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The immunohistochemical expression of endocrine gland-derived-VEGF (EG-VEGF) as a prognostic marker in ovarian cancer

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Abstract

Ovarian cancer-related angiogenesis is a complex process orchestrated by many positive and negative regulators. Many growth factors are involved in the development of the tumor-associated vasculature, and from these, endocrine gland-derived vascular endothelial growth factor (EG-VEGF) seems to play a crucial role. EG-VEGF is the first organ-specific angiogenic factor and its effects are restricted to the endothelial cells of the endocrine glands. Although EG-VEGF was detected in both normal and neoplastic ovaries, its clinical significance remains controversial. In the present study, we analyzed 30 patients with epithelial ovarian cancer, and the immunohistochemical expression of EG-VEGF was compared with the conventional clinico-pathological parameters of prognosis. Neoplastic cells of the ovarian carcinoma expressed EG-VEGF in 73.33% of the cases, as a cytoplasmic granular product of reaction. We found a strong correlation between the expression of EG-VEGF at protein level and tumor stage, grade, and microscopic type. The expression of EG-VEGF was found in patients with stage III and IV, but not in stage II. The majority of serous adenocarcinoma, half of the cases with clear cell carcinoma and two cases with endometrioid carcinoma showed definite expression in tumor cells. No positive reaction was found in the cases with mucinous carcinoma. Our results showed that EG-VEGF expression is an indicator not only of the advanced stage, but also of ovarian cancer progression. Based on these data, we concluded that EG-VEGF expression in tumor cells of the epithelial ovarian cancer is a good marker of unfavorable prognosis and could be an attractive therapeutic target in patients with advanced-stage tumors, refractory conventional chemotherapy.

***Keywords*:** ovarian carcinoma, EG-VEGF, angiogenesis, immunohistochemistry, prognosis.

Introduction

Ovarian cancer is the leading cause of death from gynecological malignancies in the Western world and the majority of patients are diagnosed at an advanced stage of disease. Moreover, many cases are admitted in the stage of peritoneal spread that frequently occurs without significant symptoms. Despite the advances made in the knowledge of the genetics and molecular biology of these tumors and the effects of new chemo-therapeutic agents, its natural evolution is poorly understood. Consequently, the 5-year survival rate for patients with ovarian cancer remains high [1].

A particular aspect that was extensively investigated in patients with ovarian cancer is tumor-associated angiogenesis. There were accumulated a lot of data that support the contribution of angiogenesis to tumor cell proliferation, tumor growth and spread. In some studies, it was shown that overexpression of the vascular endothelial factor (VEGF) in ovarian carcinoma stimulates not only the formation of new blood vessels, but also induces the neoplastic transformation of epithelial cells of the ovarian surface epithelium [2]. In clinical studies, it was shown that overexpression of VEGF is related to poor prognosis. Blockade of VEGF using humanized monoclonal antibody was thought to be a powerful therapeutic option in advanced-stage ovarian cancer, but on the other hand, results of some clinical trials based on this strategy were disappointing [3]. This is explained in part by the contribution of other growth factors, like fibroblast growth factor or platelet-derived growth factor that support the proliferation of both tumor cells and endothelial cells [4, 5].

It is more likely that angiogenesis in ovarian carcinoma is orchestrated by many growth factors and its intensity and effects on the further evolution of the tumor strongly depends on the balance between proangiogenic and antiangiogenic substances. Although many studies were conducted on ovarian cancer-related angiogenesis, the basic mechanisms of this process with this particular location remain less understood.

The first organ-specific proangiogenic molecule was isolated and characterized almost ten years ago, that was called endocrine gland derived-VEGF (EG-VEGF) [6]. EG-VEGF induces the proliferation, migration and fenestration in capillary endothelial cells associated with endocrine glands, while it has little or no effect on other endothelial cells. EG-VEGF does not belong to the VEGF family, but is a member of a new protein family with multiple regulatory functions [7]. EG-VEGF has been detected in the adrenal cortex, in the ovary, testis

and placenta and low levels of EG-VEGF mRNA have been also demonstrated in the brain, colon, small intestine, liver, spleen and thymus. Recently, it has been shown for the first time that EG-VEGF is strongly expressed in the normal adenohypophysis [8].

The presence of EG-VEGF has been demonstrated also in pathological conditions, such as human cancers, including ovarian carcinoma [9], colorectal cancer [10], pancreatic adenocarcinoma [11], and benign lesions, such as polycystic ovaries [12]. The angiogenic role of EG-VEGF is supported by the correlation found between its expression and microvascular density in all these tumors. On the other hand, the prognostic value of EG-VEGF expression by tumor cells is still a matter of debate, as no significant differences were reported between patients with high and low levels, in terms of overall survival. In addition, the clinical significance of EG-VEGF in ovarian cancer is still controversial, despite some studies found a relationship between overexpression and unfavorable prognosis.

In the present study, we investigated the expression of EG-VEGF at protein level and we looked for a correlation with conventional parameters of prognosis in a series of patients with epithelial ovarian carcinomas.

Materials and Methods

There were investigated 30 patients admitted with ovarian cancer, aged between 56 and 74 years. Specimens were taken from the primary tumors and after fixation in buffer formalin, they were processed using the routine histological procedure. Five-µm thick sections were stained with the conventional Hematoxylin–Eosin method to assess the pathological type of the tumor and the degree of differentiation. The patients were stratified according the stage of the tumor. There were six cases in the stage II, 16 in the stage III, and eight in the stage four. The pathological examination showed serous adenocarcinoma in 19 cases, clear cell carcinoma in six cases, mucinous carcinoma in three cases and endometrioid carcinoma in two cases. Tumors were graded according the requirements of *World Health Organization* [13], and we found well differentiated tumors in four cases, moderate differentiate in 18 cases and undifferentiated in four. Apparently, normal ovarian tissue adjacent to the tumor was available in 21 cases.

Additional slides containing 3-µm thick sections were stained with EG-VEGF polyclonal goat anti-human antibody, clone T16, dilution 1:100 (Santa Cruz Biotechnology Inc., Santa Cruz, USA). Briefly, deparaffinized slides were hydrated, submitted to antigen retrieval in citrate buffer pH 6 for 30 minutes, and incubated with the primary antibody for 30 minutes. The working system used in this study was LBAB+/HRP, and the final product of reaction was visualized with diaminobenzidine. Nuclei were stained with Lillie's modified Hematoxylin.

Antigen retrieval was performed using the PTlink module and the full immunohistochemical procedure was developed using the automated system Dako AutostainerPlus (DakoCytomation, Glostrup, Denmark). Examination was performed with the microscope Eclipse E60 Nikon, and images were acquired with Lucia G soft for microscopic image analysis.

EG-VEGF immunoreactivity was estimated as percent of positive cells according to this score: 0 (0% positive cells); 1 (<10%); 2 (10–50%); 3 (>50%). EG-VEGF immunoreactivity was also evaluated in terms of intensity of reaction, by using a scale from 0 to 3 for a negative, weak, moderate or strong reaction, accordingly. A final score was attributed ranging between 0 and 6, and samples that scored between 0–2 (+1) were considered negative, while samples that scored between 3–6 (+2 and +3) were considered positive. Slides obtained from the normal deep human adrenal gland, which strongly express EG-VEGF (the score was estimated as +3) were used as positive controls.

Statistical analysis was performed using the commercially available SPSS version 17.0. We applied Student's *t*-test and $p<0.05$ was considered as significant.

Results

In the outer control slides, the positive reaction was restricted to the cortex of the adrenal gland and the pattern of reaction was granular cytoplasmic (not shown). In the normal ovary, the positive reaction was restricted to the covering epithelium, as a weak to moderate but homogeneous reaction (Figure 1).

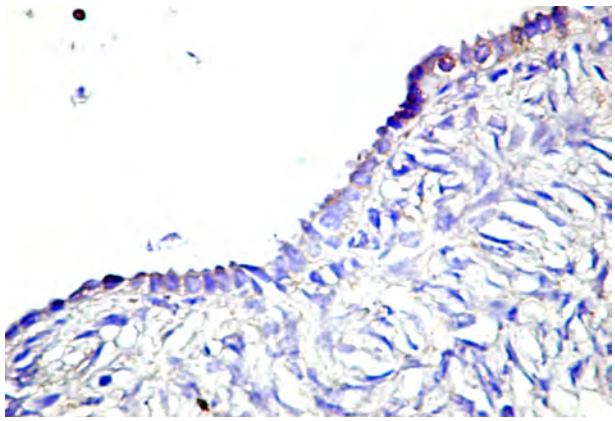

Figure 1 – ***EG-VEGF expression in the covering epithelium of the normal ovarian tissue. Original magnification, ×400.***

The pattern of distribution of the final product of reaction for EG-VEGF in ovarian carcinoma was mostly heterogeneous. We describe here three different models of the positive reaction in tumor cells: isolated cells that strongly express EG-VEGF (Figure 2a), positive cells arranged in small nests, as it was particularly in the case of clear cell carcinoma (Figure 2b), and heterogeneous (Figure 2c). Based on the scoring system detailed above, we found an overall positive reaction for EG-VEGF in 22 cases (73.33%). From these, 14 cases were scored as +2 and eight cases as +3.

An aspect with particular prognostic significance is related to the distribution of positive tumor cells at the interface between the tumor and stroma (Figure 3). The number of positive tumor cells and the intensity of

reaction gradually decreased in the deep tumor area. All these cases were diagnosed in the stage III and IV. We noticed a statistic significant relationship between the expression of EG-VEGF and the tumor stage (p<0.003).

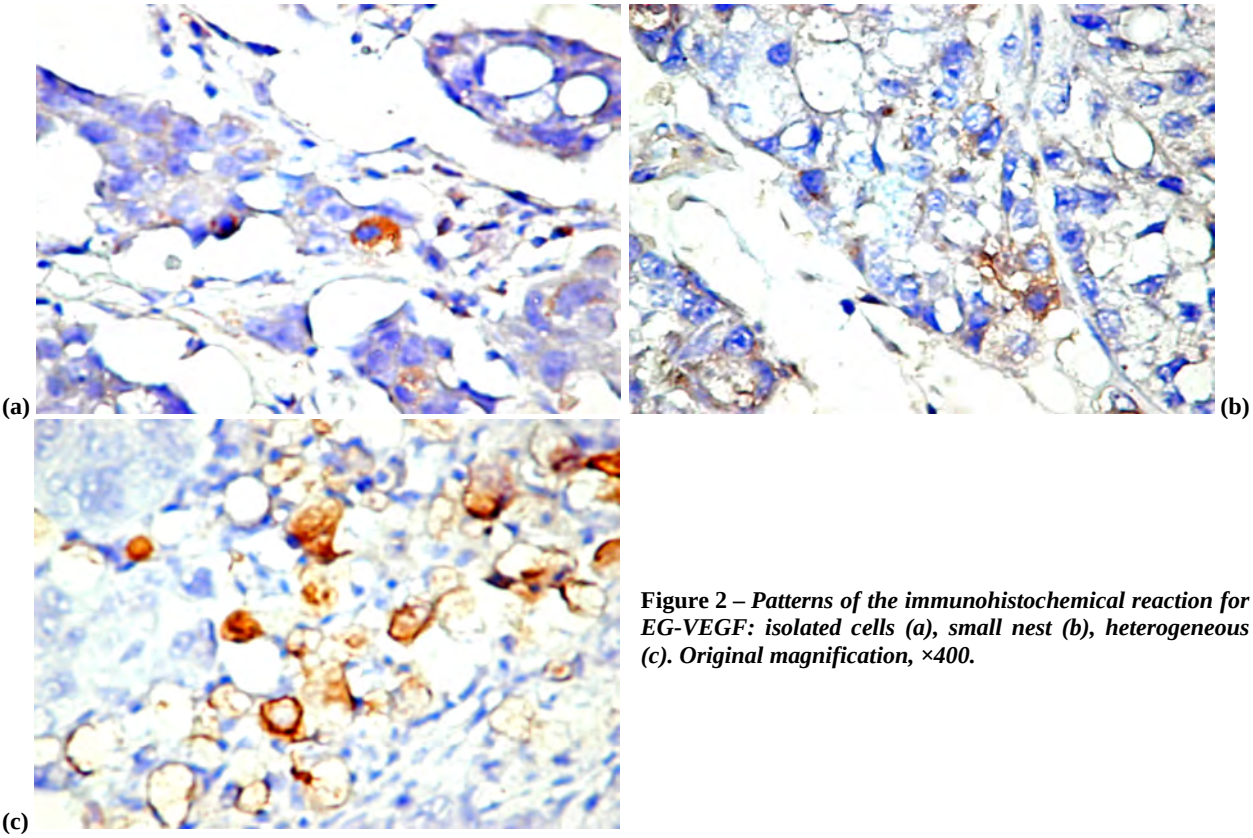

Figure 2 – ***Patterns of the immunohistochemical reaction for EG-VEGF: isolated cells (a), small nest (b), heterogeneous (c). Original magnification, ×400.***

We also found a relationship between EG-VEGF expression and the pathological type of ovarian carcinoma. Positive reaction was observed in 16 from 19 serous adenocarcinomas, in three from six cases with clear cell carcinoma, and in the two cases with endometrioid carcinoma. All the cases with mucinous carcinoma were negative for EG-VEGF. This relationship was statistically significant for p<0.0001.

The intensity of EG-VEGF expression was significantly stronger in less differentiated tumors than in the others. The pattern of the final product of reaction was diffuse (Figure 4a) or heterogeneous (Figure 4b), and the majority of tumor cells were intensely labeled. The correlation between the tumor grade and EG-VEGF expression had statistic significance (p<0.0021).

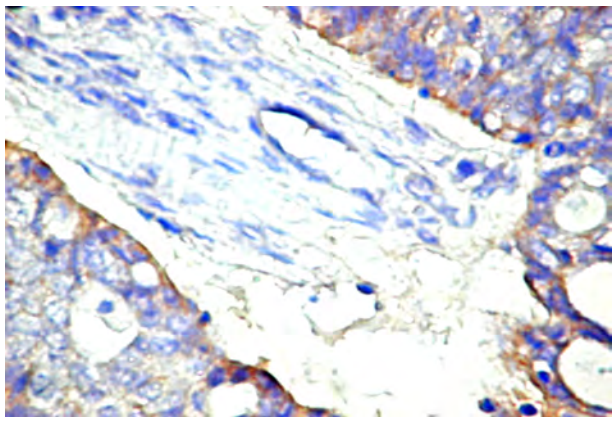

Figure 3 – ***Distribution of positive tumor cells at the interface between tumor and stroma. EG-VEGF staining. Original magnification, ×400.***

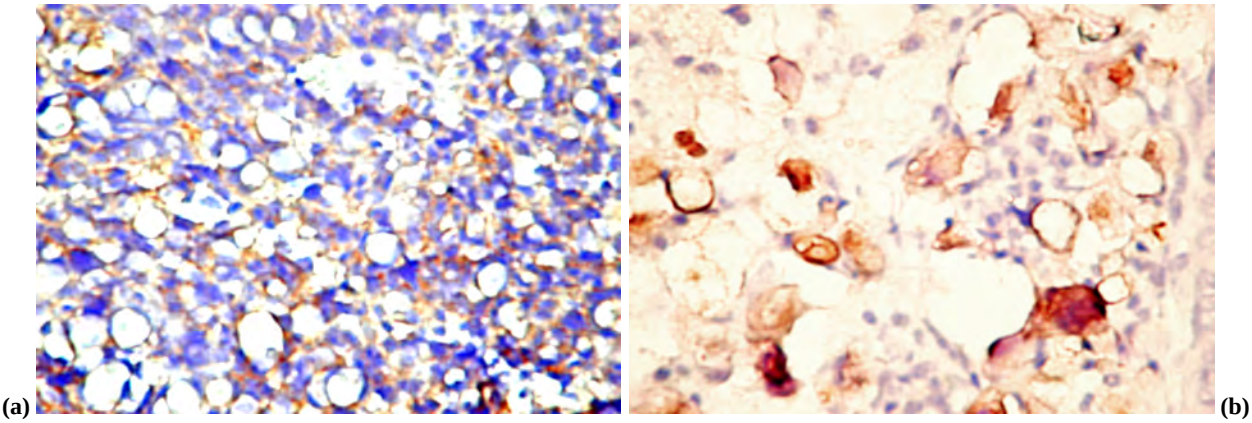

Figure 4 – ***High-grade ovarian carcinomas with strong expression of EG-VEGF. Diffuse (a) and heterogeneous (b) patterns of the final product of reaction. Original magnification, ×400.***

Discussion

Prokineticins are a novel family of secreted peptides with diverse regulatory roles, one of which is their capacity to modulate immunity in humans and in other species [14]. EG-VEGF belongs to this family and is expressed predominantly in steroidogenic tissues [15]. In humans, EG-VEGF expression is largely restricted to ovary, testis, adrenal cortex and placenta [16]. This factor is certified to be involved in normal and pathologic angiogenesis [17] and was recently reported to be responsible for the regulation of tumor cell growth and survival [18]. If the angiogenic function of EG-VEGF is well established in human normal and pathologic ovary [19], its non-angiogenic functions is still not well characterized.

In 2003, Zhang L *et al.* [9] reported that EG-VEGF was undetectable by reverse transcription-PCR in 17 established epithelial ovarian cancer cell lines or in cultured human ovarian surface epithelial cells [9]. Our results proved the EG-VEGF positive reaction in ovarian surface epithelium. This finding is in contradiction with previous ones and could explain the overexpression of EG-VEGF in all ovarian cancer, which arise from this epithelium. Also, Fraser HM *et al.* found a cyclic changes in the expression of endocrine gland vascular endothelial growth factor in the human luteal body [20]. This could partially explain the heterogeneous expression observed in our study in ovarian surface epithelium between cases and also different EG-VEGF distribution between epithelial cells from the same ovarian surface epithelium.

EG-VEGF was tested in several tumors as colon cancer [21], pancreatic cancer [22] or prostate cancer [15]. Despite of the certified deep involvement of EG-VEGF in human normal ovarian physiology, expression and distribution of EG-VEGF in corresponding ovarian tumors are less mentioned in the literature [9]. We found a high overall percentage of ovarian tumors positive for EG-VEGF with three different expression patterns. Our study mentioned for the first time EG-VEGF expression patterns specifically nominated for various histopathologic types of ovarian carcinomas. Moreover, observation concerning the distribution of positive tumor cells predominantly between tumor mass and stroma strongly suggests a potential role of EG-VEGF in progression and metastasis of ovarian carcinomas. This hypothesis is supported by previous findings of Nagano et al, in colorectal carcinoma [21]. They found that its positive expression was more frequently associated with hematogenous metastasis, and was associated with poor survival rate.

Other members of heparin binding growth factors family (which also include EG-VEGF) were studied in ovarian cancer as potential targets for tumor cells suppression. One of them, heparin-binding EGF-like growth factor exerts its biological activity through activation of the EGFR. Several lines of evidence have indicated that heparin binding-EGF plays a key role in the acquisition of malignant phenotypes, such as tumorigenicity, invasion, metastasis and resistance to chemotherapy [23]. We showed that all cases diagnosed in stage III and IV have EG-VEGF positive cells groups distributed at the periphery of the tumor, next to invasive front, and this particular aspect strongly suggests the involvement of EG-VEGF as a potential prognostic factor in ovarian carcinoma.

Conclusions

The immunohistochemical expression of EG-VEGF in 30 patients with ovarian carcinoma showed a positive reaction in 73.33%. We found statistic significant correlation between EG-VEGF expression and stage, grade, and pathological type of the tumors. Our results support the use of EG-VEGF as a predictor of invasion and local progression as promoter of angiogenesis, and could represent an attractive therapeutic target in refractory ovarian cancer.

Acknowledgments

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