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RESEARCH ARTICLE

Changes in the Expression Profile of VEGF-A, VEGF-B, VEGFR-1, VEGFR-2 in Different Grades of Endometrial Cancer

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Abstract: ***Background*:** VEGF-A, VEGF-B, VEGFR-1 and VEGFR-2 are important proteins involved in the induction and development of a new blood vessel network through which the tumor is properly nourished and oxygenated.

***Objectives*:** The aim of the study was to evaluate changes in VEGF-A, VEGF-B, VEGFR-1 and VEGFR-2 expression in endometrial cancer depending on its grade and to determine the VEGFR-1 to VEGFR-2 concentration ratio.

***Methods*:** The study group consisted of 45 patients diagnosed with endometrial cancer (G1, 17; G2, 15; G3, 13). The control group included 15 patients. VEGF-A, VEGF-B, VEGF-R1, VEGFR-2 expression was assessed using the immunohistochemical method. Statistical analysis was carried out using the Statistica 12 PL program (StatSoft, Cracow, Poland). It included the one-way ANOVA and Tukey's post-hoc test ($p<0.05$).

***Results*:** Statistically significant differences in the level of VEGF-A, VEGF-B, VEGF-R1, VEGFR-2 were observed between the majority of analyzed groups (except for VEGF-B; G3 *vs*. G1, p=0.997700). The expression pattern of VEGF-A, VEGF-R1, VEGFR-2 was as follows: G3>G2>G1>C; VEGF-B: G2> G3> G1>C. A lower concentration of VEGFR-1 than VEGFR-2 was found regardless of the cancer grade.

***Conclusion*:** VEGF-A, VEGF-B, VEGF-R1, VEGFR-2 are key proteins involved in tumor angiogenesis. The analysis of the entire panel of proteins participating in a given process is an important element of modern diagnostics. The concentration ratio of VEGFR-1 to VEGFR-2 appears to be a determining factor in the patients' survival prognosis.

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1. INTRODUCTION

Tumor vasculature can be created from pre-existing vessels (angiogenesis), as well as can arise from precursors of endothelial cells (vasculogenesis) [1]. In the initial stages of development, tumors take the form of clusters of cancer cells without vessels (in situ), the volume of which does not exceed 1-2 mm^3, and the inflow of nutrients occurs by diffusion. Their further development depends on the ability of tumor cells to produce and release proangiogenic factors [2]. When the volume reaches the critical value, the acquisition of necessary substances through diffusion is insufficient.

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Tumor environment undergoes hypoxia and acidification, leading to its necrosis. Cancer cells prevent this and, with the help of host cells, stimulate the development of their new blood vessels using angiogenesis [3-7]. This allows cancer cells to colonize distant organs and metastasize [8].

VEGF-A has been studied more frequently than other proteins in this family and has several different variants (VEGF121, VEGF145, VEGF148, VEGF165, VEGF183, VEGF189 and VEGF206) that occur due to alternative splicing and differ in the specificity and receptor function [9]. VEGF receptor tyrosine kinases include VEGF-R1 (also known as Flt1), VEGF-R2 (known as Flk1 or KDR) and VEGF-R3 (also known as Flt4) [10]. Although the expression of these receptors was initially considered to be limited to endothelial cells, it is now known that most of them are expressed in many types of tumors and correlate with clinical parameters. VEGFR-2 is the main receptor tyrosine kinase (RTK) that indirectly participates in VEGF signaling in endothelial cells and VEGF-induced angiogenesis [10]. Interestingly, some cancer cells express VEGFR-2, which plays a major role in mediating VEGF signaling [11, 12], but the response of other tumor cells to VEGF appears to be RTK-independent [13, 14], indicating that other receptors mediate VEGF signaling in these cells. The main receptor for VEGF-A is VEGFR-2, expressed primarily on vascular endothelial cells. Activation of this receptor is initiated by two important pathways. One of them stimulates cell division and the other is associated with the survival of endothelial cells. Activation of VEGFR-2 also increases vascular permeability, affects the migration of endothelial cells as well as recruitment of endothelial progenitor cells [15, 16]. In contrast, VEGF-B has the ability to interact only with VEGFR-1 and its role is mainly described in the maintenance of newly formed blood vessels [17, 18]. VEGF-B extends the lifetime of endothelial and smooth muscle cells by stimulating effects on genes encoding anti-apoptotic proteins [19]. Overexpression of VEGF-B has been reported in some cancers, *e.g*. ovarian, colorectal, prostate and kidney cancer. It is believed that its expression correlates with the progression of cancer and VEGFR-1 level [20, 21]. Endometrioid endometrial cancer is one of the most frequently diagnosed gynecological tumors. According to the WHO, it is classified into three histological grades, which differ in the degree of cell differentiation: G1 (well-differentiated), G2 (moderately differentiated) and G3 (poorly differentiated). Frequency of occurrence and high mortality are the reasons for constant search for new therapies with greater effectiveness and fewer side effects, prolonging survival and improving the patient's life comfort. At the same time, the search for markers allowing early detection of neoplastic changes continues [22].

The aim of the study was to evaluate changes in VEGF-A, VEGF-B, VEGFR-1 and VEGFR-2 expression in endometrial cancer depending on its grade and to determine the VEGFR-1 to VEGFR-2 concentration ratio.

2. MATERIALS AND METHODS

The assessment of VEGF-A, VEGF-B, VEGFR-1, VEGFR-2 expression was performed in 45 patients with endometrial cancer (study group; G1, 17; G2, 15; G3, 13) and 15 patients without neoplastic changes (control group). Exclusion criteria from the study group includes diagnosed endometriosis or adenomyosis, non-endometrioid endometrial cancer, co-existing cervical cancer, the use of hormone replacement therapy 24 months prior to surgery, BMI>40. The study was approved by the Bioethics Committee of the Medical University of Silesia in Katowice (KNW/0022/KB/237/16).

The determination of the level of studied proteins was performed based on the immunohistochemical reaction with following mouse monoclonal antibodies: anti-VEGF-A (Novus Biological, USA), anti-VEGF-B (Santa Cruz Biotechnology, Inc., USA), anti-VEGFR-1 (Novus Biological, USA) and rabbit polyclonal anti-VEGFR-2 antibody (Novus Biological, USA). The Laboratory of Pathomorphology of Beskid Center of Oncology in Bielsko-Biała provided paraffin blocks from which slides were prepared. Three slides were made (every fifth cut section) from one paraffin block. To retrieve the antigens, slides were incubated in Tris-EDTA (pH 9.0) or citrate buffer (pH 6.0) in water bath for 30 min at 95°C. Then they were treated with a solution of hydrogen peroxide (0.3% H_2O_2, 0.1% NaN_3 in PBS) for 10 minutes to block endogenous peroxidase activity. Non-specific binding was blocked with 1% BSA solution in PBS (30 min at room temperature). The next step included incubation with primary antibodies in a humidified chamber (20 h at 4°C). The avidin-biotin complex (ABC) method was used according to the manufacturer's instructions (Vectastain Elite ABC Kit, Vector Laboratories). The slides were stained with Gill's hematoxylin, dehydrated and coverslipped. Negative control was performed by replacing the primary antibody with appropriate IgG. Photographic documentation was prepared using Nikon Eclipse E200 light microscope with Nikon DS-Fi1 digital camera. A total of 15 photographs were taken from each patient under 200x magnification. NIS-AR (Nikon) program was used to assess the optical density of a reaction product in fields where a positive reaction occurred.

The data obtained on the protein level was validated by microarray technique (HG-U133; Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. The procedures were described in detail in our previous work [22].

The statistical analysis was carried out using the Statistica 12 PL software (StatSoft, Cracow, Poland). Results were presented as mean ± standard deviation. The obtained data met the assumptions of the normal distribution, which was verified by the Shapiro-Wilk test. Therefore, the next steps of analysis were performed using the parametric tests. In order to determine whether the observed differences in VEGF-A, VEGF-B, VEGFR-1, VEGFR-2 expression in the control group and in a given grade of endometrial cancer are statistically significant, the one-way ANOVA test was carried out. In the case where the test result was $p < 0.05$, Tukey's post-hoc test was conducted. In addition, the possible correlation between the expression of analyzed protein each other was evaluated based on Pearson's correlation parameter (r). The data of microarray experiment was analyzed using DNA Microarray Integromics analysis platform PL-Grid Infrastructure (http://www.plgrid.pl/en).

Table 1. Descriptive statistics for VEGF-A, VEGF-B, VEGFR-1, VEGFR-2 in control and individual grades of endometrial cancer (G1-G3), the fold and direction of expression change in the analyzed groups.

Protein	Group	M	SD	mRNA	
				FC	Up/Down
VEGF-A	C	72.31	8.74		
	G1	106.78[a]	9.85	+1.2857459	up
	G2	151.79[b,d]	12.53	+1.7453628	up
	G3	176.47[c,e]	13.27	+1.1008087	up
VEGF-B	C	0.00[c]	0.00		
	G1	100.69[a]	9.93	+1.0664871	up
	G2	159.30[b,d]	11.49	+1.0476109	up
	G3	100.85[c,e]	9.69	-1.710149	down
VEGFR-1	C	89.96	8.72		
	G1	123.54[a]	11.37	+1.4850428	up
	G2	150.18[b,d]	12.32	+1.0072452	up
	G3	161.54[c,e]	14.12	+1.0032461	up
VEGFR-2	C	103.63	10.01		
	G1	134.50[a]	12.74	+1.0757967	up
	G2	145.25[b,d]	12.50	+1.136022	up
	G3	177.15[c,e]	15.02	+1.1246978	up

M, mean; SD, standard deviation; FC, fold change;

Letters indicate statistically significant changes at the protein level at $p < 0.05$ between:

[a]G1 *vs.* control; [b]G2 *vs.* control; [c]G3 *vs.* control; [d]G1 *vs.* G2; [e]G2 *vs.* G3. The result of the Tukey's post-hoc test for each comparison was p=0.000008.

3. RESULTS

VEGF-A and VEGF-B are well expressed in the uterine glands in both control and endometrial cancer samples (Figs. **1** and **2**). Their expression at the transcriptome and protein levels is higher in endometrial cancer regardless of its differentiation compared to the control (Table **1**). It can be concluded that with the dedifferentiation of tumor cells, the expression of VEGF-A increases. The largest differences in VEGF-A levels are observed between G2 and G3 endometrial cancer. In turn, no VEGF-B expression was reported in the control. There is an increase in the level of VEGF-B in G1 and G2, however, in G3 samples a decrease in its expression is observed, reaching a level similar to that in G1. At the transcriptome level, *VEGF-B* was silenced in G3 cancer compared to the control.

In the case of VEGFR-1 and VEGFR-2 expression, the level of both receptors is higher in endometrial cancer than in the control. In addition, a lower concentration of VEGFR-1 compared to VEGFR-2 was observed in all analyzed groups. In control, the expression of receptors was visualized in stromal cells, while in endometrial cancer samples in tumor cells (Figs. **3** and **4**). The results of the microarray analysis of changes in *VEGFR-1* and *VEGFR-2* expression are similar to those obtained by IHC staining. Table **1** includes descriptive statistics (M, mean; SD, standard deviation) for each of the analyzed proteins and changes in the transcriptional activity of genes encoding VEGF-A, VEGF-B, VEGFR-1, VEGFR-2 compared to the control (FC, fold change). The footnote shows the results of ANOVA and Tukey's post-hoc test. Figs. (**1-4**) illustrate the expression of these proteins in G1-G3 endometrial cancer and control. The arrows show the places where the expression of analyzed protein was observed. Analysis of the possible correlation between the analyzed proteins showed four statistically significant ($p<0.05$) correlations: in G1 between VEGF-B and VEGFR-1 (r=-0.199525), in G2 between VEGFR-1 and VEGFR-2 (r=0.154821), in G3 between VEGF-A and VEGF-B (r=0.289199), VEGF-B and VEGFR-1 (r=0.163459).

4. DISCUSSION

In recent years, we have been observing the intensification of activities undertaken to develop new drugs used in targeted therapy. This therapy is based on the selective inhibition of signaling pathways [23] that play a key role in oncogenesis and/or cancer progression [24].

VEGF-A is expressed mainly in vascular cells and has the ability to bind primarily to VEGFR-2. However, it appears to induce significantly weaker tyrosine kinase activity, which is a consequence of the presence of an inhibitory sequence in the membrane domain that suppresses VEGFR-1 activity [25]. VEGFR-1 is characterized as a negative regulator of angiogenesis [26]. Using this observation, a model for

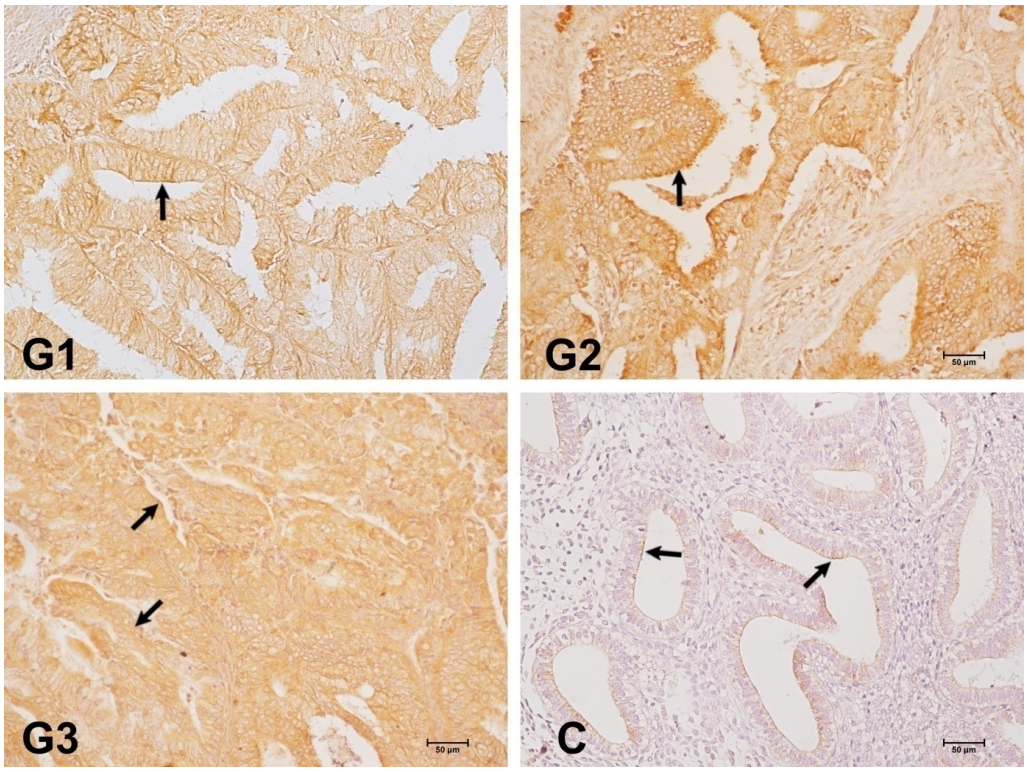

Fig. (1). Immunohistochemical localization of VEGF-A in different grades of endometrial cancer and control. C, control; G, grade of endometrial cancer; The arrows show the places where the expression of analyzed protein was observed. Positive reaction - brown color. 200x magnification.

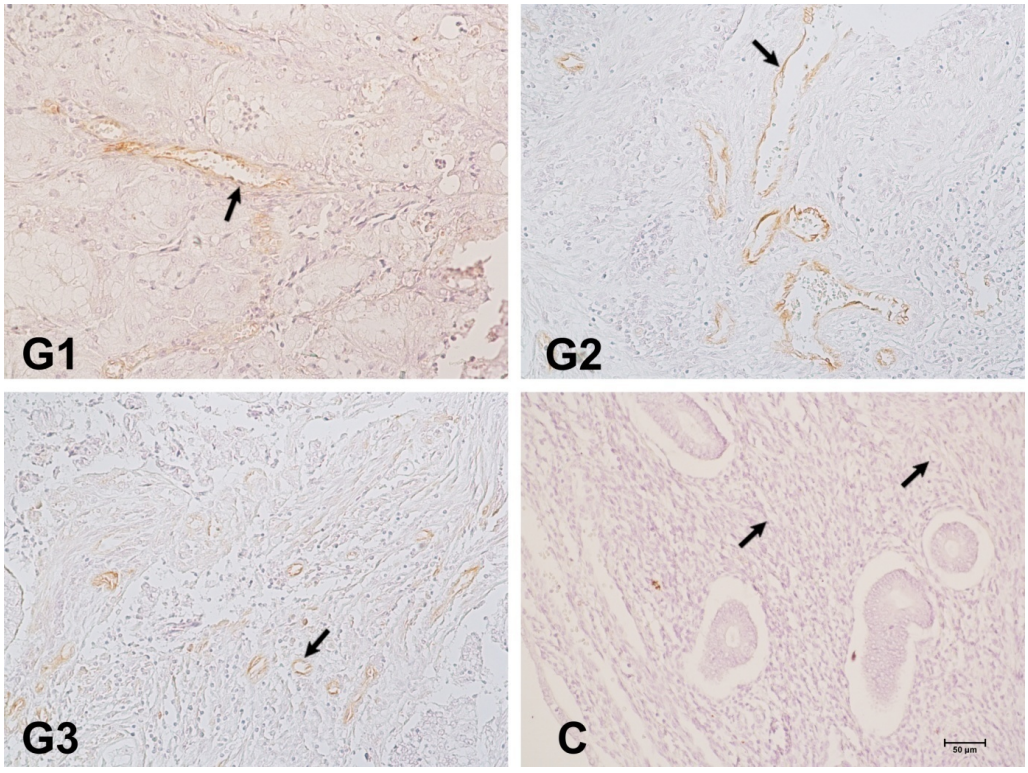

Fig. (2). Immunohistochemical localization of VEGF-B in different grades of endometrial cancer and control. C, control; G, grade of endometrial cancer. The arrows show the places where the expression of analyzed protein was observed. Positive reaction - brown color. 200x magnification.

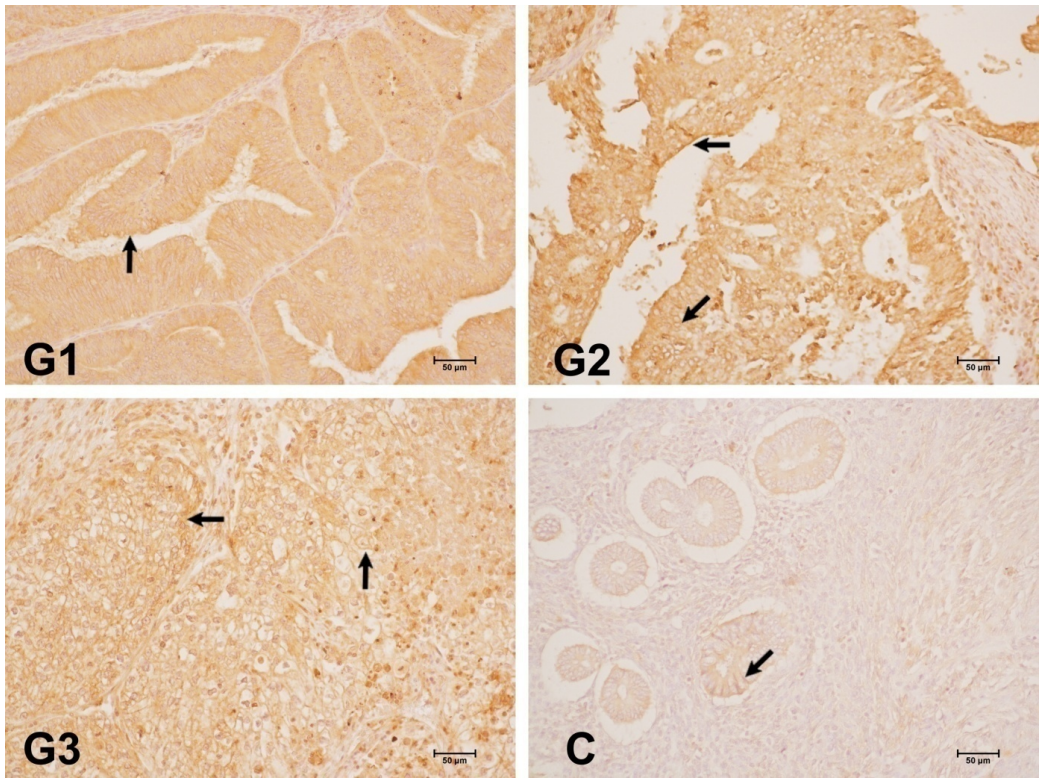

Fig. (3). Immunohistochemical localization of VEGFR-1 in different grades of endometrial cancer and control. C, control; G, grade of endometrial cancer. The arrows show the places where the expression of analyzed protein was observed. Positive reaction - brown color. 200x magnification.

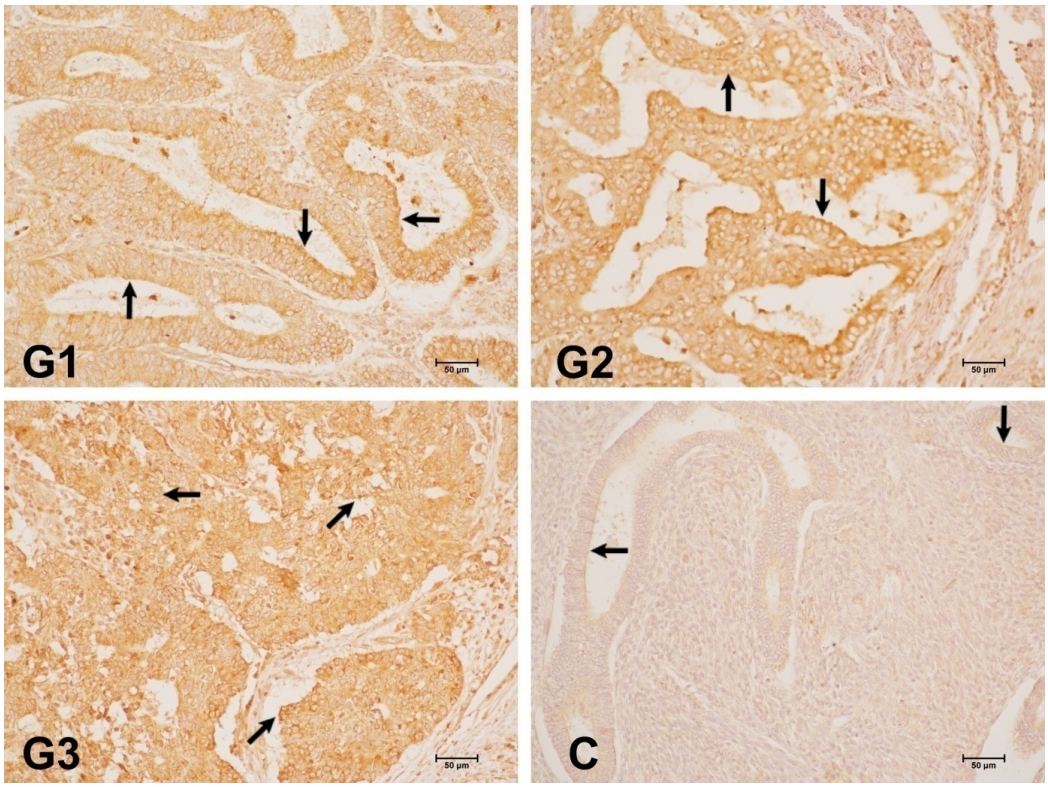

Fig. (4). Immunohistochemical localization of VEGFR-2 in different grades of endometrial cancer and control. C, control; G, grade of endometrial cancer. The arrows show the places where the expression of analyzed protein was observed. Positive reaction - brown color. 200x magnification.

VEGFR-1 was developed in which it could act as a decoy receptor that modulates angiogenesis through its ability to isolate VEGF-A. In turn, VEGF-B has the ability to interact only with VEGFR-1 and its role is described in the context of stabilization of newly formed blood vessels [17, 18]. Interestingly, it was shown that VEGF-C together with VEGF-A and some proangiogenic cytokines are released from macrophages flowing into the tumor cells. It is believed that their infiltration is at least partially responsible for the relationship between angiogenesis and cancer. As a consequence, the shaken balance of pro- and antiangiogenic factors favors the proangiogenic phenotype of the tumor [27-30]. In our studies, VEGF-A was expressed both in control as well as in endometrial cancer, regardless of its grade. Analysis of the VEGF-A expression pattern showed that as the degree of endometrial cancer differentiation decreased, its expression increased (G3>G2>G1). Holland *et al.* analyzed changes in VEGF-A and VEGF-B level in benign endometrium, atypical complex hyperplasia (ACH) and endometrioid endometrial cancer (G1/G2, 4 patients; G3, 9 patients). They observed the expression of VEGF-A in all grades of endometrial cancer, 1 of 5 cases of ACH and none in benign endometrium. In turn, VEGF-B expression was observed in all 5 cases of ACH, 3 of G1/G2 and 4 of G3 cancer. They also checked changes in *VEGF-B* transcriptional activity using RTqPCR. The VEGF-B expression was higher in benign endometrium ($n = 7$) than in atypical hyperplasia ($n = 3$, $p = 0.13$) and endometrial cancer ($n = 17$, $p = 0.04$) [31]. Combining our results with their observations, it can be concluded that the more aggressive the endometrial cancer is, the higher is the expression of VEGF-A. We also found that VEGF-B was not expressed in normal tissues, therefore its overexpression was observed in cancer cells compared to the control. Differences in the expression profile of VEGF-B at the mRNA and protein levels might be caused by microRNA (miRNA) [23]. We would like to emphasize that at every stage of the molecular analysis, all procedures were followed and the analysis was carried out with the utmost care and attention. During the IHC staining, positive and negative controls were performed, which made it possible to verify the correctness and accuracy of the assay. It is worth noting that in G3 endometrial cancer, the level of VEGF-B is similar to that in G1. This may indicate that VEGF-B is likely to be involved in maintaining normal cellular interactions in the endometrium and that the loss of its expression may contribute to tumor progression. Therefore, lower expression of VEGF-B in G1 and G3 suggests that it has the most aggressive character in these two grades of endometrial cancer.

Kamat *et al.* evaluated changes in VEGF-A expression in endometrial cancer in a group of 111 patients and an *in vitro* model. They indicated a high level of this protein in 46 samples of G1/G2 endometrial cancer and 16 G3 samples, while a low level was observed in 42 G1/G2 and 7 G3 samples. They pointed out that the elevated level of VEGF is associated with a worse prognosis and emphasized the effectiveness of including anti-vascular treatment in oncology [32]. Our results confirm this conclusion. The higher the concentration of VEGF-A, the less the endometrial cancer was differentiated, and thus it was characterized by higher metastatic capacity.

Interesting observations are also provided by studies on a group of 118 patients with endometrial cancer carried out by Kotowicz *et al.*, in which they analyzed the expression profile of VEGF-A, VEGFR-1, VEGFR-2 and IL-8. They emphasize the clinical value of IL-8 and VEGFR-2 as potential prognostic factors and that increased VEGF-A activity may be useful in pre-operative assessment of para-aortic lymph node status [33]. Mathur *et al.* observed elevated serum level of VEGF-B in early, advanced and persistent cervical cancer, as well as in ovarian and endometrial cancer. They suggest that VEGF-B can be an independent molecular marker of gynecological cancers [34]. Our observations coincide with those made by Guidi *et al.* who did not report strong *VEGF-A* expression in benign atrophic endometrium [35]. In a study conducted by Hanrahan *et al.*, it is suggested that VEGF-A and VEGF-B are responsible for the initiation of the neoplastic process, whereas VEGF-A and VEGF-C play a key role in its progression [36]. By analyzing the expression pattern of VEGFR-1 and VEGFR-2, we observed a lower concentration of the first receptor compared to the second in G1 and G3 cancer. The opposite situation can be observed in the case of G2 cancer, although the difference is relatively small. VEGFR-2 is responsible for the induction of new blood vessel formation, it determines their permeability, cell division and promotion of their survival [37]. It has been reported that in epithelial cells of breast and non-small cell lung cancers, the increased expression of VEGFR-1 is associated with short survival time [38-40]. In contrast, reduced expression of VEGFR-1 in bile duct cancer and diffuse large B-cell lymphoma (DLBCL) is considered a negative prognostic factor [41, 42]. Immunohistochemical studies of other research centers also showed that VEGFR-1 was stained in tumor cells [43, 44]. D'Hanae *et al.* found lower expression of VEGFR-1 compared to VEGFR-2 in colorectal cancer. They also emphasized the value of determining changes in their expression, while stressing that it would be reasonable to simultaneously assess the level of both receptors. This will allow to determine the relationship between receptors and increase the sensitivity and precision of the assay [45]. In turn, Kopparapu *et al.* observed a higher level of *VEGF-A*, *VEGFR-1*, *VEGFR-2* in all bladder cancer samples compared to the control. They also noticed that the level of VEGFR-2 protein was significantly higher in all cancer samples in comparison to benign urothelial mucosa ($p = 0.001$) [46]. Fuijaki *et al.* observed a relationship between the cyclooxygenase-2 (COX-2) level and the VEGF concentration. They reported that VEGF-dependent signaling contributes to the increase in COX-2 expression as well as the level of vascularization of neoplastic lesions within the endometrium [47]. These observations indicate the existence of a network of interrelations between different proteins in carcinogenesis. It seems reasonable to conclude that cancer can be included in diseases with proinflammatory etiology. The last step of our study included the search for a possible correlation between expression pattern of VEGF-A, VEGF-B, VEGFR-1, VEGFR-2 in a given grade of endometrial cancer (G1-G3). Four statistically significant ($p<0.05$) correlations were found: in G1 between VEGF-B and VEGFR-1 ($r=-0.199525$), in G2 between VEGFR-1 and VEGFR-2 ($r=0.154821$), in G3 be-

tween VEGF-A and VEGF-B (r=0.289199), VEGF-B and VEGFR-1 (r=0.163459). The obtained results indicate that although these correlations are statistically significant, they are either weak or low. Therefore, the relationship between the levels of analyzed proteins should be interpreted carefully.

Molecular studies, including the analysis of changes in the expression profile of VEGF-A, VEGF-B, VEGFR-1, VEGFR-2 are helpful in the development of individual diagnostic and therapeutic strategies. Further detailed investigation of complex mechanisms determining angiogenesis in cancer is necessary for more accurate assessment of prognosis for oncological patients.

CONCLUSION

VEGF-A, VEGF-B, VEGFR-1 and VEGFR-2 are important proteins involved in the induction and development of a new blood vessel network through which the tumor cells are properly nourished and oxygenated. In our study, we showed an increased level of all four analyzed proteins in endometrial cancer compared to the control. Our observations of the expression ratio of the examined receptors coincide with the reports from other research centers. Due to the complexity, plasticity and dynamics of changes during the entire process of carcinogenesis, we share the opinions of other researchers that entire panels of genes/proteins involved in a given process should be analyzed simultaneously. Investigation of molecular mechanisms of tumor angiogenesis will contribute to better determination of patients' prognosis and understanding of changes observed at the phenotypic level.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study is approved by the Bioethical Committee of the Medical University of Silesia, No. KNW/0022/KB/237/16 has been obtained for this study.

HUMAN AND ANIMAL RIGHTS

No animals were used in this study. The reported experiments were performed in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the *Helsinki Declaration* of 1975, as revised in 2013 (http://ethics.iit.edu/ecodes/node/3931).

CONSENT FOR PUBLICATION

Informed consent was obtained from all of the patients recruited.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this study are available within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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All authors were responsible for the concept and design of the study, collection and collation of data, analysis and interpretation of data, writing of the article, reviewing, and final reviewing of this article and graphics performance.

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