

ORIGINAL**Clinical Significance of Vascular Endothelial Growth Factor Expression and Microvessel Density in Invasive Cervical Cancer**Yasuyo Saijo¹, Hiroyuki Furumoto^{1,2}, Kanako Yoshida¹, Masato Nishimura¹, and Minoru Irahara¹¹Department of Obstetrics and Gynecology, Institute of Health Biosciences, the University of Tokushima Graduate School ; ²Department of Obstetrics and Gynecology, Tokushima Red Cross Hospital, Tokushima, Japan

Abstract : To determine whether vascular endothelial growth factor (VEGF) expression and microvessel density are predictive of prognosis in cases of invasive cervical cancer, correlations among VEGF expression, microvessel density, and clinicopathological parameters were identified. VEGF expression was evaluated in 50 cervical cancer samples by immunohistochemical staining. Microvessel density was assessed by immunostaining for CD31-positive endothelial cells in the most vascularized areas of tumors. VEGF expression and microvessel density were significantly higher in adenocarcinomas than in squamous cell carcinomas. However, in cases of adenocarcinoma, no significant correlations were found among VEGF expression, microvessel density, and clinicopathological parameters. In contrast, for squamous cell carcinomas, microvessel density was significantly higher in cases at an advanced stage and in those with several other poor prognostic factors. The finding that cervical adenocarcinomas exhibited greater VEGF expression and microvessel density than squamous cell carcinomas may explain the poorer prognosis of adenocarcinoma compared with squamous cell carcinoma. Moreover, microvessel density in squamous cell carcinomas was significantly correlated with poor prognostic factors. Therefore, there is possibility that bevacizumab, a humanized monoclonal antibody against VEGF-A, may be useful in the initial treatment targeting angiogenesis for early-stage cervical cancer. *J. Med. Invest.* 62 : 154-160, August, 2015

Keywords : cervical cancer, vascular endothelial growth factor, microvessel density, bevacizumab, angiogenesis

INTRODUCTION

Cervical cancer is still associated with high mortality rates among women worldwide (1). Tumor neovascularization plays a crucial role in the development of solid tumors including cervical cancers. Vascular endothelial growth factor (VEGF) is a potent mitogen responsible for the induction of angiogenesis (2). VEGF mRNA expression and microvessel density are significantly increased in invasive carcinomas and high-grade intraepithelial lesions compared with low-grade intraepithelial and benign squamous epithelium lesions (3). High microvessel density is also significantly associated with increased VEGF mRNA expression.

In cases of advanced stage cervical cancer, there are no significant correlations between VEGF mRNA expression and prognostic factors including histological type and lymph node metastasis, although a significant relationship between VEGF mRNA expression and microvessel density was observed (4). Furthermore, no significant correlation could be demonstrated between VEGF expression and clinicopathological parameters in cases of cervical cancer (5). However, several studies have demonstrated that increased microvessel density and VEGF expression are significantly correlated with poorer prognosis in cases of cervical cancer (6-9).

Bevacizumab, an antibody targeting VEGF, exhibits antitumor properties by inhibiting angiogenesis. Currently, it is used as an effective drug in the treatment of lung, colorectal, and ovarian cancers. Recent research has focused on the effect of bevacizumab

against cervical cancer. In the GOG 240 trial, bevacitumab significantly improved the overall survival when added to chemotherapy for patients with persistent, recurrent, or metastatic cervical cancer (10). Moreover, the U.S. Food and Drug Administration (FDA) has approved bevacizumab for use in the patients with aggressive and late-stage cervical cancer (11). Although VEGF can be a target in the treatment of cervical cancer, it is unclear in which cases bevacizumab will be the most effective. Therefore, we investigated potential correlations among VEGF expression, microvessel density, and clinicopathological features in this study to determine whether VEGF expression and microvessel density in invasive cervical cancer lesions are predictive of prognosis.

MATERIALS AND METHODS*Clinical materials*

Fifty samples of invasive uterine cervical cancer tissues were obtained from 26-70-years-old patients who provided informed consent. The patients underwent surgical treatment at the Tokushima University Hospital between 1998 and 2002. Tissue sections were fixed in paraformaldehyde and embedded in paraffin for histopathological examination and immunohistochemical staining. All cases were staged according to the 1994 International Federation of Gynecology and Obstetrics criteria as follows : two patients were stage Ia, 30 were stage Ib, five were stage IIa, and 13 were stage IIb. Tumors were classified histologically on the basis of the World Health Organization criteria as follows : 32 squamous cell carcinomas, 17 adenocarcinomas, and one adenosquamous carcinoma.

Immunohistochemistry

Tissue sections of 4- μ m thickness were cut, dried overnight at

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40°C on poly-L-lysine-coated slides, deparaffinized, and rehydrated. The methods used for antigen retrieval are described in Table 1. After washing with distilled water, the slides were incubated for 10 min in 3% hydrogen peroxide to inactivate endogenous peroxidase activity. We stained the tissue sections according to the standard avidin-biotin-peroxidase complex method using a LSAB2 Kit employing peroxidase (Dako, Glostrup, Denmark). After incubation for 10 min with 0.25% casein in phosphate-buffered saline (PBS) containing carrier protein to eliminate nonspecific background staining, the slides were covered with primary antibody solution and incubated for 120 min at room temperature. The primary antibodies used are listed in Table 1. After washing with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TTBS), the slides were incubated for 30 min with a solution containing biotinylated anti-mouse and anti-rabbit immunoglobulin G at room temperature. They were then incubated in peroxidase-conjugated avidin solution for 20 min and washed with TTBS. Color was developed using diaminobenzidine, and counterstaining was performed with Mayer's hematoxylin. The positive control treatments for VEGF (12) are described in Table 1. Samples treated with normal serum in place of primary antibodies served as the negative control. VEGF staining specificity was confirmed by the preabsorption of the primary antibody with VEGF peptides (Fig. 1).

Semiquantification of immunostaining results

The immunostained sections were semiquantitatively evaluated

Table 1. Immunostaining : Antigen retrieval, Primary antibody, and Positive control

Antigen retrieval	
VEGF	0.4% pepsin in 0.2M HCl solution, 37°C, 15 min
CD31	proteinase K, room temperature, 8 min (Dako, Glostrup, Denmark)
Primary antibody	
VEGF	rabbit anti-human VEGF (C-1), monoclonal, at 15 µg/mL (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
CD31	mouse anti-human CD31, monoclonal (clone JC70A, prediluted : Dako, Glostrup, Denmark)
Positive control	
VEGF	normal placenta

by investigators who had no knowledge of the clinical status of the patient. VEGF expression in tumor cells was evaluated as previously described (13, 14). After screening each section as a whole, microscopic fields were selected using a random point grid and the mean percentage of specifically brown immunostained tumor cell areas in five microscopic fields at 200× magnification (1.33 mm² per field) was determined. The scores were as follows : 0%=0 ; 1-25%=1 ; 26-50%=2 ; 51-75%=3 ; and 76-100%=4. The mean intensities of the immunostained areas based on the arbitrary numerical values were recorded as follows : none=0 ; weak=1 ; moderate=2 ; and strong=3. We judged that moderate staining was comparable to the staining intensity observed for positive control sections (Figs. 2 and 3). To obtain semiquantitative values for VEGF antigens per tissue, the score for the percentage of positively stained cells and intensity of the staining obtained for each section were multiplied (range, 0-12) and the overall antigen expression was categorized as follows : 0, negative ; 1-4, low ; and 6-12, high.

Microvessel density

According to a previously described method, the sections were scanned at low magnification (40-100×) to identify the most vascularized areas within the tumor samples (15). Within these areas, microvessels were counted in a 200× magnified field. Any area of brown-stained endothelial cells that was clearly separated from the adjacent microvessels, tumor cells, and other connective tissue elements was considered a single countable microvessel. Microvessel density was calculated by adding the numbers of microvessels in 15 fields for each sample.

Statistical analysis

Statistical analysis was performed using Mann-Whitney U tests and Fisher's exact probability tests. The correlation between VEGF expression and microvessel density was examined using the Spearman's rank correlation test. The Kaplan-Meier method and log rank tests were used to clarify whether VEGF expression and microvessel density are predictive of prognosis in cases of cervical cancer.

RESULTS

Correlations between VEGF expression and clinicopathological parameters

VEGF expression was observed in all 50 samples (Figs. 3A, B). The average semiquantitative VEGF expression values in cases of squamous cell carcinoma and adenocarcinoma were 4.4±1.8 and

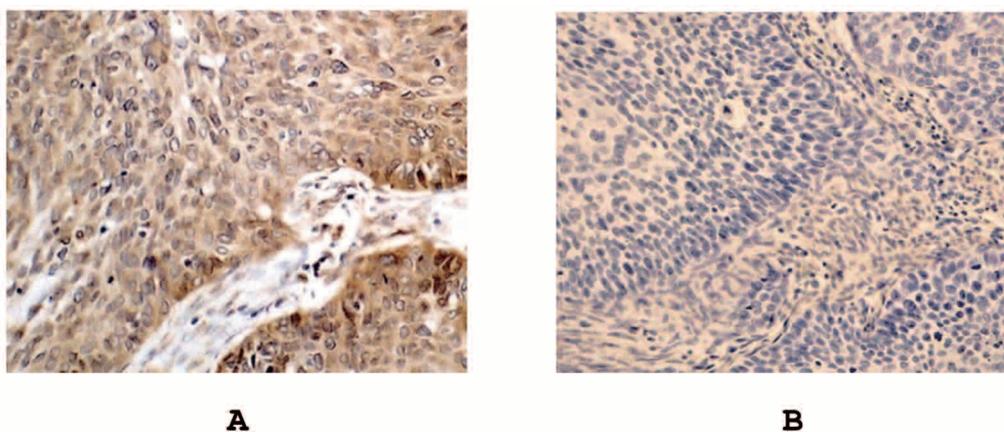


Figure 1. Antibody absorption test using VEGF peptides

The specificity of immunostaining was confirmed by testing absorption between the primary antibody and VEGF peptides. (A) Positive staining for VEGF, (B) staining of VEGF absorbed to VEGF peptides. Magnification, 200× in (A), (B).

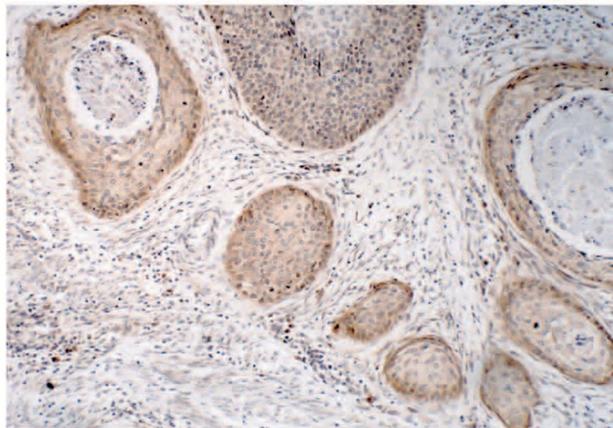
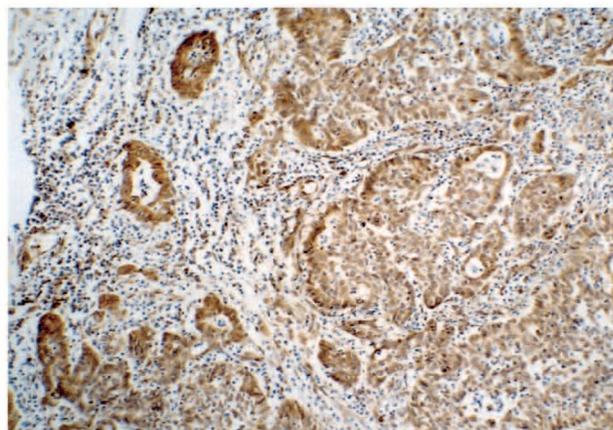
**A****B**

Figure 2. Representative examples of VEGF immunostaining. The intensity of immunostaining was semiquantified as none : 0, weak : 1, moderate : 2, and strong : 3. (A) Typical example of weak staining (squamous cell carcinoma), and (B) typical example of moderate staining (adenocarcinoma). Magnification, 100 \times .

7.3 \pm 1.8, respectively, and VEGF expression was significantly higher in adenocarcinomas than in squamous cell carcinomas ($P < 0.001$; Table 2). In addition, elevated VEGF expression was observed in a significantly larger proportion of adenocarcinomas compared with squamous cell carcinomas [88.24% (15 cases) vs. 18.75% (6 cases), $P < 0.001$; Table 3]. However, there were no significant correlations between VEGF expression and the assessed clinicopathological parameters (Table 4).

Table 2. VEGF expression and microvessel density by histological types

	VEGF expression score	Microvessel density
Squamous cell carcinoma (n=32)	4.4 \pm 1.8	488.6 \pm 189.4
Adenocarcinoma (n=17)	7.3 \pm 1.8	678.5 \pm 242.5
	$P < 0.001^*$	$P = 0.013^*$

*Mann-Whitney's U test ($P < 0.05$)

Data are presented as mean \pm standard deviation.

Table 3. VEGF expression score by histological types

	VEGF expression score	
	Low (score 1-4)	High (score 6-12)
Squamous cell carcinoma (n=32)	26 cases (81.25%)	6 cases (18.75%)
Adenocarcinoma (n=17)	2 cases (11.76%)	15 cases (88.24%)
	$P < 0.001^*$	

*Fisher's exact probability test ($P < 0.05$)

Correlations between microvessel density and clinicopathological parameters

Microvessel density was evaluated by detecting CD31-positive cells and counting the corresponding vessels (Figs. 3D, E). Microvessel density was significantly greater in adenocarcinoma samples than that in squamous cell carcinoma samples ($P = 0.013$; Table 2). In cases of adenocarcinoma, no significant correlations were observed between microvessel density and the assessed clinicopathological parameters. In contrast, microvessel density was significantly greater in squamous cell carcinoma samples than that in advanced stage carcinoma samples and in those with deeper myometrial invasion, vaginal involvement, lymph node metastasis, lymphovascular space invasion, and a high recurrence rate (Table 5).

Correlations among VEGF expression, microvessel density, and prognosis

A weak but significant correlation was observed between VEGF expression and microvessel density (data not shown). Using the Kaplan-Meier method and log rank tests, no significant correlations were observed between VEGF expression and overall survival or disease-free survival or between microvessel density and overall or disease-free survival. These same results were obtained for both adenocarcinoma and squamous cell carcinoma (data not shown).

DISCUSSION

Angiogenesis plays a key role in tumor growth and metastasis. Although angiogenic factor expression and microvessel density have been reported to be markers of tumor malignancy, their utility in cervical cancer remains controversial. A significant correlation was reported between VEGF protein expression and prognostic factors in cervical cancer lesions (6). However, another study reported no significant correlations between VEGF mRNA expression levels and prognostic factors in cervical cancer tissues (4). The correlation between microvessel density and cervical cancer prognosis is also controversial. Several studies have demonstrated that high microvessel density is a useful indicator of cervical cancer prognosis (16, 17). Randall *et al.* reported that increased tumor angiogenesis measured according to CD31-positive microvessel density is an independent prognostic marker of improved progression-free and overall survival. They hypothesized that increased microvessel density is associated with improved treatment response due to better chemotherapeutic delivery and enhanced radiation therapy in well-oxygenated tumors (18). Cervical adenocarcinoma is associated with lower survival compared with squamous cell carcinoma (19-21). Another study reported that microvessel density and VEGF mRNA expression were significantly higher in cervical adenocarcinoma than in squamous cell carcinoma, although no

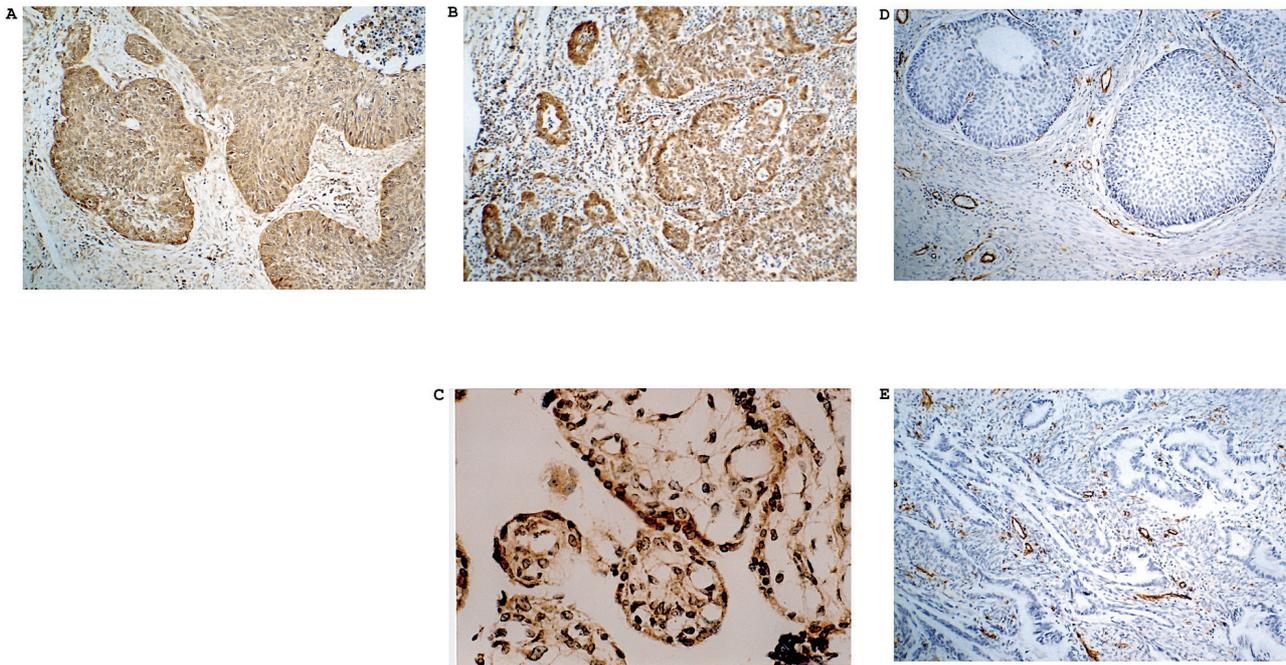


Figure 3. Immunohistochemical staining for VEGF in squamous cell carcinoma (A), in adenocarcinoma (B), in normal placenta as a positive control (C), and staining for CD31 in squamous cell carcinoma (D) and adenocarcinoma (E). VEGF expression and microvessel density were significantly greater in adenocarcinomas than in squamous cell carcinomas. Magnification, 100× in (A), (B), (D), (E) and 400× in (C).

Table 4. Clinicopathological parameters and VEGF expression score (mean±S.D.)

Variable	n	Squamous cell carcinoma		n	Adenocarcinoma	
		VEGF expression score	P value*		VEGF expression score	P value*
Stage (FIGO)						
I	19	3.9±1.4	NS	13	7.6±1.4	NS
II	13	5.1±2.1		4	6.5±3.0	
Cervical infiltration depth						
<2/3	13	4.6±1.9	NS	9	7.4±1.8	NS
≥2/3	19	4.0±1.7		8	7.3±2.0	
Parametrial involvement						
Positive	7	4.9±2.3	NS	4	8.0±0	NS
Negative	25	4.2±1.7		13	7.2±2.1	
Vaginal involvement						
Positive	9	4.6±2.2	NS	6	7.0±2.4	NS
Negative	23	4.3±1.7		11	7.5±1.5	
Pelvic lymph node metastasis						
Positive	11	4.2±1.4	NS	4	8.0±0	NS
Negative	21	4.5±2.0		13	7.2±2.1	
Lymphovascular permeation						
Positive	22	4.2±1.7	NS	9	7.4±1.7	NS
Negative	10	4.7±2.1		8	7.3±2.1	
Recurrence						
Yes	7	4.6±1.5	NS	4	8.0±0	NS
No	25	4.3±1.9		13	7.2±2.1	

*Mann-Whitney's U test (P< 0.05)

Table 5. Clinicopathological parameters and microvessel density (mean±S.D.)

Variable	Squamous cell carcinoma			Adenocarcinoma		
	n	Microvessel density	P value*	n	Microvessel density	P value*
Stage (FIGO)						
I	19	441.3±198.4	0.043	13	705.4±249.0	NS
II	13	557.8±158.0		4	591.0±228.6	
Cervical infiltration depth						
<2/3	13	376.4±165.5	0.004	9	629.0±264.5	NS
≥2/3	19	565.4±168.0		8	734.1±218.5	
Parametrial involvement						
Positive	7	588.7±183.4	NS	4	646.5±173.0	NS
Negative	25	460.6±185.0		13	688.3±265.4	
Vaginal involvement						
Positive	9	598.0±132.2	0.023	6	582.5±177.7	NS
Negative	23	445.8±193.4		11	730.8±264.1	
Pelvic lymph node metastasis						
Positive	11	624.0±161.1	0.005	4	743.8±290.9	NS
Negative	21	417.7±165.2		13	658.4±253.3	
Lymphovascular permeation						
Positive	22	542.1±187.8	0.015	9	693.2±236.3	NS
Negative	10	371.0±137.3		8	661.9±264.5	
Recurrence						
Yes	7	650.7±189.0	0.027	4	774.8±269.5	NS
No	25	443.2±166.2		13	648.8±237.1	

*Mann-Whitney's U test (P<0.05)

prognostic significance could be demonstrated for microvessel density or VEGF expression (22). In the present study, we observed a significant correlation between VEGF expression and microvessel density and both were significantly higher in adenocarcinoma samples than in squamous cell carcinoma samples. These findings may explain the aggressive behavior of adenocarcinomas. Moreover, in cases of squamous cell carcinoma, microvessel density was significantly greater in the presence of poor prognostic factors, including advanced stage carcinoma, deeper myometrial invasion, vaginal involvement, lymph node metastasis, lymphovascular space invasion, and a high recurrence rate. However, no significant correlations were observed between these factors in cases of adenocarcinoma. One explanation for this discrepancy is that the number of adenocarcinoma samples (n=17) was too small to yield significant results. Another possibility is that most cases of adenocarcinoma demonstrated high VEGF expression levels and high microvessel density, making it difficult to detect differences with statistical significance.

Currently, the optimal treatment strategy for stage Ib-IIb early cervical cancer remains unknown. Many countries accept concurrent radiotherapy (CCRT) as a standard initial treatment, and it is sometimes used postoperatively as well (23-26). Radiotherapy (RT) is becoming somewhat indispensable but for maximizing the long-term quality of life of a patient with cervical cancer, the typical late side effects of RT, such as lower extremity edema and gastrointestinal obstruction, must be considered and minimized. Cervical cancer treatment requires adjustments according to the

circumstances of each case, and such adjustments can include avoiding RT as an initial treatment, performing surgery after a pre-operative course of neoadjuvant chemotherapy, selecting chemotherapy (CT) for postoperative treatment, and resorting to RT in cases of subsequent recurrence of localized cancer within the pelvis. Using these treatment approaches, a favorable progression-free survival rate was reported for the treatment of patients with stage Ib2-IIb cervical cancer (27).

For cervical adenocarcinoma, there is no solid evidence regarding the most effective treatment approach (28). Many studies report that the use of RT within the pelvis is more effective for suppressing squamous cell carcinoma than adenocarcinoma (29-32). A recent study reported no appreciable difference in the 5-year survival rate when RT, including CCRT, was compared with CT alone in the postoperative treatment of stages Ib-IIb cervical adenocarcinoma (33). Thus, avoiding RT and applying surgery and CT in treating early cervical adenocarcinoma should be thoroughly considered.

Bevacizumab (Avastin®), a humanized monoclonal antibody directed against VEGF-A, has been reported to be effective in cases of recurrent squamous cell carcinoma of uterine cervix (34). In the present study, squamous cell carcinoma tissues clearly exhibited high microvessel density, suggesting the potential applicability of bevacizumab. In addition, VEGF expression in adenocarcinoma was significantly greater than that in squamous cell carcinoma along with a high microvessel density, which further indicates the potential effectiveness of bevacizumab for treating adenocarcinoma. In

August 2014, the FDA approved bevacizumab for cervical cancer treatment in cases of recurrence or progression (11). Because increased VEGF expression was observed in this study in cases of early invasive cancer (stage I and II), targeting angiogenesis as an initial strategy in cervical cancer treatment appears promising and raises new expectations for improvements in long-term survival among patients with a condition for which such expectations have not existed for many years.

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