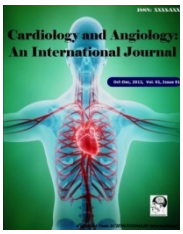

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Assessment of Mean Vessel Density and Angiogenesis in Endometrial Carcinomas

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Authors' contributions

This work was carried out in collaboration between all authors. Author KA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors RKS and NA managed the analyses of the study. Authors DG and GM managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Objective: To evaluate the role of angiogenesis in tumor growth by the assessment of mean vessel density and to quantify angiogenesis as an important variable in endometrial cancers.
Material and Methods: 53 cases of endometrial malignancies (epithelial tumors-36 cases and metastatic tumors-17 cases), were analysed for histological types, grades and features like depth of invasion and vascular invasion. Microvessel counts were performed by examining the microvessels thoroughly in terms of count, morphology and density after staining the tissues by hematoxylin & eosin stain, reticulin and immunostain (Antifactor VIII Ag).
Results: On H&E stain - Microvessel density (MVD) in endometrial malignancy ranged from 3.0 - 13.5 and mean MVD was 8.78. On Reticulin stain - MVD ranged from 3.5 - 15.2 and mean MVD was 9.76. Antifactor VIII sections showed very small microvessels or even single endothelial cells with the highest total counts and the MVD ranged from 6.5-16.8 with Mean MVD of 11.7. The counts increased with the grade of the tumor in the absence of necrosis or haemorrhage. MVD counts also increased with the stage, being

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8.12 in Stage I disease, 8.65 in Stage II and 10.8 in stage III disease. Atypical hyperplasia was found to be associated with epithelial tumors in 8 cases, making it a significant finding.
Conclusion: Role of angiogenesis assumes greater significance with increasing severity of lesions, higher grade and stage of the tumor and seems to have an important diagnostic and prognostic significance.

Keywords: Angiogenesis; endometrial carcinoma; microvessel density.

1. INTRODUCTION

Angiogenesis has an important part to play in endometrial growth and is essentially needed for regenerative, hyperplastic and neoplastic conditions as well as tumor growth and metastasis, as it allows the tissue to increase in size beyond the constraints of its original blood supply as well as for metastasis.

Recent experiments have begun to yield direct evidences that tumor growth is angiogenesis dependent [1,2]. Tumor growth is limited to a few millimeters in greatest dimension before neovascularization, but is rapid and nearly exponential after neovascularization. Such neovascularization may be stimulated by factors released from the tumor cells, tumor-associated inflammatory cells, and/or from the extracellular matrix.

In the present study, we have studied the mean vessel density (MVD) and the status of angiogenesis and tried to evaluate the role of angiogenesis in tumor growth in endometrial cancers. We have also tried to study the association between angiogenesis and histological grade and histological type of the malignancy as well as vascular invasion.

2. MATERIALS AND METHODS

This study was conducted on a total of 53 cases of endometrial malignancies (Primary Epithelial-36 cases, comprising of 15 cases of well differentiated endometrioid adenocarcinoma, 12 cases of moderately differentiated and 09 cases of poorly differentiated adenocarcinoma and metastatic tumors-17 cases, which included 7 cases of metastatic ovarian adenocarcinoma and 10 cases of direct extension from squamous cell carcinoma of cervix), in the departments of Obstetrics & Gynaecology and Pathology, J N Medical College, AMU, Aligarh. Tissues obtained from hysterectomy specimens were fixed in formalin and processed in automatic tissue processor. Sections of 3-5 micron thickness were cut from paraffin embedded blocks and subjected to Hematoxylin & Eosin stain and Reticulin stain.

Malignant lesions were analysed for histological types, grades and any other relevant feature like depth of invasion, vascular invasion, necrosis and haemorrhage, wherever possible in the given specimen. Microvessel counts were done in all the samples by observers blinded to the clinicopathological datas. Basement membrane was delineated properly by the reticulin stain, so the blood vessels were identified easily. Microvessels were examined thoroughly in terms of count, morphology and density. We also counted MVD in 40 cases of eutopic endometrium from non cancerous patients, which served as controls.

Immunohistochemical (IHC) staining for endothelial cells using (Antifactor VIII Ag) antibody was performed by avidin-biotin-peroxidase complex method. Procedure for IHC: The sections were dewaxed in xylene, rehydrated in graded alcohol, and rinsed in water. To inhibit endogenous peroxidase activity, the sections were treated by immersion in five changes of 0.3% H_2O_2 in absolute methanol (5 min each change) and rinsing in water. For antigen retrieval, the sections were immersed in 10 mM sodium citrate buffer (pH 6.0) and boiled twice for 12 min in a high-intensity microwave oven. The slides were incubated with primary monoclonal antibody for Antifactor VIII Ag (Antifactor VIII Ag /Abs4, diluted 1:40) in a humidified chamber at 4ºC overnight, followed by incubation with biotinylated anti-rabbit antibody and avidin-biotin –peroxidise complex at 37ºC for 30 min. Careful rinses were performed, with several changes of PBS between each stage of the procedure. The sections were then incubated with diaminobenzidine, then counterstained lightly with Harris Hematoxylin, dehydrated in graded alcohol, allowed to dry in air, and mounted with Permount mounting medium. Positive staining was identified as strong dark brown staining of endothelial cells.

2.1 Counting Procedure

In all cases, most discrete microvessels which appeared as lumina lined by endothelial cells were counted in high power using 40x objective lens and 10x eye piece (X400 magnification) in 10 fields and average microvessel density (MVD) was calculated for every case. In very small biopsies, maximum possible fields were evaluated. The range of MVDs was recorded and further the mean of MVDs of total number of cases was calculated. Simultaneously we also observed the morphology (size, shape & thickening of vessel wall) and distribution of blood vessels. To avoid bias, counting was done by two different observers blinded to the clinicopathological datas. Finally data from different stages and grades of the tumor were compared to assess, if the observations had any statistical significance by using student t-test and one way analysis of variance (ANOVA). The data was interpreted as significant if the value of $p < 0.05$ and not significant if $p > 0.05$.

3. RESULTS

On H&E stain - MVD in 53 cases of endometrial malignancies ranged from 3.0 - 13.5 with Mean MVD of 8.78 and on Reticulin stain - MVD ranged from 3.5 - 15.2 with Mean MVD of 9.76. On immunostained sections of endometrial malignancies, very small microvessels or even single endothelial cells were observed, with the highest total counts and the MVD ranged from 6.5-16.8 with Mean MVD of 11.7. The statistical differences in MVD counts of endometrial malignancy with eutopic endometrium (on H&E stain - MVD ranged from 2.1- 6.6 with Mean MVD of 3.92 and on Reticulin stain - MVD ranged from 2.2-7.2 with Mean MVD of 3.98) were analysed and found to be statistically significant ($p < 0.0001$).

The MVD counts in epithelial tumors, with periglandular arrangement of microvessels (Fig. 1), were further analysed to assess the difference in values in different grades of the epithelial tumors (Table1).

Table 1. MVD count in different grades of epithelial tumors

Tumor grade	No. of cases	H&E stain		Reticulin stain		Immunostain	
		Range	Mean±SD	Range	Mean± SD	Range	Mean± SD
Grade I	15	5.4-11.9	8.08±0.01	6.1-12.3	9.01±0.05	7.2-13.4	10.3±0.10
Grade II	12	8.6-12.1	10.1±0.06	8.9-12.7	10.42±0.10	9.5-14.3	11.9±0.13
Grade III	09	4.8-12.7	8.95±0.03	5.2-13.8	9.43±0.06	6.8-15.4	11.1±0.11

P value: Grade 1:2 = 0.1061; Grade 2:3=0.1052; Grade 1:3=0.1058

The cases were categorized according to modified FIGO grading system into three grades: Grade I - 5% or less tumor showed a solid growth. Grade II - Between 5% - 50% tumor showed a solid growth. Grade III - > 50% tumor showed a solid growth pattern. We found that in grade I tumor (15 cases), counts ranged from 5.4-11.9 on H&E with mean of 8.08, on reticulin stain, the counts ranged from 6.1-12.3 with mean of 9.01 and Anti-Factor VIII MVD ranged from 7.2-13.4 with mean of 10.3. In grade II tumor (12 cases), counts ranged from 8.6-12.1 on H&E with mean of 10.1, on reticulin stain, the counts ranged from 8.9-12.7 with mean of 10.4 and Anti-Factor VIII MVD ranged from 9.5-14.3 with mean of 11.9. In grade III tumors (09 cases), counts on H&E were 4.8-12.7 with mean of 8.95, on reticulin stain, the counts were in the range of 5.2-13.8 with mean of 9.4 and Anti-Factor VIII MVD ranged from 6.8-15.4 with mean of 11.1. The counts increased with the grade of the tumor in the absence of necrosis or haemorrhage. But, the overall the MVD count in grade III was lower than grade II tumors. The reason behind it could be massive necrosis or haemorrhage in a few cases of solid or higher grade tumors resulting in reduced counts, as counting was not possible in necrosed or haemorrhagic areas. We also observed that the mean MVD count of viable Grade III tumors was higher than the mean of Grade II tumors, when calculated after excluding the cases with necrosis or haemorrhage.

To assess the prognostic significance of angiogenesis, the values of MVDs were observed in different stages of endometrial carcinoma (Table 2).

Table 2. MVD count in different stages of endometrial carcinoma

Stage	No. of cases	MVD	
		Range	Mean± SD
I	25	7.1 - 9.0	8.12± 0.01
II	17	8.3 - 9.0	8.65± 0.02
III	11	10.0 - 11.6	10.8± 0.11

p values: I:II=0.0452; II:III=0.0573; I:III=0.0158

We observed that MVD counts increased with the stage of the tumor, being 8.12 in Stage I disease, 8.65 in Stage II and 10.8 in stage III disease of endometrial carcinoma. Though the MVD counts progressively increased with increasing stage of endometrial carcinoma, the difference was statistically significant only between stage I and III endometrial carcinoma ($p = 0.0158$). Different sizes and shapes of the vessels were appreciated, from very small to very large and from regular circulo-oval, longitudinal to irregular (saccular, dilated and branching) shaped vessels (Fig. 2).

In well differentiated carcinomas, they were in close proximity to the glands but in solid variants, no particular pattern of distribution of vessels could be seen. Anti-Factor VIII immunostain was of great help to identify these malformed microvessels easily and total counts were higher than those counted on H&E stain. Anti-Factor VIII immunostain sections

showed very small microvessels or even single endothelial cells, so the total counts thus obtained were highest amongst the three stains done. Counts were higher in the higher grades and stages of endometrial tumors than the lower grades or stages respectively, thus signifying a worse prognosis. Immunological response in the form of lymphocytic infiltrate was seen in all the cases, which was pronounced at the advancing front of the tumor.

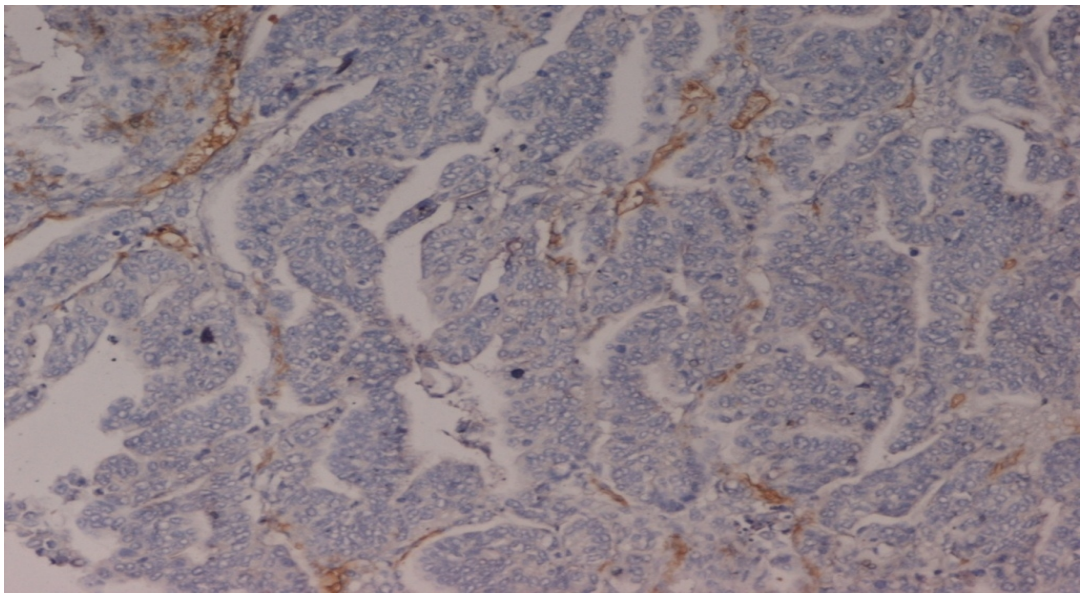

Fig. 1. Well differentiated Endometrioid adenocarcinoma with periglandular arrangement of microvessels. Anti-Factor VIII immunostain x 100

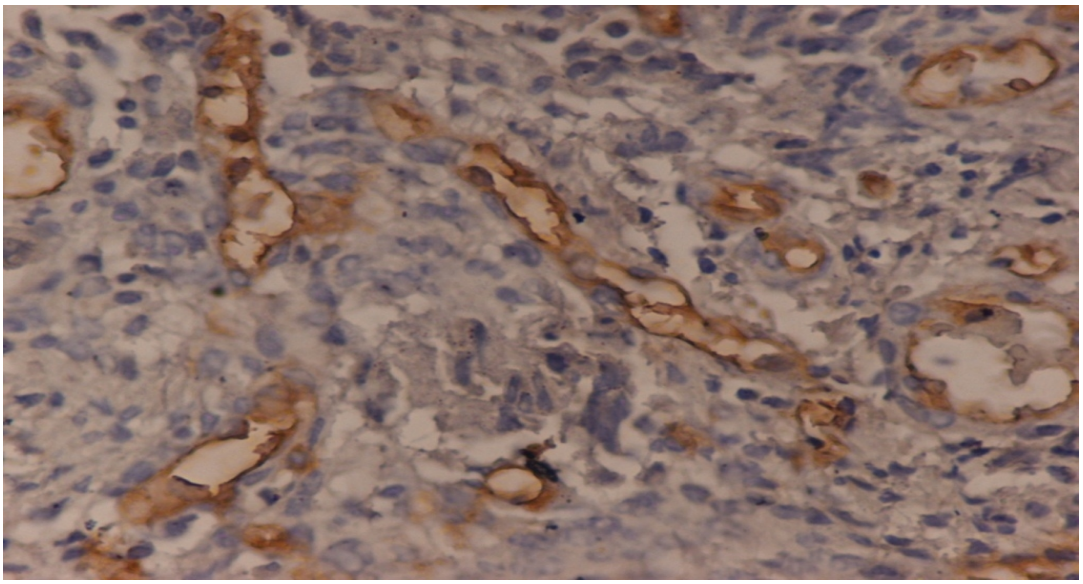

Fig. 2. Endometrioid adenocarcinoma with numerous malformed, saccular, dilated and branching microvessels. Anti-Factor VIII immunostain x 400

4. DISCUSSION

We observed in our study, that MVD counts were markedly raised in malignancy in comparison to normal endometrium. These values were highly significant when compared to those observed in normal endometrium ($p = < 0.0001$). Morgan et al. [3] have also found highly increased MVD counts in endometrial carcinoma in comparison to normal and hyperplastic endometrium in their study. They concluded that though the endometrial stroma was inconspicuous in carcinoma, it was intensely vascular than normal or hyperplastic endometrium.

Our findings on evaluation to quantitate angiogenesis by assessing MVD in endometrial lesions by anti-CD34 correlated well with the histological results of Czekierdowski et al. [2], who concluded that MVD assessed with CD-34 was almost twice as high in women with endometrial cancer than in women with a benign endometrium (antifactor VIII MVD = 41.8 vs. 27.6, $p=0.004$). Saad et al. [4] also found that the average numbers of microvessels per high power field (HPF) stained with CD31 and CD105 were higher in women with endometrial cancer than in benign endometrium (30.8/HPF vs. 13.3/HPF) respectively.

Our study showed that counts were higher in the higher grades of endometrial tumors than the lower grades, thus signifying a worse prognosis. Quite similarly, Kaku et al. studied to determine the relationship of MVD to several clinicopathologic parameters and correlated the MVD count with metastasis and survival in patients with endometrial carcinomas [5]. They also found that microvessel counts in endometrial carcinomas were significantly higher than those in the control group (antifactor VIII MVD 60 vs. antifactor VIII MVD 47; $p = 0.0071$). Erdem et al. compared survival rates with MVD in endometrial cancer samples and noted that, women with high anti-CD-105 MVD count had a significantly worse prognosis than patients with low MVD assessed with this immune marker [6].

We evaluated the pattern of angiogenesis in different grades of the tumor and observed that the counts increased with the grade of the tumor, if there was no associated necrosis or haemorrhage. The mean of MVD counts in grade III tumors was lower than in grade II tumors overall, which may be due to massive necrosis or haemorrhage in a few cases of solid or higher grade of the tumor. Decreased angiogenesis could itself result in necrosis of the tumor, as in the absence of vascular support, tumors may become necrotic or even apoptotic [7].

We noted that MVD counts were higher in the higher stages of endometrial tumors than the lower stages, leading to a worse clinical outcome. Ozuysal et al. [8] performed a study to see any correlation between angiogenesis in endometrial carcinoma with survival and clinicopathologic risk factors, by staining tissues immunohistologically, from hysterectomy specimens with factor VIII-related antigen. The mean MVD count noted by them was 26.2 (range 6-68), and it was considered as high, moderate and low, when the MVD was >30, between15-30 and <15 respectively. Also there was positive correlation between increase in MVD and surgicopathological stage ($p < 0.05$). A significant difference was seen between increased MVD and lymph node metastasis ($p < 0.05$), but they observed no correlation between MVD and age, histological type, grade and lympho-vascular invasion. Also, MVD did not have any association with depth of myometrial invasion. There was a significant difference in mean values of survival between the low and high MVD groups ($p = 0.01$). Ozuysal et al. [8] finally stated that, increased angiogenesis was found to be associated with advanced stage and decreased survival in endometrial carcinomas, a finding concordant with our study. Ozalp et al. [9] also correlated MVD with high surgical stage ($p < 0.001$),

cervical involvement ($p = 0.01$), adnexal involvement ($p = 0.04$), lympho-vascular space involvement ($p = 0.02$), pelvic and para-aortic lymph node metastasis ($p < 0.001$) and positive peritoneal cytology ($p < 0.001$).

In our study, we encountered higher MVD counts in all the grades of tumor than normal endometrium but the differences in counts within different grades of tumor were not statistically significant. De Gois Speck et al. [10] did a study to see relationship between angiogenesis and grade of histologic differentiation in endometrial adenocarcinoma. They reported mean vessel count of 15.3 in Grade I tumors; 19.0 in Grade II and 22.7 in Grade III adenocarcinomas with 12.6 in the control group. Less differentiated adenocarcinomas presented with greater angiogenesis than normal and well-differentiated carcinoma. In contrast, moderately differentiated carcinoma showed greater angiogenicity as related to normal endometrium, but did not differ from other tumoral endometria.

We also observed that increased vascular proliferation was associated with tumors exhibiting aggressive behaviour and higher counts were seen in high tumor stage. However we noted a low MVD count in presence of necrosis, as vessel count could not be elicited in necrotic tumoral tissues. Stefansson et al. did a study to examine the significance of angiogenic markers, especially vascular proliferation (by Ki-67/factor VIII staining) and the degree of pericyte coverage [by α-smooth muscle actin (α-SMA)/factor VIII staining], in a large and population-based series of endometrial carcinoma with complete follow-up [11]. They concluded that, increased vascular proliferation was associated with features of aggressive tumors, such as presence of necrosis and high tumor stage.

5. CONCLUSION

Our study showed that the role of angiogenesis assumes greater significance with increasing severity of lesions, higher grade and stage of the tumor. Angiogenesis seems to have an important assessment factor in endometrial carcinomas, particularly to the stage of the tumor.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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