresponsive to therapy (40,41). In the present study, when compared to group O, group Y had a significantly higher incidence of squamous cell carcinoma and early cancer (Table I). However, there was no significant difference in the overall survival between the two groups (Fig. 1A and B). Among patients with advanced cancer beyond stage Ib2, the prognosis was significantly poorer for group Y than for group O (Fig. 1C and D). Tumor size is also considered to be a prognostic factor for uterine cervical cancer; tumor size is part of the FIGO clinical staging system (40-42).

Many studies have reported a correlation between tumor size and vascular invasion (43,44); this suggests that vascular invasion is related to the poor prognosis in young women with advanced cancer beyond stage Ib2.

The VEGF family is a group of specific angiogenic agents that act to increase vessel permeability, as well as endothelial cell growth, proliferation and differentiation (4,5). Recent studies have shown that higher VEGF expression is correlated with a poor prognosis in lung cancer (13,14), breast cancer (15), adenocarcinoma of the gastrointestinal tract (16), hepatocellular carcinoma (17), renal cell carcinoma (18), ovarian cancer (19), endometrial carcinoma (20), and uterine cervical cancer (21-23). In our study, in the advanced cases beyond stage Ib2, VEGF expression was significantly greater in group Y than in group O (Fig. 3B). These findings suggest that VEGF expression is related to poor prognosis in young women with advanced uterine cervical cancer beyond stage Ib2.

MMPs are thought to be critical for stromal invasion and subsequent metastasis, because these proteases can degrade many ECM components and other substances (24,25). Elevated expression of MMP-2 and MMP-9 has been reported in ovarian carcinoma (26), rectal cancer (27), gastric carcinoma (28), head and neck carcinoma (29), and uterine cervical cancer (30,31). Elevation of MMP-7 expression has been reported in uterine endometrial carcinoma (45) and gastric carcinoma (46). In the present study, in advanced cases beyond stage Ib2, MMP-2 expression was significantly greater in group Y than in group O (Fig. 4B), and MMP-7 expression was much greater in group Y than in group O (Fig. 5B). These findings suggest that expression of MMP-2 and MMP-7 is related to the poor prognosis of young women with advanced uterine cervical cancer beyond stage Ib2. There was no significant difference in MMP-9 expression among the stages (Fig. 6), but MMP-9 expression was significantly greater in group Y than in group O, and there was a significant difference in prognosis between group Y and group O. These findings suggest that MMP-9 expression may be related to the poor prognosis of young women with uterine cervical cancer.

COX-2 is a key enzyme that is involved in the conversion of arachidonic acid to prostaglandins. It plays a significant role in cell-cycle regulation, inhibition of apoptosis, and pathological angiogenesis. COX-2 promotes carcinogenesis, tumor proliferation, and the spread of disease (32,33). Several studies have shown that COX-2 is up-regulated in many human malignancies (34-36), including uterine cervical cancer (37,38). COX-2 expression was significantly greater in group Y than in group O in those with advanced uterine cervical cancer beyond stage Ib2 (Fig. 7B). These findings suggest that COX-2 expression is related to the poor prognosis of young women with advanced uterine cervical cancer beyond stage Ib2.

The results of the present study suggest that VEGF, MMPs and COX-2 are related to the poor prognosis of young women with uterine cervical cancer. However, the mechanism by which these factors affect survival has not yet been clarified. At present, we are in the process of clarifying the mechanism and examining therapy that targets these proteins.

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