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Published online: 17 May 2019.

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ORIGINAL ARTICLE

VEGF expression and microvascular density in relation to high-risk-HPV infection in cervical carcinoma – An immunohistochemical study

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Abstract *Background:* The poor outlook of cervical carcinoma, a human papillomavirus (HPV)-related cancer mandates the search for new treatment modalities. Therapeutic targeting of tumor vasculature is a promising strategy. The aim was to study angiogenesis in cervical carcinoma in terms of VEGF expression and assessment of microvascular density (MVD) in relation to HPV antigen expression.

Methods: Thirty paraffin blocks of cervical carcinoma were studied for the immune expression of VEGF and MVD utilizing CD34 monoclonal antibody. Statistical analysis of these immunopheno-types in relation to tumor type, grade and HPV antigen expression was performed.

Results: This retrospective study comprised of 17 squamous cell carcinomas, 11 adenocarcinomas and two adenosquamous carcinomas. Eleven cases were low grade and 19 were high-grade cases. VEGF expression was detected in 100% of cases. The relation between carcinoma grade and VEGF expression and MVD was statistically significant. There was no relation between VEGF intensity and tumor type although more intense VEGF staining tended to occur in cervical adenocarcinomas. VEGF density was significantly lower in squamous cell carcinomas compared to adenocarcinomas. Mean MVD was 50.37 ± 20.0. The relation between MVD and VEGF expression was statistically significant. HPV immune expression was detected in 93.33% of cases. The relation between HPV

antigen expression and each of tumor histotype and grade was not statistically significant. There was a statistically significant relation between HPV antigen expression and each of MVD and VEGF intensity. Multivariate statistical analysis showed MVD as an independent predictor of carcinoma grade.

Conclusion: VEGF was expressed in 100% of studied cervical carcinoma. There was a statistically significant relation between VEGF expression and MVD. Since HPV antigen expression was significantly correlated with MVD and VEGF staining intensity, we provide evidence that HPV infection may augment tumor angiogenesis in cervical carcinoma. MVD emerged as an independent predictor of cervical carcinoma grade and hence of progressive behavior.

1. Introduction

Cervical cancer is a worldwide public health problem being the second most common cancer in females after breast cancer.[1] In Egypt, the latest census by the National Cancer Institute in Cairo in 2003 showed predominance of cervical over endometrial carcinoma.[2]

The latest WHO classification of cervical tumors [3] classifies epithelial malignancies mainly into four main groups: squamous carcinomas; adenocarcinomas; neuro-endocrine tumors; and others including adenosquamous carcinomas.[4]

Carcinoma of the cervix is considered a sexually transmissible disease.[5] At the present time, viral agents particularly human papillomavirus are believed to be the most likely factors incriminated in the pathogenesis.[6]

Approximately 200 types of human papillomavirus (HPV) have been identified, of which 85 have been genotypically characterized.[7] About 40 different types of HPV specifically infect the genital area.[8] According to their malignant potential, these viruses can be classified as high- or low-risk types. The high-risk types (predominantly HPV-16 and -18) are related to high-grade intraepithelial lesions and invasive cervical carcinoma.[9]

However, most HPV infections either regress spontaneously or progress to cancer after a long period of latency.[10] Therefore, it was inferred that additional requirements other than HPV infection are likely to be needed for neoplastic transformation of cervical epithelial cells.[10]

There is accumulating evidence that angiogenesis plays a pivotal role in cervical carcinogenesis.[11] However, little is known about the role of viral oncoproteins in regulating angiogenesis.[12]

Angiogenesis is the development of new blood vessels from pre-existing vasculature.[13] When a tumor launches the growth of new vessels, it is said to have undergone an ''angiogenic switch''. The principal trigger for this event appears to be hypoxia. Newly formed blood vessels will further promote tumor cell proliferation [14] and increase the possibility of metastasis.[15]

A group of angiogenic stimulators and inhibitors regulate the process of angiogenesis [16] Angiogenic stimulators include vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), acidic and basic fibroblast growth factors (FGF), transforming growth factors-α and β (TGF α and β) and others.[17] On the other hand, angiogenic inhibitors as angiostatin and endostatin exist to prevent excessive neoangiogenesis. Under normal circumstances, there is an equilibrium between pro and anti-angiogenic factors, this balance is disrupted in malignancy thereby favoring promotion of tumor angiogenesis.[18]

A great deal of attention has been focused on therapy that can interrupt angiogenesis. Anti-angiogenic therapy may target endothelial cells directly, suppress the production and or action of pro-angiogenic peptides or augment the release of antiangiogenic factors within the tumor.[19]

Microvascular density (MVD) is a commonly used marker for estimation of angiogenesis.[20] Measuring MVD necessitates labeling the vessels to be counted using antibodies against any of the antigens naturally expressed by endothelial cells like F-VIII, CD31, CD34 and CD105.[20] Different methods have been used for counting MVD, including manual counting of vascular hotspots and computerized image analysis.[21]

VEGF represents the major inducer of angiogenesis.[16,22] It is encoded by the human *VEGF-A* gene, on chromosome 6p21.3 [22] and belongs to the VEGF superfamily of growth factors which also includes VEGF-B, C, D, E and placental growth factor (PlGF).[23] VEGF exerts its biologic effect through interaction with transmembrane tyrosine kinase receptors (VEGFR). In tumors, VEGF expression is induced in response to hypoxia, genetic and cytokine stimulation.[24]

Several functions have been described for VEGF including acting as a potent mitogen for vascular endothelial cells,[25] increasing microvascular permeability,[26] degradation of extracellular matrix,[23] acting as an antiapoptotic factor for endothelial cells[24,26] in addition to mobilizing bone marrow-derived endothelial cell precursors.[23]

In the present work we aimed at studying the role of angiogenesis as measured by microvascular density and expression of vascular endothelial growth factor (VEGF) in relation to the status of HPV infection in some cases of cervical carcinoma.

2. Methods

This retrospective study was approved by the Ethics Committee of Alexandria Faculty of Medicine. It consisted of 30 consecutive cases of clinically and histologically proven cervical carcinomas. Patients were diagnosed and treated at the Alexandria University Hospital, Alexandria, Egypt, between May 2007 and April 2009. All tissue samples were formalin fixed and paraffin-embedded. Data about tumor stage were not always available so this parameter was not considered in analysis.

2.1. Histopathological examination

Five micron-thick sections cut from archival paraffin blocks were H&E stained and examined microscopically to determine tumor histologic type and grade. Squamous cell carcinomas were graded according to the modified Broders' grading system

(grades I–IV) that takes into account nuclear anaplasia in addition to the degree of differentiation.[27] Adenocarcinomas were graded according to the recommendations of the Association of Directors of Anatomic and Surgical Pathology[28] (grades I–III) taking into consideration architectural as well as nuclear criteria. For statistical purposes, cases of grades I and II were grouped together as low-grade tumors (11 cases, 36.7%), while grades III and IV cases were grouped together as high-grade tumors (19 cases, 63.3%).

2.2. Immunohistochemistry

Heat-induced epitope retrieval was performed by microwaving the sections in EDTA (1 mM, pH 8.0) for VEGF and in citrate buffer (10 mM, pH 6.0) (Thermo Fisher, Fremont, USA) for CD34 and HPV at 700W, 4 times, 2 min each. Endogenous peroxidase activity and nonspecific binding were blocked.

Three primary, mouse monoclonal antibodies were employed; VEGF antibody (Ab-7 Cat. #MS-1467-R7), human papillomavirus antibody (Ab-3, Clone K1H8, Cat. #MS-1826-R7) and CD34 antibody (Ab-1, Clone QBEnd/10, Cat. #MS-363-R7). All three primary antibodies were supplied in a pre-diluted format and were incubated with tissue sections at 4 °C overnight. (Thermo Fisher Scientific Inc., Fremont, CA, USA). The reaction product was visualized using the streptavidin–biotin peroxidase system with Diaminobenzidine as a chromogen (UltraVision Detection System Anti-Mouse, HRP/DAB, Cat #TM-015-HD, Thermo Fisher, Fremont, CA, USA) according to the manufacturer's protocol.

Sections without primary antibodies served as negative controls. Blood vessels in normal cervical tissue adjacent to the tumor served as internal positive controls for VEGF and CD34 immunostaining whereas commercially available positive control slides were used as positive control for HPV immunostaining (Cat. #MS-1826-CS7, 1=0?>Thermo Fisher, Fremont, CA, USA).[29,30]

Finally, sections were counterstained with hematoxylin, dehydrated and mounted.

2.3. Assessment of immunostaining

2.3.1. VEGF immunostaining
Positive VEGF immunostaining was defined as a cytoplasmic and or membranous staining of tumor cells.[31] VEGF-stained sections were studied at 100× magnification to assess VEGF staining density and intensity. VEGF density was defined as percentage of VEGF-stained tumor cells scored according to a 2-teired scoring system[31,32] whereby score 1 designated positive cytoplasmic staining in ⩽50% and score 2 in >50% of tumor cells. VEGF staining intensity was scored according to a 4-teired system[32] into score 0: negative, 1: weak, 2: moderate and 3: strong staining. All sections were stained in a single run to avoid procedural variations affecting the credence of comparing VEGF intensity readings.

2.3.2. HPV-immunostaining
This was evaluated as either positive or negative, whereby any number of tumorous or non-tumorous cells showing brown nuclear staining qualified as a positive result.

2.3.3. CD34 immunostaining
A case was considered positive if any number of tumor vessel endothelial cells showed cytoplasmic brown staining.

2.4. Assessment of MVD

MVD was calculated according to the method described by Weidner et al.[21] CD34-stained sections were examined under low magnification (100×) to determine the area of highest vascularity (hot spot). Ten consecutive high power fields (HPFs) (400×) were assessed within a hot spot and vessel count was estimated in each HPF using the framework of an optical grid mounted onto the eye piece. Ten MVD readings were obtained for each tumor, the mean of which was recorded and expressed as the number of vessels HPF.[20,21]

Microvessels were identified as any CD34 positive endothelial cell or cluster of cells with or without visible lumen. In dense vascular networks, each distinct branch was interpreted as a single vessel. Large anastomosing sinusoidal vessels were counted as a single vessel.[33]

3. Statistical analysis

Statistical analysis was performed using SPSS (Statistical Package for Social Sciences, version 13.0, Chicago, USA).

3.1. Bivariate analysis

Continuous variables were expressed as mean ± standard deviation (SD), while categorical variables were expressed as numbers and percentages. Statistical relations between two categorical variables were tested using Chi-square (χ^2) or Fisher exact tests. Relations between categorical and continuous variables were tested using t test and ANOVA. The level of significance was set at $P \leqslant 0.05$.

3.2. Multivariate analysis

In order to identify possible predictors of biological behavior of cervical carcinoma, all statistically significant variables with cervical carcinoma types or grade in the bivariate analysis were introduced into a logistic regression model. Odds ratio and 95% confidence intervals (CI) were calculated.

ROC curves were used to evaluate the specificity, sensitivity and overall accuracy of the predictive variables. The value that showed the highest overall accuracy was used as the cut-off points of the continuous variables.

4. Results

In the current study, 30 cases of cervical carcinoma were included. The mean age was 49.79 + 4.67 years ranging between 40 and 60 years. Histologically, 17 cases were squamous cell carcinomas (56.7%), 11 adenocarcinomas (36.7%) while two cases (6.7%) were adenosquamous carcinomas.

Cases of squamous cell carcinoma were classified into a case (5.8%) of grade I, three cases (17.6%) of grade II, eight cases (47.1%) of grade III and five cases (29.4%) of grade IV. Adenocarcinomas were classified into grade 1 in five cases (45.4%), grade II in two cases (18.1%) and grade III in four cases (36.8%).

Both cases of adenosquamous carcinoma were high grade (poorly differentiated glandular component).

Overall, 11 cases (36.7%) of low-grade carcinoma and 19 cases (63.3%) of high-grade carcinoma were included in the present study.
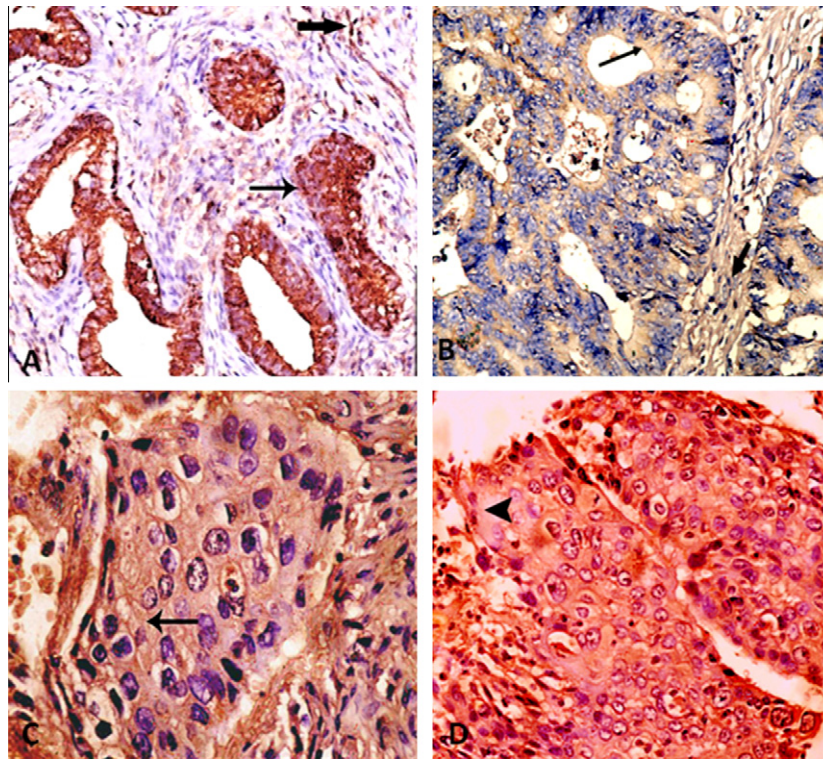
Figure 1 VEGF immunostaining. (A) Grade II adenocarcinoma showing strong cytoplasmic VEGF immunostaining in both neoplastic epithelial (thin arrow) and stromal cells (thick arrow). (B) Grade II adenocarcinoma showing moderate cytoplasmic VEGF immunostaining ((A and B) anti-VEGF, 200×). (C) Grade III squamous cell carcinoma showing moderate cytoplasmic VEGF immunostaining in epithelial cells. (D) Grade III squamous cell carcinoma showing heterogeneous cytoplasmic VEGF immunostaining with weak staining in areas (arrow head) and strong intensity in others ((C and D) anti-VEGF, 400×).

4.1. Immunohistochemistry

4.1.1. VEGF immunostaining

Heterogeneous VEGF positive cytoplasmic and membranous staining was seen both in neoplastic epithelial cells and in stromal cells (Fig. 1A). In addition, endothelial lining of intratumoral blood vessels showed positive cytoplasmic staining (internal control).

The difference in VEGF staining intensity among the three histologic types of cervical carcinoma was statistically insignificant ($P = 0.315$), while the difference in VEGF staining density was statistically significant ($P = 0.002$, Fisher exact test) with the highest VEGF density scores recorded in adenocarcinomas and lowest values recorded in squamous cell carcinomas (Tables 1 and 2).

The relation between cervical carcinoma grade and VEGF expression was statistically significant ($P \leqslant 0.0001$) (being direct with VEGF staining intensity and inverse with VEGF density) (Fig. 1A–D) (Tables 3 and 4).

4.1.2. CD34 immunostaining and assessment of MVD

CD34 positive cytoplasmic staining was seen in endothelial cells lining intratumoral blood vessels and vessels in adjacent normal cervical tissue (internal positive control). MVD values ranged between 17.9 and 90.2 vessel/HPF with a mean of 50.37 ± 20.02.

The highest MVD values were recorded in adenosquamous carcinomas (65.70 ± 10.60) (Fig. 2C), while the least values

Table 1 Relation between VEGF intensity and histologic subtypes of cervical carcinoma.

	VEGF intensity scores			Total	P
	1	2	3		
Histologic subtypes					
Squamous cell carcinoma					
No.	3	5	9	17	0.315
%	42.9	83.3	52.9	56.7	
Adenocarcinoma					
No.	4	1	6	11	
%	57.1	16.7	35.3	36.7	
Adenosquamous carcinoma					
No.	0	0	2	2	
%	0	0	11.8	6.7	

P: statistically significant at < 0.05.

were recorded in squamous cell carcinomas (47.32 ± 18.42) (Fig. 2A). However, the difference in MVD between the three histologic types was statistically insignificant ($P = 0.450$).

The difference in mean MVD values between low-grade (30.74 ± 8.25) and high-grade (61.74 ± 15.38) carcinomas was statistically significant (t test: 7.18, $P \leqslant 0.0001$).

4.1.3. HPV immunostaining

HPV positive nuclear staining was seen in infected neoplastic and non-neoplastic cells in 28 cases (93.3%).

Table 2 Relation between VEGF staining density and histologic subtypes of cervical carcinoma.

| | VEGF density scores | | Total | P |
	1	2		
Histologic subtypes				
Squamous cell carcinoma				
No.	14	3	17	
%	82.4	23.1	56.7	
Adenocarcinoma				
No.	2	9	11	0.002*
%	11.8	69.2	36.7	
Adenosquamous carcinoma				
No.	1	1	2	
%	5.9	7.7	6.7	

* *P*: statistically significant at < 0.05.

Table 3 Relation between VEGF staining intensity and cervical carcinoma grade.

| | Cervical carcinoma grades | | Total |
	Low grade	High grade	
VEGF intensity scores			
1			
No.	7	0	7
%	63.6	0	23.3
2			
No.	2	4	6
%	18.2	21.1	20.0
3			
No.	2	15	17
%	18.2	78.9	56.7
Total			
No.	11	19	30
%	100.0	100.0	100.0
χ^2	16.66		
P value	< 0.0001*		

* *P*: statistically significant at < 0.05.

Table 4 Relation between VEGF staining density and cervical carcinoma grade.

| | Cervical carcinoma grades | | Total |
	Low grade	High grade	
VEGF density scores			
1			
No.	1	16	17
%	9.1	84.2	56.7
2			
No.	10	3	13
%	90.9	15.8	43.3
Total			
No.	11	19	30
%	100.0	100.0	100.0
P of Fisher exact test	< 0.0001*		

* *P*: statistically significant at < 0.05.

relation between VEGF staining intensity and HPV expression was statistically significant ($P = 0.042$); however, the relation between VEGF staining density and HPV expression was not ($P = 0.179$). (Tables 8 and 9).

4.4. Results of multivariate statistical analysis

In a logistic regression analysis, including cervical carcinoma grade, MVD, VEGF intensity and density of expression; MVD emerged as an independent predictor of cervical carcinoma grade hence implying a poorer outcome. ($P = 0.042$) (Table 10).

The sensitivity, specificity and overall accuracy of MVD were 92.9%, 75% and 85.5%, respectively and the value of MVD that recorded the highest overall accuracy (MVD = 40.01 vessel/HPF) was chosen as the cut-off point. According to this value, cervical carcinomas with MVD values ⩽40.01 vessel/HPF were of expected lower grade and carcinomas with MVD >40.01 vessel/HPF were of expected higher grade. ROC curve (Fig. 3) showed that the area under the curve is 0.790 (confidence interval = 0.623–0.957, $P = 0.007$). Hence, MVD could be used as a predictive test for cervical carcinoma grade.
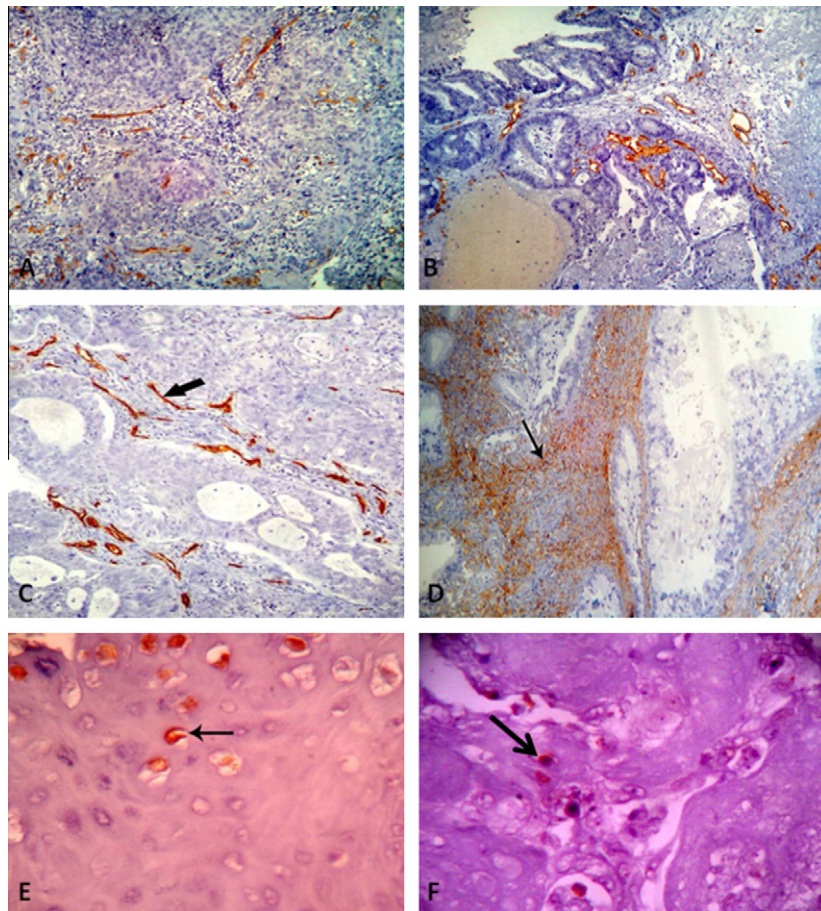
5. Discussion

Cancer of cervix is the second most common cancer among females worldwide with a ratio of mortality to incidence of 52%.[34] The annual number of new cervical cancer cases in Egypt in 2008 was 514 projected to increase by 93% to 846 in 2025.[35]

The poor outlook of cervical carcinoma necessitates the search for new prognostic factors and therapeutic strategies. One such promising strategy is targeting tumor blood vessels. However, till the present time, the prognostic import of biological determinants of angiogenesis and their putative role in treatment of cervical carcinoma are not settled.[36]

The poor prognostic implication of MVD in cervical cancer is well established,[37] however; so far, reports concerning the relation between VEGF and MVD in cervical cancer are profoundly contradictory.[38,39]

VEGF expression in cervical carcinoma has been studied at many levels including serum VEGF level,[40] VEGF mRNA[36]

Fifteen cases of squamous cell carcinoma (88.23%) (Fig. 2E), all adenocarcinomas (11 cases) (Fig. 2F) and both adenosquamous carcinomas (two cases) were positive. However, no statistical significance was found between HPV expression and histologic type ($P = 0.304$) or tumor grade ($P = 0.13$). (Table 5).

4.2. Relationship between VEGF expression and MVD

The relation between VEGF staining intensity and MVD of cervical carcinoma was statistically significant ($P = 0.0001$) (Table 6) and so was the inverse relation between VEGF staining density and MVD ($P = 0.028$). (Table 7).

4.3. Relationship between HPV expression and each of MVD and VEGF expression

The relation between HPV expression and MVD was statistically significant ($t = 7.541$, $P \leqslant 0.0001$). Furthermore, the

Figure 2 Assessment of MVD using CD34 antibody and HPV antigen immune expression. (A) Grade IV squamous cell carcinoma, (B) grade II adenocarcinoma, (C) adenosquamous carcinoma, (A–C) showing CD34 positive-tumor blood vessels, (D) grade I adenocarcinoma showing positive CD34 staining of individual endothelial cells forming no visible lumen (arrow). ((A–D) anti-CD34, 200×). (E) Grade II squamous cell carcinoma showing strong positive nuclear HPV immunostaining (arrow). (F) Grade II adenocarcinoma showing strong positive nuclear HPV immunostaining (arrow). ((E and F) anti-HPV, 400×).

Table 5 Relation between HPV expression and cervical carcinoma grade.

	Cervical carcinoma grade		Total
	Low grade	High grade	
HPV expression			
Positive			
No.	9	19	28
%	81.8	100	93.3
Negative			
No.	2	0	2
%	18.2	0	6.7
Total			
No.	11	19	30
%	100.0	100.0	100.0
P of Fisher exact test	0.13		

P: statistically significant at <0.05.

and tissue VEGF protein.[36] The latter detected immunohisto-chemically was the method adopted in the present study being the most practically applicable.[41] Moreover, expression of VEGF mRNA was not always found to parallel VEGF protein expression, possibly due to epigenetic modification.[36]

In the present study, VEGF expression was invariably detected in all cases which points to the crucial role played by VEGF in cervical carcinogenesis.[41] Cytoplasmic VEGF staining in tumor cells and adjacent stromal cells was previously reported.[42] Immunoreactive stromal cells are most probably considered to be macrophages that were attracted to the tumor to phagocytose VEGF positive-tumor cell debris.[42]

In the present work, expression of VEGF by endothelial cells lining intratumoral microvessels was in keeping with previous observations[43,44] In these articles, such endothelial cells expressed mRNA of VEGF receptors implying that VEGF molecules released by tumor cells became bound to their receptors onto tumor vessel-endothelium. This receptor–ligand interaction may provide a mechanism of retaining and concentrating VEGF, thereby maximizing its activity within the tumor locale while preventing its diffusion elsewhere. This finding stands in contrast to the ubiquitous distribution of other pro-angiogenic peptides and thereby strongly favors

Table 6 Relation between VEGF intensity and MVD.

VEGF intensity	N	Mean	S.D.	Min.	Max.	ANO-VA	P value
Score 1	7	28.1200	8.03770	17.90	39.40	12.301	0.0001[*]
Score 2	6	45.9167	15.26832	26.40	70.20		
Score 3	17	61.1082	16.82993	34.50	90.20		
Total	30	50.3727	20.02574	17.90	90.20		

[*] P: statistically significant at < 0.05.

Table 7 Relation between VEGF density and MVD.

VEGF density	Mean	S.D.	Min.	Max.	t	P
⩽50%	57.2782	16.97857	26.40	90.20	5.368	0.028[*]
>50%	41.3423	20.70976	17.90	77.20		
Total	50.3727	20.02574	17.90	90.20		

[*] P: statistically significant at < 0.05.

Table 8 Relation between HPV expression and VEGF intensity.

	VEGF intensity score			Total
	1	2	3	
HPV				
Positive				
No.	5	6	17	28
%	71.4	100.0	100.0	93.3
Negative				
No.	2	0	0	2
%	28.6	0	0	6.7
Total				
No.	7	6	17	30
%	100.0	100.0	100.0	100.0
P	0.042[*]			

[*] P: statistically significant at < 0.05.

Table 9 Relation between HPV expression and VEGF density.

	VEGF density score		Total
	1	2	
HPV			
Positive			
No.	17	11	28
%	100.0	84.6	93.3
Negative			
No.	0	2	2
%	0	15.4	6.7
Total			
No.	17	13	30
%	100.0	100.0	100.0
P of Fisher exact test	0.179		

P: statistically significant at < 0.05.

the use of anti-VEGF therapy as specific anti-angiogenic agents with minimal side effects.[44]

Most of the studied cases showed heterogeneous VEGF staining intensity within the same tumor. The same finding was previously reported by Tokumo et al.[45] who attributed such heterogeneity to regional ischemia stemming from regional differences in tumor cell growth. In addition, such staining heterogeneity may be ascribed to varying binding affinity and degradation of VEGF isoforms released within the same tumor.[46] In the current study, like in others,[47,48] a statistically significant positive correlation was found between VEGF intensity and cervical carcinoma grade. Increasing tumor grade is coupled with augmented proliferation rate which calls for increased outgrowth of vascular supply. Such stresses generate a state of chronic hypoxia within the tumor. Hypoxia, known to be the most potent angiogenic stimulator, switches on tumor angiogenesis via up regulating a set of genes including the VEGF gene.[49]

On the other hand, the statistically significant relation between VEGF density and cervical carcinoma grade was inverse. Other authors previously reporting the same finding[45] posed that once a certain microvascular density is obtained, growth reaches a plateau and the demand for VEGF declines, causing a decrease in its expression (negative feedback).[50] This explanation can be further extrapolated to decipher the inverse statistically significant relation between VEGF density and MVD ($P = 0.028$) which we reported as well as others.[51] MVD is assumed to reflect past angiogenic activity whereby the vascular network will remain long after the expression of VEGF has ceased.[52] This might explain why VEGF expression – unlike MVD – had no prognostic value in the work of Tjalma et al.[50]

We were able to prove that VEGF density was higher in adenocarcinomas compared to squamous cell carcinomas and the relation was statistically significant. Such a finding is in line with results reported by Tjalma et al.[50] This can be ascribed to the fact that in the present study, most cases of squamous cell carcinoma were of high grade (and hence of low VEGF density) while most cases of adenocarcinoma were of low grade (and hence of higher VEGF density).

Table 10 Logistic regression analysis.

	Wald χ^2	P value	Odds ratio	95.0% C.I.	
				Lower	Upper
MVD	3.560	0.053[*]	1.570	0.983	2.508
HPV	0.01	0.98	231.34	0.03	456.11
VEGF intensity	0.60	0.44	2.58	0.24	28.03
VEGF density	0	0.99	1.74	0.99	3.123

[*] P: statistically significant at < 0.05.
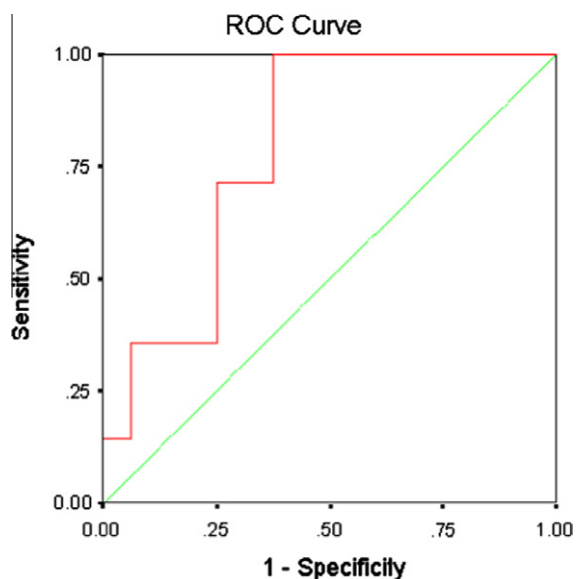
Figure 3 ROC curve for accuracy of MVD to predict grade of cervical carcinoma.

More intense VEGF staining was observed in cervical adenocarcinoma compared to other histotypes; however, no statistical significance was reached although previously reported.[45] This could possibly be ascribed to the small number of adenocarcinomas enrolled in the present study.

MVD is considered a measure of tumor angiogenesis and a significant prognostic factor that correlates with increased likelihood of metastasis and worse prognosis in many tumor types.[36]

In our work, MVD showed significant correlation with tumor grade. This finding comes in accord with previous reports[48] but still contradicts with others.[53] Such discrepancy may be attributed to variation in the studied histologic types of cervical carcinoma, different methodologies adopted in measuring MVD and the immunohistochemical markers used for highlighting endothelial cells.

The highest values of MVD that we recorded were in adenocarcinomas and the lowest values were in squamous cell carcinomas. Both findings were previously reported.[4,37] We again ascribe our inability to reach statistical significance to the small number of adenocarcinomas included in the present study.

Highlighting blood vessels immunohistochemically is an essential prerequisite for measuring MVD. Several antibodies have been employed for that purpose including factor VIII,

CD105, CD34 and CD31 antibodies.[54] Our selection of CD34 was based on its superior sensitivity[55] with detection of a greater number of microvessels in cervical tumors compared to other antibodies.[56] Furthermore, it stains neoplastic endothelium a deeper shade than normal endothelium.[55] However, compared to other antibodies as CD31 and factor VIII, it was found to be less specific in evaluating angiogenesis as it stains non-endothelial cells (e.g. hematopoietic progenitor cells) as well.[57] For these considerations, it is imperative for the evaluator to interpret the results of immunostaining in the proper morphological context to better assess MVD as an index of angiogenesis.

Since infection by high-risk types of HPV is strongly associated with cervical carcinoma,[7,8] we investigated the possible association between virus infection and tumor angiogenesis. HPV expression in cervical carcinoma has been studied at many levels including estimation of serum HPV virus load,[58] HPV DNA by PCR, in situ hybridization[59] and immunohistochemical detection of HPV antigens.[60]

In the current work we were able to immunohistochemically detect HPV expression in 93.3% of the studied cervical carcinomas which is comparable to other data reported from Muslim and Middle East countries as Morocco, (70.5%)[61] and Iran (85.5%).[62] In a study covering 22 countries and coordinated by the International Agency for Research on Cancer (IARC), the overall detection rates of HPV DNA were found to vary little among different regions of the world (83–89%).[63] Furthermore, in an Egyptian study,[64] prevalence of HPV infection (detected by PCR) among Egyptian females 35 years and above was 2.6% while HPV infection was positive in 94.3% of cervical neoplastic lesions (pre-invasive and invasive). Actually, some authors allege that HPV causes practically 100%[65] of cervical carcinomas, and ascribe the underestimation of its prevalence in these tumors to "limitations of study methodologies".[66]

All cases of cervical adenocarcinoma examined were positive for HPV which further endorses the notion that positivity for HPV can be used to confirm the endocervical origin of an adenocarcinoma[67].

In the current work, HPV immune expression of the studied cases of cervical carcinoma showed positive statistically significant correlation with VEGF intensity. This is a novel finding on the immunohistochemical level. The same import, however, was reached by Song et al. who reported that VEGF expression is related to HPV load detected by PCR.[68] The concept of higher VEGF expression in HPV-16 E6 positive cells compared to HPV-16 E6-negative ones was previously reported.[69] The latter study group explained that HPV-16 E6 oncoprotein might contribute to tumor angiogenesis by direct stimulation

of the VEGF gene promoter.[69] In the same context, other researchers added that HPV-16 E6 oncoprotein interfered with the ubiquitin mediated degradation of HIF-1α, a transcription factor involved in activating VEGF gene promoter in response to hypoxia.[49] Furthermore, it has been pointed out that the HPV-16 E7 oncoprotein enhanced the release of VEGF[70] while, HPV-16 E5 oncoprotein up-regulated VEGF expression through the activation of a diversity of signaling pathways.[71]

Not only did HPV immune expression correlate significantly with VEGF intensity but also with MVD of studied tumors, a notion which lends credibility to the new concept claiming that HPV infection stimulates tumor angiogenesis in cervical carcinoma.

6. Conclusions

VEGF was expressed in all studied cases of cervical carcinoma. There was a statistically significant relation between VEGF expression and MVD. Since HPV immune expression was significantly correlated with MVD and VEGF staining intensity, we hereby provide further evidence that HPV infection may augment tumor angiogenesis in cervical carcinoma. In cervical carcinoma, MVD could be used as an independent predictor of cervical carcinoma grade hence implying a poorer outcome especially if anti-VEGF therapy is to be considered.

Conflict of interest

The authors have no conflict of interest to declare.

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