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ORIGINAL ARTICLE

Tissue expression of VEGF in cervical intraepithelial neoplasia and cervical cancer

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Summary

Purpose: *To examine the expression of vascular endothelial growth factor (VEGF) in the cervical tissue of individuals divided into the control group (normal cervix), group A (HSIL lesions), and group B (cervical cancer, FIGO stage I-IIA). Analyzed was also the expression of VEGF between groups and subgroups in group A and B. The expression of VEGF was also compared with histopathological parameters in group B.*

Methods: *Examined was the histopathological material taken from 109 operated patients. The patients were divided into 3 groups based on the definitive histopathological findings: control group (30 patients), group A (33 patients), and group B (46 patients). Immunohistochemistry was performed to examine the expression of VEGF.*

Results: *The expression of VEGF was negative in the control group, while in 11 patients (33.33%) from group A and 28 patients (60.87%) from group B it was significantly different ($p<0.05$) compared to the control group. There was neither statistically significant difference in the expression of VEGF in group A regarding the type of intraepithelial lesion, nor in group B regarding the FIGO disease stage ($p>0.05$). In patients with poor histopathological prognostic parameters such as tumor diameter > 2 cm (24/46), depth of stromal invasion > 10 mm (32/46), positive lymph nodes (17/46), and with infiltration of the uterine body (11/46) a statistically significant difference was confirmed regarding the expression of VEGF.*

Conclusion: *The increased VEGF expression in groups A and B compared with the control group indicated the importance of VEGF as a proangiogenic factor in neoangiogenesis in precancerous and cancerous changes in the cervix. The frequent expression of VEGF in the subgroup of patients with poor histopathological prognostic factors (group B) indicated the importance of the activity of proangiogenic factors in the process of cervical cancer neoangiogenesis. Further investigations should be aimed at these markers as prognostic factors in the high risk group of patients with cervical cancer who should receive adjuvant therapy after radical operation and consider using antiangiogenic drugs as part of adjuvant treatment.*

Key words: *cervical intraepithelial neoplasia, neoangiogenesis, uterine cervical neoplasms, vascular endothelial growth factor*

Introduction

Tumor angiogenesis is defined as the formation of neovessels from preexisting vascular structures, mainly capillaries and venules, under the influence of a malignant tumor [1,2].

In 1971, Folkman reported that angiogenesis is mediated by angiogenic molecules, inducing the growth of a close capillary network that surrounds and invades tumors [3,4]. This hypothesis has been supported by indirect and direct evidence from many studies [5-10].

Tumor angiogenesis is regulated by a balance of stimulators (e.g., VEGF, bFGF) and inhibitors of angiogenesis (e.g., angiostatin, endostatin, angiostatic steroids). The stimulation of angiogenesis during carcinogenesis is a result of the rupture of the balance between pro- and antiangiogenic factors. The important activator of the overexpression of angiogenic factors that breaks the balance

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between proangiogenic factors and inhibitors in the tumor microenvironment is hypoxia that induces the hypoxia-inducible factor 1α (HIF-1α) [11,12]. Recent studies show the importance of tumor angiogenesis as a key factor that affects the patients' survival and the malignant potential of some gynecological malignant tumors [13].

Among many proangiogenic factors, the most potent is VEGF [14]. VEGF is a proangiogenic peptide that stimulates growth and development of vascular endothelial cells, stimulates proliferation and differentiation of the new vascular net, prolongs the lifetime of existing vessels, and helps the tumor growth. VEGF has 6 subtypes of structurally similar proteins that regulate differentiation and growth of the vascular system [15].

The purpose of the present study was to examine the expression of VEGF in the cervical tissue of individuals divided into the control group (normal cervix), group A (high-grade squamous intraepithelial lesions [HSIL]), and group B (cervical cancer, FIGO stages I-IIA). Analyzed was also the expression of VEGF between groups and subgroups in group A and B. Also the expression of VEGF was compared with histopathological parameters in group B.

Methods

Histological material taken from 109 patients was analyzed. The patients had undergone hysterectomy with or without adnexectomy because of benign uterine lesions (myomas) or conization due to dysplastic changes or radical hysterectomy due to cervical carcinoma. The patients were divided into 3 groups based on the definitive histopathological findings.

Control group

This group consisted of 30 patients who had undergone total hysterectomy due to benign lesions of the uterus and/or ovaries.

Criteria for excluding patients from this group were:

a) Previous excision or ablation of the cervix before hysterectomy
b) Diagnosis of precancerous or malignant lesions of the cervix
c) Verified chronic inflammation of the cervix
d) Verified malignancy of the genital tract
e) Verified malignant disease of any localization

Group A

This group consisted of 33 patients diagnosed with HSIL changes in the cervix and samples for pathology were obtained by any of excisional methods.

Criteria for excluding patients from this group were:

a) Patients with previously diagnosed HSIL and treated by any ablative or excisional procedure
b) Verified malignancy of the genital tract
c) Verified cervical intraepithelial dysplasia I (CIN I)
d) Verified malignant disease of any localization

Group B

This group included 46 patients with verified cervical cancer, FIGO stages I-IIA, and having Piver class III radical hysterectomy with lymphadenectomy.

Criteria for excluding patients from this group were:

a) Verified cervical cancer and previous treatment with radiotherapy or neoadjuvant chemotherapy
b) Previous ablation or excision treatment of the cervix
c) Verified malignancy of the genital tract of other localization
d) Verified malignant disease of any localization

Patients were informed about the purpose of this study and they signed written informed consent. The study was approved by the Hospital Ethics Committee.

Immunohistochemical analyses

In group A, samples for immunohistochemical analyses were selected from the cervix conization. In the control group and group B (radical hysterectomy) the samples were obtained from the cervix. Four micron-thick sections of formalin-fixed paraffin-embedded (FFPE) tissue blocks were cut and mounted on coated slides. The sections were deparaffinized in xylene, rehydrated in descending ethanol grades, and incubated for 5 min in 3% hydrogen peroxide to block endogenous tissue peroxidase. Antigen retrieval was performed by using citrate buffer in microwave oven. Immunostaining was done by using the standard streptavidin-biotin-peroxidase complex according to standard procedure of Dakocytomation-DAKO (Glostrup, Denmark).

The slides were incubated for 30 min at room temperature with primary antibody followed by incubation with biotinylated antimouse antibodies and streptavidin-biotin-peroxidase complex for 30 min each. 3-amino-9-ethylcarbazole (AEC,DAKO) was used as chromogen. The samples were rinsed in tris buffer solution (TBS; 0.05M, pH 7.6) after each incubation and the slides were counterstained with hematoxylin.

The tissue samples in which the primary antibody was omitted during treatment served as a negative control for each antibody. All analysis of immunohistochemically processed tumor tissue samples was done by means of light microscopy, qualitative and semi qualitative methods, and expressed as percent of positive cells compared to the total cell number in

representative samples. After immunohistochemical analysis was done the VEGF expression was compared among the groups. In group A, the VEGF expression was compared with the grade of dysplasia. In group B, the VEGF expression was compared with the stage of disease and the following histopathological parameters: grade of differentiation, depth of stromal infiltration, stromal lymphocytic invasion, infiltration of the vaginal margin, lymph node metastasis, number of metastatic lymph nodes, and lymphovascular invasion.

Statistics

We used descriptive statistics (frequency, percent, mean value, and standard deviation) for statistical data processing. Column charts and box-and-whisker diagrams were used for graphical presentation of data and results. Student's t-test and analysis of variance (ANOVA) were used for comparison of numerical features and Pearson's chi-square test, Fisher's exact test for non numerical features. Statistical significance was set at $p<0.05$. The software program STATISTICA 9.0 (STATSOFT, Tulsa, USA) was used for statistical analyses.

Results

The average patient age was 46.12±10.183 years. Of 109 studied patients 30 (27.5%) belonged to the control group, 33 (30.3%) to group A, and 46 (42.2%) to group B (Figure 1).

No VEGF expression was observed in the control group of patients, contrary to group A where it was observed in 11 (33.33%) patients and group B where it was observed in 28 (60.87%) patients (Figure 2).

Comparison of VEGF expression among the studied groups of patients showed statistically significant differences (Figure 3 and Table 1).

Table 1. Comparison of VEGF expression among the studied groups

VEGF expression	*Chi-square*	*p value*
Control group vs Group A	12.115	0.0005
Control group vs Group B	28.913	0.0001
Group A vs Group B	5.829	0.0015

No VEGF expression was observed in all studied samples of the control group and further comparison of VEGF was done between the samples of group A and B. In group A, no statistically significant VEFG expression was found in relation to the type of CIN (p=0.056). VEGF expression was evident only in the subgroup of patients with CIN 3 changes (Figure 4).

In group B, no statistically significant difference of VEGF expression was found in relation to FIGO stage and histopathological parameters ($p>0.05$). In the subgroup of patients with poor histopathological prognostic factors such as tumors > 2 cm, stromal infiltration > 10 mm, positive lymph nodes, and infiltration of the uterine isthmus a statistically significant difference was confirmed in relation to the presence of VEGF expression (positive VEGF - 66.66, 66.66, 70.56, and 63.64% compared to negative VEGF – 33.34, 33.34, 29.44, and 39.13%; $p<0.05$) (Figure 5).

Grade of differentiation, lymphovascular invasion, and presence of stromal lymphocytic invasion were not correlated with VEGF expression

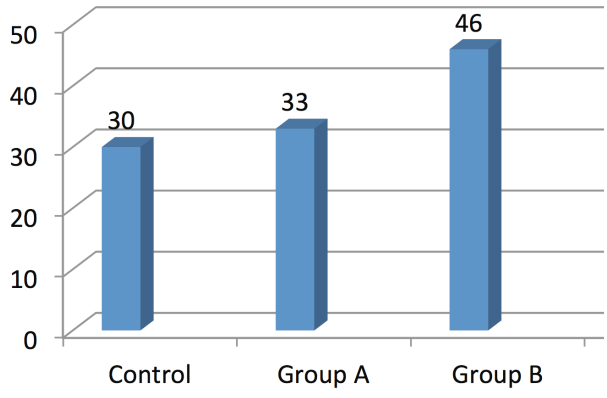

Figure 1. Patient number distribution according to groups.

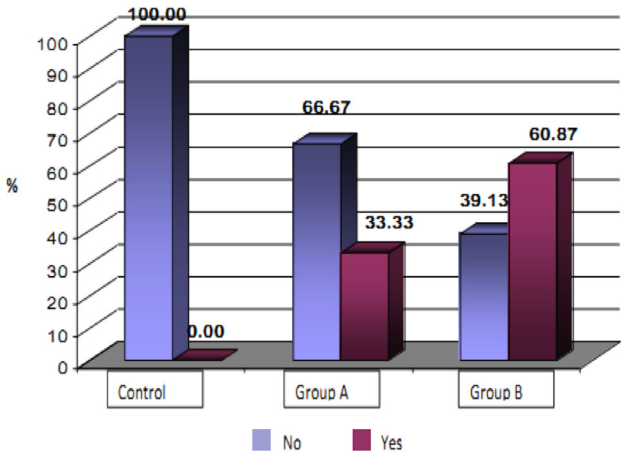

Figure 2. Percent VEGF expression according to patient groups.

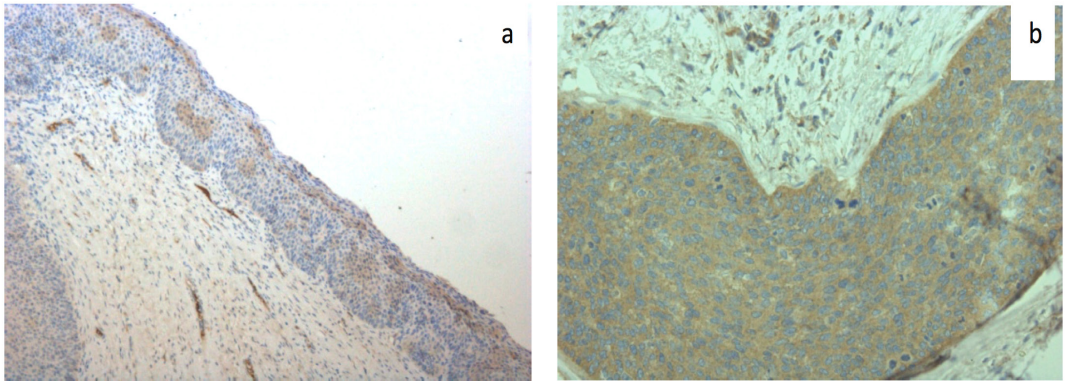

Figure 3. Immunohistochemical expression of VEGF in group A **(a)** x100 and B **(b)** x100.

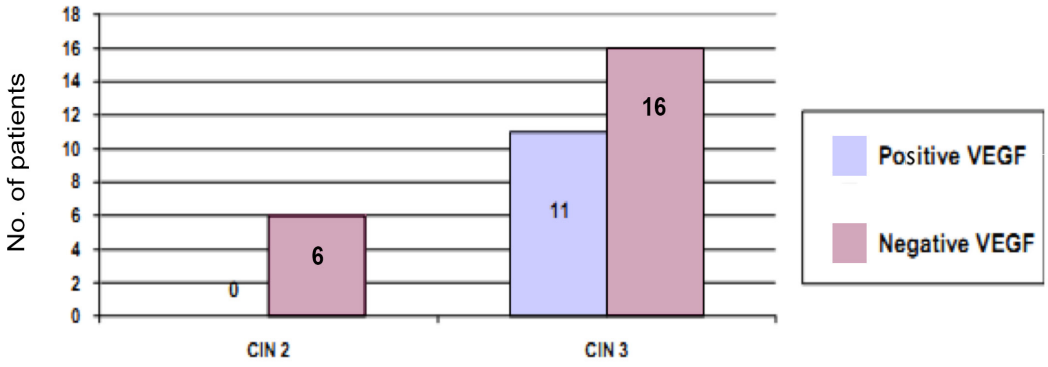

Figure 4. Number of patients with of VEGF expression in group A according to the type of cervical intraepithelial neoplasia.

(p>0.05). No statistical evaluation was done in the subgroup of patients who had infiltration of the parametrium and vaginal margin because of the small number of samples (3 and 4 patients, respectively).

Discussion

Mortality from cervical cancer ranks third among all cancers in females. Over 85% of deaths due to cervical cancer are registered in less developed countries. Implementation of screening programs in 1960 has decreased the incidence and mortality of this disease except in least developed countries without screening programs where these indices are still high. Compared to European countries, Serbia ranks fifth in incidence (19.6/100 000) and third in mortality (8.6/100 000) due to cervical cancer [16].

Neoangiogenesis is an important factor in

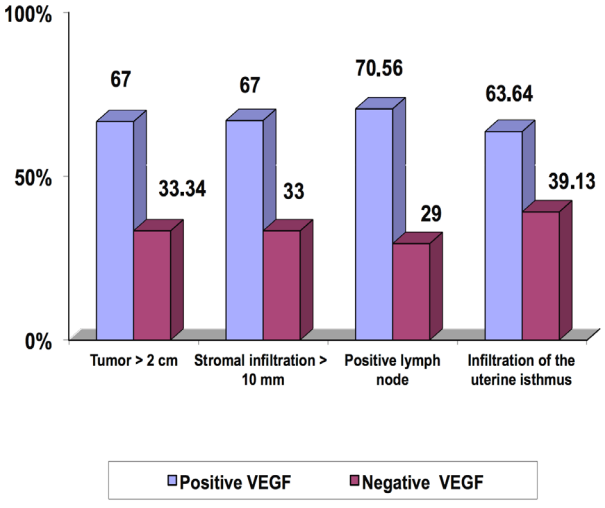

Figure 5. Percent VEGF expression in group B patients with poor histopathological parameters (tumor size >2cm, depth of stromal infiltration >10mm, lymph node metastasis).

the development of cervical cancer. Obermair et al. demonstrated that the expression of VEGF significantly correlates with microvessel density in CIN. Angiogenic parameters, such as microvessel density, and the expression of VEGF increase with the grade of cervical dysplasia. Microvessel density and VEGF expression in the adjacent normal epithelium remain nearly constant at a significantly lower level [17]. Guidi et al. showed VEGF mRNA expression and microvessel density to be significantly increased in patients with invasive cervical cancer and in high-grade intraepithelial lesions compared with those observed in low-grade lesions and benign epithelium [18]. Kodama et al. [19] found significant relationship between microvessel density and the level of VEGF mRNA ($p<0.01$). The highest level of VEGF mRNA expression was observed in early invasive cervical cancer. Except for stage IV B, the stage of disease inversely correlated with the level of VEGF mRNA ($p<0.05$) These findings provide evidence that the expression of VEGF is involved in the promotion of angiogenesis in cervical cancer and plays an important role in early invasion. There was no significant difference in the level of VEGF mRNA with respect to lymph node metastasis, depth of stromal invasion, tumor size, parametrial involvement, or vaginal involvement among these patients [19]. In our study, VEGF expression was confirmed in group A (33.33%) and group B patients (60.87%) but not in the control group. A statistically significant difference was confirmed comparing the VEGF expression between patients with HSIL changes (group A, 33.33%) and patients with cervical cancer (group B, 60.87%) ($p<0.05$). No statistically significant difference was observed in patients within group A, but it should be noted that VEGF expression was higher in grade 3 dysplasia. In the subgroup of patients with CIN 3 VEGF expression was found in 11 of 27 patients. Borderline results obtained by chi-square test ($p=0.056$) suggested that if we had a larger number of samples in group A, a statistically significant difference for VEGF expression in relation to the grade of CIN would probably emerge. Regarding the VEGF expression in the subgroup of patients with poor histopathological parameters (tumors > 2 cm, stromal infiltration > 10 mm, positive lymph nodes, and infiltration of the uterine isthmus) we confirmed a positive correlation between VEGF expression and the above-mentioned histopathological parameters (66.66, 66.66, 70.56, and 63.64%, respectively; $p<0.05$, contrary to the grade of differentiation, lymphovascular invasion, and the presence of stromal lymphocytic infiltration; $p>0.05$).

Dai et al. studied COX-2, VEGF, and prostaglandin expression in CIN changes and cervical cancer in a material consisting of 20 cervical samples with no pathological changes, 20 cervical samples with inflammation, 20 samples with CIN changes, and 40 samples of cervical cancer. Their results confirmed positive VEGF expression in cervical cancer (58%) compared to the normal cervical samples, inflammatory cervical samples and cervical samples with CIN changes (0, 5, and 15%, respectively). The authors did not investigate a subgroup regarding the grade of CIN changes. In addition, they did not notice statistically significant difference regarding histopathological changes (tumor size, histological grade, and disease stage). Positive VEGF expression was verified in 72.2% (8/11) of patients with tumor size ≥4cm compared to 51.7% (15/29) of patients with tumors <4cm [20]. In our study, VEGF expression in the subgroup of patients with tumors >2 cm (24/46) was found in 66.66% of the patients vs 57.12% found in the subgroup with tumors < 2 cm (21/46) ($p<0.05$).

Goncharuk et al. [21] presented 75 patients with cervical cancer FIGO stages I-IV and investigated VEGF expression semiquantitatively in relation to histopathological changes and survival. VEGF expression was confirmed in 89% of the patients (20% of patients had weak VEGF expression, 29% had moderate expression, and highest expression was found in 51% of the patients). The study confirmed a statistically significant difference of VEGF expression in relation to FIGO disease stage (stage I 40%, stage II 80%, stage III 80.67%, and stage IV 90%). No statistically significant difference was noticed in relation to the histological type of the tumor (squamous cell carcinoma - 55%, adenocarcinoma - 65%); this could be explained by the small number of patients with adenocarcinoma. A statistically significant difference in VEGF expression was confirmed in relation to histological grade (G2 53.5%, G3 63.4%) and to the status of lymph node (positive lymph nodes – 75.3%, negative lymph nodes 50.5%). Comparison of VEGF expression with the 5-year survival showed a statistically significant difference (>5 years 12.5%, <5 years 84%) [21]. It should be emphasized that in this study comparison of VEGF expression in relation to histopathological parameters was performed for all included patients regardless of disease stage (I-IV). In our study, however, the comparison was done in re-

lation to FIGO stage I disease. In addition, Hellberg et al. found a high level of VEGF expression (≥50%) among patients with IB/IIA stage of the disease (77.9%) vs patients with disease stage IIB/IV (59.7%) [22].

Hammes et al. investigated VEGF expression in relation with proto-oncogene macrophage stimulating factor (c-fsm) and COX-2 in the carcinogenesis of cervical cancer. The study population included 26 patients with benign changes of the cervix, 20 patients with CIN 1, 30 patients with CIN 3 , and 28 patients with cervical squamous cell carcinoma. Positive VEGF expression in normal cervical tissue, with CIN 1, HSIL changes, and carcinoma of the cervix were 11.5, 39.3, 53.3, and 75%, respectively. VEGF expression showed a statistically significant correlation with c-fms and COX-2 expression in case of HSIL changes and carcinoma of the cervix [23]. Investigation did not include the verification of the expression in HISL group but it was considered as a unique group regardless of the degree of dysplasia. In addition, the group of patients with cervical cancer was not studied according to the stage of disease. The results of this study regarding VEGF expression in the group of patients with no histopathological changes of the cervix, patients with HSIL changes, and patients with cervical cancer were in agreement with the results of our study.

Lee et al. investigated a group of 117 patients with stage IB2 carcinoma of the cervix and found a correlation of VEGF expression with the depth of stromal invasion, lymph nodes, status and tumor size. Intensity of VEGF expression was negatively correlated with overall survival [24]. Shi et al. confirmed high-level of expression of VEGF-c and COX-2 in cervical cancer compared to chronic cervicitis and CIN changes. Using multivariate analysis they showed significant correlation of VEGF-c subtype with metastases to the lymph nodes [25]. In our previous study COX-2 expression in the group with cervical cancer compared to the control group suggested a potential impact of COX-2 in the carcinogenesis of cervical cancer together with VEGF [26]. In the Zusterzeel et al. [27] study the serum VEGF level also correlated significantly with the disease free interval (DFI) and overall survival (OS). Multivariate Cox regression analysis confirmed that serum VEGF is a prognostic factor for DFS (p=0.03) and OS (p=0.04) [27]. In conclusion, the authors emphasized the correlation of serum VEGF levels and disease dissemination and considered it as a possible prognostic factor for patients with carcinoma of the cervix [27]. Taken all these studies together the role of neoangiogenesis in the development of invasive cervical carcinoma is crucial.

Conclusion

The linear elevation of VEGF expression in the groups with HSIL lesions and cervical cancer compared to the control group showed the role of this proangiogenic factor in the mechanism of neoangiogenesis in precancerous and cancerous changes of the cervix. The finding of more frequent VEGF expression in the subgroup of patients with poor histopathological parameters (group with cervical cancer) indicated the importance of the activity of VEGF in the process of neoangiogenesis of cervical cancer and its progression. Additional studies should be directed to the investigation of neoangiogenesis markers as predictive factors for adjuvant therapy in the group of high-risk, surgically-treated patients and potential use of anti-angiogenesis drugs as a new therapeutic approach.

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