Pol J Pathol 2011; 1: 31–40

Clinical significance of VEGFR-2 and VEGFR-3 expression in ovarian cancer patients

Dagmara Klasa-Mazurkiewicz[1], Mirosław Jarząb[2], Tomasz Milczek[3], Barbara Lipińska[2], Janusz Emerich[4]

[1]Department of Perinatology, Medical University of Gdańsk
[2]Department of Biochemistry, University of Gdańsk
[3]Department of Gynaecologic Oncology, Medical University of Gdańsk
[4]Head of Department of Gynaecologic Oncology (1996 to 2009), Medical University of Gdańsk

The vascular endothelial growth factor (VEGF) family and VEGF receptors (VEGFR) play an essential role in angiogenesis and lymphangiogenesis. The aim of this study was to clarify the prognostic significance of VEGFR expression in ovarian carcinoma. Levels of VEGFR-2 and VEGFR-3 tissue expression in human ovarian tumours were assayed by immunoblotting and the correlations between analysed factors and clinicopathological features were examined. Tissue samples consisted of 42 benign and 10 borderline (low malignant potential – LMP) tumours, 76 ovarian carcinomas, 8 Krukenberg tumours and 32 normal ovarian tissues. The highest relative level of VEGFR-2 was detected in cases with at the early stages of cancer development. The highest level of VEGFR-3 was detected advanced cancer stages and those with Krukenberg tumours. Overexpression of VEGFR-3 was found to correlate with the debulking status ($p = 0.02$) and positive response to chemotherapy ($p = 0.04$). A statistically significant longer progression free survival (PFS) was observed in women with a low than with a high expression of VEGFR-3 ($p = 0.01$). Increased levels of VEGFR-2 expression at the early stages of ovarian cancer may indicate the significance of neoangiogenesis at these stages. Overexpression of VEGFR-3 reflects the aggressiveness of ovarian carcinoma spread and has a predictive value for identifying high-risk patients with poor prognosis.

Key words: ovarian cancer, VEGFR-2, VEGFR-3, prognostic factors, angiogenesis, lymphangiogenesis.

Introduction

Diagnosis and subsequent treatment of ovarian cancer is a great challenge for oncologic gynaecology. Ovarian cancer is the cause of the largest number of deaths among all gynaecological malignancies in women. The world statistics note over 200 000 new ovarian cancer cases annually, as well as 115 000 deaths caused by it [1]. Unfavourable prognoses in ovarian cancer cases are the inducement for the search for more efficient and less toxic treatment methods. In parallel, research aimed at finding new biomarkers is being conducted, which would allow for more efficient screening as well as identifying patients who may benefit from more "aggressive" treatment or target treatment.

The key stages in the development of cancer are the passage of the carcinoma cells through the basic membrane – the acquisition of invasive properties, infiltration of adjacent tissues, and creation of new blood and lymphatic vessels to sustain the growth of the tumour, which leads to the appearance of metastasis [2]. Angiogenesis and lymphangiogenesis are processes which are taken into consideration in early diagnosis and prognosis of the course of the cancer [3, 4]. Increasing evidence shows that the VEGF (vascular endothelial growth factor) family proteins play an important role during metastatic spread of

cancer, which can occur via blood or lymphatic vessels [4]. The biological functions of the VEGFs are mediated by a family of tyrosine kinase receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4). Signalling through VEGFR-2 and VEGFR-3 is involved in angiogenesis and lymphangiogenesis, respectively [5, 6].

Vascular endothelial growth factor receptor-2 (VEGFR-2) mediates the majority of the downstream effects of VEGF-A (referred to as a vascular permeability factor), which is an endothelial specific mitogen with a diverse range of angiogenic activities. VEGFR-2 is involved in microvascular permeability, endothelial cell proliferation, invasion, migration and survival. It has been demonstrated that inactivation of the *VEGFR-2* gene in mice resulted in death *in utero*, caused by defects in blood island formation and vascular formation. The specificity of VEGFR-2 expression, its location on the surface of the tumour vessels, and its predominant role in tumour angiogenesis make it a highly desirable target for the development of both antiangiogenic and vascular targeting drugs. Various VEGFR-2 inhibitors including receptor-specific antibodies and low molecular weight chemicals have been developed and one of the latter (BAY43-9006) has been approved by the FDA for the treatment of renal cancer patients [6, 7].

Vascular endothelial growth factor receptor-3 (VEGFR-3) was the first lymphatic-specific growth factor receptor identified [8]. It is found almost exclusively in lymphatic endothelium in human adults. VEGFR-3 is also expressed in endothelial cells of tumour blood vessels, suggesting its implication in tumour angiogenesis [9]. Several studies show that VEGFR-3 is required for cardiovascular development during embryogenesis. In mouse development VEGFR-3 is expressed in venous endothelium at sites of lymphatic vessel sprouting, while in normal adult tissues, its expression is restricted to lymphatic endothelium [9]. Inactivation of the *VEGFR-3* gene resulted in defective blood vessel development in early mouse embryos [10]. Several reports suggest that VEGFR-3 signalling pathway may be a potential target for anti-cancer therapy [11, 12]. Roberts *et al*. [12] have shown that inhibition of VEGFR-3 activation by antagonistic antibodies suppressed lymph node and organ metastases in a mouse breast carcinoma model.

Because of the role of the VEGF receptors in tumorigenesis and as drug targets in cancer therapy, it is important to assess their expression and prognostic significance as well as correlation with clinicopathological features in specific types of cancer.

The aim of this work was to evaluate the expression of VEGFR-2 and VEGFR-3 in human ovarian tumours and assess the relationship between the expression of the analysed proteins and the selected clinicopathological features, and the results of treatment among the ovarian cancer patients.

Material and methods

Patients and tissue samples

168 ovarian specimens were obtained during surgeries from patients who were treated in the Gynaecologic Oncology Department, Medical University of Gdańsk (from April 2004 to July 2007, Head of Department – professor Janusz Emerich). Collecting of tissues was supervised by a pathologist. One part of every tissue sample was immediately frozen in liquid nitrogen, stored at –70°C and subsequently used for protein assay; the second part was fixed in formalin and then histopathologically diagnosed. Specimens consisted of 32 normal tissues (obtained from patients who underwent surgery for gynaecologic diseases other than ovarian tumours), 42 benign tumours, 10 borderline (low malignant potential – LMP) tumours, 76 malignant epithelial ovarian neoplasms and 8 Krukenberg tumours. The study was approved by the local Ethics Committee (no. NKEBN/556/2006).

The mean age of all the patients was 54.0 years. The mean age for women with benign tumours was 49.2 years (range: 19 to 80 years), 46.6 years (range: 25 to 76 years) for women with LMP tumours, 57.5 years (range: 24 to 86 years) for patients with invasive ovarian carcinomas and 62.0 years (range: 48 to 80 years) for patients with Krukenberg tumours. The mean age for women who underwent surgery for gynaecologic diseases other than ovarian tumours was 51.7 years (range: 34 to 73 years).

Among of the benign lesions, 12 were classified as serous cystadenomas, 10 as endometrioses, 8 as mucinous cystadenomas, 8 as ovarian dermoid cysts, 2 as thecomas and 2 as corpus luteum. Among the borderline tumours, 4 were serous cystadenomas, 4 mucinous cystadenomas, one Brenner's borderline malignant tumour and one endometrioid tumour. Among Krukenberg tumours 6 were mammogenes and 2 metastatic from the digestive system.

The stage of the disease in patients with ovarian carcinoma was established according to the International Federation of Gynecology and Obstetrics (FIGO) staging system. Histological classification was defined according to the World Health Organization (WHO) system. Tumours were graded as well-, moderately- or poorly-differentiated.

Patient medical documents were reviewed to obtain data regarding age, diagnosis, histology, FIGO stage, presence of ascites, residual disease after tumour cytoreductive surgery, response to primary chemotherapy, time to recurrence, and demise. Optimal

cytoreduction was defined as <1 cm of residual disease following the surgery. Primary chemotherapy with paclitaxel and carboplatin or cisplatin was used to treat all patients with advanced invasive carcinoma (67 patients). Response to primary chemotherapy was evaluated during a second-look laparotomy or according to the Response Evaluation Criteria in Solid Tumours [13].

Preparation of tissue extracts

Protein extracts were prepared from frozen tissue samples by homogenising (homogenizer Ultra-Turvax T8, IKA-WERKE) 100 mg of tissue in 300 µl of ice-cold buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100) containing a cocktail of protease inhibitors (1 mM AEBSF, Sigma). The homogenates were then centrifuged at 10 000 × *g* for 15 min at 4°C to pellet cellular debris and total protein concentration was determined by Bradford method using bovine serum albumin (BSA) to generate standard curve.

Western-blotting analysis

Tissue lysates containing equal amounts of total protein were separated by SDS-PAGE. Separated proteins were electroblotted to Immobilon P (Millipore) and then probed with the primary and the secondary antibodies. To detect proteins of interest, enhanced chemiluminescence system was used according to the supplier's protocol (Lumi-Light Western Blotting substrate; Roche). Relative levels of proteins were estimated densitometrically using β-actin as internal reference. Each assay was repeated three times and the differences between assays did not exceed 10%.

Monoclonal anti-VEGFR-2/3 antibodies were purchased from Santa Cruz Biotechnology (C-20); monoclonal anti-β-actin antibodies were obtained from Sigma. As secondary antibodies, anti-rabbit or anti-mouse HRP-conjugated immunoglobulins were used (Sigma).

Densitometric and statistical analysis

To quantify the levels of Western-blotting products, densitometric analysis was performed using the *1Dscan EX 3.0* program (Scanalytics, Inc).

Correlations between VEGFR-2/3 expression and patients' clinicopathologic variables were analysed using the χ^2 test or Fisher's exact test. The association between protein levels and tissue types was evaluated by the nonparametric Kruskal-Wallis test. Kaplan-Meier method was used to generate survival curves, and differences in survival were analysed using the log-rank test, based on the VEGFR-2/3 expression status. Progression-free survival (PFS) time was calculated as the time in months from the date of the surgery to the progression of the disease or death for non-censored events, or to the date of the last contact for censored events when the woman was still alive without the evidence of disease progression. Overall survival (OS) time was calculated as the time in months from the date of the surgery to death for non-censored events, or to the date of the last contact for censored events when the woman was still alive. The follow-up was censored in June 2008. Univariate and multivariate analyses were done using the Cox proportional hazards model. Probability values of <0.05 were considered statistically significant. All analyses were performed using statistical analysis software: StatSoft, Inc. (2005). STATISTICA, version 7.1, Analyse-it (Analyse-it Software, Ltd), Microsoft Excel (Microsoft Corp.).

Using exploratory statistical analysis, patients with ovarian carcinoma were dichotomized into two groups, high and low VEGFR-2/3 expression based on a cut-off value determined by the receiver-operating characteristic curve (ROC), which yielded the highest hazard ratio. The area under the ROC curve for VEGFR-3 was 0.63 (95% CI: 0.50-0.75, $p = 0.02$), and for VEGFR-2 it was 0.57 (95% CI: 0.45-0.68, $p = 0.12$). Next, the results of the initial exploratory data were confirmed by conducting an independent validation test.

Results

Western-blotting analysis of VEGFR-2/3 expression in ovarian tissue

As a result of immunoblotting with the use of anti-VEGFR-2 monoclonal antibodies, a dominating protein band of ca. 85 kDa was found in the tissue lysates, which is in agreement with molecular weight of the non-glycosylated VEGFR-2 form [14] (Fig. 1). Protein bands of 85 kDa were discovered in all analysed tissues. In normal tissues, the relative protein level ranged from 0.07 to 4.33, in benign tumours – from 0.12 to 9.0, in borderline tumours – from 0.02 to 7.34, in ovarian cancer – from 0.14 to 7.84, and in Krukenberg tumours – from 0.69 to 3.03. No statistically-significant differences regarding the relative protein level in the analysed tissue groups were found ($p = 0.6$). The highest VEGFR-2 concentration was found at the earlier stages of the ovarian cancer progression (median of 2.01) (Fig. 2A).

Immunoblotting with the use of anti-VEGF-R3 monoclonal antibodies revealed protein bands of ca. 85 kDa, which corresponds to the non-glycosylated form of VEGFR-3 [15] (Fig. 1).

Protein bands of 85 kDa were discovered in 29 out of the 32 normal tissues (90%) and the relative protein levels ranged from 0.02 to 2.33, in 35 out of

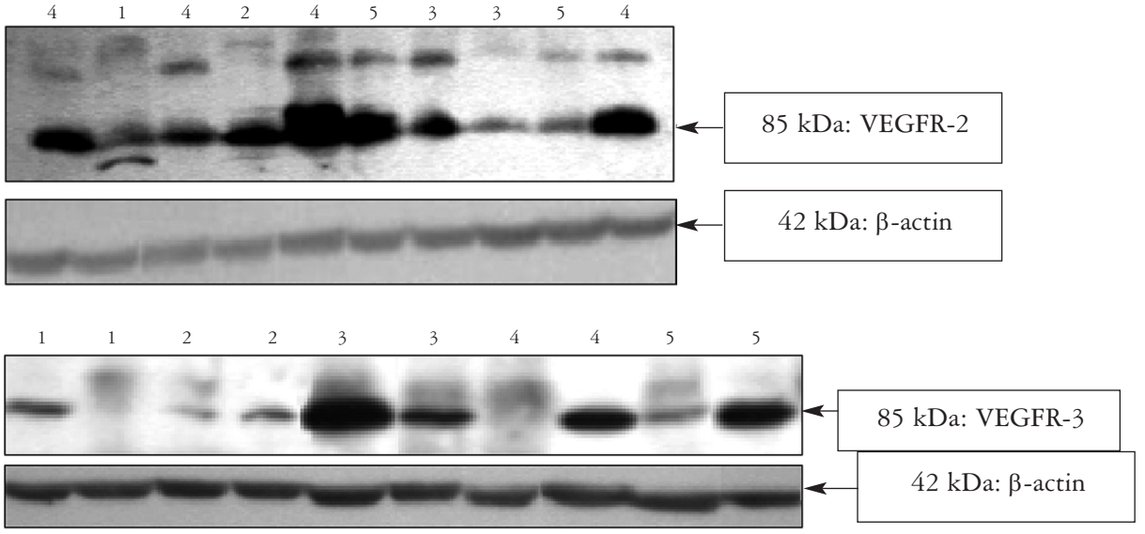


Fig. 1. Representative results of Western-blotting analysis of VEGFR-2 and VEGFR-3 expression in ovarian samples: (1 – normal tissues, 2 – benign tumours, 3 – borderline tumours, 4 – ovarian carcinoma, 5 – metastatic tumours). Proteins were immunodetected with antibodies against VEGFR-2, VEGFR-3 or β-actin

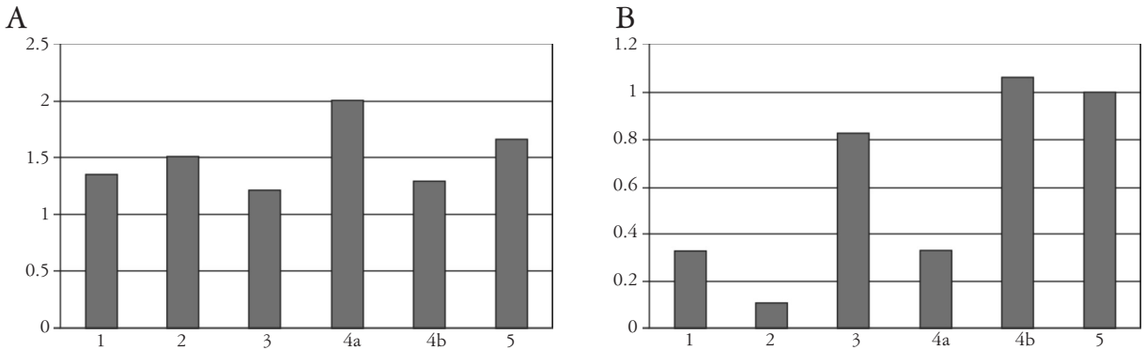


Fig. 2. Relative level of VEGFR-2 (A) and VEGFR-3 (B) in ovarian samples (1 – normal tissues, 2 – benign tumours, 3 – borderline tumours, 4a – ovarian carcinomas: early stages, 4b – ovarian carcinomas: advanced stages, 5 – metastatic tumours). Median values are presented

the 42 benign tumours (83.3%, 0.01-6.09), in 10 out of the 10 borderline tumours (100%, 0.02-3.78), in 70 out of the 76 ovarian cancer tissues (92.1%, 0.05-4.21) and in all (8) Krukenberg tumours (100%, 0.01-3.05). Statistically-relevant differences regarding the relative protein level in the analysed tissue groups were found ($p = 0.000$). The highest VEGFR-3 level was found at the later stages of the ovarian cancer progression (median of 1.07) and in Krukenberg tumours (median of 1.0) (Fig. 2B).

Relation between VEGFR-2/3 expression and clinicopathological features of ovarian carcinomas

In the current cohort of ovarian cancer patients the mean age at diagnosis was 57.5 years (range: 24 to 86 years). Serous cystadenocarcinoma was the most common histological type (60.5%). All patients were treated surgically, of which 50% had optimal cytoreduction. Clinicopathological staging showed that the majority of patients (77.6%) were in the advanced stage (III and IV). According to the histological grading, 43.4% of the tumours were poorly-differentiated (grade 3). 55.2% of the patients completely responded to primary chemotherapy (complete pathological or clinical remission). The correlations of VEGFR-2/3 expression with various clinical variables are listed in Table I.

A high expression of VEGFR-2 at the early stages of ovarian cancer progression was noted significantly more often ($p = 0.002$). A comparison of ovarian cancer histological types revealed a more frequent occurrence of high VEGFR-2 expression in cancer

Table I. VEGFR-2/3 expression and clinicopathological characteristics in ovarian cancer. The correlations between VEGFR-2/3 expression and clinicopathological features in patients with ovarian carcinoma were analysed using the χ^2 test or Fisher's exact test (*). N – number of patients

CHARACTERISTICS	CASES (N)	VEGFR-2 EXPRESSION		P-VALUE	VEGFR-3 EXPRESSION		P-VALUE
		LOW N (%)	HIGH N (%)		LOW N (%)	HIGH N (%)	
Patient's age							
≤50	25	6 (24.0)	19 (76.0)	0.48	4 (16.0)	21 (84.0)	0.26
>50	51	14 (27.4)	37 (72.6)		14 (27.4)	37 (72.6)	
FIGO stage							
I + II	17	0 (0)	17 (100)	0.002	10 (58.8)	7 (41.2)	0.02
III + IV	59	20 (33.9)	39 (66.1)		8 (13.6)	51 (86.4)	
Tumour grade							
G1 + G2	43	8 (18.6)	35 (81.4)	0.069	9 (20.9)	34 (79.1)	0.51
G3	33	12 (36.4)	21 (63.6)		9 (27.3)	24 (72.7)	
Histological subtype							
serous	46	17 (36.9)	29 (63.1)	0.007	9 (36.9)	37 (63.1)	0.44
other	30	3 (10.0)	27 (90.0)		9 (30.0)	21 (70.0)	
Debulking status							
optimal	38	6 (15.8)	32 (84.2)	0.03	13 (34.2)	25 (65.8)	0.02
suboptimal	38	14 (36.8)	24 (63.2)		5 (13.2)	33 (86.8)	
Ascites							
yes	45	12 (26.7)	33 (73.3)	0.57	10 (32.3)	21 (67.7)	0.14
no	31	8 (25.8)	23 (74.2)		8 (17.8)	37 (82.2)	
Response to chemotherapy							
yes	37	11 (57.9)	26 (54.2)	0.7	11 (78.6)	26 (49.1)	0.04
no	30	8 (42.1)	22 (45.8)		3 (21.4)	27 (50.9)	

cases other than serous ($p = 0.007$). A high expression of VEGFR-2 was noticeably more common in patients who underwent optimal cytoreduction (0.03). No correlation between the expression of VEGFR-2 and the patient's age, the grade, presence of ascites or positive response to first-line chemotherapy was noticed.

While assessing the correlation between the expression of VEGFR-3 and the degree of the disease's clinical progression, high expression of VEGFR-3 was observed more often in stages III and IV ($p = 0.02$). More frequent high expression of VEGFR-3 was also noted in patients where optimal cytoreduction was not performed during initial surgery (0.02). Statistically-significant connection between the response to first-line chemotherapy and the VEGFR-3 expression level was discovered. Among those patients who exhibited a low expression of VEGFR-3, positive response to chemotherapy was more frequent.

VEGFR-2/3 expression and prognosis

Kaplan-Meier method was used to explore the impact of VEGFR-2/3 expression on the outcome of patients with epithelial ovarian cancer. No difference in progression-free survival in the context of VEGFR-2 expression was observed. While assessing overall survival rates in the context of VEGFR-2 expression, no statistically-significant differences between the groups assessed have been found. While assessing progression-free time in relation to VEGFR-3 expression, statistically-significant differences were observed. Progression occurred more frequently in patients with high VEGFR-3 expression. Differences in overall survival rates depending on the expression of VEGFR-3 have been observed. Longer survival rates appeared in patients with a low expression of VEGFR-3; however, this difference did not achieve statistical relevance in the log rank test ($p = 0.1$).

Multivariate Cox proportional hazard model analysis concerning the impact of all patients characteristics and analysed protein expression on overall survival

The Cox univariate and multivariate proportional hazard regression model was used to evaluate the effects of the traditional prognostic factors and VEGFR-2/3 expression on survival rates (Table II). No correlations between the age at diagnosis, histological type of tumour, grade, presence of ascites and survival have been found. Positive response to primary chemotherapy was significantly associated with increased survival ($p = 0.00003$). In multivariate analysis only, the response to chemotherapy retained an independent prognostic factor for OS ($p = 0.0002$).

Discussion

Angio- and lymphangiogenesis are the critical factors in the growth, progression, and metastatic spread of solid tumours [5, 16]. Furthermore, they have been correlated with prognosis in patients with ovarian cancer. The pathogenesis of the angio- and lymphogenic events in ovarian cancer, however, is not well defined. In this study, we assayed the levels of the VEGF receptors, VEGFR-2 and VEGFR-3, in cancer tissues, using immunoblotting.

As a result of immunoblotting with the use of anti-VEGF-R2 monoclonal antibodies, a dominant protein of ca. 85 kDa was found in the tissue samples. In most tissues, proteins of 120 kDa could also be seen. We assume that they correspond to the unglycosylated and glycosylated forms of the VEGFR-2 receptor, respectively [14].

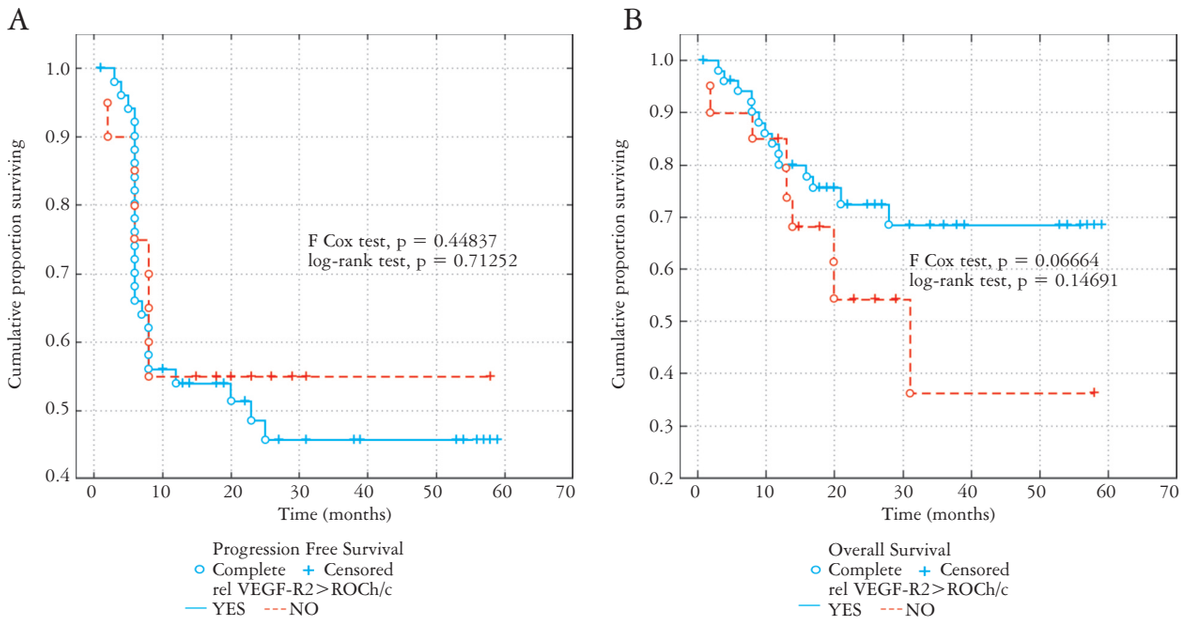
Relative levels of VEGFR-2 (the 85 kDa variant) in the individual samples were comparable. The only difference in the level of VEGFR-2 expression was found in correlation with the clinical stage of ovarian cancer. Higher VEGFR-2 levels were noted in tissues collected from patients with early stages of ovarian cancer patients. While comparing histological types of ovarian cancer, a high expression of VEGFR-2 was observed more frequently in cancer types other than serous. A high VEGFR-2 expression was also more frequent in patients who underwent optimal cytoreduction. No correlation between the level of VEGFR-2 expression and the patient's age, the grade, the existence of ascites, positive response to chemotherapy or overall survival has been found.

The higher VEGFR-2 expression in carcinoma tissues when compared with benign tumours or normal tissues was previously found in cancers of prostate [17], urinary bladder [18], colon [19], and kidney [20], as well as in sarcoma and carcinoma of the corpus uteri [21, 22].

The correlation between the expression of VEGFR-2 and prognosis was found in lung cancer patients [23], chronic lymphatic leukaemia [24], pancreatic cancer [25] and breast cancer [26]. However, other studies showed no correlation between the expression of VEGFR-2 and prognosis. These included research

Table II. Multivariate Cox proportional hazard models for OS

PROGNOSTIC FACTOR	HAZARD RATIO	95% CONFIDENCE INTERVAL	P
Univariate analysis			
Patient's age (≤50 : > 50)	0.856	0.37-1.978	0.7152
Clinical stage (I + II : III + IV)	2.966	1.528-5.751	0.00013
Grading (G1+G2 : G3)	1.007	0.436-2.329	0.98580
Ascites (no : yes)	1.549	0.655-3.663	0.3192
Histological subtype (other : serous)	1.386	0.586-3.278	0.4568
Debulking status (≤1 cm : > 1 cm)	2.337	0.99-5.517	0.0528
Response to chemotherapy (yes : no)	38.3072	6.434-364.232	0.00003
VEGFR-2 overexpression (yes : no)	1.845	0.795-4.279	0.1537
VEGFR-3 overexpression (no : yes)	2.999	0.703-12.799	0.1379
Multivariate analysis step 1, $p < 0.1$			
Clinical stage (I + II : III + IV)	0.767	0.255-2.309	0.6376
Response to chemotherapy (yes : no)	57.662	6.244-532.492	0.0003
Debulking status (≤1 cm : > 1 cm)	1.674	0.631-4.440	0.3002
Multivariate analysis step 2, $p < 0.05$			
Response to chemotherapy (yes : no)	48.408	6.434-364.232	0.0002



VEGFR-2 expression	*High (yes)*	*Low (no)*
Data completed	26 (46.4%)	9 (45.0%)
Data censored	30 (53.6%)	11 (55.0%)

VEGFR-2 expression	*High (yes)*	*Low (no)*
Data completed	14 (25.1%)	9 (45.0%)
Data censored	42 (75.0%)	11 (55.0%)

Fig. 3. Kaplan-Meier progression-free survival (A) and overall survival (B) analysis for VEGFR-2 expression categorized as low or high according to a cutoff value determined by the ROC curve in women with ovarian cancer. These graphs illustrate the estimated progression-free or overall survival function for women with high VEGFR-2 expression as compared with those with low VEGFR-2 expression. The significance of the log rank test to evaluate the equality of progression-free or overall survival distributions was $p = 0.44$ or $p = 0.1$, respectively

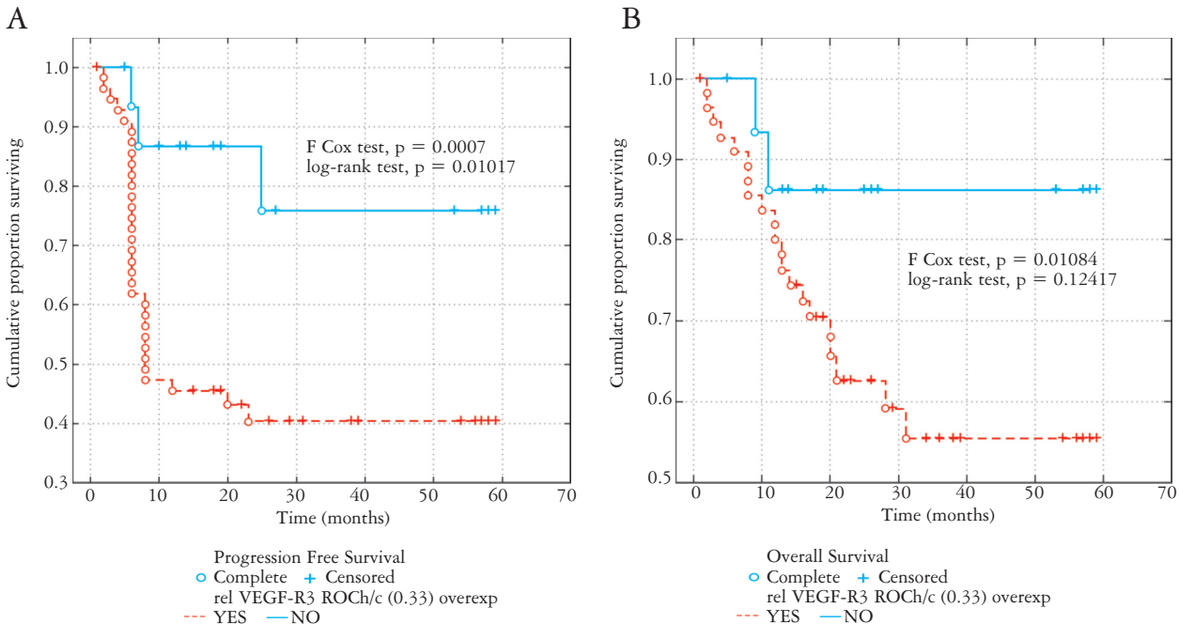
on larynx cancer [27], malignant melanoma [28], acute leukaemia [29], oesophagus cancer [30], breast cancer [31], sarcoma of the corpus uteri [21], cancer of the corpus uteri [22] and pancreatic cancer [32].

Only a few authors assessed the significance of this receptor expression in ovarian cancer patients. Boocock *et al*. [33] were the first to indicate the existence of VEGFR-2 in endothelial and carcinoma cells in samples collected from ovarian cancer tissues as well as from metastases. Abu-Jawdeh *et al*. [34] assayed VEGFR-2 mRNA and showed an increased expression of this receptor in ovarian cancer and borderline ovarian tumours, as compared with benign tumours and normal tissues.

Orre *et al*. assessed the expression of VEGFR-2 in 19 benign and 37 malignant ovarian tumours [35]. The authors noted higher levels of VEGFR-2 in benign tumours when compared with ovarian cancer tissues. The researchers, compared areas and high microvessel density (MVD) with average MVD areas and stated no difference in the expression of the marker in ovarian cancer tissues. Inan *et al*. [36] assessed the expression of VEGFR-2 in ovarian borderline tumours and ovarian carcinomas, using the immunohistochemical method. The authors showed the presence of VEGFR in both endothelial cells and carcinoma cells, with a higher expression of the scrutinised protein exhibited by the ovarian cancer tissues.

Available literature mentions only one study showing the effect of VEGFR-2 expression on ovarian cancer prognosis. Nishida *et al.* [37] examined immunohistochemically VEGFR-2 expression levels in 80 ovarian cancer patients. The researchers showed a positive correlation between the high expression of VEGFR-2 and the appearance of distant metastases, lymph node metastases and the appearance of carcinoma cells in ascites. They did not find any correlation between the expression of the analysed receptor and the clinical stage, the grade or the patient's age. While assessing the prognostic significance with the use of multivariate analysis, the authors pointed out to significantly longer survival among those patients who exhibited a low expression of VEGFR-2.

In conclusion, the results concerning the VEGFR-2 expression in ovarian cancer, and their prognostic value,



VEGFR-3 expression	*High (yes)*	*Low (no)*
Data completed	32 (55.2%)	3 (16.7%)
Data censored	26 (44.8%)	15 (83.3%)

VEGFR-3 expression	*High (yes)*	*Low (no)*
Data completed	21 (36.2%)	2 (11.1%)
Data censored	37 (63.8%)	16 (88.9%)

Fig. 4. Kaplan-Meier progression-free survival (A) and overall survival (B) analysis for VEGFR-3 expression categorized as low or high according to a cutoff value determined by the ROC curve in women with ovarian cancer. These graphs illustrate the estimated progression-free or overall survival function for women with high VEGFR-2 expression as compared with those with low VEGFR-2 expression. The significance of the log rank test to evaluate the equality of progression-free or overall survival distributions was p = 0.01 or p = 0.1, respectively

are diverse and indicate that further research is required to clarify the role of VEGFR2 in this type of cancer.

As a result of protein immunodetection with the use of anti-VEGF-R3 monoclonal antibodies, a protein band of ca. 85 kDa was found in the tissue samples, which corresponds to the unglycosylated form of VEGF-R3 [15]. Statistically-significant differences in relative protein levels in the analysed tissue samples were observed. The highest expression level of VEGFR-3 was noted at the advanced stages of the disease and in Krukenberg tumours. More frequent occurrence of the high expression of VEGFR-3 was also noted in ovarian cancer patients who did not undergo optimal cytoreduction at the initial surgery. Statistically-relevant correlation between positive response to first line chemotherapy and VEGFR-3 expression was observed. Among patients with low VEGFR-3 expression, positive response to chemotherapy was more frequent. Comparison of the progression-free periods in reference to the level of VEGFR-3 expression also showed statistical significance. Progression was more frequent among patients with high VEGFR-3 expression. The above results indicate that a high expression of VEGFR-3 reflects the aggressiveness of the tumour spread and has a predictive value for identifying high-risk patients with poor prognosis.

Previously, VEGFR-3 expression was detected in several tumour cells such as colorectal adenocarcinoma [38], prostatic cells [39], myeloid leukaemia cells [40] and pancreatic endocrine malignant cells [41]. Moreover, VEGFR-3 expression was gradually increased with tumour stages in human cutaneous melanoma [42] and cervical cancer [43]. It was also shown that VEGFR-3 expression correlated with clinical metastasis and patient survival [44]. The significance of VEGFR-3 over-expression as a negative prognostic factor has been shown in lung cancer [45, 46], breast cancer [47], stomach cancer [48], endometrial cancer [49], melanoma [50], prostate cancer [51] and larynx cancer [52].

However, only a few authors have published research results pertaining to the significance of VEGFR-3 in ovarian cancer [37, 53]. Yokoyama *et al.* marked the level of this receptor in benign and borderline tumours, and ovarian carcinoma [53]. They did not find any difference in the VEGFR-3 protein level between benign and borderline tumours, but showed a higher expression of this

receptor in ovarian carcinoma. No correlation between VEGFR-3 expression and patient's age, histological type or subtype and the existence of distant metastases has been noted. Also, no significance of this marker as a prognostic factor in ovarian cancer patients has been shown.

Nishida *et al.* [37] found a higher level of VEGFR-3 in ovarian carcinoma tissues when compared with benign tumours. The authors did not, however, show any correlation between the level of VEGFR-3 and selected clinicopathological factors, such as the patient's age, clinical stage of the disease, histological subtype, the grade, or the presence of metastases into the lymph nodes. The analyzed marker did not have any statistical significance in the ovarian cancer patient group.

The results presented in this paper are the first available in the subject literature, which show a correlation between the expression of VEGFR-3 and response to chemotherapy as well as ovarian cancer prognosis. The results point out to an important role of lymphangiogenesis in the development of this type of cancer. At the same time these results indicate that further research on the relevance of lymphangiogenesis in ovarian cancer is necessary, since it could lead to introduction of new anti-lymphangiogenic drugs, inhibiting the development of the carcinoma.

Summarizing: an increased level of VEGFR-2 at the early stages of ovarian cancer may indicate the significance of neoangiogenesis at the early stages of ovarian cancer. No relationship between the expression of VEGFR-2 and positive response to chemotherapy and the overall survival rate among ovarian cancer patients has been found. Overexpression of VEGFR-3 is a disadvantageous prognostic and predictive factor in ovarian cancer patients.

Acknowledgement

This work was supported by the Polish Ministry of Science and Higher Education (Grant No. N 40306631/3077). The authors declare that there is no conflict of interests. Presented in part at ASCO Annual Meeting, Chicago, 4-9 June 2010.

References

1. Ozols RF. Challenges for chemotherapy in ovarian cancer. Ann Oncol 2006; 17: 181-187.
2. Christiansen JJ, Rajasekaran AK. Reassessing epithelial to mesenchymal transiton as a prerequisite for carcinoma invasion and metastasis. Cancer Res 2006; 66: 8319-8326.
3. Lenz HJ. Antiangiogenic agents in cancer therapy. Oncology 2005; 19: 1-11.
4. Achen MG, Stacker SA. Tumor lymphangiogenesis and metastatic spread – New players to emerge. Int J Cancer 2006; 119: 1755-1760.
5. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol 2005; 23: 1011-1027.
6. Shibuya M. Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. J Biochem Mol Biol 2006; 39: 469-478.
7. Ran S, Huang X, Downes A, Thorpe PE. Evaluation of novel antimouse VEGFR-2 antibodies as potential antiangiogenic of vascular targeting agents for tumor therapy. Neoplasia 2003; 5: 297-307.
8. Kaipainen A, Korhonen J, Mustonen T, et al. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci U S A 1995; 92: 3566-3570.
9. Pepper MS. Lymphangiogenesis and tumor metastasis: myth or reality? Clin Cancer Res 2001; 7: 462-468.
10. Dumont DJ, Jussila L, Taipale J, et al. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. Science 1998; 282: 946-949.
11. Sundar SS, Ganesan TS. Role of lymphangiogenesis in cancer. J Clin Oncol 2007; 25: 4298-4307.
12. Roberts N, Kloos B, Cassella M, et al. Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. Cancer Res 2006; 66: 2650-2657.
13. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumours. European Organisation for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000; 92: 205-216.
14. Robinson ChJ, Stringer SE. The splice variants of vascular endothelial growth factor (VEGF) and their receptors. J Cell Sci 2001; 114: 853-865.
15. Pajusola K, Aprelikova O, Korhonen J, et al. FLT4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines. Cancer Res 1992; 52: 5738-5743.
16. Stacker SA, Achen MG. From anti-angiogenesis to anti-lymphangiogenesis: emerging trends in cancer therapy. Lymphat Res Biol 2008; 6: 165-172.
17. Pallares J, Rojo F, Iriarte J, et al. Study of microvessel density and the expression of the angiogenic factors VEGF, bFGF and the receptors Flt-1 and FLK-1 in benign, premalignant and malignant prostate tissues. Histol Histopathol 2006; 21: 857-865.
18. Liu L, Zhu D, Gao R, Guo H. Expression of vascular endothelial growth factor, receptor KDR and p53 protein in transitional cell carcinoma of the bladder. Urol Int 2008; 81: 72-76.
19. Giatromanolaki A, Koukourakis MI, Sivridis E, et al. Activated VEGFR2/KDR pathway in tumour cells and tumour associated vessels of colorectal cancer. Eur J Clin Invest 2007; 37: 878-886.
20. Tsuchiya N, Sato K, Akao T, et al. Quantitative analysis of gene expressions of vascular endothelial growth factor-related factors and their receptors in renal cell carcinoma. Tohoku J Exp Med 2001; 195: 101-113.
21. Arita S, Kikkawa F, Kajiyama H, et al. Prognostic importance of vascular endothelial growth factor and its receptors in the uterine sarcoma. Int J Gynecol Cancer 2005; 15: 329-336.
22. Talvensaari-Mattila A, Soini Y, Santala M. VEGF and its receptors (flt-1 and KDR/flk-1) as prognostic indicators in endometrial carcinoma. Tumour Biol 2005; 26: 81-87.
23. Seto T, Higashiyama M, Funai H, et al. Prognostic value of expression of vascular endothelial growth factor and its flt-1 and KDR receptors in stage I non-small-cell lung cancer. Lung Cancer 2006; 53: 91-96.

24. Ferrajoli A, Manshouri T, Estrov Z, et al. High levels of vascular endothelial growth factor receptor-2 correlate with shortened survival in chronic lymphocytic leukemia. Clin Cancer Res 2001; 7: 795-799.
25. Büchler P, Reber HA, Büchler MW, et al. VEGF-RII influences the prognosis of pancreatic cancer. Ann Surg 2002; 236: 738-749.
26. Ghosh S, Sullivan CA, Zerkowski MP, et al. High levels of vascular endothelial growth factor and its receptors (VEGFR-1, VEGFR-2, neuropilin-1) are associated with worse outcome in breast cancer. Hum Pathol 2008; 39: 1835-1843.
27. Marioni G, Giacomelli L, D'Alessandro E, et al. Laryngeal carcinoma recurrence rate and disease-free interval are related to CD105 expression but not to vascular endothelial growth factor 2 (Flk-1/Kdr) expression. Anticancer Res 2008; 28 1B: 551-557.
28. Lin EY, Piepkorn M, Garcia R, et al. Angiogenesis and vascular growth factor receptor expression in malignant melanoma. Plast Reconstr Surg 1999; 104: 1666-1674.
29. Verstovsek S, Estey E, Manshouri T, et al. Clinical relevance of vascular endothelial growth factor receptors 1 and 2 in acute myeloid leukaemia and myelodysplastic syndrome. Br J Haematol 2002; 118: 151-156.
30. Kato H, Yoshikawa M, Miyazaki T, et al. Expression of vascular endothelial growth factor (VEGF) and its receptors (Flt-1 and Flk-1) in esophageal squamous cell carcinoma. Anticancer Res 2002; 22, 6C: 3977-3984.
31. Dales JP, Garcia S, Bonnier P, et al. Prognostic significance of VEGF receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) in breast carcinoma]. Ann Pathol 2003; 23: 297-305.
32. Chung GG, Yoon HH, Zerkowski MP, et al. Vascular endothelial growth factor, FLT-1, and FLK-1 analysis in a pancreatic cancer tissue microarray. Cancer 2006; 106: 1677-1684.
33. Boocock CA, Charnock-Jones DS, Sharkey AM, et al. Expression of vascular endothelial growth factor and its receptors flt and KDR in ovarian carcinoma. J Natl Cancer Inst 1995; 87: 506-516.
34. Abu-Jawdeh GM, Faix JD, Niloff J, et al. Strong expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in ovarian borderline and malignant neoplasms. Lab Invest 1996; 74: 1105-1115.
35. Orre M, Lotfi-Miri M, Mamers P, Rogers PA. Increased microvessel density in mucinous compared with malignant serous and benign tumours of the ovary. Br J Cancer 1998; 77: 2204-2209.
36. Inan S, Vatansever S, Celik-Ozenci C, et al. Immunolocalizations of VEGF, its receptors flt-1, KDR and TGF-beta's in epithelial ovarian tumors. Histol Histopathol 2006; 21: 1055-1064.
37. Nishida N, Yano H, Komai K, et al. Vascular endothelial growth factor C and vascular endothelial growth factor receptor 2 are related closely to the prognosis of patients with ovarian carcinoma. Cancer 2004; 101: 1364-1374.
38. Witte D, Thomas A, Ali N, et al. Expression of the vascular endothelial growth factor receptor-3 (VEGFR-3) and its ligand VEGF-C in human colorectal adenocarcinoma. Anticancer Res 2002; 22: 1463-1466.
39. Li R, Younes M, Wheeler TM, et al. Expression of vascular endothelial growth factor receptor-3 (VEGFR-3) in human prostate. Prostate 2004; 58: 193-199.
40. Fielder W, Graeven U, Ergün S, et al. Expression of FLT4 and its ligand VEGF-C in acute myeloid leukemia. Leukemia. 1997; 11: 1234-1237.
41. Hansel DE, Rahman A, Hermans J, et al. Liver metastases arising from well-differentiated pancreatic endocrine neoplasms demonstrate increased VEGF-C expression. Mod Pathol 2003; 16: 652-659.
42. Clarijs R, Schalkwijk L, Hofmann UB, et al. Induction of vascular endothelial growth factor receptor-3 expression on tumor microvasculature as a new progression marker in human cutaneous melanoma. Cancer Res 2002; 62: 7059-7065.
43. Van Trappen PO, Steele D, Lowe DG, et al. Expression of vascular endothelial growth factor (VEGF)-C and VEGF-D, and their receptor VEGFR-3, during different stages of cervical carcinogenesis. J Pathol 2003; 201: 544-554.
44. Su JL, Yang PC, Shih JY, et al. The VEGF-C/Flt-4 axis promotes invasion and metastasis of cancer cells. Cancer Cell 2006; 9: 209-223.
45. Arinaga M, Noguchi T, Takeno S, et al. Clinical significance of vascular endothelial growth factor C and vascular endothelial growth factor receptor 3 in patients with nonsmall cell lung carcinoma. Cancer 2003; 97: 457-464.
46. Kojima H, Shijubo N, Yamada G, et al. Clinical significance of vascular endothelial growth factor-C and vascular endothelial growth factor receptor 3 in patients with T1 lung adenocarcinoma. Cancer 2005; 104: 1668-1677.
47. Mylona E, Alexandrou P, Mpakali A, et al. Clinicopathological and prognostic significance of vascular endothelial growth factors (VEGF)-C and -D and VEGF receptor 3 in invasive breast carcinoma. Eur J Surg Oncol 2007; 33: 294-300.
48. Jüttner S, Wissmann C, Jöns T, et al. Vascular endothelial growth factor-D and its receptor VEGFR-3: two novel independent prognostic markers in gastric adenocarcinoma. J Clin Oncol 2006; 24: 228-240.
49. Yokoyama Y, Charnock-Jones DS, Licence D, et al. Expression of vascular endothelial growth factor (VEGF)-D and its receptor, VEGF receptor 3, as a prognostic factor in endometrial carcinoma. Clin Cancer Res 2003; 9: 1361-1369.
50. Mouawad R, Spano JP, Comperat E, et al. Tumoural expression and circulating level of VEGFR-3 (Flt-4) in metastatic melanoma patients: Correlation with clinical parameters and outcome. Eur J Cancer 2009; 45: 1407-1414.
51. Li R, Younes M, Wheeler TM, et al. Expression of vascular endothelial growth factor receptor-3 (VEGFR-3) in human prostate. Prostate 2004; 58: 193-199.
52. Wang Z, Chen Y, Liang C, et al. Expression of VEGF-C and VEGFR-3 in human laryngeal squamous cell carcinomas and cervical metastatic lymph node tissues. Lin Chuang Er Bi Yan Hou Ke Za Zhi 2004; 18: 664-667.
53. Yokoyama Y, Charnock-Jones DS, Licence D, et al. Vascular endothelial growth factor-D is an independent prognostic factor in epithelial ovarian carcinoma. Br J Cancer 2003; 88: 237-244.

Address for correspondence

Dagmara Klasa-Mazurkiewicz
Department of Perinatology
Medical University of Gdańsk
ul. Kliniczna 1a
80-402 Gdańsk
tel. +48 58 349 34 61
fax +48 58 341 80 03
e-mail: dklasa@gumed.edu.pl