RESEARCH ARTICLE

Serum levels of VEGF and VEGF-C in patients with endometrial cancer

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ABSTRACT. Endometrial cancer (EC) is the most common type of uterine cancer. A dualistic model of endometrial tumorigenesis serves as a useful way of categorizing these cancers in terms of both etiology and clinical behavior. There are two types of EC: type I and type II. Type I is so-called estrogen-dependent, and appears mostly in pre- and perimenopausal women, it is well differentiated and therefore has a better prognosis. Type II EC is estrogen-independent, diagnosed mostly in postmenopausal women, thin and fertile women, or in women with normal menstrual cycles. It is aggressive and has a worse prognosis than type I. The aim of this study was to evaluate the relationship between the pretreatment serum levels of VEGF and VEGF-C and the outcome of EC patients. A total of 98 patients treated between 1999 and 2003 were included in this study. Circulating VEGF and VEGF-C levels were determined using ELISA kits. VEGF levels among the 76 patients with type I, and the 22 patients with type II EC were significantly higher than those found in the healthy control subjects ($p < 0.001$). The differences in mean values of VEGF-C were highly significant in both types of tumor examined compared to the control ($p < 0.001$). The results demonstrate that serum VEGF concentration correlated significantly with advanced FIGO stage in type II EC ($p < 0.001$). The preoperative VEGF-C level correlated with advancing tumor stages in type I EC ($p < 0.05$). An elevated preoperative VEGF-C was an independent risk factor for disease-specific survival in patients with type II tumors. Thus, in type II EC patients with high preoperative levels of VEGF-C, pelvic and para-aortic lymphadenectomy should be performed. However, the value of longitudinal measurements of the markers used is yet to be determined.

Key words: vascular endothelial growth factor (VEGF), vascular endothelial growth factor-C (VEGF-C), endometrial cancer (EC), enzyme-linked immunosorbent assay (ELISA)

Cancer of the uterus is the seventh most commonly diagnosed cancer that occurs in women, with 189,000 new cases and 45,000 deaths occurring worldwide each year. About 60% of these occur in more developed countries. The highest incidence rates are in the USA and Canada. The age-adjusted incidence rate in the USA was 23.3 per 100,000 women per year [1]. Other regions with age-standardized rates in excess of 10 per 100,000 include Europe, Australia and New Zealand, the southern part of South America, and the Pacific Island nations. Low rates occur in Africa (Uganda 3.3 per 100,000) and Asia (China 3.8 per 100,000) [2]. In Poland, the age-adjusted incidence was 13.7 per 100,000 women per year [3].

Most endometrioid carcinomas are well-to-moderately differentiated, and arise against a background of endometrial hyperplasia. These tumors, also known as type I (low-grade) endometrial carcinomas, have a favorable prognosis. They are associated with long-duration, unopposed estrogen stimulation. About 10% of endometrial cancers are type II (high-grade) lesions. Women with such tumors are at high risk of relapse and metastatic disease. These tumors are not estrogen-driven, and most are associated with endometrial atrophy; surgery is commonly followed by adjuvant therapy [4].

In the last decade, several studies have assessed the clinical relevance of different biological variables evaluated in serum/plasma/tissue samples from patients with endometrial cancer in order to detect markers capable of predicting either the response to adjuvant therapy or outcome [5-9].

Circulating angiogenic and lymphangiogenic growth factors have been investigated in malignant tumors whose lymph node status detection is crucial in terms of treatment planning.

VEGF-A is a key molecule in the induction of angiogenesis and vasculogenesis. It causes proliferation, sprouting, migration and tube formation of endothelial cells. The

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VEGF-A gene is located on chromosome 6p21.3, and is encoded by eight exons separated by seven introns. VEGF induces angiogenesis in a variety of physiological and pathological conditions including embryogenesis, corpus luteum formation, tumor growth, wound healing, and compensatory angiogenesis in the heart [10, 11].

VEGF-C is a key regulator of lymphangiogenesis and an important mediator of tumor metastasis to lymph nodes. The VEGF-C gene is located on chromosome 4q34. VEGF-C genes comprise over 40 kb pairs of genomic DNA, and consist of seven exons. VEGF-C is produced as a precursor protein and is proteolytically activated in the extracellular space by proteases to generate a homodimeric protein with high affinity for both VEGFR- 2 and VEGFR-3. VEGF-C induces mitogenesis, migration and survival of endothelial cells. VEGF-C is expressed in the heart, small intestine, placenta, ovary, and the thyroid gland in adults [11].

VEGF-C specifically activates the VEGF receptor-3 (VEGFR-3), a cell-surface tyrosine kinase receptor expressed on lymphatic endothelial cells as well as on other cell types such as cancer cells. Expression of VEGF-C by tumor cells in xenograft or transgenic cancer models increased the abundance of lymphatic vessels at the tumor periphery and sometimes within the tumor, promoted metastasis to local lymph nodes and, in some models, facilitated distant organ metastasis [12].

Several angiogenic and lymphangiogenic factors have been identified and are believed to be involved in physiological as well as pathological angiogenesis in the human endometrium [13, 14]. It has been shown to be significantly upregulated in endometrial cancers and to be associated with tumor angiogenesis and disease outcome [10, 15, 16]. The expression of VEGF is four to 10 times higher at the invading tumor front than in the central tumor areas [17]. Indeed, it has been shown that overexpression of VEGF and its receptors are related to poor prognosis in patients with endometrial carcinomas [18-21]. Circulating VEGF levels seem to be particularly useful as a prognostic tool in clinical subsets of patients, such as those with early-stage or lymph node-negative cancers, for which current clinicopathological parameters are limited in their prognostic capacity [22-24].

Therefore, the aim of this study was to evaluate the relationship between the pretreatment serum levels of VEGF and VEGF-C, and the outcome for endometrial cancer patients.

DONORS AND METHODS

Patients and clinical samples

A total of 98 patients with endometrial cancers (aged between 49-72 years; median: 61.7 years), treated at the Department of Gynecology and Septic Obstetrics, Medical University of Bialystok and Department of Gynecology District Hospital in Bialystok, between 1999 and 2003, were included in this study. None of the patients had received chemotherapy, hormone therapy or radiotherapy prior to surgery. All patients had primary cancers and were receiving their first treatment. Cases selected in the current study presented the same stage, both clinically and surgically. All tumors were staged according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. All patients underwent abdominal hysterectomy and bilateral oophorectomy. Pelvic and para-aortic lymphadenectomy was performed at stage II (34 cases) and at stage III (16 cases). Adjuvant chemotherapy was added to the treatment for stage III patients.

Clinicopathological information was obtained from medical charts. Histopathological examination was performed according to the WHO classification. Representative samples of hysterectomy specimens were stained with H&E for light microscopy study and evaluated to confirm tumor stage, and to assess depth of myometrial invasion, grade, histological type and presence or absence of lymphovascular space invasion.

Preoperative, 5 mL blood samples were collected and serum was separated immediately by centrifugation at 3,000 g for 10 min and stored at -70°C until assay. Circulating VEGF and VEGF-C levels in the serum samples were determined quantitatively using the VEGF (Quantikine human VEGF; R&D Systems, Minneapolis, MN, USA) and VEGF-C (IBL International GmbH, Hamburg, Germany) ELISA kits according to the manufacturers' instructions.

In brief, 100 μL of recombinant human VEGF, standard or serum sample, were added to a microtiter plate coated with murine monoclonal antibody specific to human VEGF. After a two-hour incubation at room temperature and then washing away of any unbound substances, a horseradish peroxidase-linked polyclonal antibody specific for VEGF was added to each well to sandwich the VEGF. Further washings were performed to remove any unbound antibody-enzyme reagent before the stop solution was added. For the VEGF-C assay, VEGF-C standard solution or each serum sample, mixed with an equal volume of diluent buffer of two-fold serial dilutions, was added to the wells of the plates precoated with rabbit antihuman VEGF-C antibodies.

The plates were ready for measurement at the optical intensity of 450 nm by ETI Sorin Biomedica ELISA reader (Sorin Biomedica, Bio-Tek, USA). Each measurement was made in duplicate. A standard curve of VEGF or VEGF-C was plotted for each assay and two calibrators of normal serum samples were included in every assay-run to adjust for plate-to-plate variance. The detection sensitivity limits of the VEGF and VEGF-C assays were 9.0 and 4.0-48.4 (mean 13.3) pg/mL, respectively, whereas the coefficient of variance of both assays was less than 5.0%.

The serum from 30 healthy women (aged between 19-71 years; mean: 52.8 years), who visited District Hospital for gynecologic malignancy screening, served as controls. Patients gave their informed consent for the study. The protocol had been previously approved by the Bioethical Committee of the Medical University of Bialystok. Follow-up data were collected until January 2010.

Statistical analysis

Continuous data are expressed as the median (range). Categorical data were compared by the χ^2 test or Fisher's exact test. The Kruskal-Wallis and Mann-Whitney *U* tests were used to evaluate differences between observations. Correlations between continuous data were evaluated by means of the Spearman rank test.

Disease-specific survival rate was calculated from the date of surgery until death due to endometrial cancer.

Kaplan-Meier curves were plotted and compared using log rank statistics. A Cox proportional hazards regression model was used for multivariable analyses. All statistical analyses were calculated using Statistica software version 9.0PL (StatSoft, Inc., StatSoft Polska Sp. z o.o., Poland). $P < 0.05$ was considered to be statistically significant.

RESULTS

The tumors were classified as follows: 76 cases were type I (endometrioid endometrial carcinomas), and 22 cases were type II (therein 19 cases of serous and three of clear-cell carcinomas). Among patients with type I endometrial cancer, 43 had tumors classified as stage I, 24 patients had tumors classified as stage II, and nine patients were classified as stage III. The type II tumors were classified as follows: five cases were in stage I, 10 cases were in stage II and seven cases were in stage III. The samples were grouped according to histological grade: 41 type I endometrial cancers were classified as grade 1, 21 were grade 2 and 14 were grade 3. All 22 type II tumors were grade 3 *(table 1)*.

All patients were followed up. At a median follow-up of 60 months (range 1-126), 37 patients had died as a consequence of cancer progression.

Both serum VEGF and VEGF-C levels were significantly increased in endometrial cancer compared with the levels in healthy controls *(figure 1)*. Preoperative serum VEGF levels among the 76 patients with type I and the 22 patients with type II endometrial cancer, were significantly higher than those in the 30 healthy control subjects: 495.7 (164.3-704.8) pg/mL and 579.4 (366.8-760.5) pg/mL *versus* 232.4 (135.5-330.7) pg/mL ($p < 0.001$). The differences in mean values for VEGF-C were highly significant in both types of tumors examined: 5,120.1 (2,030.4-8,460.6) pg/mL in type I and 7213.2 (4,531.8-10,831.1) pg/mL in type II *versus* 4,223.5 (1,821.9-6,910.2) pg/ml in control ($p < 0.001$) (*table 2*).

The correlation between the FIGO stage and pretreatment serum values for VEGF and VEGF-C are shown in *Table 3*. The results demonstrate that serum VEGF concentration correlated significantly with the FIGO advanced stage in type II endometrial cancer ($p < 0.001$). Conversely, it did not correlate with the FIGO stage in type I tumors. In patients with type I endometrial cancers, the pretreatment serum VEGF-C levels correlated with the FIGO stage ($p < 0.05$), but did not correlate with staging in type II cancers.

Among those patients with high pretreatment levels of VEGF (> 495.7 pg/mL), in type I endometrial cancer, the Kaplan-Meier survival estimates of the 1-year, 5-year, and 10-year survival rates were 91.9%, 87.2%, and 89.2%, respectively, while the respective rates among patients with low levels (< 495.7 pg/mL) were 94.9%, 94.9%, and 94.9%. Statistically significant differences were not observed between survival rates over time (log-rank test; $p = 0.57$) *(figure 2)*.

In the group with type II endometrial cancer with high preoperative levels of VEGF (> 579.4 pg/mL), the Kaplan-Meier survival estimates of the 1-year, 5-year, and 10-year survival rates were 70.0%, 60.0%, and 60.0%, respectively, while the respective rates among endometrial cancer patients with low levels (< 579.4 pg/mL) were 83.3%, 75.0%, and 75.0%. No statistically significant difference was observed between survival rates over time (log-rank test; $p = 0.48$) *(figure 3)*.

In type I endometrial cancer patients with low VEGF-C levels of 5,120.1 pg/mL or less, the 1-year, 5-year, and 10-year survival rates were 90.4%, 88.6%, and 88.6%, compared with those with levels of more than 5,120.1 pg/mL (97.0%, 94.0%, and 94.0%, respectively); the differences in survival were not significant (log-rank test; $p = 0.56$) *(figure 4)*.

Among those patients with high levels of VEGF-C (> 7,213.2 pg/mL) in type II endometrial cancer, the Kaplan-Meier survival estimates of the 1-year, 5-year, and 10-year survival rates were 50.0%, 0%, and 0%, respectively, while the respective rates among patients with low levels (< 7,213.2 pg/mL) were 60.0%, 50.0%, and 50.0%. A statistically significant difference was observed between survival rates over time (log-rank test; $p = 0.0197$) *(figure 5)*. The cumulative disease-specific survival curves of patients grouped according to VEGF and VEGF-C levels are shown in *figures 2-5*.

Table 1
Patients and tumor characteristics.

Characteristics	Patients n (%)	
	Type I	Type II
	76 (77.6)	22 (22.4)
Age		
≤ 60 years	45 (59.2)	3 (13.6)
> 60 years	31 (40.8)	19 (86.4)
Stage		
I	43 (56.6)	5 (22.7)
II	24 (31.6)	10 (45.5)
III	9 (11.8)	7 (31.8)
Histological grade		
G1	41 (53.9)	-
G2	21 (27.7)	-
G3	14 (18.4)	22 (100)

Table 2
Comparison of serum VEGF and VEGF-C levels between patients with type I and II endometrial cancer.

	No. of cases	VEGF (pg/mL) median (range)	p-value	VEGF-C (pg/mL) median (range)	p-value
Controls	30	232.4 (135.5-330.7)		4,223.5 (1,821.9-6,910.2)	
Type I	76	495.7 (164.3-704.8)	< 0.001	5,120.1 (2,030.4-8,460.6)	< 0.001
Type II	22	579.4 (366.8-760.5)	< 0.001	7,213.2 (4,531.8-1,0831.1)	< 0.001

VEGF: vascular endothelial growth factor; VEGF-C: vascular endothelial growth factor C; type I endometrial cancer: endometrioid endometrial cancer; type II endometrial cancer: non-endometrioid endometrial cancer.

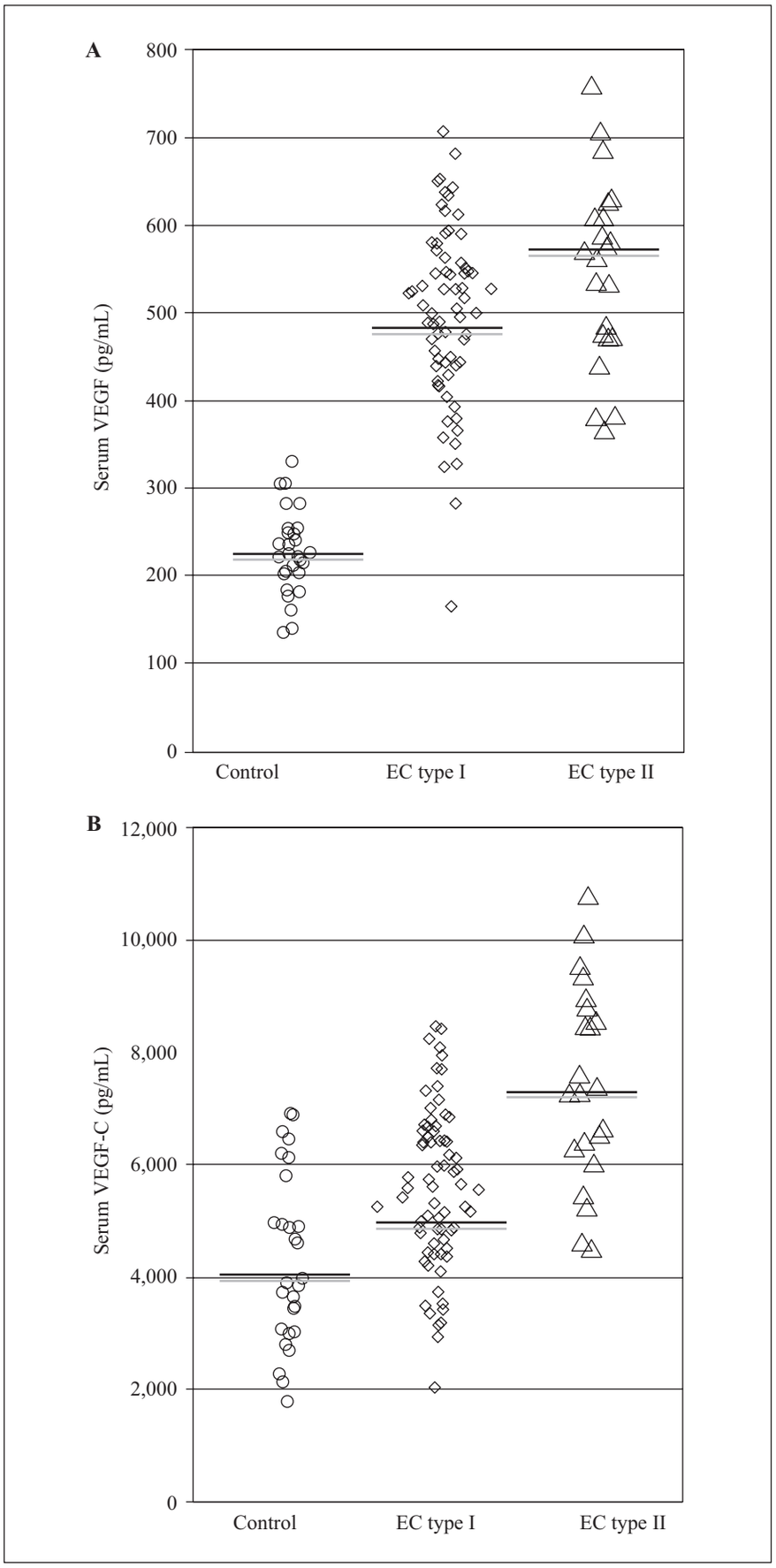

Figure 1
Dot plots of preoperative serum VEGF **(A)** and VEGF-C **(B)** levels in patients with endometrial cancer (EC) type I and II. The horizontal line in dot clusters of each group indicates the mean values.

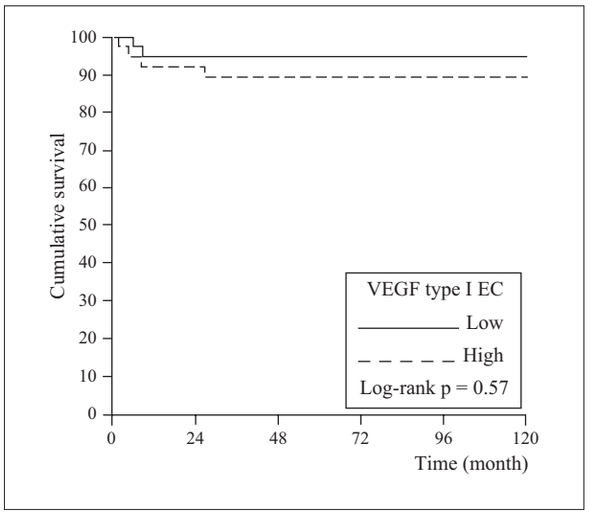

Figure 2
Kaplan-Meier survival analysis concerning VEGF serum levels and cumulative disease-specific survival in patients with type I EC (p = 0.57).

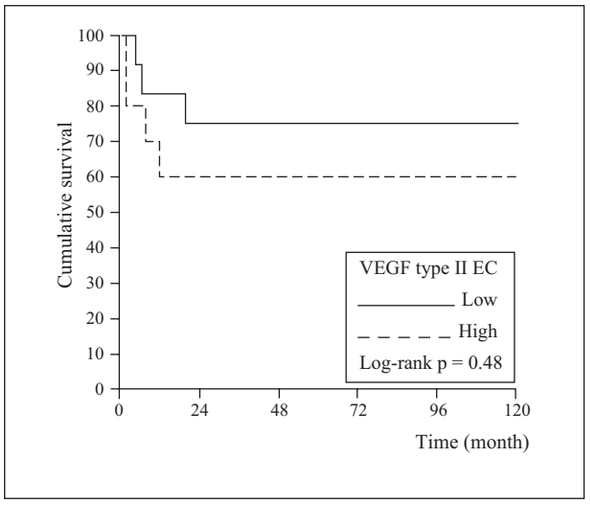

Figure 3
Kaplan-Meier survival analysis concerning VEGF serum levels and cumulative disease-specific survival in patients with type II EC (p = 0.48).

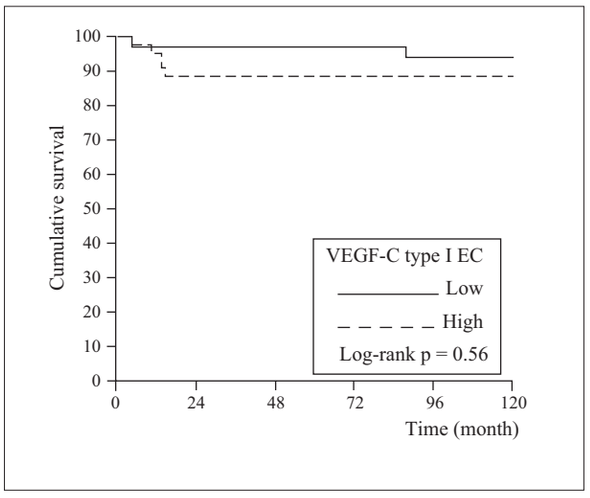

Figure 4
Kaplan-Meier survival analysis concerning VEGF-C serum levels and cumulative disease-specific survival in patients with type I EC (p = 0.56).

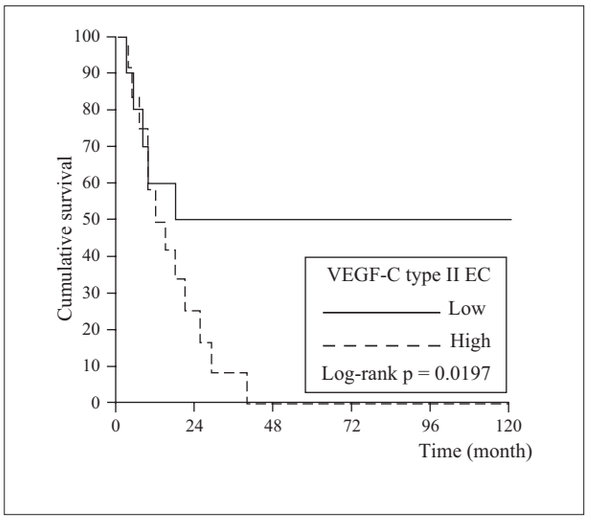

Figure 5
Kaplan-Meier survival analysis concerning VEGF-C serum levels and cumulative disease-specific survival in patients with type II EC (p = 0.0197).

DISCUSSION

Studies involving circulating VEGF in cancer patients have so far focused mainly on their prognostic implication in relation to tumor status and outcome. Although the results of many studies have not been completely homogeneous, there is compelling evidence that VEGF and VEGF-C levels are of prognostic significance [25-28].

Apart from providing prognostic value, the level of these circulating factors may also have important therapeutic implications in the selection of patients for adjuvant therapy. The use of preoperative circulating VEGF and VEGF-C levels before surgical resection to predict invasiveness of a tumor, such as the presence of vascular invasion and lymph node metastasis, is particularly attractive in that it may help in the selection of patients for neoadjuvant therapy [24].

Although endometrial cancer is the most common type of gynecological cancer in the developed world, the details of its progression are still not well understood. In contrast to other malignancies with a high potential for neovascularization and distant metastatic potential, the lack of clinical relevance of VEGF in endometrial cancer could be attributed to the relatively low angiogenic potential of the cancer, which rarely metastasizes by the hematogenous route and has a low incidence of distant metastases [29]. On the other hand, there was a tendency for endometrial cancer patients with distant metastases to have higher VEGF levels [27].

In endometrial cancer, a single study has examined the value of measuring plasma/serum/tissue/cytosol VEGF and VEGF-C levels as a predictive factor for cancer progression and outcome. In other studies, the up-regulation of tissue VEGF and VEGF-C mRNA and protein expression has been described. Data from these papers however, cannot be extrapolated to circulating levels of these angiogenic factors in endometrial cancer [30].

In the present study, both preoperative levels of circulating angiogenic factor, VEGF and lymphangiogenic factor, VEGF-C were measured, and the differences between VEGF and VEGF-C were characterized regarding the relationship with the clinicopathological features of

Table 3
Correlation between preoperative serum VEGF and VEGF-C levels and clinical stage of endometrial cancer.

	No. of cases	VEGF (pg/mL)	VEGF-C (pg/mL)
		median	median
Type I			
Stage I	43	483.1	4,710.3
II	24	500.2	5,212.8
III	9	466.7	5,733.2
p value		NS	$p < 0.05$
Type II			
Stage I	5	490.7	7,123.4
II	10	581.3	7,121.9
III	7	699.5	7,212.3
p value		$p < 0.001$	NS

Type I endometrial cancer: endometrioid endometrial cancer; type II endometrial cancer: non-endometrioid endometrial cancer.

endometrial cancer patients. However, the role of circulating VEGF in endometrial cancer patients remains controversial. One study focusing on endometrial cancer pathogenesis and tumor growth found no significant difference in VEGF levels between patients and normal individuals, whereas other studies have shown the opposite [23, 31]. In our previous immunohistochemical study, highly VEGF-positive tumors showed a poorer prognosis than VEGF-negative tumors. There was a trend towards an association between the highly positive expression of VEGF and 5-year, disease-free survival. These results suggest that VEGF may be an important, clinically relevant inducer of angiogenesis in type I endometrial cancer [32]. However, methodological problems such as inter- and intraobserver variability, and the selection of the area of the most intense VEGF immunoexpression remain unresolved. Thus, quantification of VEGF from patient serum would be easier and more objective.

Stage is an independent risk factor for endometrial cancer patients. In our present study, type I tumors with an advanced clinical stage did not show elevated VEGF levels, but type II showed significantly higher VEGF levels. This suggests that tumor biology, other than tumor angiogenesis, may also influence local invasiveness in type I endometrial carcinoma. VEGF-C concentrations were higher in patients with advanced stages of type I endometrial cancer; therefore, VEGF-C could reflect disease progression. This suggests that VEGF-C-promoted lymphangiogenesis may be continuous, and becomes greater during type I cancer growth.

An increased preoperative VEGF-C level was the only risk factor for the occurrence of nodal metastases, and might be potentially of use in predicting the presence of clinically relevant, nodal metastases. Measurement of preoperative VEGF-C can be used to predict any lymph node metastases, but whether this marker could be applied to facilitate a more selective application of abdominal lymphadenectomy in endometrial cancer is questionable [33, 34].

In conclusion, preoperative VEGF and VEGF-C levels were substantially higher in endometrial cancer patients compared with control subjects. The preoperative VEGF-C level correlated with advancing tumor stages in type I endometrial cancer. An elevated preoperative VEGF-C was the independent risk factor for disease-specific survival in patients with type II tumors. We suggest that in type II endometrial cancer patients with preoperative high levels of VEGF-C, pelvic and para-aortic lymphadenectomy should be performed. However, the value of longitudinal measurements of the markers used is yet to be determined.

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