

## RESEARCH ARTICLE

# The Association of Parametrial Invasion with the Expression of Vascular Endothelial Growth Factor-C and Other Factors in Squamous Cell Cervical Carcinoma Stage IB and IIA

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## Abstract

**BACKGROUND:** The presence of parametrial invasion in early stage squamous cell cervical cancer (SCCC) indeed indicates worse prognosis and need more adjuvant treatment. This study aimed to investigate the association between parametrial invasion and clinicohistopathology variables.

**METHODS:** This retrospective study used specimens of squamous cell carcinoma stage IB-IIA. The inclusion criteria were cervical cancer stage IB-IIA and post radical hysterectomy with lymph node dissection and exclusion criteria was damaged and or insufficient histological preparations. The clinicohistopathology variables included age, tumour size and stage, differentiation and inflammation grade, lymphatic vascular and parametrial invasion, and lymph node metastasis. Histopathology staining, immunohistochemistry examination, and vascular endothelial growth factor-C (VEGF-C) expression were

evaluated according to the standard procedure. The independent-T, Chi square, and Fisher's exact test were applied to evaluate the association. The significance was set at  $p < 0.05$ .

**RESULTS:** Seventy-five cases were eligible. Parametrial invasion was found in 15 cases (20%). Three variables demonstrated a significant association with parametrial invasion, tumor size  $>4$  cm (OR 4.32, 95% CI 1.29-14.38,  $p=0.01$ ), lymphatic node metastasis (OR 3.90, 95% CI 1.17-13.03,  $p=0.02$ ), and VEGF-C (OR 0.75, 95% CI 0.65-0.87,  $p=0.03$ ).

**CONCLUSION:** Tumor size of  $>4$  cm and lymph node metastasis (LNM) had a higher risk of parametrial invasion in SCCC stage IB-IIA.

**KEYWORDS:** cervical carcinoma, parametrial invasion, vascular invasion, peritumoral stroma, VEGF-C expression

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## Introduction

The high incidence and mortality of the cervical cancer occur in developing countries. Approximately 85% of the prevalence and 88% of the deaths due to cervical cancer are found in those countries. (1) Mortality of the cervical cancer is also high in Indonesia. There were an estimated 9,498 deaths from cervical cancer annually, which was the

second leading cause of death among Indonesian women. (2) The highest mortality was related to cancer progression and poor prognosis as indicated by tumor invasion and lymph node metastasis (LNM). Parametrial tissue is the most frequent site of tumor cells dissemination. The presence of parametrial invasion indicates worse prognosis and more radical surgery is needed in early stage cervical cancer. (3,4)

The vascular endothelial growth factor C (VEGF-C) is a member of vascular endothelial growth factor (VEGF)

family and has a role in angiogenesis. It is produced by tumor cells and peritumor.(5,6) The VEGF-C plays an essential role in lymphangiogenesis predominantly via vascular endothelial growth factor receptor-3 (VEGFR-3). (7) By promoting new lymph vessels, the VEGF-C can accelerate tumor cells metastasis to lymph nodes and distant organs.(8) Prior studies reported that increased VEGF-C was correlated with poor prognosis in several types of cancers. (9,10) A prior study reported that VEGF-C was proven to be involved in lymphangiogenesis in cervical cancer and its gene expression increase according to the increase in the clinical stage.(11)

Lymphatic changes due to lymphangiogenesis intratumoral and peritumoral have been believed to cause distinct biological effect on tumor behaviour and prognosis. However, the results vary between studies. Study in gastric cancer revealed that the intratumoral lymphatic vessel density (LVD) was more associated with the presence of LNM in early stage whereas peritumoral LVD was an independent risk factor for LNM and prognosis.(12,13) Study on the expression of VEGF-C in peritumoral lymphatic has been performed. The results found increased expression of VEGF-C at the edge compared with the center of tumor were correlated with high peritumoral LVD, lymphovascular invasion (LVI), and nodal metastasis. These indicated that the peritumoral lymphatic invasion was linked with tumor metastasis and expected to be an independent prognostic factor in cervical cancer.(14) On the contrary, study on gastric cancer reported that intratumoral, but not peritumoral, lymphatic vessel density (LVD) was closely related with LNM and poor prognosis.(15)

To the best of our knowledge, there may be fewer studies that investigate the association between parametrial invasion and other prognostic factors such as lymphatic invasion in peritumoral in cervical cancer. In addition, the relationship between parametrial invasion, VEGF-C expression, peritumoral lymph vessels with and LNM, and clinicopathological outcomes also have not been firmly established yet. The aim of the present study is to investigate the association of parametrial invasion with VEGF-C expression, LVI, LNM in cervical cancer stage IB-IIA.

## Methods

This was a retrospective study. Tumor specimens were obtained from the Cipto Mangunkusumo Hospital, Jakarta and Hasan Sadikin Hospital, Bandung from September 2014 to January 2016. The inclusion criteria were set as

follows: cervical cancer stage IB-IIA according to the 1998 Federation Internationale des Gynaecologistes et Obstetristes (FIGO) staging system and patients had undergone radical hysterectomy with lymph node dissection. Damaged and or insufficient amounts of paraffin blocks were excluded. The recent study was approved by the Research Ethic Committee of Faculty of Medicine, Universitas Indonesia (No. 564/UN2.F1/ETIK/2014).

### Histopathologic Examination

Histopathologic preparation was made with standard procedure. Initially, the tissues from specimens were fixed at 10% buffered formalin. The next sequential steps were paraffin-embedding and hematoxylin and eosin (H&E) staining. Evaluations of H&E slides of the cases were performed to confirm the diagnosis. The histopathologic features including histologic type, tumor staging and size, tumor differentiation, the presence of LVI, parametrium invasion, and LNM were assessed.

### Immunohistochemistry

Deparaffinization of the 4- $\mu$ m-thick sections were performed in xylene, followed by rehydration in graded concentration of ethanol. The endogenous peroxidase activity within tissue was destructed by mixing with 3% H<sub>2</sub>O<sub>2</sub> in phosphate buffer solution (PBS) for 20 minutes. Antigen retrieval was attained by immersing the sections in 0.01 M citrate buffer, pH 6.0 and heated in a decloaking chamber for 35 minutes. Nonspecific binding sites were blocked in phosphate-buffered saline (PBS) for 20 minutes at room temperature. Primary antibodies for VEGF-C (1:150, Abcam, Cambridge, UK) was used and the slides were incubated overnight at 4°C. After rinsing twice with phosphate buffered saline (PBS) for 10 min, the sections were then incubated with a secondary solution Starr trek Universal HRP Detection system® (Biocare, Birmingham, UK) for 30 min at room temperature. Ultimately, the 3,3'-diaminobenzidine (DAB) was used according to the manufacturer's instructions to visualize the peroxidase activity. Meyers hematoxylin was used to counterstain the sections. A positive staining was confirmed when brown color appeared. The sections were then cleaned using dH<sub>2</sub>O. The positive control was a single slide with squamous cell cervical carcinoma (SCCC) showed strong whose the sections were stained with VEGF antibody. The negative control was achieved by omitting the primary antibody.

Cytoplasmic and membrane immunoreactivity in peritumor was evaluated semiquantitatively. The numbers of brown color stromal cell at peritumor area were evaluated

under light microscopic Olympus CX21 (Olympus, Tokyo Japan) and digital camera Optilab (Optilab, Phoenix, USA) at five random high power field. The expression of VEGF-C was defined as 'low' if there was no staining of inflammatory cells or staining of less than 5%, and defined as 'high' if the staining was more than 5%. The labeling was evaluated by two pathologists independently. If the results were not in agreement, they had to discuss to reach an agreement.

### Statistical Analysis

Numerical data were presented as mean  $\pm$  standard deviation (SD) whereas categorical data as frequency. Comparison between two groups was analyzed using an independent sample of T-test. The association between LNM and VEGF-C expression with clinicopathology features, was analyzed using Chi-Square or Fisher's exact test. The odds ratio (OR) and 95% confidence interval (CI) were also determined. A *p*-value of  $<0,05$  was considered significant. Statistical analysis was computed by using the Statistical Package for the Social Sciences (SPSS) software version 17.

## Results

A total of seventy-five specimens were eligible. Table 1 presented the clinicopathologic variables. Most of the cases were tumor in stage IB with size of  $\leq 4$  cm, well-moderate differentiated, and had well-moderate inflammation grade. Parametrial invasion and lymph nodes metastasis were found only in 15 (20%) and 18 (24%) patients, respectively. LVI was identified in half of the cases.

The VEGF-C showed heterogeneous immunohistochemical expression within the tumor. In peritumoral stroma VEGF-C showed moderate and strong staining intensity (Figure 1). VEGF-C expression in the peritumoral stroma was a predominant low expression (60 cases or 80%), whereas only 15 (20%) cases showed high VEGF-C expression. In 15 cases with high VEGF-C expression, only 1 case showed the presence of LNM and no cases of parametrial invasion.

Table 2 demonstrated the comparison between parametrial invasion and its association with the clinicopathological features. Age was not different between positive and negative parametrial invasion ( $p=0.65$ ). Parametrial invasion had association with tumor size, LNM, and VEGF-C expression. The OR for tumor size, LNM, expression of VEGF-C were 4.32 (95% CI 1.29-14.38,  $p=0.01$ ), 3.90 (95% CI 1.17-13.03,  $p=0.02$ ), 0.75 (95% CI 0.65-0.87,  $p=0.03$ ).

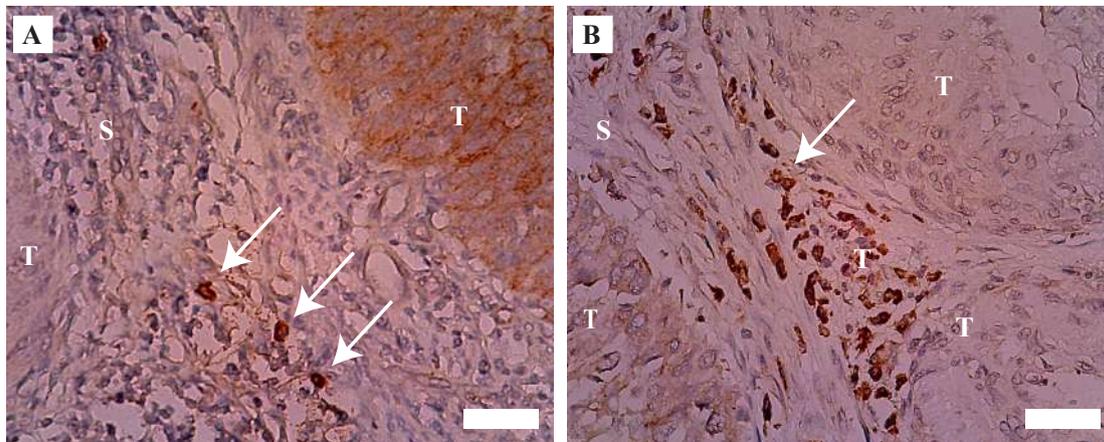
**Table 1. Clinicohistopathology variables.**

Clinicohistopathology Variables	Frequency (%)	
Age (years, mean $\pm$ SD)	46.2 $\pm$ 7.9	
Stage		
FIGO IB1-IB2	53	70.70%
FIGO IIA1-IIA2	22	29.30%
Tumour size		
$\leq 4$ cm	46	61.30%
$> 4$ cm	29	38.70%
Differentiation grade		
Well-moderate	58	77.30%
Poor	17	22.70%
Inflammation grade		
Mild-moderate	43	57.30%
Severe	32	42.70%
Lymphatic vascular invasion		
Negative	37	49.30%
Positive	38	50.70%
Parametrial invasion		
Negative	60	80.00%
Positive	15	20.00%
Lymph node metastasis		
Negative	57	76.00%
Positive	18	24.00%

The association between VEGF-C expression and clinicopathology variables was shown in Table 3. The results show there was no association between age, stage, tumor size, histological grade, degree of inflammation and LVI with VEGF-C expression. The same results were demonstrated with LNM variable, there was a tendency of decreased expression of VEGF-C in peritumoral cervical SCC with LNM compared without LNM ( $p=0.09$ ). A tendency between low expression of VEGF-C and LNM was noted, however, the association was not statistically significantly ( $p=0.10$ ). The expression of VEGF-C is only related with parametrial invasion (in Table 2,  $p=0.03$ ). There was no relationship between VEGF-C expression and other clinicohistopathology factors.

## Discussion

Parametrial invasion indicates a poor prognosis and has implications for the need for more radical surgery.



**Figure 1. Expression of VEGF-C in peritumoral stroma cervical SCCC.** A: low expression, less than 5% inflammatory cells positive VEGF-C (arrow); B: high expression, more than 5% inflammatory cells positive VEGF-C (arrow). T: tumor, S: stroma; white bar: 10  $\mu$ m.

Parametrial tissue resection during radical hysterectomy, however may cause partial denervation of the autonomic nerve resulting in postoperative complications. The factors related to parametrial invasion in the early stage of SCCC should be identified for determining the risk of parametrial involvement. Our study found parametrial invasion occurred

in 20% (15 cases). Parametrial invasion was related to tumor size of >4 cm, LNM, and low expression of VEGF-C but not with other variables. Tumor size >4 cm, LNM, and high expression of VEGF-C had OR 4.32, 3.90 and 0.75, respectively. However, the association with VEGF-C was reversed and did not match with what was expected.

**Table 2. The association between clinicopathology characteristics and parametrial invasion.**

Clinicopathological Characteristics	Parametrial Invasion		p- value	OR (95%CI)
	Positif	Negative		
Age (years)	45.5 $\pm$ 7.8	46.5 $\pm$ 8.0	0.65	
Stage				
FIGO IB1-IB2	11	42	0.99	0.85 (0.24-3.02)
FIGO IIA1-IIA2	4	18		
Tumour size (cm)				
$\leq$ 4 cm	5	41	0.01	4.32 (1.29-14.38)
> 4 cm	10	19		
Differentiation grade				
Well-moderate	11	47	0.73	1.32 (0.36-4.82)
Poor	4	13		
Inflammation grade				
Mild-moderate	2	13	0.72	1.80 (0.36-9.00)
Severe	13	47		
Lymphatic vascular invasion				
Negative	5	32	0.17	2.29 (0.70-7.49)
Positive	10	28		
Lymph node metastasis				
Negative	8	49	0.02	3.90 (1.17-13.03)
Positive	7	11		
VEGF-C expression				
Low	15	45	0.03	0.75 (0.65-0.87)
High	0	15		

**Table 3. The association between VEGF-C expression and clinicohistopathology variables.**

Clinicopathologic variables	VEGF-C expression		p- value
	Low (n=60)	High (15)	
Stage			
FIGO IB1-IB2	45	8	0.12
FIGO IIA1-IIA2	15	7	
Tumour size (cm)			
≤ 4 cm	36	10	0.64
> 4 cm	24	5	
Histological grade			
Well differentiated	46	12	1
Moderate-poorly differentiated	14	3	
Lymphatic vascular invasion			
Negative	31	6	0.42
Positive	29	9	
Lymph node metastasis			
Negative	43	14	0.09
Positive	17	1	

In addition, VEGF-C has no association significantly with any variables.

Parametrium is the most frequent site of local spread of cervical cancer and its metastatic spread mainly lymphatic. (16) The tumor dissemination to parametrial tissue need resection, which is a more radical surgery causing known complications such as dysfunction of the bladder, erectile organ, and anorectal peristalsis. (17) Infertility is the most concerning complication for reproductive women who undergone radical surgery. (16) Previous studies have been performed to identify the risk factors for parametrial metastasis. This can help identify patients with low risk of parametrial metastasis and perform less radical surgery. Study on cervical carcinoma reported that patients with stage IB1 accompanied by superficial invasion (<5 mm) were at very low risk of parametrial invasion. Some previous studies demonstrate that parametrial invasion are related to tumor size, histologic grade, LNM, and LVI. The incidence of parametrial involvement is negative or low in primary tumor size of less than 2 cm, no LVI, no LNM. (18-21) In agreement with some previous studies, this study reported any association between parametrial invasion with tumor size, LNM, and VEGF-C expression.

The association between parametrial invasion and VEGF-C expression was not evident in this study. With a significant *p*-value, the association was not as expected, in

which parametrial invasion was more pronounced in low expression rather than in high expression of VEGF-C. The mechanism is not clearly understood, but it might be related to tumor cells invasion. Spreading tumor cells into lymphatic vessels could destroy the lymphatic endothelial lead to low expression of VEGF-C. However, this finding needs further evaluation to confirm whether VEGF-C indeed associate with parametrial invasion.

VEGF-C specifically acts on lymphatic endothelial cells through its recepto, VEGFR-3 to form new lymphatic vessels. (22,23) Study in cervical carcinoma indicated that VEGFR-3 expression was increased and correlated significantly with peritumoral LVD. (23) A similar results was reported which also found an increased VEGV-C expression was in accordance with clinical stage in SCCC. (11) Our study was not in agreement with those previous studies. The expression of VEGF-C had no association with LVI. We did not have not some definite explanation, but it might be related to histologic staining methods. With histologic appearance that resembles blood vessel, lymphatic vessel invasion could not be distinguished by the H&E staining method. (24) To date, some more specific staining method (for example: D2-40, a monoclonal antibody) has been considered to have better capability to distinguish the vessels because it selectively stain lymphatic endothelial and exclusively found in the peritumoral area. (23,25-27)

Both intra- or peritumoral lymphatic have a role on tumor cells dissemination and clinicopathology but the results vary between studies. It has been reported that peritumoral lymphatic vessels could be a potential prognostic marker for endometrial carcinoma.(28) A meta-analysis study reported that peritumoral lymphatic vessels was thought to be the main dissemination route in breast cancer.(29) On the other hand, a previous study found a conflicting result in which intratumoral LVD had a strong association with LNM and prognosis.(15) However, a more recent study reported that both intratumoral and peritumoral LVD correlate with LNM.(30) We examined peritumoral lymphatic as it was considered to be more often associated with nodal metastasis and clinicopathological features. In addition, intratumoral lymphatic is sometimes collapse because it has been invaded and destroyed by tumor cells. (31,32) Our study did not find any correlations between LVI with LNM and VEGF-C. LVI has been regarded as a strong prognostic factor in breast cancer.(33,34) Yet, previous study evaluating the relationship between LVD, LVI, and LNM demonstrated that LVD correlated moderately and LVI correlated weakly with LNM.(29)

Not every tumor types demonstrates the association of VEGF-C with LNM. Research during recent years has provided more understanding towards the mechanism of local invasion and lymphatic spread. Nowadays, two lymphangiogenic growth factors, VEGF-C and VEGF-D, are recognized. They are produced by both tumor and stromal cells. These growth factors induce proliferation and migration of cancer cells through signaling via VEGFR-3 receptor found in lymphatic endothelial cells.(23) Secreted in an active form, VEGF-C and VEGF-D have an ability to bind VEGFR-3. The VEGF-C and VEGF-D will be processed by protease into an active form with higher affinity for VEGFR-3 in the extracellular environment. (21,24) There was an indication of overlapping functions between these two growth factors, because a deficiency of VEGF-C can be replaced by VEGF-D.(24) Similar evidence was shown in colorectal cancer with decreased VEGF-D, in which the carcinoma may enhance the elevated levels of VEGF-C to bind with VEGFR-3 easily. The balance of different VEGF family members changes significantly, in which the ratio of VEGF-C and VEGF-D is essential for lymphatic invasion and metastasis.(22)

Limitations of this study have been documented. First, we did not examine LVD as an indicator for lymphatic invasion. Second, we did not use D2-40 staining even though it is a more specific staining for endothelial lymphatic vessels. The use of D2-40 could improve detection rates and

avoid the false positive or negative as well. Third, VEGF-D expression was not examined. The VEGF-D, like VEGF-C, is also a potential regulator for lymphangiogenesis. The presence of VEGF-D expression is needed to confirm the lymphatic vascular invasion which could not be detected by VEGF-C.

## Conclusion

In conclusion, this study indicates that parametrial invasion has association with tumor size and LNM, but not with VEGF-C expression. Tumor size of >4 cm and positive LNM in stage IB-IIA squamous cell carcinoma increased the risk of parametrial invasion. Even with a significant result, the association of parametrial invasion and the VEGF-C is not conclusive because the direction of association is not appropriate. A further investigation is needed to confirm the association.

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