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Vascular endothelial growth factor expression in ovarian serous carcinomas and its effect on tumor proliferation

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Abstract

Introduction: Vascular endothelial growth factor (VEGF), an endothelial mitogen, acts through VEGF receptors (VEGFRs) on the endothelial cells. During neoplastic transformation, it is hypothesized that the tumor expresses VEGF and also acquire VEGF receptor, enabling VEGF action in an autocrine and paracrine manner with varied effects on the tumor growth and progression. This study on ovarian serous carcinomas (OSCs) was done to determine the expression of VEGF and to correlate it with tumor proliferation. **Material and Methods:** Forty cases of OSCs were included. Immunohistochemistry was performed for VEGF and Ki-67. The VEGF slides were assigned an immunohistochemical score based on the staining intensity (a) and the percentage of tumor cells staining (b). The sum of both (a) and (b) ranged from 0-6. VEGF was considered positive when the score was more than 2. For Ki-67, maximally immunostained areas were selected; 500 cells counted and positive fraction determined. Mann Whitney test was used to determine the difference in the median value of Ki-67 between VEGF positive tumors and VEGF negative tumors. **Results:** Of the 40 cases, 32 cases had a VEGF score of >2 (positive) and 8 cases had VEGF score <2 (negative). The Ki-67 score ranged from 2-98%, with mean of 51%. The median Ki-67 index was much higher in VEGF positive cases as compared to VEGF negative tumors (57.5% vs. 40%). However, the difference in the two categories did not reach statistical significance ($P = 0.45$, Mann Whitney test). **Conclusion:** Ovarian serous carcinomas express VEGF in a significant number of cases (80% in the present study) although its potential mitogenic effect on tumor cells was not confirmed.

Key words: Ki-67, ovarian serous carcinomas, tumor proliferation, vascular endothelial growth factor

Introduction

Vascular endothelial growth factor (VEGF) also known as vascular permeability factor is a dimeric protein which was first isolated from bovine pituitary folliculostellate cells.[1] There are 4 isoforms of VEGF namely VEGF 121, 165, 189 and 206 which differ in their molecular mass and in biological behavior and are transcribed from a single gene on human chromosome 6 as a result of alternate splicing.[2] The VEGFs are produced by normal cell types like smooth muscle, corpus leuteum, adrenal cortex cells and exerts its effect by binding to a family of VEGF receptors, namely Flt, Flt-1/KDR and Flt-4 which are predominantly expressed on the endothelial cells. This suggests that only the endothelial cells are assigned to carry the mitogenic VEGF signals to the cell nuclei.

It is now known that many human tumors, like breast and colon, overexpress VEGF,[3-5] and this enhances tumor angiogenesis, by autocrine and paracrine mechanisms. Some studies have hypothesized the expression of VEGF receptors in tumor cells (acquired during neoplastic transformation) thereby, facilitating VEGF action on them in an autocrine manner.[6] By this mechanism, it is thought that VEGFs carry out their mitogenic activity in the tumor cells thereby enhanching tumor proliferation.

In ovarian carcinomas, VEGF has an additional role, causing tumor associated ascitis due to its ability to increase the vascular permeability. With the above considerations and a very few studies available in literature, relating VEGF expression and proliferation in ovarian serous carcinomas it is compelling to study the effect of VEGF expression on tumor proliferation.

In the present study, the VEGF expression in ovarian serous carcinoma cells was studied and correlated with proliferative index (Ki-67 labeling index) using immunohistochemical methods.

Materials and Methods

Forty patients who underwent staging laparotomy and diagnosed as primary ovarian serous carcinoma during the period 2000 to 2008, were randomly selected from the case files of Department of Pathology and included in this study. Cases with incomplete staging and post neoadjuvant chemotherapy were excluded from the study. The hospital records were reviewed for patient data including patient's age, FIGO (the International Federation of Gynecology and Obstetrics) stage, debulking status and lymph node status. The initial histopathological sections stained with hematoxylin and eosin was reviewed by two pathologists. Grading was done according to the 2 tired system. From each case a representative tissue block with viable and assessable tumor tissue was chosen for immunohistochemical analysis.

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Immunohistochemistry was performed by manual method for VEGF and Ki-67 using tissue sections of 4-5 micron thickness, floated on sialinised slides and incubated overnight at 60C. Deparaffinised sections were stained with standard streptavidin-biotin-peroxidase technique. Antigen retrieval was by steam treatment in a citrate buffer and the sections quenched for 10 minutes in hydrogen peroxide. The slides were coated and incubated at room temperature for 30 minutes with primary antibodies (ready to use): VEGF (polyclonal rabbit IgG antibody, BioGenex, San Ramon,(USA) and Ki-67 (monoclonal Ki-88, BioGenex, San Ramon, USA). Subsequently, the slides were incubated with secondary antibody and streptavidin peroxidase for 30 minutes each with PBS (phosphate buffer saline) rinses in between. Reactivity was detected using 3,3'Diaminobenzidine (DAB) as chromogen and were counterstained with Harris' Hematoxylin. For negative controls, the primary antibody was omitted from the staining protocol and positive control of corpus luteum for VEGF and reactive lymphnode for Ki-67 were included with each batch of staining.

The VEGF slides were assigned an immunohistochemical score based on the intensity of staining and the percentage of tumor cells staining. A total score corresponding to the sum of both were taken [Table 1]. The sum of both (a) and (b) ranged from 0-6. VEGF was considered positive when the score was more than two.[7]

For Ki-67 immunostain, the maximally immunostained areas were selected and Ki-67 positive and negative cells were counted at X-400 magnification. All the cells showing positive immunostaining were counted regardless of the intensity. In each case a minimum of 500 cells were counted and the percentage of positive cells were determined. The Institutional Ethical Board approval was obtained before the study was commenced and this study was funded by the research society.

Statistical Methods

To determine the association of VEGF with Ki-67 in ovarian serous carcinomas, the difference in the median value of Ki-67 between VEGF positive tumors and VEGF negative tumors was analysed by Mann Whitney test (non-parametric test).

Results

Forty cases of primary ovarian serous carcinomas were studied. The patient's age ranged from 21 years to 85 years with a mean age of 53.6 years. FIGO grading and staging was done: 90% were of high grade morphology and were in advanced stage (FIGO stage III and IV). VEGF immunostaining was predominantly noted in the cytoplasm of the neoplastic cells. The intensity of staining ranged from 1 + to 3 + in each case. According to the histoscore assigned to each tumor, 32 cases had a score of >2 and were considered to have significant VEGF expression. 35% of the cases showed strong expression of VEGF with a score of 6 [Figure 1]. The distributions of the cases according to the VEGF score is shown in the Table 2.

The proliferative activity showed a wide variation within a given tumor and ranged from 2-98%, when estimated as nuclear staining in maximally stained areas [Figure 2]. The mean Ki-67 score was 51%. The median Ki-67 index was much higher in VEGF positive cases as compared to VEGF negative tumors (57.5% vs.40%). However, the difference in the proliferative index in the two categories did not reach statistical significance (P = 0.45, Mann Whitney test).

Discussion

Vascular endothelial growth factor (VEGF) is a potent endothelial mitogen that acts through the various VEGF receptors (VEGFRs) that are present on the endothelial cells of the blood vessels and lymphatics. Many human tumors like those of the lung, breast and colorectum are known to over express VEGF and acquire the VEGFRs during the neoplastic transformation.[6] Although the prime

Table 1: Immunohistochemical assessment of vascular endothelial growth factor histoscore

Score	Intensity (*a*)	Percentage of tumor cells staining (*b*) (%)
0	Negative	0 positive cells
1	Weak staining	<25 positive cells
2	Intermediate staining	26-50 positive cells
3	Strong staining	>50 positive cells

Table 2: Distribution of cases according to vascular endothelial growth factor score

VEGF score	No. of cases	VEGF status
Score 0	7	VEGF negative=8/40 cases (20%)
Score 1	0	
Score 2	1	VEGF positive=32/40 cases (80%)
Score 3	4	
Score 4	2	
Score 5	12	
Score 6	14	

VEGF=Vascular endothelial growth factor

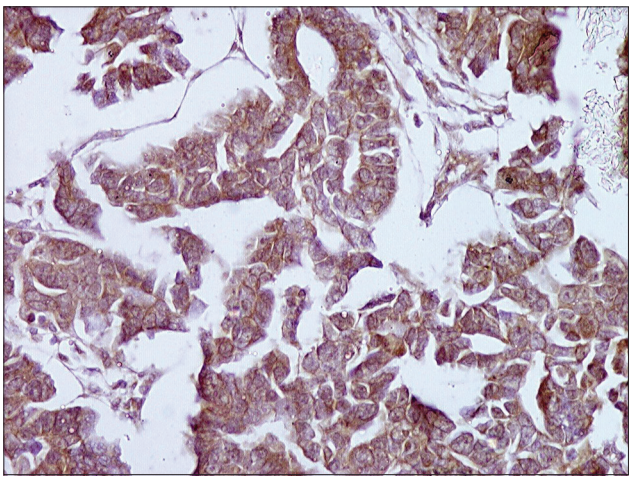

Figure 1: Immunohistochemistry showing cytoplasmic positivity for VEGF in the neoplastic cells of ovarian serous carcinoma (×400)

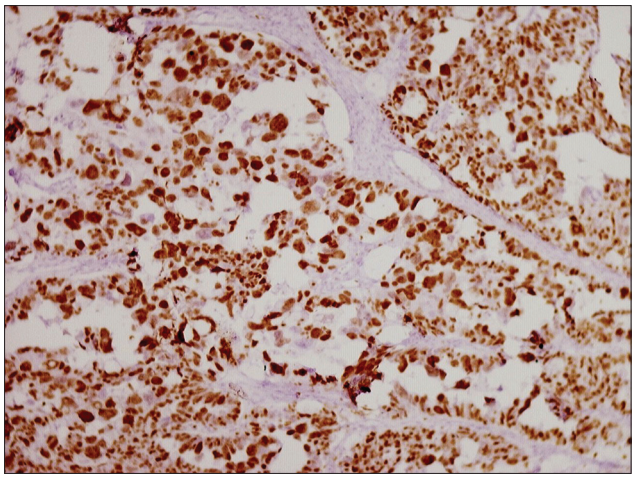

Figure 2: Immunohistochemistry showing nuclear positivity for Ki-67 in the maximally immunostained areas (×200)

role of VEGF in a neoplastic proliferation is promoting tumor angiogenesis, its different isoforms act on the different VEGFRs in the neoplastic cells by autocrine and paracrine mechanisms, leading to neoplastic growth and progression.

Although the effect of VEGF on tumor angiogenesis is well documented, the other effects of VEGF in tumorigenesis vary in different tumors. A very few studies in literature have reported VEGF overexpression in ovarian cancers[8,9] and its role in ovarian serous carcinogenesis is not well established. Thereby, we have tried to study the expression of VEGF by the neoplastic cells of ovarian serous carcinomas and correlate its expression with the proliferative activity of the tumor.

Studies done on human cancers, correlating VEGF expression and tumor proliferation show conflicting results. Fan *et al.,* study on colorectal carcinoma cell lines found no association between VEGF expression and tumor cell proliferation, but proved that the VEGF ligands help in tumor progression and metastasis.[10] In Dordvic's study on renal cell carcinomas, double immunohistochemical staining with Ki-67 and VEGF did not show a co-localization of the makers indicating no association between VEGF and Ki-67.[11] Whereas Brustmann and Naude's study on 45 ovarian serous carcinomas and 10 benign serous cystadenomas, found a significant positive relationship between VEGF expression and mitotic activity and concluded that VEGF expression could be a indicator of neoplastic proliferation in ovarian tumors.[12] Similar finding was also seen in the study by Garzetti *et al*.[13] In our study on 40 cases of ovarian serous carcinomas we could not find a significant association between VEGF expression and tumor proliferation.

One possible explanation for this could be the nature of the VEGFRs that are expressed on the tumor cells. As previously stated, VEGF acts on several receptors which are expressed on the tumor cells as well as on endothelial cells during neoplastic transformation. There are threedifferent kinds of VEGFRs namely: VEGFR-1 (Flt), VEGFR-2 (Flt-1/KDR) and VEGFR-3 (Flt-4). The role of these three receptors varies. VEGFR-1 helps in tumor migration, VEGFR-2 helps in mitogenic signaling and VEGFR-3 is present predominantly on the endothelial cells of the lymphatics.[3,6] Therefore the type of receptor expressed by the neoplastic cells determines the effect of VEGF in the tumor. The expression of these receptors is variable in human cancers. Lallas study on head and neck squamous cell carcinomas showed that the cells expressed all three receptors.[3] However, F Fan *et al*., in their study on colorectal carcinoma cell lines showed expression of VEGFR-1 only and the VEGF expression did not show an association with proliferation. Therefore, they concluded that VEGF plays a role in tumor migration and metastasis.[10]

Massod *et al.* in their study on VEGF and their receptors in various cancer cell lines stated that antibodies to VEGFR-1 and VEGFR-2 were more potent inhibitors of tumor cell proliferation than either alone.[6] Therefore, the knowledge of VEGFR status while assessing the effect of VEGF expression on tumor proliferation would be more informative.

Another possible explanation for these conflicting results, could be attributed to the difference in the technique of assessing VEGF expression and proliferative activity in the tumor cells. VEGF can be detected at the mRNA level by *in situ* hybridization technique or real time PCR methods and at the protein level using immunohistochemistry.[1] There are 4 different isoforms of VEGF which differ in their molecular weight and the mRNA detection methods are more sensitive in detecting all the known isoforms of VEGF, namely VEGF 121, VEGF 156, VEGF189, VEGF206. Whether the imunohistochemical detection of VEGF isoforms parallels the detection by *in situ* hybridization needs to be assessed. Dordevic *et al.* study on the VEGF and NF-kB expression in renal cell carcinomas demonstrated an association between the pattern of immunohistochemical staining of VEGF and its mRNA levels. The perimembranous pattern of staining was associated with up regulation of VEGF mRNA and the diffuse staining pattern was not.[11] In the present study, all the cases showed diffuse cytoplasmic staining pattern. Whether mRNA was actually upregulated or not is not known and probably mRNA detection methods will explain our results.

Likewise, proliferative activity in a tumor can be detected by various methods. Although MIB-1 index by immunohistochemistry is used widely for estimating proliferation in many human tumors, detecting proliferation dependent gene (histone H3) as done by Mattern *et al.* may be a superior method, as the gene *per se* is detected.[14]

It is a well-known fact that any solid tumor needs adequate vascular supply for delivering oxygen and nutrients and also to remove the waste products. Hypothetically vascular density should have its influence on tumor proliferation. Contrary to this hypothesis, studies have found no correlation between microvessel density and tumor proliferation in epidermoid cancers of the lung,[7] breast cancer[15] and esophageal cancers.[16] These data

suggest that the growth factors controlling tumor growth are not the same as those involved in endothelial cell growth. Therefore, VEGF may not directly act on the tumor cells to increase tumor proliferation, but may act through indirect mechanisms as it is well known that in any neoplastic transformation and progression multiple genetic mutations are involved and they may have pleiotropic effects on the tumor growth.

Conclusion

The present study shows that ovarian serous carcinomas express VEGF in a significant number of cases (80% in the present study) although its potential mitogenic effect on tumor cells was not confirmed. However, the lack of a positive association between VEGF and Ki-67, in the present study could be attributable to (i) the technical methods used for detecting the expression of VEGF and Ki-67, where the mRNA detection methods could probably have given a more significant results. (ii) Studying the effect of VEGF on tumor proliferation in conjunction with VEGFRs, where the type of the receptor expressed would explain the effect of VEGF. Also knowing the affinity of the receptors to VEGF and the autocrine regulatory loop specific to VEGF signaling, all of which would play a crucial role in carrying out the mitogenic signals of VEGF.

Though the proliferative activity in ovarian serous carcinomas is influenced by many growth factors, in the light of the present study, we do not completely exclude the possible mitogenic effect of VEGF on tumor cells. Furthermore, VEGF has an indirect effect on tumor proliferation by increasing intra tumoral microvasculature; thereby, increasing tumor proliferation needs to be studied by assessing intratumoral micro-vascular density.

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