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CORRELATION BETWEEN VASCULAR ENDOTHELIAL GROWTH FACTOR-C EXPRESSION AND INVASION PHENOTYPE IN CERVICAL CARCINOMAS

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The correlation between vascular endothelial growth factor (VEGF)-C gene expression and *in vitro* invasive activity and matrix metalloproteinase (MMP)-2 or 9 gene expression and proteolytic activity in 11 cervical carcinoma cell lines, was investigated. Immunohistochemical expression of VEGF-C in 52 cervical carcinoma tissues was also correlated with tumor aggressiveness with respect to clinicopathologic features, tumor vascularity, MMP-2 expression and patient outcome. Expression of VEGF-C mRNA differed remarkably among the cell lines and there was a statistical correlation between VEGF-C gene expression and the number of invaded tumor cells ($p = 0.0009$) and MMP-2 gene expression and activity ($p < 0.05$). Anti-VEGF-C antibody inhibited the invasive and proteolytic activity of tumor cells in a concentration-dependent manner. VEGF-C or MMP-2 expression in clinical tissue samples was well correlated with depth of myometrial invasion, endometrial invasion, pelvic lymphnode metastasis and tumor vascularity ($p < 0.05$) and there was a close relation between VEGF-C and MMP-2 expression ($p < 0.0001$) in cervical carcinomas. Overall survival rates for 14 patients with strong VEGF-C staining tumors were lower than those for 38 patients with weak VEGF-C staining tumors ($p = 0.0132$) and VEGF-C tissue status emerged as an independent prognostic parameter ($p = 0.0232$). These results suggest that VEGF-C expression is closely related to invasion phenotype and affects the patient's survival in cervical carcinomas.

Key words: *VEGF-C; MMP; invasion; angiogenesis; cervical carcinoma*

Tumor angiogenesis is essential for tumor development. Prevascular tumors may remain dormant *in situ* for months to years and the switching from a subgroup of prevascular tumor cells to an angiogenic phenotype enables rapid growth, progression and metastasis.[1] Angiogenesis is induced by factors produced by the tumor or the nonmalignant cells that infiltrate the tumor. Various peptide growth factors, such as vascular endothelial growth factor (VEGF),[2,3] basic fibroblast growth factor (bFGF)[4,5] and thymidine phosphorylase (dThdPase)[6–8] have been found to stimulate the proliferation and motility of endothelial cells, thus inducing new blood vessel formation. VEGF-A, also known as vascular permeability factor, is considered to play an important role in tumor angiogenesis.[3] Recently, 3 new members of the VEGF family, VEGF-B, -C and -D, have been discovered and characterized.[9–11] It has been suggested that VEGF family members, bFGF and dThdPase are expressed in a variety of human tumors in different ways.

Metastatic spread of the solid tumor depends on a critical cascade of events that includes tumor cell adhesion to a distant site, extracellular matrix degradation, migration, proliferation and ultimately neovascularization.[12] The primary tumor with a high proportion of angiogenic cells is likely to give rise to metastatic implants that are already angiogenic, enabling them to grow in lymphnodes and distant organs.[13] Tumors that produce a higher level of angiogenic factors may have a more aggressive behavior than tumors negative for those factors in the process of invasion and metastasis. Among various angiogenic factors, VEGF-C is ranked first as a lymphoangiogenic factor,[14] which induces lymphatic proliferation and spread of solid tumors. Lymph node status remains the strongest prognostic factor in a variety of human malignant tumors including uterine cervical carcinomas and lymph node metastasis is promoted by tumor cell aggressiveness, such as abilities of motility and invasiveness. There have, however, been very few reports on the direct correlation between the expression of VEGF-C gene and invasive activity in tumor cells.[15–17]

In our present study, we investigated the mRNA expression levels of VEGF-C gene in various cervical carcinoma cell lines and correlated them with invasion phenotype of the cells. Moreover, we examined the correlation between VEGF-C expression and tumor aggressiveness in cervical carcinomas with respect to clinicopathologic features and patient outcome.

MATERIAL AND METHODS

Cell culture

Experiments were conducted using 7 human cervical squamous cell carcinoma (SKG-I, SKG-II, SKG-IIIa, SKG-IIIb, OMC-1, YUMOTO and QG-U) and 4 adenocarcinoma (HOKUG, NUZ, OMC-4 and CAC-1) cell lines. The OMC-1[18] and OMC-4[19] cell lines were established in our laboratory. The SKG-I,[20] SKG-II,[21] SKG-IIIa and SKG-IIIb[22] cell lines were kindly provided by Dr. Shiro Nozawa, Keio University, Tokyo. The YUMOTO,[23] QG-U[24] and NUZ[25] cell lines were kindly provided by Dr. Naotake Tanaka, Chiba University, Chiba. The HOKUG[26] and CAC-1[27] cell lines were provided by Dr. Isamu Ishiwata, Ishiwata Hospital, Mito and Dr. Osamu Hayakawa, Sapporo Medical College, Sapporo, respectively. All of the 11 cell lines, except for the YUMOTO, QG-U and NUZ cell lines, were maintained as monolayer cultures in Ham's F-12 medium (Flow Laboratories Inc., Irvine, Scotland) supplemented with 10% FBS (Mitsubishi Chemical Co., Tokyo) at 37°C in a humidified incubator with 5% CO_2 in air. The YUMOTO, QG-U and NUZ cell lines were cultured in RPMI-1640 medium (GIBCO BRL, Bethesda, MD) supplemented with 10% FBS as described above. The cells were grown in 75-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark), washed with phosphate-buffered saline (PBS) and then harvested after a brief treatment with 0.1% trypsin solution containing 0.02% EDTA (Flow). The cell viability was determined by trypan blue dye exclusion before use.

Tumor conditioned medium (TCM) was prepared from the culture supernatant of the cells. Briefly, confluent monolayers of tumor cells grown in 6-cm plastic dishes (Corning 25010, Iwaki Glass, Tokyo) were rinsed twice with serum-free Ham's F-12 medium and incubated at 37°C for 24 hr with 4 ml of serum-free medium. The TCM samples thus obtained were condensed 5-fold by using Centricon-10 microconcentrators (AMICON, Division of W.R. Grace & Co., Columbia, MA). The protein concentration of

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TABLE I - PCR PRIMERS AND CONDITIONS

Gene	Primer	Tm (°C)	Product size (bp)
VEGF-C	5'-AGACTCAATGCATGCCACG-3' 5'-TTGAGTCATCTCCAGCATCC-3'	57	435
MMP-2	5'-CCACGTGACAAGCCCATGGGGCCCC-3' 5'-GCAGCCTAGCCAGTCGGATTTGATG-3'	58	480
MMP-9	5'-GTGCTGGGCTGCTGCTTTGCTG-3' 5'-GTCGCCCTCAAAGGTTTGGAAT-3'	60	303
β-actin	5'-CTTCTACAATGAGCTGCGTG-3' 5'-TCATGAGGTAGTCAGTCAGG-3'	60	305

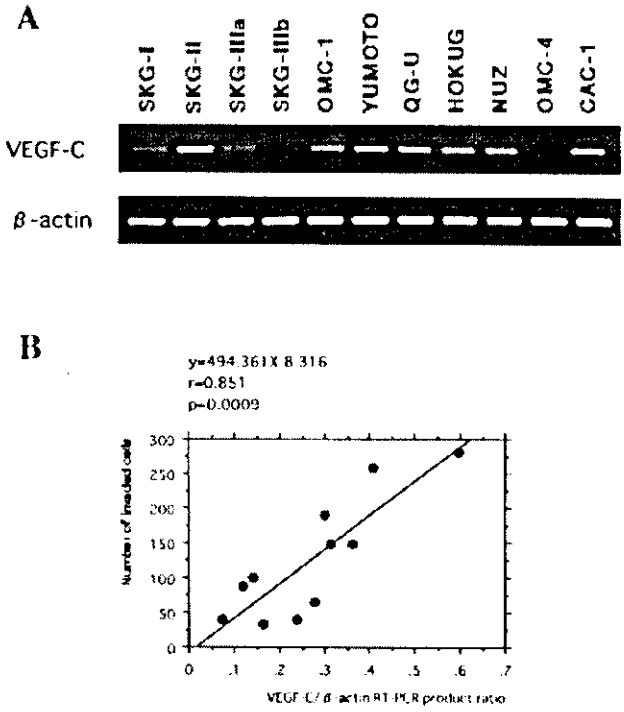

FIGURE 1 – RT-PCR analysis of VEGF-C in 11 human cervical cancer cell lines (*a*) and correlation between VEGF-C:β-actin RT-PCR product ratio and the number of invaded tumor cells (*b*). The primers and the expected sizes from the reported cDNA sequence are shown in Table I. Each point represents the mean of triplicates.

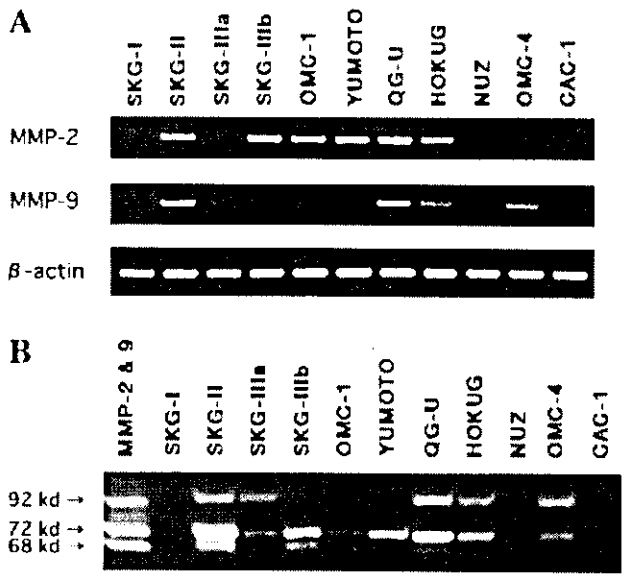

FIGURE 2 – RT-PCR analysis of MMP-2 and 9 in 11 human cervical cancer cell lines (*a*) and the gelatinolytic activities of TCM from the cells (*b*). The primers and the expected sizes from the reported cDNA sequence are shown in Table I. Numbers indicate the molecular weight of the proteinase bands.

TCM was determined by using a DC protein Assay Kit (BioRad Laboratories, Hercules, CA) and then stored at −80°C until use.

RNA isolation and cDNA preparation

RNA was extracted from the cells by SV-total RNA isolation kit (Promega Corp., Madison, WI) according to the supplier's protocol. Contaminating residual genomic DNA was removed by digestion with RNase free DNase (Promega). cDNAs were prepared using at least 2 μg of total RNA and SUPERSCRIPT II reverse transcriptase (GIBCO BRL Life Technologies, Gaithersburg, MD) with random hexamers as primers and were finally dissolved in diethyl pyrocarbonate-treated water and then frozen at −20°C until use.

Reverse transcription-polymerase chain reaction analysis

Oligonucleotide primers for reverse transcription-polymerase chain reaction (RT-PCR) were designed using published sequences of VEGF-C, matrix metalloproteinase (MMP)-2, 9 and β-actin genes and synthesized by the solid-phase triester method. The primers used in our study and the expected sizes from the reported cDNA sequence are shown in Table I. For PCR, cDNA aliquots were diluted in sterile water, depending on transcript abundance. Five cDNA concentrations for target genes/β-actin primer combinations were used. For accurate quantification using this method, measurements must be taken in the linear phase of the reaction, in which cDNA concentration is directly proportional to signal intensity. A range of cDNA concentrations was used to determine the linear phase of the PCR. cDNA template was amplified by PCR in a final volume of 20 μl reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM dNTP, 0.5 μM each primer and 1.25 U Taq polymerase (Perkin-Elmer, Norwalk, CN). After an initial denaturation at 96°C for 3 min, various cycles of denaturation (94°C for 1 min), annealing (60°C for β-actin and 57–60°C for other genes for 1 min) and extension (72°C for 2 min) for the respective target genes were carried out on a Perkin-Elmer GeneAmp PCR System 9700. The final extension was performed at 72°C for 10 min. The number of cycles in the RT-PCR was determined so as to obtain logarithmic amplification of each gene for semi-quantitative analysis of the expression levels of the genes. After visualization of the RT-PCR products by 1.5% agarose gel electrophoresis with ethidium bromide staining, gel images were obtained using the ATTO densitograph UV-image analyzer (ATTO Corp., Tokyo) and the densities of the products were quantified using ATTO densitometry software version 2. The relative expression levels were calculated as the density of the product of respective target

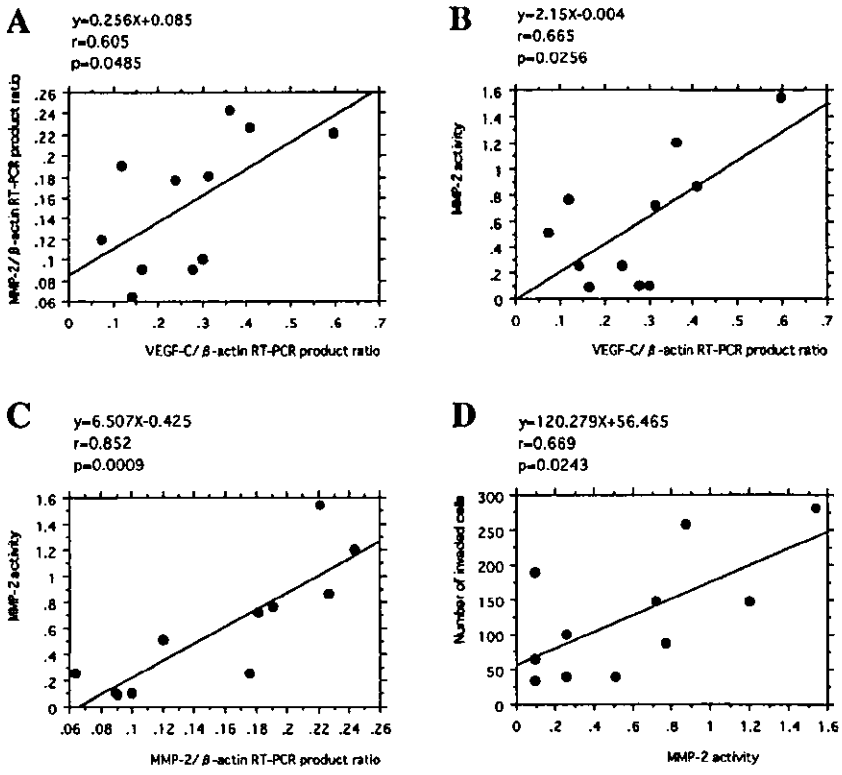

FIGURE 3 – Correlation between VEGF-C and MMP-2 gene expression (*a*), VEGF-C gene expression and MMP-2 activity (*b*), MMP-2 gene expression and activity (*c*) and MMP-2 activity and the number of invaded tumor cells (*d*). Each point represents the mean of triplicates.

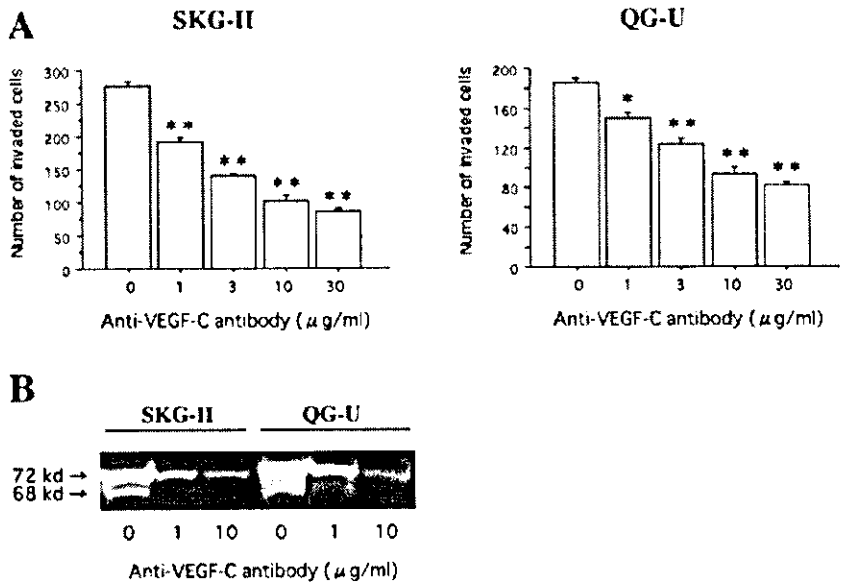

FIGURE 4 – Effects of anti-VEGF-C antibody on the invasive activity of SKG-II and QG-U cells into reconstituted basement membrane components (*a*) and the gelatinolytic activities of TCM from these cells (*b*). Numbers indicate the molecular weight of the proteinase bands. Scale bars: SE, $*p = 0.0006$ vs. control; $**p < 0.0001$ *vs.* control.

genes divided by that of β-actin from the same cDNA. Each analysis was performed in triplicate.

Haptoinvasion assay

The invasive activity of tumor cells was assayed in Chemotaxicell culture chambers (Kurabo, Osaka) according to the method reported by Albini *et al.*[28] with some modifications as described previously.[29] Polyvinylpyrroridone-free polycarbonate filters with 8.0 μm pore size were precoated with 10 μg of fibronectin in a volume of 50 μl of PBS on the lower surface and dried overnight at room temperature under a hood. The matrigel diluted to 500 μg/ml with cold PBS was then applied to the upper surface of the filters (5 μg/filter) and dried again. Log-phase cell cultures of tumor cells were harvested with 0.1% trypsin containing 0.02% EDTA and resuspended to a final concentration of 3.0×10^6/ml in growth medium. Cell suspension (200 μl) was added to the upper compartment and 600 μl of growth medium was immediately added to the lower compartment. The chambers were then incubated for 24 hr at 37°C in a 5% CO_2 air. The filters were fixed with ethanol and stained with hematoxylin. The cells on the upper

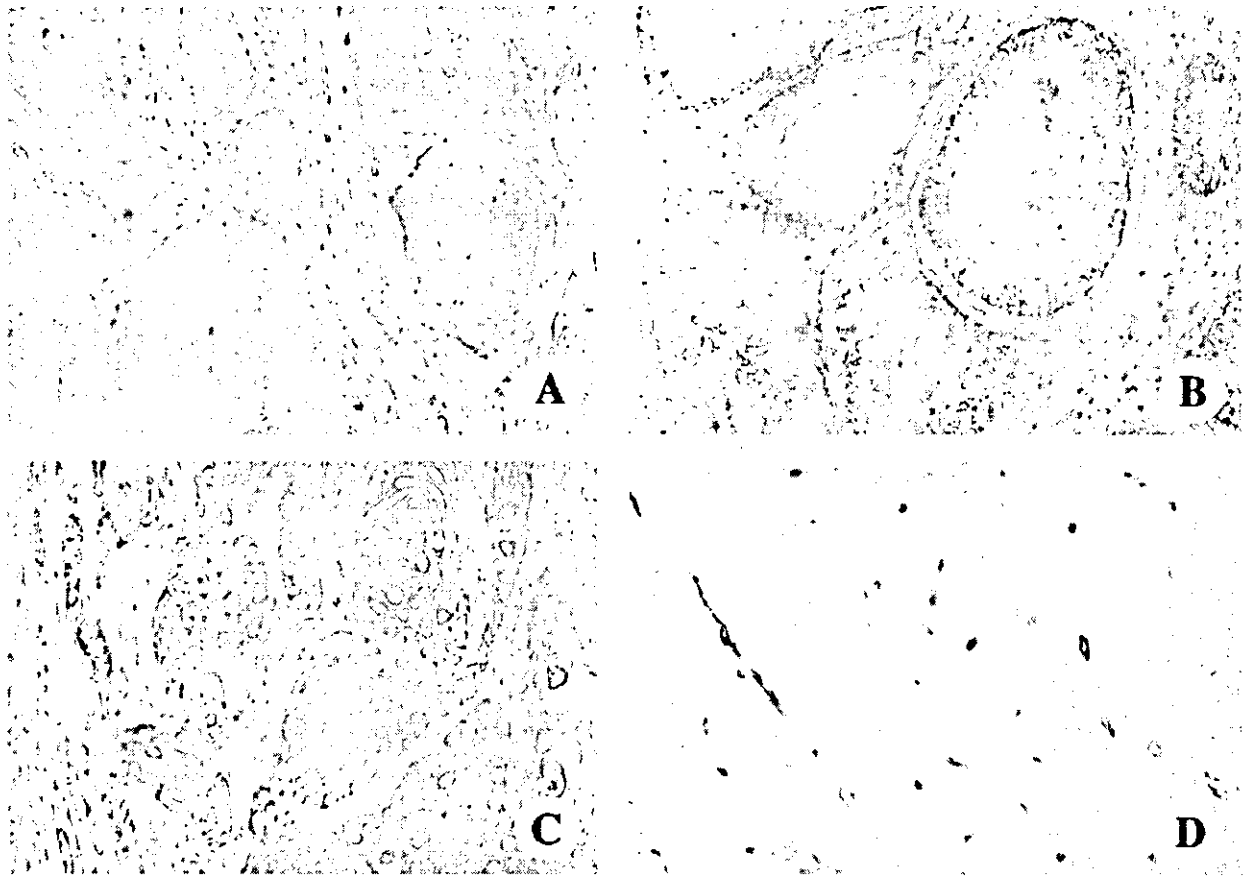

FIGURE 5 – VEGF-C and MMP-2 expression and intratumoral microvessels in cervical carcinoma. Immunoreactivity of VEGF-C and MMP-2 is identified in the cytoplasm of tumor cells. There are 2+ VEGF-C-positive staining in squamous cell carcinoma (*a*) and adenocarcinoma (*b*) and 2+ MMP-2-positive staining in squamous cell carcinoma (*c*). Intratumoral microvessels are detected as consistent staining of endothelial cells using anti-CD34 antibody (*d*). Magnification ×200 (*a–d*).

surface of the filters were removed by wiping with a cotton swab. The cells that had invaded to various areas of the lower surface were manually counted under a microscope at a magnification of ×400. Each assay was performed in triplicate.

Zymograms

The proteolytic activity of TCM was examined by electrophoresis in a gelatin-embedded polyacrylamide gel, based on the methods described by Heussen and Dowdle.[30] The equal protein concentration of each TCM sample was mixed with sodium dodecyl sulfate (SDS) sample buffer containing 1 mM phenylmethylsulfonyl fluoride and applied, without heating or reducing, to polyacrylamide gels containing 1 mg/ml of gelatin. After electrophoresis, the gels were washed twice with 2.5% Triton X-100 for 60 min to remove the SDS, incubated in the incubation buffer containing 0.15 M NaCl, 50 mM Tris-HCl, 10 mM $CaCl_2$, 0.05% NaN_3 for 30 hr and then stained in 0.1% Coomassie Blue. Gel images of unstained bands were obtained using the ATTO densitograph and their densities were quantified using ATTO densitometry software version 2. The relative gelatinolytic levels were calculated as the density of each unstained band divided by that of purified MMP-2 or 9 simultaneously examined. Each assay was performed in triplicate.

Effects of anti-VEGF-C antibody on invasion phenotype of tumor cells

Effects of anti-VEGF-C antibody (C-20, Santa Cruz, CA) on the invasive and proteolytic activity of tumor cells were examined. Log-phase cell cultures of SKG-II and QG-U cells were harvested and resuspended to a final concentration of 3.0×10^6/ml in growth medium. Cell suspension (200 μl), with or without various amounts of anti-VEGF-C antibody, was added to Chemotaxicell culture chambers and the cells that had invaded were counted as described above. In addition, gelatinolytic levels of TCM samples from SKG-II and QG-U cells incubated with or without anti-VEGF-C antibody, were also analyzed by zymograms as described above. Each assay was performed in triplicate.

Patients and tissue samples

A total of 52 primary cervical carcinomas, which had been resected in our department were used in our study. Patients had received neither chemotherapy nor radiation therapy before surgery. All patients underwent radical hysterectomy with bilateral adnexectomy and bilateral pelvic lymphadenectomy. Clinical stages and pathological diagnosis were decided according to the classification of the International Federation of Gynecology and Obstetrics.[31] Of these patients, 19 had Stage Ib, 4 had Stage IIa and 29 had Stage IIb disease. Data concerning patient outcome, including overall survival and development of metastasis, were available for all 52 patients. Tumor specimens were fixed in 10% buffered formalin and embedded in paraffin wax. Histological features in resected tumors were assessed using standard hematoxylin and eosin-stained sections. Serial sections including the greatest diameter of the tumors from the operative specimens were used for the present study.

TABLE II – CORRELATION BETWEEN VEGF-C EXPRESSION AND CLINICOPATHOLOGICAL FEATURES

Variable	−/1+ Expression (%) (n = 38)	2+ Expression (%) (n = 14)	p value[1]
Age			
≤50 years	17 (44.7)	10 (71.4)	NS
>50 years	21 (55.3)	4 (28.6)	
Clinical stage			
Stage 1	13 (34.2)	6 (42.9)	NS
Stage 2	25 (65.8)	8 (57.1)	
Histologic type			
Squamous	30 (78.9)	8 (57.1)	NS
Adenosquamous and adeno	8 (21.1)	6 (42.9)	
Histologic grade			
Well differentiated	11 (28.9)	5 (35.7)	NS
Moderately and poorly differentiated	27 (71.1)	9 (64.3)	
Depth of invasion[2]			
1–2	32 (84.2)	4 (28.6)	0.0001
3–4	6 (15.8)	10 (71.4)	
Endometrial invasion			
Negative	33 (86.8)	8 (57.1)	0.0200
Positive	5 (13.2)	6 (42.9)	
Pelvic lymphnode metastasis			
Negative	34 (89.5)	7 (50.0)	0.0020
Positive	4 (10.5)	7 (50.0)	
Ovarian metastasis			
Negative	37 (97.4)	14 (100)	NS
Positive	1 (2.6)	0 (0)	
Vaginal infiltration			
Negative	27 (71.1)	9 (64.3)	NS
Positive	11 (28.9)	5 (35.7)	
IMVD			
<57.3	27 (71.1)	2 (14.3)	0.0003
>57.3	11 (28.9)	12 (85.7)	
MMP-2			
−/1+ Expression	32 (84.2)	1 (7.1)	<0.0001
2+ Expression	6 (15.8)	13 (92.9)	

[1]NS, no significant difference.–[2]Both the uterine cervix and parametrium are halved equally, and the deepest section of the primary tumor is classified from 1–4 of myometrial invasion.

Immunohistochemical staining for VEGF-C or MMP-2

An immunohistochemical study for VEGF-C or MMP-2 was performed using the avidin-biotin-peroxidase complex method. De-waxed and rehydrated tissue sections were incubated overnight at 4°C with goat polyclonal anti-VEGF-C (Santa Cruz) or mouse monoclonal anti-MMP-2 antibody (Fuji, Toyama, Japan) at a 1:50 dilution and then washed with PBS. Biotinylated rabbit anti-goat or horse anti-mouse immunoglobulin (DAKO, Kyoto, Japan) was then added to the sections for 30 min at room temperature. Peroxidase-conjugated avidin (DAKO) was applied after the sections were washed with PBS. Peroxidase activity was detected by exposure of the sections to the solution of 0.05% 3,3'-diaminobenzidine and 0.01% H_2O_2 in Tris-HCl buffer at pH 7.6 for 3–6 min at room temperature. The sections were counter-stained with hematoxylin. Normal goat or mouse IgG was used as a substitute for the primary antibody for the negative controls. The immunoreactivity of VEGF-C or MMP-2 is expressed as a percentage of positively stained cancer cells/total number of cancer cells and assigned to 1 of 3 subgroups: −, <10%; 1+, 10–50%; 2+, >50%. Characterization of VEGF-C or MMP-2 expression was performed independently by the 2 investigators who had no knowledge of clinicopathologic features or outcomes.

Determination of intratumoral microvessel density

To highlight endothelial cells,[32] de-waxed and rehydrated tissue sections were incubated overnight at 4°C with mouse monoclonal anti-CD34 antibody (QB-END/10, Novocastra Laboratory, Newcastle, UK) at a 1:50 dilution and then washed with PBS. The following steps were the same as those used for the anti-VEGF-C or MMP-2 protocol. The sections showed a frequently heterogeneous staining pattern for anti-CD34 antibody. For the determination of intratumoral microvessel density (IMVD), the 5 most vascular areas within a section were selected and counted under a light microscope with a 200-fold magnification (*i.e.*, ×20 objective lens and ×10 ocular lens; 0.7386 mm^2 per field) as described by Weidner *et al.*[13] Vessel counting was also performed independently by the 2 investigators as described above. The average numbers were recorded as IMVD for each case.

Statistical analysis

All statistical calculations were carried out using StatView statistical software. The Spearman rank correlation coefficient was used to analyze the relation between 2 different values. The significance of differences between groups was calculated by applying a non-parametric test. The clinical characteristics and IMVD of the patients were compared to the expression of VEGF-C or MMP-2 in the tumor cells and checked by the Mann-Whitney and χ^2 tests. The survival curves were plotted according to the Kaplan-Meier method and their statistical differences were analyzed by the log-rank test. A multivariate Cox proportional hazard model analysis was used to evaluate the statistical strength of independent association between selected covariates and patient survival. A level of $p < 0.05$ was accepted as statistically significant.

RESULTS

VEGF-C gene expression and invasive activity of cervical carcinoma cell lines

As shown in Figure 1*a*, the relative expression levels of VEGF-C gene in comparison with β-actin expression differed remarkably among the cell lines and ranged from 0.073 in OMC-4 cells to 0.596 in SKG-II cells. Interestingly, as shown in Figure 1*b*, there was a statistically significant correlation between VEGF-C gene expression and the number of invaded tumor cells, with a coefficient correlation of 0.851 ($p = 0.0009$).

TABLE III - CORRELATION BETWEEN MMP-2 EXPRESSION AND CLINICOPATHOLOGICAL FEATURES

Variable	−/1+ Expression (%) (n = 33)	2+ Expression (%) (n = 19)	*p* value[1]
Age			
≤50 years	17 (51.5)	10 (52.6)	NS
>50 years	16 (48.5)	9 (47.4)	
Clinical stage			
Stage 1	11 (33.3)	8 (42.1)	NS
Stage 2	22 (66.7)	11 (57.9)	
Histologic type			
Squamous	26 (78.8)	12 (63.2)	NS
Adenosquamous and adeno	7 (21.2)	7 (36.8)	
Histologic Grade			
Well differentiated	9 (27.3)	7 (36.8)	NS
Moderately and poorly differentiated	24 (72.7)	12 (63.2)	
Depth of invasion[2]			
1–2	29 (87.9)	7 (36.8)	0.0001
3–4	4 (12.1)	12 (63.2)	
Endometrial invasion			
Negative	29 (87.9)	12 (63.2)	0.0356
Positive	4 (12.1)	7 (36.8)	
Pelvic lymphnode metastasis			
Negative	29 (87.9)	12 (63.2)	0.0356
Positive	4 (12.1)	7 (36.8)	
Ovarian metastasis			
Negative	32 (97.0)	19 (100)	NS
Positive	1 (3.0)	0 (0)	
Vaginal infiltration			
Negative	23 (69.7)	13 (68.4)	NS
Positive	10 (30.3)	6 (31.6)	
IMVD			
<57.3	24 (72.7)	5 (26.3)	0.0012
>57.3	9 (27.3)	14 (73.7)	

[1]NS, no significant difference.–[2]Both the uterine cervix and parametrium are halved equally, and the deepest section of the primary tumor is classified from 1–4 of myometrial invasion.

MMPs gene expression and activity of cervical carcinoma cell lines

Because proteinase expression is closely related to invasion phenotype of tumor cells, we next examined MMP-2 and 9 gene expression in 11 cervical carcinoma cell lines by RT-PCR analysis. As can be seen in Figure 2*a*, the relative gene expression levels of MMPs in comparison with β-actin expression also differed remarkably among the cell lines. A higher relative expression level of MMP-2 was observed in QG-U cells (0.243) and of MMP-9 in SKG-II cells (0.712). To further investigate the MMPs activity of tumor cells, the enzymatic degradation of gelatin substrate by TCM from the cells was examined in zymograms. As shown in Figure 2*b*, the relative gelatinolytic levels in comparison with purified MMP-2 or 9 activity again differed remarkably among the cell lines. A higher relative gelatinolytic level of MMP-2 (72 kd type IV collagenase) was observed in SKG-II cells (1.542) and of MMP-9 (92 kd type IV collagenase) in QG-U cells (1.200).

Correlation between VEGF-C gene expression and invasion phenotype in cervical carcinoma cell lines

There was a close correlation between VEGF-C gene and MMP-2 gene expression levels (Fig. 3*a*) and activity (Fig. 3*b*), with a coefficient correlation of 0.605 ($p = 0.0485$) and 0.665 ($p = 0.0256$), respectively. MMP-2 activity was well correlated with MMP-2 gene expression (Fig. 3*c*) and the number of invaded tumor cells (Fig. 3*d*), with a coefficient correlation of 0.852 ($p = 0.0009$) and 0.669 ($p = 0.0243$), respectively. Although MMP-9 gene expression levels were well correlated with their activity, there was no statistical correlation between VEGF-C gene and MMP-9 gene expression levels and activity (data not shown).

Effects of anti-VEGF-C antibody on invasion phenotype of tumor cells

As shown in Figure 4*a*, the invasive activity of SKG-II and QG-U cells into reconstituted basement membrane components (Matrigel) was inhibited by the presence of 1–30 μg/ml of anti-VEGF-C antibody in a concentration-dependent manner. Moreover, as can be seen in Figure 4*b*, the gelatin zymography showed that the treatment of these tumor cells with anti-VEGF-C antibody resulted in a decrease of MMP-2 activity in a concentration-dependent manner.

Expression of VEGF-C or MMP-2 and tumor vascularity in cervical carcinomas

The immunoreactivity of VEGF-C or MMP-2 was observed in the cytoplasm of tumor cells (Fig. 5*a–c*). In contrast to the immunoreactivity of VEGF-C or MMP-2, that of anti-CD34 antibody was located only on the cytoplasm of endothelial cells and not on tumor cells or interstitial cells (Fig. 5*d*). The negative control slides for VEGF-C, MMP-2 and CD34 exhibited no specific staining. IMVD for the 52 tumors ranged from 24.8–118.6 with a median value of 57.3 for CD 34 staining. A median value of 57.3 was taken as the cutoff point for discrimination of the 52 patients into 2 subgroups: 29 patients with hypovascular and 23 with hypervascular tumors.

Correlation between VEGF-C expression and clinicopathologic features, tumor vascularity and MMP-2 expression

The clinicopathological data, tumor vascularity and MMP-2 expression from 14 patients whose tumors had 2+ VEGF-C-positive staining and 38 whose tumors had negative or 1+ VEGF-C-positive staining, were compared. As shown in Table II, the immunoreactivity of VEGF-C was well correlated with depth of myometrial invasion ($p = 0.0001$), endometrial invasion ($p = 0.02$), pelvic lymphnode metastasis ($p = 0.002$), tumor vascularity ($p = 0.0003$) and MMP-2 expression ($p < 0.0001$).

Correlation between MMP-2 expression and clinicopathologic features and tumor vascularity

The clinicopathological data and tumor vascularity from 19 patients whose tumors had 2+ MMP-2-positive staining and 33

whose tumors had negative or 1+ MMP-2-positive staining, were compared. As shown in Table III, the immunoreactivity of MMP-2 was well correlated with depth of myometrial invasion (p = 0.0001), endometrial invasion (p = 0.0356), pelvic lymphnode metastasis (p = 0.0356) and tumor vascularity (p = 0.0012).

VEGF-C or MMP-2 expression and patient outcome

Of the 52 patients examined in our study, 12 patients (23.1%) experienced recurrences and 6 (11.5%) had died due to relapse of the disease during follow-up period. The overall survival rates for 14 patients whose tumors had 2+ VEGF-C-positive staining were significantly lower than those for 38 whose tumors had negative or 1+ VEGF-C-positive staining (p = 0.0132) (Fig. 6*a*). The immunoreactivity of MMP-2 and tumor vascularity, however, failed to have statistical correlation with the patient's survival (Fig. 6*b*,*c*). When 4 clinico-pathologic parameters, IMVD, MMP-2 and VEGF-C tissue status, which have been considered as the good prognostic indicators, were entered into multivariate Cox proportional hazard model analysis, only VEGF-C tissue status emerged as an independent prognostic parameter (p = 0.0232) as shown in Table IV.

DISCUSSION

Growth of solid tumors depends on angiogenesis, the process by which new blood vessels develop from the endothelium of a pre-existing vasculate. Tumors promote angiogenesis by secreting various angiogenic factors and newly formed blood vessels induce tumor cell proliferation and invasiveness. VEGF-C is a ligand for VEGF receptor (VEGFR)-3 (Flt-4), a tyrosine kinase receptor that is predominantly expressed in the endothelium of lymphatic vessels.[10] Experimental results with the VEGF-C-transgenic mouse have shown that VEGF-C expression is associated with hyperplasia of lymphatic vessels.[14] The newest member of the VEGF family, VEGF-D, is 48% identical with VEGF-C and VEGF-D can also bind to and activate VEGFR-3.[11] To date, no other growth factor involved in the development of lymphatic vessels has been reported. It is conceivable that VEGF-C and -D might play a crucial role in lymphatic proliferation and spread of solid tumors. Recently, some investigators have demonstrated the close relation between VEGF-C expression and lymphatic invasion and lymphnode metastasis in gastric,[15] breast[16] and thyroid[17] carcinomas. Moreover, it is generally accepted that tumor cell invasion into the lymphatic vessels is established by the destruction of stroma around the lymphatic vessels via activation of matrix-digesting enzymes, which are produced by tumor cells or fibroblasts.[33,34] Therefore, it could reasonably be expected that VEGF-C expression in tumor cells is directly linked to invasion phenotype of the cells.

In our present study, expression of VEGF-C mRNA differed remarkably among the cell lines and there was a statistical correlation between VEGF-C gene expression and the number of invaded tumor cells into reconstituted basement membrane. VEGF-C gene expression was also well correlated with MMP-2 gene expression and activity. MMP-2 (72 kd type IV collagenase) has been considered to play a central role in the process of invasion and metastasis of gynecological tumors.[29,35–39] Moreover, anti-VEGF-C antibody inhibited the invasive and proteolytic activity of tumor cells in a concentration-dependent manner. Tumor cells that produce VEGF-C may have a higher invasive and metastatic potential because of their capacity to pass through tissue barriers by degrading extracellular matrices (ECM) with MMP-2.

VEGF-C requires proteolytic cleavage to produce the fully active form of the factor, that of the central VEGF homology domain.[40] The stepwise proteolytic processing of VEGF-C generates several VEGF-C forms with increased activity toward VEGFR-3, but only the fully processed VEGF-C is able to activate VEGFR-2 (KDR/Flk-1). Like VEGF, mature VEGF-C increases migration and proliferation of vascular endothelial cells. We have recently found that VEGFR-1 (Flt-1) and VEGFR-2 genes are

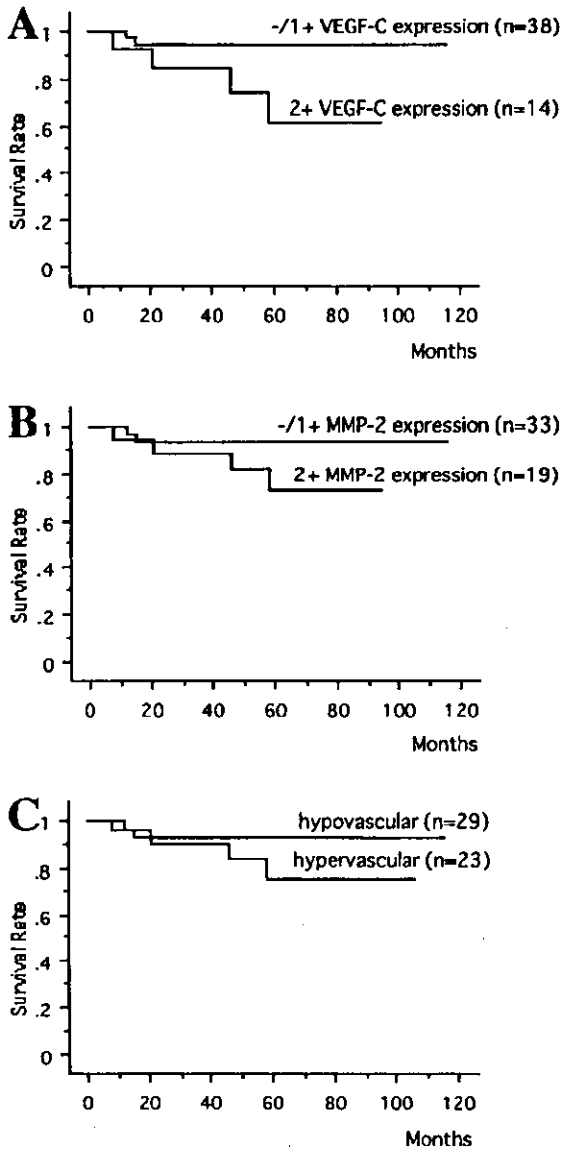

FIGURE 6 – Kaplan-Meier analysis of survival rates of 52 patients according to VEGF-C and MMP-2 expression and tumor vascularity. The overall survival rates for 14 patients whose tumors have 2+ VEGF-C-positive staining are significantly lower than those for 38 whose tumors have negative or 1+ VEGF-C-positive staining (p = 0.0132) (*a*). The immunoreactivity of MMP-2 and tumor vascularity has no statistical correlation with the patient's survival (*b*,*c*).

expressed in several cervical carcinoma cell lines we used here (unpublished data), which gives the possibility of regulating biological functions of the cells with an autocrine loop of VEGF-C. Although our present results indicated the blockade of invasion phenotype of tumor cells by anti-VEGF-C antibody, further studies are needed to demonstrate the link between the expression of several VEGF-C forms and invasive behavior of the cells.

Abundant evidence supports the concept that tumors can induce angiogenesis through a variety of angiogenic molecules,[2–8] and the grade of angiogenesis, expressed as IMVD, is reported to be a strong prognostic indicator in a variety of malignancies.[41] VEGF-A and bFGF have emerged as central regulators of the angiogenic process in physiological and pathological conditions,[2–5] and their expression in tumor cells has been considered to

TABLE IV - MULTIVARIATE COX PROPORTIONAL HAZARD MODEL

Variable	Risk ratio	95% CI[1]	*p* value
Histologic type			
Squamous	0.909	0.441–1.872	0.7948
Adenosquamous and adeno			
Depth of invasion[2]			
1–2	0.395	0.154–1.017	0.0541
3–4			
Endometrial invasion			
Negative	0.806	0.315–2.064	0.6532
Positive			
Pelvic lymphnode metastasis			
Negative	1.050	0.394–2.799	0.9221
Positive			
IMVD			
<57.3	1.924	0.944–3.920	0.0715
>57.3			
MMP-2			
−/1+ Expression	2.841	1.000–8.076	0.0501
2+ Expression			
VEGF-C			
−/1+ Expression	3.305	1.178–9.273	0.0232
2+ Expression			

[1]CI, confidence interval.–[2]Both the uterine cervix and parametrium are halved equally, and the deepest section of the primary tumor is classified from 1–4 of myometrial invasion.

reflect the aggressive biological characteristics of the tumor. Several workers have found that the expression of dThdPase also shows a significant correlation with tumor angiogenesis and we have demonstrated that dThdPase gene and protein expression is closely associated with angiogenesis and invasion phenotype in cervical carcinomas.[39,42,43] To our knowledge, however, there has been no report describing the possible association among VEGF-C expression, tumor aggressiveness and patient outcome in cervical carcinomas.

Our study clearly demonstrated that VEGF-C expression was well correlated with tumor invasion, lymphnode metastasis and MMP-2 expression in cervical carcinoma tissues. These results are consistent with the findings of our *in vitro* study. Tumor cells producing both VEGF-C and MMP-2 may have a higher potential for degradation of ECM and intravasation into the lymphatic vessels, resulting in muscular invasion and lymphnode involvement. Interestingly, there was a close correlation between immunohistochemical expression of VEGF-C and tumor vascularity. Several VEGF-C forms are ligands for VEGFR-3 and the fully processed VEGF-C can bind to and activate VEGFR-2.[10,40] In certain circumstances, proteolytic processing would release mature VEGF-C.[40] VEGF-C secreted by tumor cells may also act as a potential angiogenic factor for blood vessels via activating VEGFR-2 on the cell membrane of endothelial cells.[16,40]

Our present results also demonstrated that the patients with strong VEGF-C staining tumors had a poorer prognosis than did those with weak VEGF-C staining tumors. Furthermore, VEGF-C tissue status was confirmed to be a significantly independent factor for poor prognosis. It has been speculated that the reason is the prevalent progression by the deeper muscular invasion and the higher lymphatic spread in VEGF-C-positive tumors than in VEGF-C-negative tumors.[15] In fact, some investigators have recently demonstrated the correlation between VEGF-C/VEGF-D/VEGFR-3 axis and lymphatic tumor spread.[44–46] It would be of interest to confirm this concept in *in vivo* cervical carcinoma models using tumor cells, such as SKG-II, with a higher expression level of VEGF-C gene. Moreover, further studies are needed to clarify the molecular events that coregulate the expression of VEGF-C and MMP-2 genes.

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Survivin Gene Expression in Endometriosis

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Survivin is a novel inhibitor of apoptosis and is expressed during fetal development and in cancer tissues, but its expression has not been reported in normal adult tissues or benign diseases. We investigated survivin gene and protein expression in a tumor-like benign disease, endometriosis, and correlated them with apoptosis and invasive phenotype of endometriotic tissues. Gene expression levels of survivin, matrix metalloproteinase (MMP)-2, MMP-9, and membrane type 1 (MT1)-MMP in 63 pigmented or nonpigmented endometriotic tissues surgically obtained from 35 women with endometriosis were compared with those in normal eutopic endometrium obtained from 12 women without endometriosis. Survivin, MMP-2, MMP-9, and MT1-MMP mRNA expression levels in clinically aggressive pigmented lesions were significantly higher than those in normal eutopic endometrium, and survivin gene expression in pigmented lesions was also higher than that in nonpigmented lesions ($P < 0.05$). There was a close correlation between survivin and MMP-2, MMP-9, or MT1-MMP gene expression levels in 63 endometriotic tissues examined ($P < 0.01$). Apoptotic cells detected by the dUTP nick-end labeling were rare in 11 ovarian endometriotic tissues, which showed positive immunohistochemical expression for survivin and MMPs. Our findings suggest that up-regulation of survivin and MMPs may cooperatively contribute to survival and invasion of endometriosis. (*J Clin Endocrinol Metab* 87: 3452–3459, 2002)

ENDOMETRIOSIS, THE PRESENCE of endometrium outside of the uterine cavity, is a common disease, causing abdominal pain, dysmenorrhea, dyspareunia, and infertility in 10–15% of menstruating women (1). Its etiology is unclear but it is thought to be due to the implantation and maintenance of disseminated uterine endometrium, predominantly on the ovary and pelvic peritoneum (2). Clinical observations have led to the assessment that endometriosis is an invasive disease (3–5). In endometriotic lesions, although derived from normal endometrium, decreased expression of adhesion molecules and increased expression of proteolytic enzymes may contribute to establishment of endometrial glands and stroma at ectopic sites, likely as a behavior of cancer cells (5–8). Normal epithelial cells undergo apoptosis when they separate from their primary tissue. However, spontaneous apoptosis of ectopic endometrial tissue is impaired in women with endometriosis, and its decreased susceptibility to apoptosis might participate in the growth, survival, and invasion of endometriotic tissue (9, 10). Although there have been some reports on the induction of apoptosis in endometriotic lesions (11, 12), there is no consensus on the mechanism of escape from apoptosis in endometriosis, and little is known on the correlation between survival activity and invasive phenotype in endometriotic cells.

Among the regulators of cell death, inhibitor of apoptosis (IAP) proteins have recently emerged as modulators of an evolutionarily conserved step in apoptosis, which may potentially involve the direct inhibition of terminal effector caspases 3 and 7 (13). Recently, a novel and structurally unique member of the IAP gene family, designated survivin, was identified (14). Unlike other IAP proteins, survivin was found during embryonic and fetal development, was completely down-regulated and undetectable in normal adult tissues, and became prominently reexpressed in all of the most common human cancers (14). However, there has been no report on the biological significance of survivin in endometriosis, an aggressive tumor-like benign disease. In the present study, we investigated survivin gene and protein expression in surgical specimens from patients with endometriosis and correlated them with apoptosis and invasive phenotype of endometriotic tissues.

Abbreviations: IAP, Inhibitor of apoptosis; MMP, matrix metalloproteinase; MT1, membrane type 1; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling.

Subjects and Methods

Patients and tissue samples

The subjects in this study were women of reproductive age undergoing laparoscopy or laparotomy for suspected endometriosis. At the time of surgery, pelvic organs were examined carefully for the presence and extent of endometriosis. Stages of the disease and macroscopic findings of endometriotic lesions were classified according to the revised American Fertility Society classification (15). Patients who received sex steroid derivatives or GnRH analogs within 1 yr before the operation were excluded in the current study. This study was approved by our institutional review board, and appropriate informed consent was obtained from all women.

Endometriotic tissues for mRNA analysis were removed with biopsy forceps under laparoscopy or laparotomy from 35 women with endometriosis. Of these patients, 2 had stage I, 1 had stage II, 8 had stage III, and 24 had stage IV disease. Control eutopic endometrial tissues were collected by endometrial curettage from 12 women without any findings of endometriosis, which was confirmed with laparoscopy. Cycle phase for each subject was assigned based on histological evaluation of eutopic endometrium or basal body temperature. All tissue specimens were immediately frozen in liquid nitrogen and then stored in −80 C until use.

Tissue samples for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) and immunohistochemistry were obtained from 11 women who underwent ovarian cystectomy or oophorectomy under the diagnosis of chocolate cyst of the ovary. Resected ovarian tissues were fixed in 10% formalin and embedded in paraffin wax. Serial sections of 5 μm each were placed on silanized slides (DAKO Corp., Kyoto, Japan) and maintained at room temperature. Eutopic endometrial tissues collected from seven women without endometriosis as described above were also used for TUNEL and immunohistochemical study.

RNA isolation and cDNA preparation

RNA was extracted from homogenized tissue samples by phenol/chloroform extraction according to the RNA STAT-60 protocol (Tel-Test, Friendswood, TX) according to the supplier's recommendation. Contaminating residual genomic DNA was removed by digestion with RNase free DNase (Promega Corp., Madison, WI). cDNAs were prepared using at least 2 μg total RNA and SUPERSCRIPT II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) with random hexamers as primers and were finally dissolved in diethyl pyrocarbonate-treated water and then frozen at −20 C until use.

RT-PCR analysis

Oligonucleotide primers for RT-PCR were designed using a published sequence of survivin (16), matrix metalloproteinase (MMP)-2 (17), MMP-9 (18), membrane type 1 (MT1)-MMP (19), and β-actin (20) genes and synthesized by the solid-phase triester method. The primers used in this study and the expected sizes from the reported cDNA sequence are shown in Table 1.

Amplification of cDNA was performed according to the method described previously (20, 21). For PCR, cDNA aliquots were diluted in sterile water, depending on transcript abundance. Three cDNA concentrations for each target gene/β-actin primer combinations were used. For accurate quantification using this method, measurements must be taken in the linear phase of the reaction, in which cDNA concentration is directly proportional to signal intensity. A range of cDNA concentrations was used to determine the linear phase of the PCR. cDNA template was amplified by PCR in a final volume of 20 μl reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM dNTP, 0.5 μM each primer, and 1.25 U Taq polymerase (Perkin-Elmer Corp., Norwalk, CT). After an initial denaturation at 96 C for 3 min, various cycles of denaturation (94 C for 1 min), annealing (57 C for survivin, 58 C for MMP-2, 62 C for MT1-MMP, and 60 C for MMP-9 and β-actin for 1 min), and extension (72 C for 2 min) for the respective target genes were carried out on a Perkin-Elmer GeneAmp PCR System 9700. The final extension was performed at 72 C for 10 min. The number of cycles in the RT-PCR was determined so as to obtain logarithmic amplification of each gene for semiquantitative analysis of the expression levels of the genes. After visualization of the RT-PCR products by 1.5% agarose gel electrophoresis with ethidium bromide staining, gel images were obtained using the ATTO densitograph UV-image analyzer (ATTO Corp., Tokyo, Japan), and the densities of the products were quantified using the ATTO densitometry software, version 2. The relative expression levels were calculated as the density of the product of each target gene divided by that of β-actin from the same cDNA. Each analysis was performed in triplicate.

DNA nick-end labeling

DNA breaks were detected *in situ* by TUNEL according to the method of Gavrieli *et al.* (22), with some modifications as described previously (23, 24). Paraffin sections were de-waxed, rehydrated through a graded alcohol series, and washed with PBS. Subsequently, the tissues were digested with 20 μg/ml proteinase K (Sigma, St. Louis, MO) for 15 min at room temperature and then washed with distilled water and subsequently with PBS. The tissues were incubated with a solution containing 2% H_2O_2 in PBS to inhibit endogenous peroxidase activity and then washed with PBS. TdT buffer solution [100 mM potassium cacodylate, 2 mM cobalt chloride, and 0.2 mM dithiothreitol (pH 7.2)] containing 0.3 U/μl TdT (Oncor, Gaithersburg, MD) and 0.04 nmol/μl digoxigenin-dUTP (Oncor) were added to cover the tissues, which were then incubated in a humidified atmosphere for 60 min at 37 C. The tissues were washed with buffer solution containing 300 mM sodium chloride and 30 mM sodium citrate for 30 min at 37 C to terminate the reaction and then washed with PBS. They were subsequently incubated with anti-digoxigenin-peroxidase complex for 30 min at room temperature and stained with a solution of 0.05% 3,3′-diaminobenzidine and 0.01% H_2O_2 in Tris-HCl buffer (DAB solution) at pH 7.6 for 3–6 min at room temperature. The sections were counterstained with hematoxylin. Negative controls were obtained by omitting TdT from the buffer solution.

Immunohistochemistry

For immunohistochemical staining, rabbit antihuman survivin polyclonal antibody (SURV11-A; Alfa Diagnostic International, San Antonio, TX) and mouse antihuman MMP-2, MMP-9, and MT1-MMP monoclonal antibodies (Fuji Photo Film Co., Ltd., Toyama, Japan) were used for avidin-biotin-peroxidase complex procedure. After deparaffinization, sections were treated with a solution containing 2% H_2O_2 in PBS to inhibit endogeneous peroxidase activity. For survivin staining, the slides were then immersed in a 10-mM citrate buffer (pH 6.0) and heated in an autoclave for 5 min at 120 C to retrieve masked antigens. The slides were incubated overnight at 4 C with primary antibodies at a 1:50 dilution and washed with PBS. Biotinylated goat antirabbit or horse antimouse immunoglobin (DAKO Corp.) was then added to the sections for 30 min at room temperature. Peroxidase-conjugated avidin (DAKO Corp.) was applied after the sections were washed with PBS. Peroxidase activity was detected by exposure of the sections to the DAB solution as described above. The sections were counterstained with hematoxylin. Normal rabbit or mouse IgG was used as a substitute for the primary antibody for the negative controls.

Evaluation of histochemical staining

Semiquantitative evaluation of TUNEL and immunohistochemical staining was performed according to the method reported by Harada *et al.* (25), with some modifications. The slides were evaluated independently by two investigators in a blind fashion. Three different fields containing 100 glandular cells per field were examined at ×400 magnification. The percentage of positive epithelial cells was scored as: 0, no positive cells; 1, 0–25% positive; 2, 25–50% positive; 3, more than 50% positive. To indicate intra- and interobserver variations, the staining result was expressed as mean ± SE for each case. For semiquantitative evaluation of the expression of survivin and MMPs in stromal components, three different fields containing at least 200 stromal cells around

TABLE 1. PCR primers and conditions

Gene	Primer	Temperature (C)	Product size (bp)
Survivin	5′-GTGAATTTTTGAAACTGGACAG-3′ 5′-CCTTTCCTAAGACATTGCTAAG-3′	57	243
MMP-2	5′-CCACGTGACAAGCCCATGGGGCCCC-3′ 5′-GCAGCCTAGCCAGTCGGATTTGATG-3′	58	480
MMP-9	5′-GTGCTGGGCTGCTGCTTTGCTG-3′ 5′-GTCGCCCTCAAAGGTTTGGAAT-3′	60	303
MT1-MMP	5′-ACATTGGAGGAGACACCCAC-3′ 5′-TAGGCAGTGTTGATGGACGC-3	62	493
β-Actin	5′-CTTCTACAATGAGCTGCGTG-3′ 5′-TCATGAGGTAGTCAGTCAGG-3′	60	305

the glands were examined and the percentage of positive stromal cells was scored as described above.

Statistical analysis

All statistical calculations were carried out using StatView statistical software (Tokyo, Japan). Gene expression levels of survivin and MMPs in endometriotic tissues were compared with those in normal eutopic endometrium, and the significance of differences was determined by the Mann-Whitney *U* test. The Spearman rank correlation coefficient was also used to analyze the relation between survivin and MMP gene expression levels in endometriotic tissues. A level of *P* less than 0.05 was accepted as statistically significant.

Results

A total of 63 endometriotic tissues for mRNA analysis was obtained from 35 women with endometriosis. They included 43 pigmented (red or black; blueberry spot, blood breb, and chocolate cyst) and 20 nonpigmented (white or yellow-brown; serous breb and surface elevation) lesions. Gene expression levels of survivin, MMP-2, MMP-9, and MT1-MMP in these endometriotic lesions were compared with those in normal eutopic endometrium obtained from 12 women without endometriosis.

Figure 1 shows an example for mRNA expression of survivin, MMP-2, MMP-9, and MT1-MMP in normal endometrium and in endometriotic tissues. Their relative gene expression levels in comparison with β-actin expression ranged widely among the lesions examined; however, there was no significant difference in the expression level of survivin and MMPs between stages of the disease and cyclic phases of the patients (data not shown). As can be seen in Fig. 2, gene expression levels of survivin, MMP-2, MMP-9, and MT1-MMP in pigmented lesions were significantly higher than those in normal eutopic endometrium ($P = 0.0056$, 0.0324, 0.0021, and 0.0349), respectively. Moreover, survivin gene expression in pigmented lesions was statistically higher than that in nonpigmented lesions ($P = 0.0446$), whereas there was no significant difference in MMP-2, MMP-9, and MT1-MMP gene expression levels between two groups. As shown in Fig. 3, a–c, there was a close correlation between survivin and MMP-2, MMP-9, or MT1-MMP gene expression levels in 63 endometriotic tissues examined, with coefficient correlations of 0.356, 0.663, and 0.411 ($P = 0.0042$, <0.0001, and 0.0008), respectively. Interestingly, relative gene expression levels of MMP-2 were also well correlated with those of MT1-MMP, with a coefficient correlation of 0.636 ($P < 0.0001$) (Fig. 3d).

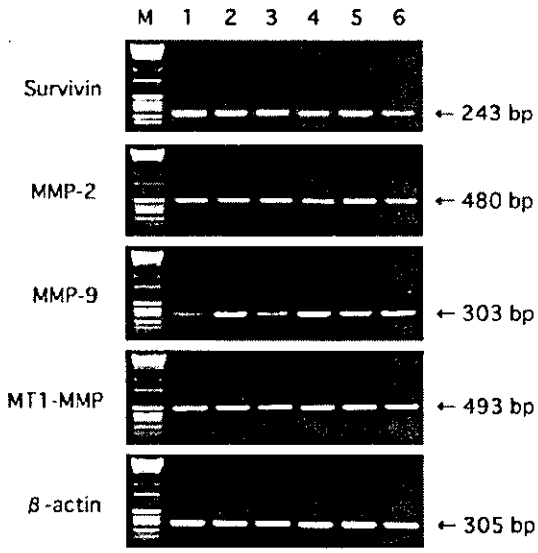

FIG. 1. RT-PCR analysis of survivin, MMP-2, MMP-9, and MT1-MMP in normal endometrium and endometriotic tissues. Lanes 1 and 2 correspond to samples from normal eutopic endometrium, lanes 3 and 4 from nonpigmented lesions, and lanes 5 and 6 from pigmented lesions, respectively. The primers and the expected sizes from the reported cDNA sequence are shown in Table 1.

Seven specimens from normal endometrium and 11 from ovarian endometriosis were examined for apoptosis and expression of survivin and MMPs. Table 2 shows the overall results of histochemical staining outcomes. TUNEL and immunohistochemical staining for cases 5 and 9 are shown in Figs. 4 and 5, respectively. The negative control slides for TUNEL, survivin, and MMPs exhibited no specific staining. The apoptotic epithelial cells positive for TUNEL were detected in 2 of 7 and only 1 of 11 cases in eutopic and ectopic endometrium, respectively. Glandular epithelial cells positive for survivin and MMPs were observed in all 11 specimens from ovarian endometriosis regardless of the menstrual cycle phase. However, survivin, MMP-2, MMP-9, and MT1-MMP expression in normal endometrial gland were relatively weak and detected in one, two, two, and three of seven specimens, respectively. On the other hand, stromal cells positive for survivin, MMP-2, MMP-9, and MT1-MMP were detected in 4, 11, 7, and 8 of 11 specimens from ovarian endometriosis, respectively, whereas MMP-2 expression in normal endometrial stroma was weak and detected in 2 of 7 specimens. Survivin, MMP-9, and MT1-MMP expression were not observed in stromal cells from normal eutopic endometrium.

Discussion

Survivin is a novel inhibitor of apoptosis and is expressed during fetal development and in cancer tissues, but its expression has not been reported in normal adult tissues or benign diseases (13, 14). Very recently, Konno *et al.* (16) demonstrated that survivin gene and protein expression was detected in normal human endometrium and that survivin could play an important role in physiological homeostasis during the normal menstrual cycle. It could be expected that survivin is also expressed in ectopic endometriotic tissues, however, there has been no investigation on the biological role of survivin in endometriosis.

MMPs, enzymes that are important for extracellular matrix turnover, have recently been implicated in invasion and development of endometriosis (8). MMPs appear to be overexpressed in endometriotic lesions and contribute to establishment of endometrial glands and stroma at ectopic sites (26–28). Although sex steroids and GnRH agonists have been shown to regulate MMP expression in a model of experimental endometriosis (27–29), limited investigation has focused on gene expression of MMPs in human peritoneal endometriotic lesions (8, 30). Moreover, it is still unclear how

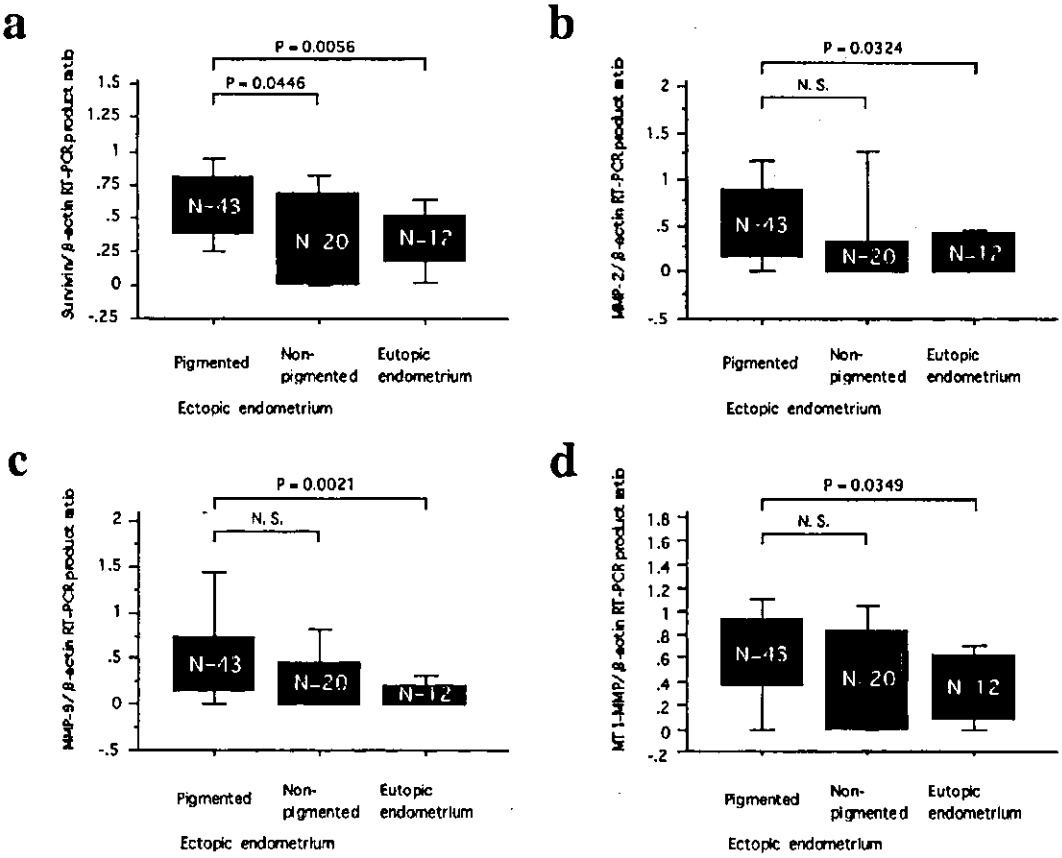

FIG. 2. Gene expression levels of survivin (a), MMP-2 (b), MMP-9 (c), and MT1-MMP (d) in pigmented and nonpigmented endometriotic tissues and normal eutopic endometrium. Values are expressed as relative expression levels of each target gene in comparison with β-actin expression. *Box*, 25–75% distribution; *bars*, 10–90% distribution. N.S., Not significant.

endometriotic cells expressing MMPs escape from apoptosis during the development of endometriosis.

In the present study, we investigated gene expression of survivin and MMPs in eutopic and ectopic endometrial tissues and found that survivin, MMP-2, MMP-9, and MT1-MMP mRNA expression levels in pigmented endometriotic lesions were significantly higher than those in normal eutopic endometrium. Moreover, survivin gene expression in pigmented lesions was higher than that in nonpigmented lesions. In patients with endometriosis, visual findings of peritoneal endometriotic foci are often recognized as typical red or black pigmented lesions and nonpigmented lesions seem to occur earlier during the development of the disease (31, 32). We previously reported that pigmented lesions, such as blueberry spot, blood breb and chocolate cyst, were clinically more active and aggressive (33). It is likely that upregulation of survivin and MMP expression may cooperatively contribute to survival and invasion of endometriotic tissues. Interestingly, there was a close correlation between survivin and MMP-2, MMP-9, or MT1-MMP gene expression levels in 63 endometriotic tissues examined. Invasive endometriotic cells may escape from apoptosis by expressing a higher level of survival gene survivin.

To confirm this hypothesis, we then performed histochemical study for eutopic and ectopic endometrial tissues. Endometriotic lesions rarely contained apoptotic epithelial cells, and only 1 of 11 cases showed positive staining for TUNEL. Jones *et al.* (34) also demonstrated that apoptotic cells were rare in ovarian endometriosis, and there was no significant difference in the number of apoptotic cells between eutopic and ectopic endometrium. Interestingly, immunohistochemical expression of survivin and MMPs in glandular epithelial cells was positive for all 11 specimens from ovarian endometriosis, and survivin expression in ectopic endometrium was extremely stronger than that in eutopic endometrium. Konno *et al.* (16) reported that survivin protein expression in normal endometrium was strongest in the late secretary phase and was not detected in the proliferative phase. In this study, survivin was identified in one normal endometrial sample in the late secretary phase, however, it could not be detected in four samples in the early secretary phase. The cyclic change of survivin protein during the normal menstrual cycle should be further elucidated.

We also evaluated survivin and MMP expression in stromal components of eutopic and ectopic endometrium. Konno *et al.* (16) demonstrated that stromal cells of eutopic endometrium were completely negative for survivin. Our present results revealed that survivin was not detected in

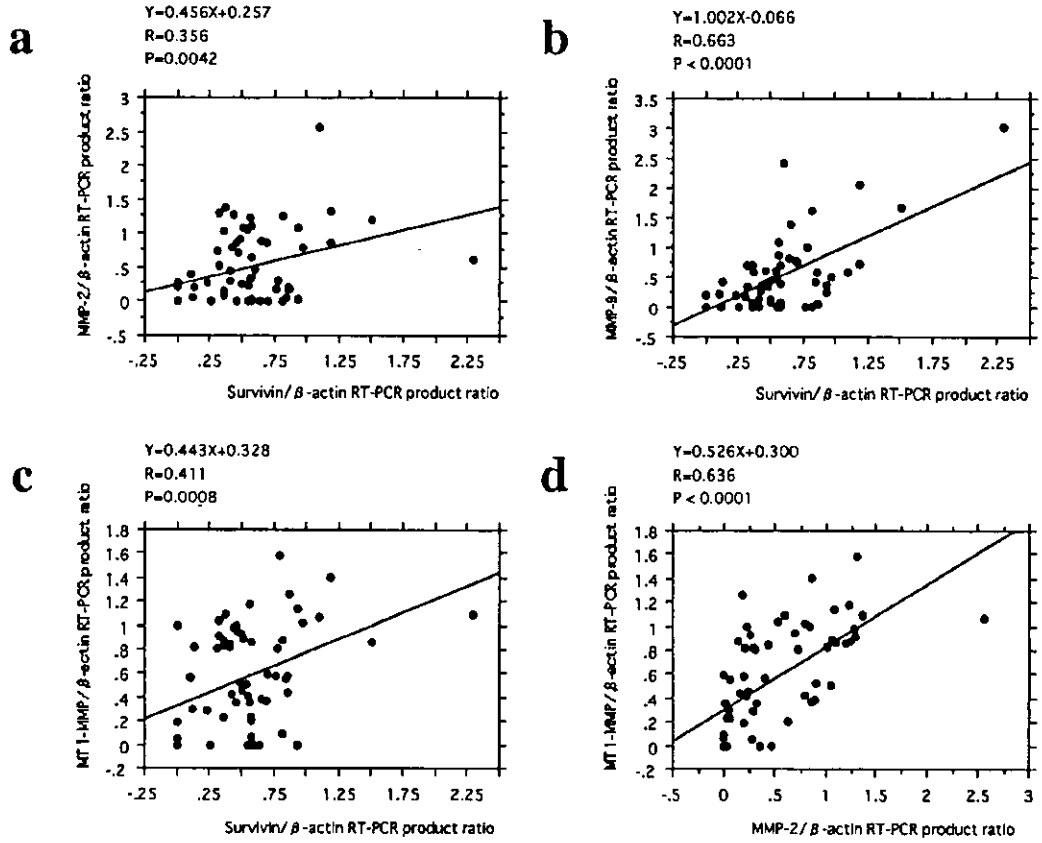

FIG. 3. Correlation between survivin and MMP-2 (a), MMP-9 (b), or MT1-MMP (c), and between MMP-2 and MT1-MMP (d) gene expression levels in 63 endometriotic tissues examined. Each *point* represents the mean of triplicates.

TABLE 2. Histochemical staining outcomes

Patient no.	Cycle	TUNEL	Survivin		MMP-2		MMP-9		MT1-MMP	
		Gland	Gland	Stroma	Gland	Stroma	Gland	Stroma	Gland	Stroma
Normal endometrium										
1	E.P.	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2	L.P.	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.3	0.3 ± 0.2	0.7 ± 0.2	0.0 ± 0.0	1.2 ± 0.4	0.0 ± 0.0
3	E.S.	0.8 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.4	0.0 ± 0.0
4	E.S.	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
5	E.S.	1.0 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
6	E.S.	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
7	L.S.	0.0 ± 0.0	0.8 ± 0.2	0.0 ± 0.0	1.0 ± 0.3	0.3 ± 0.2	0.8 ± 0.2	0.0 ± 0.0	0.8 ± 0.3	0.0 ± 0.0
Ovarian endometriosis										
8	E.P.	0.0 ± 0.0	1.0 ± 0.3	0.0 ± 0.0	1.2 ± 0.2	1.0 ± 0.3	1.0 ± 0.0	0.8 ± 0.2	1.0 ± 0.3	0.5 ± 0.2
9	L.P.	0.0 ± 0.0	2.5 ± 0.2	1.7 ± 0.3	2.7 ± 0.2	2.3 ± 0.3	2.5 ± 0.2	2.2 ± 0.3	2.2 ± 0.2	1.7 ± 0.4
10	L.P.	0.0 ± 0.0	1.0 ± 0.5	0.0 ± 0.0	1.0 ± 0.3	0.8 ± 0.2	0.8 ± 0.2	0.0 ± 0.0	0.7 ± 0.2	0.0 ± 0.0
11	L.P.	0.0 ± 0.0	1.2 ± 0.6	0.0 ± 0.0	1.2 ± 0.4	1.0 ± 0.0	1.2 ± 0.4	0.3 ± 0.2	0.8 ± 0.2	0.3 ± 0.2
12	E.S.	0.0 ± 0.0	1.3 ± 0.2	0.0 ± 0.0	2.5 ± 0.2	2.0 ± 0.0	1.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.0	0.7 ± 0.2
13	E.S.	0.0 ± 0.0	1.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.3	1.2 ± 0.4	1.2 ± 0.3	0.0 ± 0.0	1.0 ± 0.3	0.0 ± 0.0
14	E.S.	0.0 ± 0.0	2.3 ± 0.2	0.7 ± 0.2	1.0 ± 0.3	0.8 ± 0.3	2.3 ± 0.3	0.8 ± 0.3	0.8 ± 0.3	0.5 ± 0.2
15	L.S.	0.7 ± 0.2	1.5 ± 0.2	0.0 ± 0.0	1.2 ± 0.3	0.7 ± 0.2	1.0 ± 0.3	0.5 ± 0.2	0.7 ± 0.2	0.2 ± 0.2
16	L.S.	0.0 ± 0.0	1.0 ± 0.0	0.0 ± 0.0	2.5 ± 0.5	2.5 ± 0.2	2.3 ± 0.3	1.5 ± 0.2	2.3 ± 0.5	1.2 ± 0.4
17	L.S.	0.0 ± 0.0	2.2 ± 0.4	0.8 ± 0.5	1.2 ± 0.4	1.0 ± 0.0	0.8 ± 0.2	0.0 ± 0.0	0.8 ± 0.3	0.0 ± 0.0
18	L.S.	0.0 ± 0.0	2.3 ± 0.3	1.5 ± 0.7	2.8 ± 0.2	2.3 ± 0.2	1.2 ± 0.3	0.7 ± 0.2	2.5 ± 0.3	0.8 ± 0.3

E.P., Early proliferative; L.P., late proliferative; E.S., early secretory; L.S., late secretory. Each value is mean ± SE.

stromal components of normal eutopic endometrium, however, 4 of 11 cases with ectopic endometrium showed positive immunostaining for survivin in stromal cells around the glands. Jones *et al.* (34) reported that bcl-2 up-regulation in stromal cells may prevent apoptosis in ectopic endometrium. We speculate that endometrial stromal cells expressing sur-

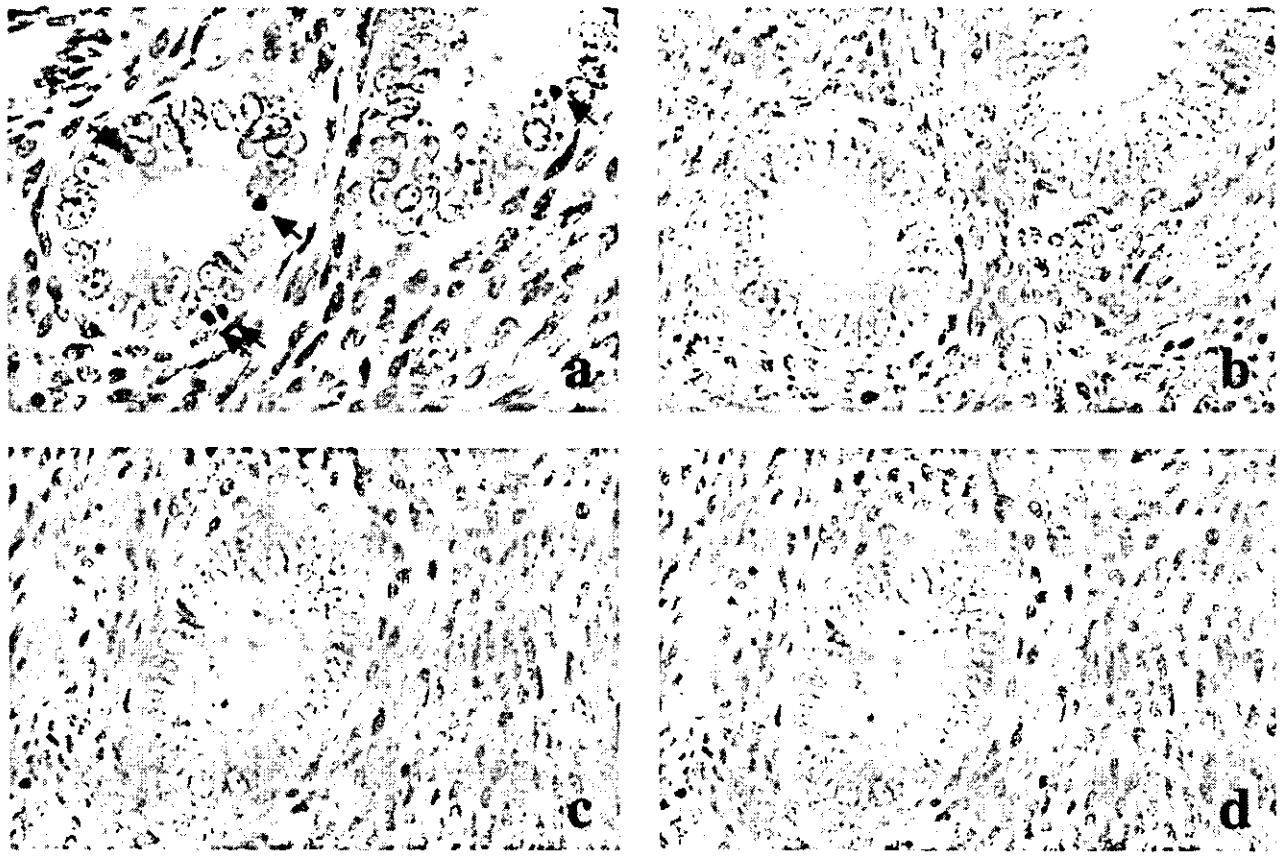

FIG. 4. Histochemical staining of normal eutopic endometrial tissue (case 5 in Table 2). Intense TUNEL signals are observed in the small nuclei of glandular epithelial cells (a; *arrows*), but the immunoreactivity of survivin (b), MMP-2 (c), and MT1-MMP (d) is negative both in epithelial and stromal cells. Original magnification, ×400.

vivin may antagonize caspase-3-mediated apoptosis and promote the development of endometriosis, but its molecular mechanism should be further elucidated. Rodgers *et al.* (35) reported a consistent expression of MMP-2 throughout the menstrual cycle in the stromal components of cycling endometrium. In contrast, Wenzl and Heinzl (36) were not able to detect MMP-2 expression in the stromal components of eutopic endometrium, but they observed strong MMP-2 immunoreactivity both in glandular epithelial and stromal cells of ectopic endometrium and stated that ectopic endometriotic tissue has a greater capacity to invade due to higher proteinase expression. In this study, 2 of 7 cases with normal eutopic endometrium showed positive immunostaining for MMP-2 in glandular epithelial and stromal cells, whereas all 11 cases with ectopic endometrium showed stronger MMP-2 expression not only in gland but also in stroma. Moreover, MMP-9 and MT1-MMP immunoreactivity in stromal cells was detected only in ectopic endometriotic tissue. MMP expression in stromal components of ectopic endometrium may be also related to up-regulation of MMP gene expression and invasive phenotype of endometriosis.

Endometriotic epithelial cells that expressed MMP-2 were also positive for MT1-MMP. MMP-2 has been shown to be activated by MT1-MMP, which possesses a functional transmembrane domain and acts as cell-surface receptor and activator for proMMP-2 (19, 37, 38). Relative gene expression levels of MMP-2 were well correlated with those of MT1-MMP in endometriotic lesions as described above. The up-regulated invasiveness of endometriotic cells by MMP-2 may be closely linked to its activation by MT1-MMP. These results suggest that overexpression of both survivin and MMPs in endometriotic tissues may contribute to escape from apoptosis and development of the disease.

The molecular interaction between survivin and MMPs in endometriotic tissues is still unclear. However, recent studies have demonstrated that highly metastatic cancers exhibit a higher resistance to apoptotic cell death compared with low metastatic forms (39, 40) and that survivin expression is closely associated with invasive phenotype of oral (41), esophageal (42), and ovarian (43) cancers. Yoshida *et al.* (43) reported that the expression of survivin enhanced invasive activity of ovarian cancer cells by up-regulation of MMP-2. Survivin expression may provide a strong advantage factor for tumor progression, both affording protection from broad apoptosis-inducing environmental stimuli and maintaining proper mitotic progression of the proliferating and invasive population (41). Additional studies are needed to clarify the

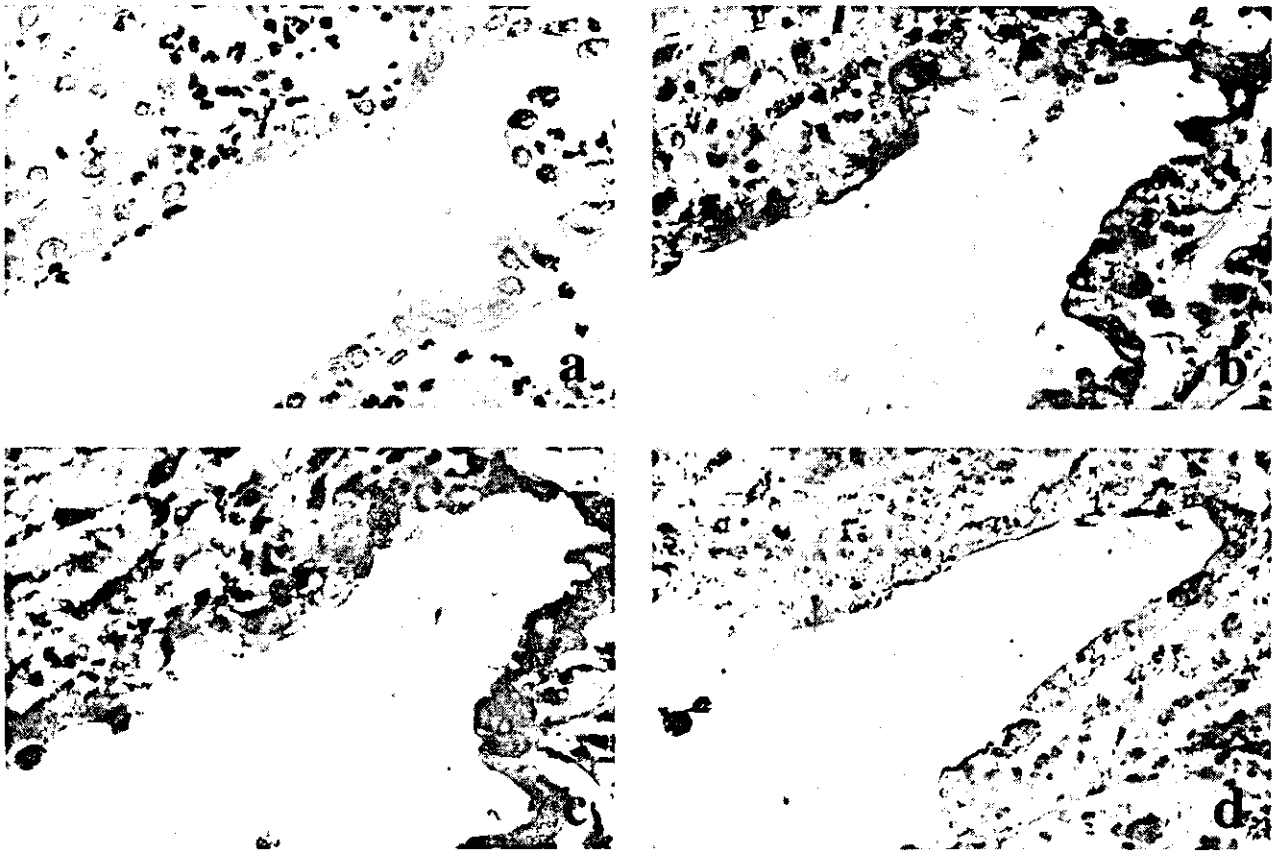

FIG. 5. Histochemical staining of ectopic endometrial tissue from ovarian endometriosis (case 9 in Table 2). Glandular epithelial cells show negative staining for TUNEL (a), but the immunoreactivity of survivin (b), MMP-2 (c), and MT1-MMP (d) is positive both in epithelial and stromal cells. The immunoreactivity of MMP-9 is similar to that of MMP-2 (data not shown). Original magnification, ×400.

molecular events that co-regulate the expression of survivin and MMP genes in the development of endometriosis.

To the best of our knowledge, this is the first report to demonstrate survivin gene expression in endometriosis and to highlight the relation between survival and invasion of endometriotic cells. These observations are potentially important in understanding the pathogenesis of an aggressive tumor-like benign disease, endometriosis.

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特　集　増えてきた子宮体癌　その診断から最新の治療まで

わが教室における子宮体癌の管理

Clinical management of uterine corpus cancer in our department

丹野 純香 *TANNO Sumika*　伊藤 潔*1 *ITOU Kiyoshi*　片平 敦子 *KATAHIRA Atsuko*

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子宮体癌は，近年増加傾向にある疾患であり，当科においても治療した子宮癌全体に占める割合が30％を越えるようになった．脂肪の過剰摂取など食事の欧米化や結婚の高齢化，出産回数の減少などライフスタイルの変化，平均寿命の高齢化などが体癌の罹患率の増加の一因と考えられている．子宮体癌の多くはⅠ，Ⅱ期の早期に診断されるため，婦人科癌のなかでは予後良好な部類に属する．しかし，進行癌や再発癌においては依然として予後不良であり，予後改善のため有効な術後補助療法，あるいは寛解導入療法の確立が必要である．

本稿では，当教室における体癌患者に対する術前診断，基本術式，術後補助療法および寛解導入療法の選択，フォローアップの仕方などについて若干の文献的考察を加えながら述べる．

Key Words　子宮体癌，手術療法，化学療法

はじめに

子宮体癌は，近年増加傾向にある疾患であり，当科においても治療した子宮癌全体に占める割合が30％を越えるようになった．脂肪の過剰摂取など食事の欧米化や結婚の高齢化，出産回数の減少などライフスタイルの変化，平均寿命の高齢化などが体癌の罹患率の増加の一因と考えられている．子宮体癌の多くはⅠ，Ⅱ期の早期に診断されるため，婦人科癌のなかでは予後良好な部類に属する．しかし，進行癌や再発癌においては依然として予後不良であり，予後改善のため有効な術後補助療法，あるいは寛解導入療法の確立が必要である．

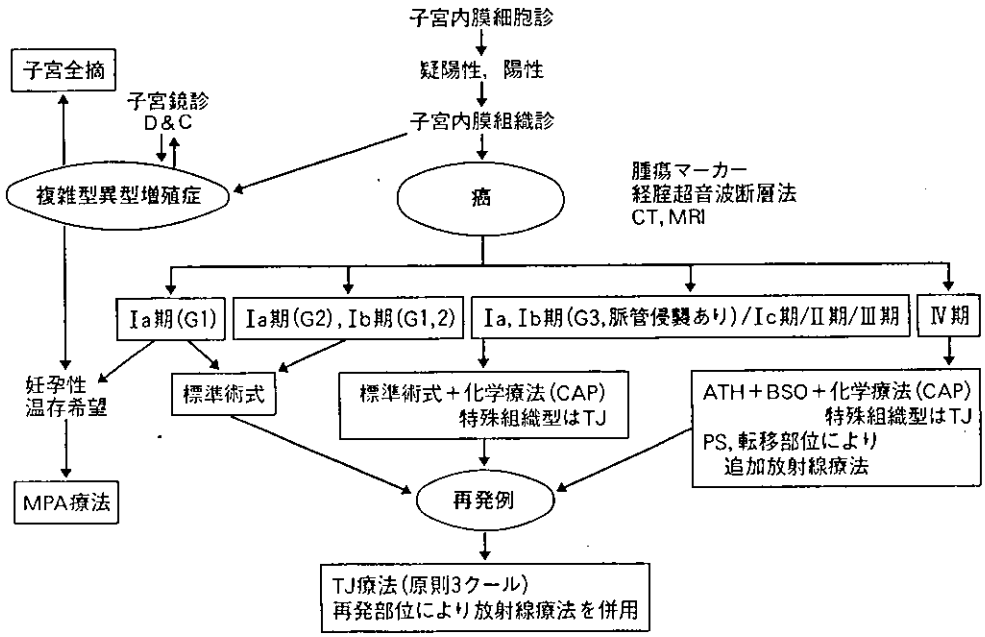
本稿では当教室における体癌患者に対する術前診断，基本術式，術後補助療法および寛解導入療法の選択，フォローアップの仕方などについて若干の文献的考察を加えながら述べる．

術前検査

子宮内膜細胞診にて疑陽性，陽性の場合，子宮内膜組織診を行い，確定診断を行う．組織診でatypical hyperplasiaの場合には，子宮鏡検査，子宮内膜全面掻爬を行い，診断を確定する．体癌であった場合には，筋層浸潤の有無およびリンパ節の腫脹，遠隔転移の有無を評価するために，MRI，CT検査を行っている．

術式の選択

体癌の治療には，手術療法，放射線療法，化学療法，ホルモン療法などがある．体癌の大部分は比較的放射線感受性の低い腺癌であり，また化学療法の効果も確実なものでないことから，手術療法が第一選択となる．しかし，その術式については，多くの議論があり，子宮頸癌におけるような臨床期別ごとの術式が確立されていない．



computed tomography; CT, Magnetic resonance imagine; MRI
標準術式; ATH＋BSO＋PLA＋PALA

図1　当科における子宮体癌プロトコール

当科における現在の術式の選択について述べる（図1）．標準術式を単純子宮全摘（ATH），両側付属器切除（BSO），骨盤リンパ節郭清（PLA），傍大動脈リンパ節郭清（PALA）としⅠ期からⅢ期まで行っている．Ⅳ期はATH＋BSOとし，化学療法を追加している．手術不適例は，BMI＞35の高度肥満，PS≧4，宗教上の理由や合併症のある症例，75歳以上の高齢者などであり，これらに対してはradiationを選択している．またBMI＞32，PS≧3，70歳以上の高齢者にはリンパ節郭清は行っていない．

そこで，問題となってくるのが，Ⅱ期症例での広汎子宮全摘術の是非と，傍大動脈リンパ節郭清の適否である．まず，広汎子宮全摘術の是非について述べる．わが国の子宮癌登録施設では，Ⅱ期癌の治療として広汎子宮全摘を行っている施設は全体の84%に及んでいる[1]．しかし，表1に示すように，桜木ら[2]，澤ら[3]，Leminenら[4]のいずれの報告においても，Ⅱ期癌に対する単純子宮全摘と広汎子宮全摘による5年生存率に有意な差はないとされている．当教室でも，1988年から1996年に子宮体癌にて手術を施行した転帰不明例や手術不能例を除く181例を対象として解析を行っている（図2，表2）．手術術式は原則的にATH＋BSO＋PLAを施行し，術後再発高危険群（Ic期以上，脈管侵襲例，G3，特殊な組織型）には放射線療法や，CAP療法（cisplatin，adriamycin，cyclophosphamide）を中心とした化学療法を追加した．解析方法として生存率の検討はKaplan-Meier法により行い，log rank testで検定した．予後因子の解析はCoxの比例ハザードモデルを用いた．予後因子の関連性はχ^2検定を行った．進行期別生存曲線と5年生存率を図2に示した．子宮体癌の5年生存率はⅠ期（95%），

表1　FIGO類（1988年）Ⅱ期における手術術式別5年生存率の比較

	n	広汎	単摘	p値
桜木ら（1997）	34	51.9%	45.5%	N.S
澤ら（1998）	48	83.7%	75.5%	N.S
Leminenら（1995）	1297	77%	65%	N.S

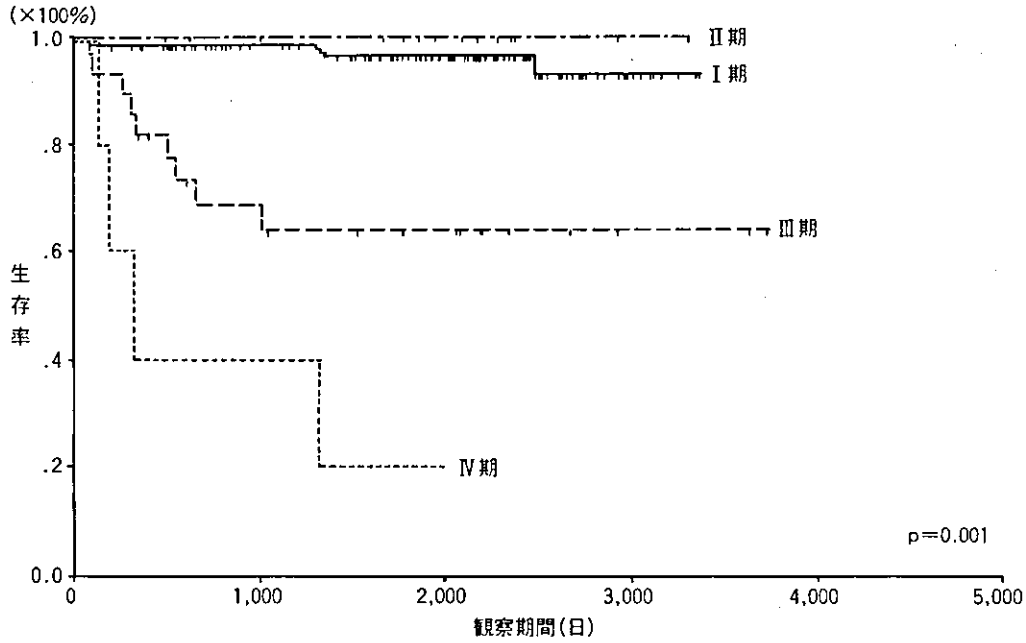


図2　進行期別累積生存率

Ⅱ期(100%)，Ⅲ期(62%)，Ⅳ期(20%)であった．頸部浸潤は，生存率や単変量解析では統計学的に有意に予後との関連がみられたが，ほかの予後因子を補正した多変量解析ではリスク比は1.1倍となり，有意差が消失した（表2）．さらに頸部浸潤は筋層浸潤の深さ，骨盤リンパ節転移の有無，組織分化度の低さ，傍子宮結合織浸潤の有無と5%の危険率で関連ありと判定された．またⅡ期体癌の5年生存率が100%で，頸部浸潤が独立した予後因子ではなかったことから，Ⅱ期体癌では単純子宮全摘術であっても，充分良好な成績が得られることが示唆された．すなわち傍子宮組織をより広汎に摘出する意義のある深い頸部浸潤の症例ではその他の予後因子も不良であり，子宮外に病変が及んでいる可能性が高い．この場合切除範囲の大小は意味を持たないと思われ，当教室では，現在すべての症例において広汎子宮全摘ではなく，単純子宮全摘を選択している．

次に傍大動脈リンパ節郭清の適否について検討した．221施設に対してのアンケート調査では骨盤リンパ節は原則として郭清する施設が71%で，症例により郭清または生検という施設を含めると98%に達していた[1]．また，傍大動脈リンパ節に関しては，44%の施設が症例によって郭清または生検を行うとの結果であったが，20%が原則として郭清を，12%が原則として生検をするという回答であった．表3[5]に部位別のリンパ節転移頻度について示す．骨盤内リンパ節，傍大動脈リンパ節のいずれにも転移がないものの割合は80%を少し越えるということになる．傍大動脈リンパ節転移の頻度をみると，おおむね10%程度かと考えられる．骨盤内リンパ節に転移を認めたグループのなかの半数弱のものは，傍大動脈リンパ節にも転移が起こっている可能性があるとい

表2　予後因子の多変量解析

因　子	RR (95%CI)	p値
年齢 (<60, ≧60)	1.3 (1.01-1.9)	0.02
進行期 (Ⅰ,Ⅱ,Ⅲ,Ⅳ)	2.3 (1.2-4.2)	0.008
分化度 (1, 2, 3)	2.0 (1.2-4.8)	ns
筋層浸潤 (0, <1/2, ≧1/2, 1)	1.2 (0.4-5.5)	ns
頸部浸潤 (−, +)	1.1 (0.3-4.2)	ns
傍結合織浸潤 (−, +)	2.6 (0.3-21.1)	ns
付属器転移 (−, +)	1.9 (0.3-11.9)	ns
骨盤リンパ節転移 (−, +)	2.1 (1.9-11.8)	0.03
洗浄細胞診 (−, +)	1.0 (1.0-0.6)	ns