Value of Quantifying the Intratumoral Microvessel Density Based on Immunohistochemical Detection of PECAM-1 and vWF in Colorectal Carcinoma from Iraqi Patients

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Abstract

Counting of newly formed microvessel may prove to be a useful tool in the early detection of metastatic potential and selection of patients for whom antiangiogenesis drugs might be beneficial.

Aim of the study: We designed this study to assess the significance of microvessel quantification in colorectal cancer with respect to different clinicopathological variables.

Subjects and Methods: forty archived paraffin embedded colorectal adenocarcinoma samples and their resection margins were enrolled in our study. Thin paraffin embedded sections (3-5µm thick) of both tumor and resection margins were prepared for each respective biopsy and were used to detect endothelial cell surface expression of PECAM-1 and vWF, by and immunohistochemistry technique.

Results: Based on the current outcome, there were significant differences in microvessel density based on PECAM-1 or vWF immunostaining when each tumor sample were compared to its respective resection margin (p<0.001and p<0.001, respectively). In addition, tumors ≥3mm^3 in size demonstrated a significant increase in their microvessel density compared to their counterparts whether PECAM-1 or vWF immunostaining was applied (p<0.001 and p<0.001, respectively). Moreover, when tumor samples were analyzed based on their depth of invasion, for intratumoral microvessel count, exclusively, vWF immunostaining analysis demonstrated significant differences among the three groups SMP, SE, and OR since the latter came up with the highest microvessel count (p<0.05). When tumor lymph node metastases was questioned, exclusively, vWF immunostaining were significantly differentiated among N0, N1, and N2 groups (p<0.05). Concerning the possible correlations between the two investigated parameters in respect to various histopathological variables; both PECAM-1 and vWF immunostaining demonstrated significant positive correlations in tumor samples (r=0.37), whereas in resection margins, these correlations were absent. Although PECAM-1 and vWF immunostaining revealed significant and positive correlations within tumor differentiation (WD: r=0.56, MD: r=0.57, and PD: r=0.89) as well as with tumor stage (A-B: r=0.39 and C-D: r=0.31), still, they seem to correlate significantly and exclusively within SE group (r=0.74), tumors <3mm^3 in size (r=0.66), N0 (r=0.36), and N1 (r=0.85) groups. However, PECAM-1 and vWF immunostaining revealed significant but negative correlation exclusively within N2 group (r= -0.38).

Conclusions: In conclusion, microvessel count could be useful as a predictor for tumor metastases in patients with colorectal adenocarcinoma. Possible interpretations of the current outcome are explained thoroughly in the text.

الخلاصة

ان حساب الاوعية الدقيقة المتكونة حديثا يمكن ان يكون اداة نافعة لغرض التحري عن احتمالية انتقال الورم ولغرض اختيار المرضى الذين يحتمل استفادتهم من العلاجات المضادة لتخليق الاوعية الدموعية. ، صُممت هذه الدراسة لغرض تقييم اهمية احتساب الاوعية الدقيقة في اورام قولونية مستقيمية غدية من مرضى عراقيين بالمقارنة مع المتغيرات المرضية السريرية. لهذا الغرض، أدرجنا في هذه الدراسة أربعون عينة لسرطان قولوني مستقيمي غدي مطمورة بالشمع مع اربعون حافة مستاصله لنسج السرطان مطمورة بالشمع أيضاً تابعة لنفس المريض. هُيئت مقاطع مطمورة بالشمع بسمك ملائم (3-5 مايكروميتر) لكل من الورم وحافة الورم المستأصل واستخدمت لتحديد مقدار كثافة الاوعية الدقيقة داخل الورم بالاعتماد على تقنية الاصطباغ الناعي النسجي الكيميائي لعلامات الخلية المبطنة للوعاء PECAM-1 و vWF .

اظهرت النتائج وجود فروقات معنوية في كثافة الاوعية الدقيقة المعتمد على الاصطباغ المناعي لكل من PECAM-1 و vWF وذلك عندما تمت المقارنة بين الورم مع ما يقابله من حافة الورم المستأصل($p<0.001$ و $p<0.001$ بالتتابع). كما أن العينات السرطانية بحجم اكبر او يساوي 3 ملم مكعب اظهرت زيادة معنوية في كثافة الاوعية الدموية الدقيقة بالمقارنة بالعينات ذات حجم اقل من 3ملم مكعب ($p<0.001$ و $p<0.001$ بالتتابع). علاوة على ذلك, عندما حللت عينات الورم بالاعتماد على عمق الغزو, فأن تحليل الاصطباغ المناعي باستخدام vWF حصرا قد اظهر فرقا معنويا بين المجموعات SMP و SE و OR ($p<0.001$ و $p<0.001$ بالتتابع), اذ أن المجموعة الاخيرة حصلت على أعلى عدد من الاوعية الدقيقة. وعندما سؤل عن الانتقال الى العقد اللمفاوية كان تحليل الاصطباغ المناعي باستخدام vWF حصرا قد اظهر فرقا معنويا بين المجاميع الثلاثة N0 و N1 و N2 ($p<0.05$ $p<0.05$, بالتتابع) .فيما يخص العلاقة المحتملة بين المعلمين المبحوثين ضمن متغيرات الهستوباثولوجيا , لوحظ ان الاصطباغ المناعي لكلا PECAM-1 و vWF يمتلك علاقة موجبة معنوية في الورم (r=0.37) بينما في الحافات المستأصلة كانت هذه العلاقة مفقودة. كذلك لوحظ وجود علاقات موجبة ومعنوية للاصطباغ المناعي لكل من PECAM-1 و vWF مع تمايز الورم , وكذلك مع مرحلة أو طور الورم , وعمق الغزو , والانتقال الى العقد اللمفاوية وكذلك حجم الورم.

نستنتج مما تقدم ان حساب الاوعية الدقيقة يمكن ان يكون منبئ مفيد لانتقال الورم القولوني المستقيمي الغدي في المرضى العراقيين.

Introduction

It is usually accepted that a correct identification of tumor-associated vessels requires the use of endothelial cells markers identified by Immunohistochemistry. Several concurrent endothelial cell markers are employed. The most popular ones are platelet-endothelial cell adhesion molecule-1 (PECAM-1) and von Willebrand factor (vWF) [1].

PECAM-1 is a 130-KD glycoprotein belongs to the immunoglobulin (Ig) superfamily of cell adhesion molecules. It is found in large quantities on the surface of endothelial cells (ECs) and is less abundant on platelets and leukocytes. It plays a major role in a number of cellular interactions, particularly in adhesion between ECs and polymorphonuclear leukocytes, monocytes, and lymphocytes during inflammation, and between adjacent ECs during angiogenesis [2]. vWF is synthesized in ECs and megakaryocytes and its function is to promote thrombus formation by mediating adhesion of platelets to the injured vessel wall and to one another [3]. All other functional site in the vWF molecules supports platelet adhesion and aggregation by binding to extracelllar matrix components or to memberane receptors [3]. The vWF is expressed at higher levels on the venous than on the arterial side of the capillary circulation and in human tissues, in the endothelium of larger

vessels and in the adult endocardium [4]. As vWF in the tissues derives uniquely from vascular endothelial cells, this feature makes vWF particularly useful to detect activation of the endothelium, an early sign of angiogenesis, in tumors [5]

In term of endothelial cells, angiogenesis can be viewed as a process in which these cells serve their initial cell-cell contacts, proliferate, and migrate into the perivascular matrix where they reestablish their cell-cell associations to form new patent vascular channels [6], although the evidence does not support a role for PECAM-1 in endothelial cell proliferation [7], a number of reports have implicated PECAM-1 in endothelial cell motility [8, 9] and in the endothelial cell-cell associations required for the organization of endothelial cells into tubular networks [10]. The early stages of angiogenesis involve the migration of endothelial cells into the surrounding perivascular matrix phenomena that is dependent on the integrin mediated endothelial cell adhesion to extracellular matrix proteins [11]. A number of studies have established that engagement of surface PECAM-1 may transduce intracellular signals that activate the adhesive function of integrins [12]. It is therefore possible that for endothelial cell, binding of endothelial PECAM-1 to one or more of its non PECAM-1 ligands, facilitates endothelial cell migration by augmenting integrin dependent adhesion [12]. Immunohistochemical detection of PECAM-1 and vWf has been used extensively to quantify angiogenesis of xenograft tumors in immunodeficient animal models carrying various human tumor cell loads [13-15]

Aim of the study: To assess the significance of microvessel quantification in colorectal cancer with respect to different clinicopathological variables based on PECAM-1 and vWF (members of endothelial cell markers) immunostaining.

Materials and Methods

Forty archived paraffin-embedded tumors and their resection margins along with the histopathological report for each patient were taken from histopathological laboratories that belong to the Gastroenterology and Hepatology Teaching Hospital, Baghdad Teaching Hospital, Al-Kadhymia Teaching Hospital as well as private hospitals. Collection of samples was concluded on one year interval 2003-2004. Twenty patients (50%) were males and 20 (50%) were females. Mean patient age was 54.75 years (range between 28 and 82 years). H and E slides were prepared form the paraffin embedded blocks and were examined again by histopathologist to confirm data. Thin paraffin embedded sections (3-5μm thick) of both tumor and resection margin tissue sections were prepared on positively charged slides for the detection of endothelial cell surface expression of PECAM-1 and vWF, by and immunohistochemistry technique.

Immunohistochemistry

Immunohistochemical detection of endothelial cell surface expression of PECAM-1 and vWF was performed by the streptavidin-biotin method. Sections (3-5 μm thick) were heat fixed (55°C, 30 min) and deparaffinized in three changes of xylene. The sections were rehydrated and antigen retrieved as instructed by the detection kit manufacturer (Dako, Denmark). To quench endogenous peroxidase, 3% H2O2 was applied to

the tissues (5 min, room temperature). A protein blocker (Dako, Denmark), was applied to the sections (10 min, room temperature). Diluted Mouse anti-PECAM-1(Clone and isotype: JC70A mouse IgG1, Kappa) monoclonal antibody or Anti-vWF (Clone and isotype: F8/86 mouse IgG1, Kappa) monoclonal antibody (Dako, Denmark) was applied to the tissues and incubated (2 h, 37°C). After a 10-min wash in phosphate-buffered saline–Tween 20, slides were incubated with a biotinylated anti-mouse IgG and washed with phosphate-buffered saline–Tween 20, and avidin-biotin complex (ABC; (Dako, Denmark)) was applied for 1hr at room temperature. The DAB (diaminobenzidine) was applied (30 min) and the sections were washed, counterstained with hematoxylin (30 sec), and mounted with mounting medium and examined microscopically. Both positive and negative controls were included for each run of immunohistochemistry. The negative control was obtained by replacing the primary antibody with PBS buffer. Tonsillar tissue was used as positive control (Parumis *et al*., 1990). Determination of monoclonal antibody concentration to be used was made through a number of standardization protocols and found to be 1:40 for anti-PECAM-1 Ab 1:50 for anti-vWF Ab.

Determination of intratumoral microvessel density (IMD)

To investigate the IMD, the method described by Weidner *et al*., (1991) was applied. The hallmark of this method is to identify regions with the highest vascularization by immunohistochemical staining of endothelial cells (called hot spots) to restrict subsequent counting of the microvessels to these hotspots. This method is internationally recognized as a routine procedure for the evaluation of IMD as a prognostic marker in solid human tumors (Vermeulen *et al*., 1996). The hotspots were selected by scanning sections at low magnification X40 (X4 objective and X10 eyepiece); where as the counting was performed at an X100 magnification (X10 objective and X10 eyepiece). Any highlighted endothelial cells or endothelial cell cluster clearly separated from adjacent microvessels, tumor cells and stroma, was considered as a single, countable microvessel. Branching structures were counted as a single vessel unless there was a break in the continuity of the structure. Five fields in the hot spot were counted and the mean of these five fields was considered to be the number of blood vessels for each patient.

Statistical analysis

For the comparison between tumor and resection margin regarding the investigated parameters, the t test of significance was conducted. The association between surface expression of PECAM-1 and vWF along with tumor differentiation, depth of invasion, and lymph node metastasis was performed by chi-square (X2) and ANOVA test as well as 95% confidence interval. On the other hand, the association between the investigated parameters and tumor stage as well as tumor size was performed by student t-test. The correlations between the two investigated parameters in respect to various clinicopathological parameters were calculated by correlation coefficient (r). Statistical significance was defined as $p<0.05$.

Results

Clinicopathological Data

Forty archived paraffin embedded colorectal adenocarcinoma

samples and their resection margins were enrolled in our study. Twenty patients (50%) were males and twenty (50%) were females with male to female ratio of 1: 1. The mean patients age was 54.75 years (range between 28 and 82 years). According to the histological differentiation, tumors were classified in to three groups: well differentiated adenocarcinoma (WD), moderately differentiated adenocarcinoma (MD), and poorly differentiated adenocarcinoma (PD). Among forty cases, there were 7 cases well differentiated adenocarcinoma, 25 cases were moderately differentiated adenocarcinoma and 8 cases poorly differentiated adenocarcinoma. On the other hand, patients were grouped depending on different histopathological criteria including, tumor stage A-B versus C-D, tumor depth of invasion {tumor invades submucosa into muscularis properia (SMP), tumor reaches serosa (SE), and tumors invade other organs (OR)}, and tumor size ($<3mm^3$ versus $\geq 3mm^3$). Other histopathological data were shown in table 1.

Intratumoral microvessel density

Microvessel count in both tumor and resection margin tissues

In this work, we have determined tumor vascularization in forty cases of colorectal cancer by immunohistochemical staining with anti-PECAM-1 and anti-vWF; their typical staining patterns were shown in figure 1 and 2, respectively. Microvessel count in both resection margins and tumor tissues were between 8-18 versus 1-130 microvessel (mv)/mm^2 for PECAM-1 immunostaining and between 11-26 versus 2-60 mv/mm^2 for vWF immunostaining. As we demonstrated earlier, we determined the 99% confidence interval for patients and use its lower limit as a cut off value. Accordingly, patients was divided into two groups, hypovascular group which have microvessel countless that the cut off value, and hypervascular group which have more than or equal to that of the cut-off value. Our data analysis based on student t-test pointed out about 2.78 and 2.13 fold increase in microvessel density in mean values of tumor samples versus their resection margin with a significant differences ($p<0.001$ and $p<0.001$, table 2) for both PECAM-1 and vWF, respectively

Association between microvessel density and histopathological variables

As shown in table 3, no significant differences were found in surface expression of PECAM-1and /or vWF among the groups of tumor differentiation and stages. However, regarding tumor depth of invasion and tumor lymph node metastasis, unlike PECAM-1 immunostaining analysis which revealed a comparable level of staining among SMP, SE, and OR groups of patients ($p=0.930$), vWF immunostaining in SMP was significantly lower than that in SE and OR groups ($p<0.05$). In addition, the mean of surface expression of vWF was significantly associate with lymph node metastasis ($p<0.05$, table 3). Moreover, there were 63% and 55% increase in the mean of microvessel density calculated in tumors ≥3mm3 in size compared to that of tumor $<3mm^3$ in size whether microvessel density were determined based on PECAM-1 or vWF immunostaining ({16.2 ± 4.2 and 18.8 ± 3.3 for tumor size <3mm3} versus {44.2 ± 5.3 and 42.2 ± 4.8 for tumor size $\geq 3mm^3$}, respectively). These differences were found to be highly significant based on student t-test ($p<0.001$ and $p<0.001$, table 3).

Correlations between PECAM-1 and vWF expression in Respect to Different Histopathological Variables

The correlation between the PECAM-1 and vWF surface expression as well as the correlation in tumor and their resection margins and within different histopathological variables was analyzed by correlation coefficient (r). In the resection margins, all the correlations were weak, positive, and not significant. Whereas, in tumor tissues, the correlation between PECAM-1 and vWF was positive and significant at the 5% level (r=0.37, table 4).

Table 1 Histopathological data of colorectal cancer patients

Variable	Patients (n=40) N(%)
Age:	
•Mean±S.E.*	54.75 ± 2.35
•Median (yrs)	55
•Range (yrs)	(28-82)
M: F	20:20
Histological type	
•WD	7 (17.5%)
•MD	25 (62.5%)
•PD	8 (20%)
TNM staging	
•T1	2 (5%)
•T2	10 (25%)
•T3	21 (52.5%)
•T4	7 (17.5%)
•N0	21 (52.5%)
•N1	12 (30%)
•N2	7 (17.5)
•M0	38 (95%)
•M1	2 (5%)
Tumor stage	
• A-B	22 (55%)
• C-D	18 (45%)
Tumor size	
• <3mm^3	13 (32.5%)
• ≥3mm^3	27 (67.5%)
Tumor depth of invasion	
• SMP	11 (27.5%)
• SE	22 (55%)
•OR	7 (17.5%)
Recurrent	2 (5%)

Table 2. Microvessel count in both tumor tissues and their resection margins

Monoclonal antibodies	*Resection margins (n=40)*	*Tumor tissues (n=40)*
Anti-PECAM-1		
Mean ±SE†	12.6 ± 0.87	*35.1* ± 4.34
Median	12.5	30
99%C.I ‡	-------	23.3 - 46.8
t-test p-value	-------	*p<0.001*
Anti-vWF		
Mean ±SE	16.2 ± 1.49	34.58 ± 3.8
Median	14.5	28.5
99%C.I.	-------	24.28 – 44.87
t-test p-value	-------	***p< 0.001***

*standard error

Furthermore, there were positive correlation between them within tumor differentiation (WD: r=0.56, MD: r=0.57, PD: r=0.89, table 4), stage (A-B: r=0.39 versus C-D: r=0.31, table 4). While the correlation within depth of invasion, was high, positive, and significant within SE (r=0.74, table 4). Within tumor size <3mm^3, the correlation was positive, high, and significant (r=0.66), whereas it was weak, not significant within size ≥3mm^3 (r=0.13, table 4).

Table 3. Microvessel density and different histopathological variables

H***istopathological variables***	**N**	***Anti-PECAM-1 mean±SE***	***Anti-vWF mean±SE***
Histological type			
•WD	7	42 ± 16.3	32.43 ± 4.13
•MD	25	33.36 ± 4.81	33.24 ± 4.86
•PD	8	34.5 ± 8.4	40.6 ± 11.4
p value		1.312	3.319
Tumor stage			
• A-B	22	33.8 ± 5.1	29.2 ± 4.4
• C-D	18	36.2 ± 6.8	39 ± 5.8
pvalue		0.78	0.19
Tumor depth of invasion	11	37.27 ± 11.8	27.91 ± 7.85
• SMP	22	33.59 ± 4.91	33 ± 4.65
• SE	7	36.43 ± 8.34	50 ± 9.2
• OR		0.93	$p<0.05$
p value			
Lymph node metastasis			
• ***N0***	21	37.38 ± 7.07	39.71 ± 5.92
• ***N1***	12	28.17 ± 4.7	23.67 ± 5.5
• ***N2***	7	40.14 ± 10.5	37.86 ± 6.57
p value		0.576	$p<0.05$
Tumor size			
• <3mm^3	13	16.2 ± 4.2	18.8 ± 3.3
• ≥3mm^3	27	44.2 ± 5.3	42.2 ± 4.8
p value		$p<0.001$	$p<0.001$

Concerning lymph node metastasis, there were positive, significant correlation within N0 and N1 groups (r=0.36 and r=0.85, respectively), but it became significant negative correlation within the third group (r=-0.38, table 4).

Discussion

It has been well established that intra-tumoral microvessel density is an expression of the density of tumor-associated vascular networks [16]. Counting of newly formed microvessel may prove to be a useful tool in the early detection of metastatic potential and selection of patients for whom anti-angiogenesis drugs might be beneficial [17]. The evaluation of intra-tumoral microvessel density implies to count all tumor-associated vessels by surface unit. This in turn, implies a reliable method for the identification of vascular structures and a reproducible means for their quantification. It is usually accepted that a correct identification of tumor-associated vessels requires the use of endothelial cell markers identified by immunohistochemistry. [16]

Several endothelial cell markers have been used; however, in our study we used PECAM-1 and vWF. Several studies were reported to determine

intra-tumoral microvessel density based on the use of either anti-PECAM-1 or anti-vWF monoclonal antibodies. Horak *et al*., (1992)[17], demonstrated that intra-tumoral microvessel density (using anti-PECAM-1 monoclonal antibodies on paraffin embedded breast cancer tissues) was significantly higher in tumor than non tumor tissues (p=0.0001), he also found that there was significant association between microvessel density and tumor size (<2cm versus 2.1-4 cm, p=0.0007).

In another studies using the same monoclonal antibody on paraffin embedded colorectal tumors, Engel *et al*., (1996)[18, 19] and Vermeulen *et al*., (1999)[19] reported that high microvessel density correlate with recurrence, shorter survival and hematogenous metastasis.

In addition, Giatromanolaki *et al*., (1999)[20], reported that high microvessel density was the only parameter that predicted a worse overall survival. On the other hand, other studies that have been used anti-vWF monoclonal antibodies including, Maeda *et al*., (1995)[21] found that prognosis of the hypervascular group of gastric carcinoma to be significantly (p<0.05) worse than that of the hypovascular group.

Also, they reported that there was no statistically significant association between microvessel density and histologic type and with depth of invasion. Another example, Tarta et al., (2002)[22] reported that there was no significant association between intra-tumoral microvessel density and histological differentiation (p=0.6), but they observed that deeper tumor invasion significantly increased the rate of high microvessel density in almost linear fashion (p=0.02).

In the current work, our data statistical analysis revealed that the intratumoral microvessel count in tumor tissues was significantly higher than the microvessel count in resection margin based on immunohistochemical staining of both PECAM-1 and vWF (p<0.001 and p<0.001, respectively, table 2). However, statistical analysis failed to demonstrate any significant differences between microvessel density and tumor differentiation (p=1.31 and p=3.31, table 4) and with tumor stage (p=0.78 and p=0.19, table 4), for both PECAM-1 and vWF, respectively. This came in contrast to Horak *et al*., (1992)[17], who reported that there was significant association between microvessel density based on immunohistochemical staining of PECAM-1 and tumor differentiation (p=0.028). It's important to note that although we didn't find significant correlation with tumor differentiation as well as with tumor stage, the means of intratumoral microvessel count were increased but not for statistical significant level.

This might be attributed to the limited number of cases within each group which was investigated where the statistical analysis becomes insensitive to detect such association. On the other hand, differential expressions of various endothelial cell markers at different stages of endothelial cell development that may affect endothelial cell surface expression of PECAM-1 and vWF and subsequently, affect intratumoral microvessel count. To confirm this we observed an important point here, there were few

differences in the intratumoral microvessel count between PECAM-1 and vWF for the same patient. The bases of these finding could be explained based on two previously speculated observations. First, the structural characteristics of tumor-associated vascular networks, depends on the properties of the pre-existing vessels from which they derive. While the second depend on tumor-specific microenvironmental influences [23].

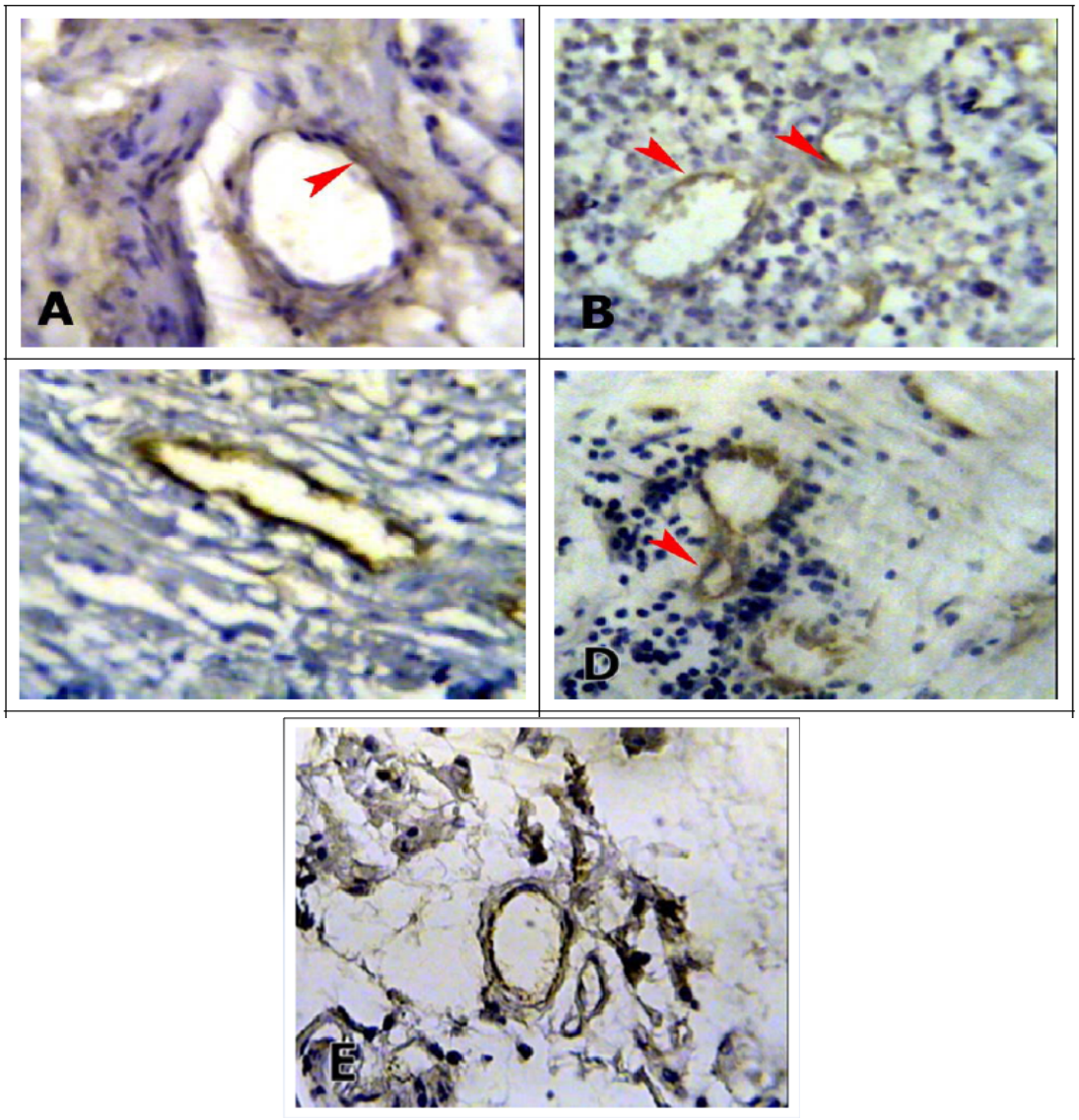

Figure 1. Immunohistochemical staining of PECAM-1 in colorectal tissue. Immunostaining of endothelial cell surface expression of PECAM-1 by peroxidase /DAB (brown) counter-stained with hematoxylin. (A) Well differentiated adenocarcinoma, Duck's stage A. (B) moderately differentiated adenocarcinoma, stage B. (C) moderately differentiated adenocarcinoma, stage C. (D) Poorly differentiated adenocarcinoma, stage D. (E) Resection m

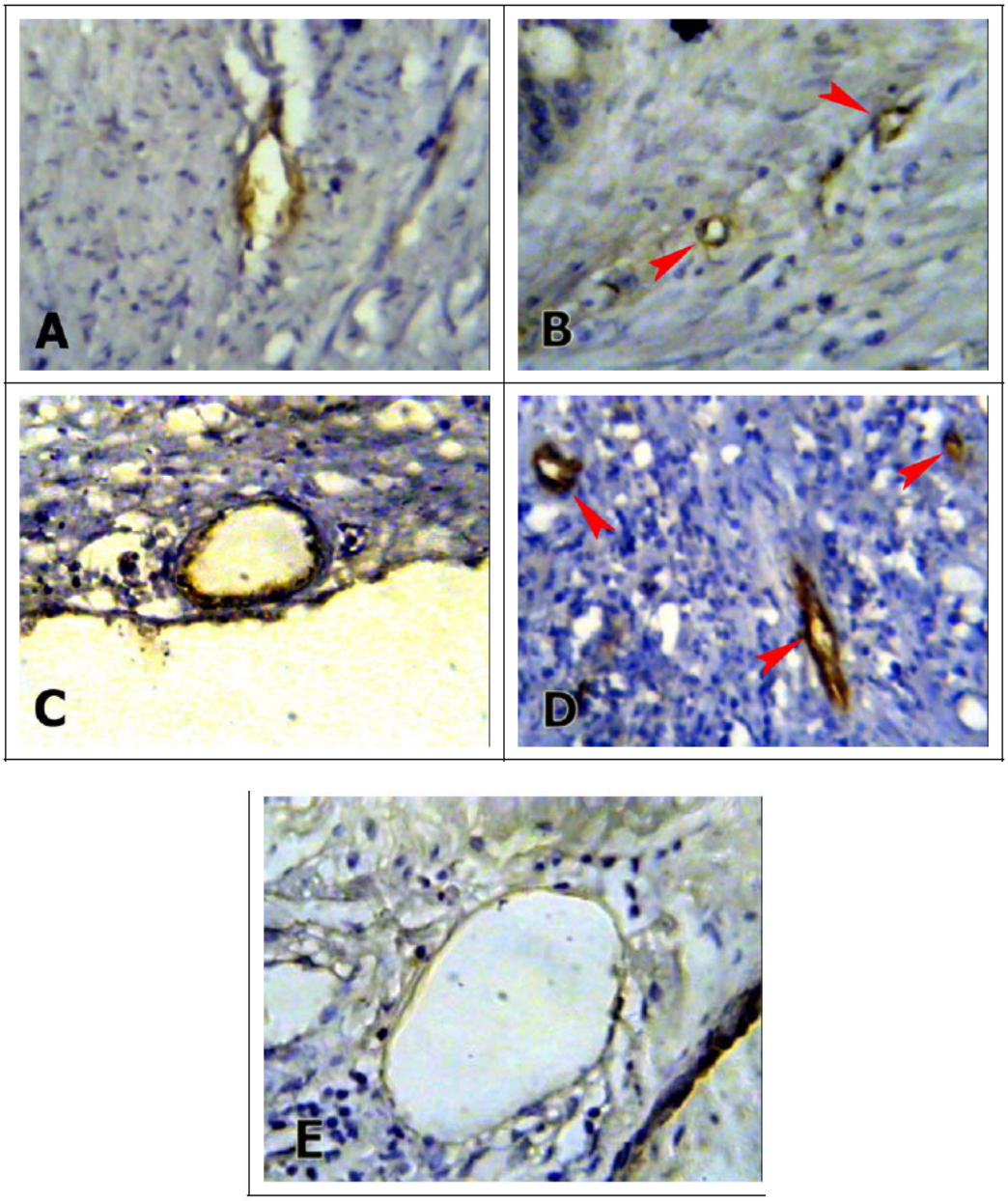

Figure 2. Immunohistochemical staining of vWF in colorectal tissue. Immunostaining of endothelial cell surface expression of vWF by peroxidase /DAB (brown) counter-stained with hematoxylin. (A) Well differentiated adenocarcinoma, Duck's stage A. (B) Moderately differentiated adenocarcinoma, stage B. (C) Moderately differentiated adenocarcinoma, stage C. (D) Poorly differentiated adenocarcinoma, stage D. (E) Resection margin. Magnification power for A-E (X400)

In the line with the first hypothesis is the high degree of heterogenesity of normal endothelia [24]. Capillary endothelial cells often present evidence of tissue-specific differentiation. One of the best examples is that of brain capillary endothelial cells, which are characterized by highly specific structural and functional properties [23]. Grafe *et al*., (1994)(25); Scholz and Schaper, (1997)(26) have reported that PECAM-1 was homogenously distributed over the entire endothelial cell surface, luminal and ubluminal as

well as lateral, both *in vivo* and *in vitro*.

Table 4. Correlations between PECAM-1, and vWF within the investigated histopathological variables

Variable	***Correlation of PECAM-1 with vWF***
Resection margins	0.195
Tumors	0.369*
Differentiation	
WD	0.568*
MD	0.57*
PD	0.891*
Tumor Stage	
A and B	0.392*
C and D	0.315*
Tumor size	
< 3mm^3	0.661*
≥ 3 mm^3	0.130
Depth of invasion	
SMP	0.103
SE	0.744*
OR	0.180
L.N metastasis	
N0	0.366*
N1	0.855*
N2	- 0.382**

*significant positive correlation, **significant negative correlation

On the other hand, vWF in the tissue originated uniquely from vascular endothelial cells. This feature makes vWF particularly useful to detect activation of the endothelium, an early sign of angiogenesis [5]. In tumors vWF is expressed at higher levels on the venous than on the arterial side of the capillary circulation and in human tissues in the endothelium of larger vessels and in the adult endocardium [4]. The distribution of vWF protein in the endothelium is regulated by such factors as blood flow and platelet number. In addition, thrombin generation may recruit non-expressing endothelial cells to produce vWF. These findings suggesting that vWF synthesis is controlled at the transcriptional level and that the extracellular environment may determine cell variations in expression levels [5]. It may be hypothesized that the heterogenecity of normal endothelial cells results in significant differences in the response to angiogenic stimuli or in the kinetics of the angiogenic process. Variations in the cell to cell expression of vWF are believed to be dependent on signals derived from the local environment [27]. It has been claimed that PECAM-1 is normally distributed widely over the surface of vascular endothelium *in vivo* but that in response to TNF-α or other types of activation, PECAM-1 is redistributed to the lateral plasma membranes [28]. Furthermore, Delisser *et al*., (1997)[29], had shown that blocking antibodies to PECAM-1 was found to block cytokine (bFGF) induced neovascularization. Therefore, our results might encourage further studies to investigate the influences of local microenvironment on tumor angiogenesis and their effect on endothelial cell markers.

Concerning the correlation between intratumoral microvessel density with tumor lymph node metastasis and with tumor depth of invasion, the current study demonstrated that, unlike PECAM-1,

which reveal no significant differences in endothelial cell surface expression along with tumor lymph node metastasis (p=0.5, table 3). This came in contrast to Horak *et al*., (1992)(17) who reported significant association (p=0.0001); vWF immunostaining reveal significant association among N0, N1, N2 groups (p<0.05, table 3). Similarly, regarding tumor depth of invasion, intratumoral microvessel count in OR group was significantly higher than that of SMP and SE groups based on immunohistochemical staining with anti-vWF (p<0.05, table 3), whereas, PECAM-1 immunostaining reveal no significant differences in microvessel count (p>0.05, table 3). This might possibly suggest that vWF is more sensitive than PECAM-1. The increased number of microvessel count which associated with tumor depth of invasion and lymph node involvement could be explained by the requirement for neovascularization to achieve tumor invasion and metastasis since the invasive tumor cells required blood vessel to support their growth with oxygen and nutrients and also increase the opportunity for tumor cells to metastasize. Therefore, intratumoral microvessel count could be used as predictor to select patients at higher risk for tumor metastasis and / or recurrences.

Regarding the correlation between intratumoral microvessel density and tumor size, our data statistical analysis reflected significant association between the intratumoral microvessel density and tumor size, (p<0.001 and p<0.001, table 3), based on immunostaining with PECAM-1 and vWF, respectively. This is in contrast to Tarta *et al*., (2002) who reported that there was non-significant association with the tumor size (p=0.3). Our results could be considered as a supportive conclusion to the hypothesis which speculated that, during the prevascular phase, the tumor is rarely larger than 2-3mm^3 and may contain a million or more cells [30]. Up to this size, tumor cells can obtain the necessary oxygen and nutrient supplies required for growth and survival by simple diffusion [31]. Thus, the tumor stays in dormant state and can be expanding beyond few millimeters before it become vascularized. In addition to that, newly formed intra tumoral blood vessels provide a way for tumor cells to enter the circulation and to metastasize to distant organs [32]. This is possibly because, tumor cells are rarely shed into the circulation before the primary tumor become vascularized [33], and newly formed capillaries have fragmented basement membrane and leak, making them more penetrable by tumor cells than mature vessels [34]. Therefore, in the hypervascular tumors, the metastatic process may be enhanced by the leaky nature of newly formed blood vessels, making the vascular invasion step easier to accomplish. Thus, our results suggested that enhanced vascular supply reflects an increased malignant potential because greater number of tumor vessels increase the opportunity for tumor cells to enter the circulation.

Its note worthy that there were three cases from seven which are in the early stages of malignancy but it have high microvessel density. We can speculate that those patients are at higher risk for metastasis and recurrence. Inversely, we have two cases from eight which are in the late stages of malignancy and have low microvessel density. Beside that, we know that the tumor to be aggressive, it must have neovascularization. This result could be possibly due to differential expression of various

endothelial cell markers as we mentioned earlier. On the other hand, our results might possibly support the hypothesis of vasculogenic mimicry. Vasculogenic mimicry is the generation of deregulated, aggressive tumor cells without participation by endothelial cells and independent of angiogenesis [(35)]. The angiogenic switch therefore could be defined both by tumor cells ability to turn on the hosts blood vessels at a given puncture, as defined by Folkman, (1995)(36). But also by some other change in aggressive tumor cells that would allow them to turn themselves into vessels that could provide microcirculation. Bergers *et al*., (1999)[(37)] studying the pancreatic islet cell carcinoma metastases, they found that during the face of treatment with angiogenesis inhibitors, that angiogenesis inhibitors, alone or in combination, did not prevent progression to the invasive carcinoma, and that the blood vessel density was not decreased. This, may support the notion that a tumor microcirculation not lined by endothelial cells and that tumor cells remain intact. This might possibly play a physiological role in the maintenance and growth of other aggressive tumors [(38)]. Therefore, our results might encourage further studies to investigate a panel of endothelial cell markers and their correlations with different histopathological variables.

To address how did the two investigated parameters correlated in tumor tissues versus resection margins, the current study also focused on whether there were any correlations between intratumoral microvessel density based on PECAM-1 and vWF surface immunostaining in respect to various histopathological variables. In general and at the resection margin level, the current data showed positive, weak, and statistically insignificant correlations PECAM-1 and vWF r=0.195, table 4). On the contrary, when tumor samples were under investigation, PECAM-1 and vWF immunostaining demonstrated a significant positive correlations (r=0.36, table 4). This might possibly attributed to normal threshold of surface expression for PECAM-1 and vWF. Since the resection margins are apparently normal tissue and there were no signs for tumorgenesis and vasculogenesis. Therefore, no need for multiple expressions of PECAM-1, and vWF. Nevertheless, the current outcome failed to pointed out any correlations among the two parameters (PECAM-1 and vWF) when they were analyzed together at the resection margins, table 4.

Concerning the correlations between PECAM-1 and vWF immunostaining along with the different histopathological variables, the current study revealed increasing positive correlations between PECAM-1 and vWF in respect to tumor differentiation (WD: r=0.56, MD: r=0.57, and PD: r=0.89, table 4), tumor stage (A-B: r=0.39 versus C-D: r=0.31, table 3-8), SE group (r=0.74, table 4), tumors $<3mm^3$ in size (r=0.66, table 4), and within N0 and N1 group (r=0.36 and r=0.85, respectively, table 4). Still, there is negative correlation within N2 group between PECAM-1 and vWF (r=-0.38, table 4). Other correlations were insignificant. These results might be due to the presence of several endothelial cell markers other than PECAM-1 and vWF which might possibly overexpressed and interfere with the expression of PECAM-1 and / or vWF during certain stage of endothelial cell development during which our detection was performed.

In conclusion, regarding intra-tumoral microvessel density, the findings of significantly increase of

microvessel count inconformity with tumor size and depth of invasion might possibly confirm the hypothesis that tumor progression might be related to angiogenesis. Thus, microvessel count could be used as a predictor for recurrences in patients with colorectal adenocarcinoma. A worth standing point is that the current study demonstrated significant associations between lymph node metastasis as well as tumor depth of invasion with intratumoral microvessel density based on vWF but not PECAM-1 immunostaining since the former found to be more sensitive compared to the latter and thus could be used alone during the assessment of intratumoral microvessel density.

References

1. Wang, D., et al.,. Biotech Histochem, 2008. **83**: p. 179-189.
2. Müller, A.M., et al.,. Exp Mol Pathol 2002. **72** p. 221–9.
3. Ruggeri, Z.M., . J Clin. Invest. , 1997. **Supplement, 100**: p. s41-s46.
4. Page, C., et al., . Am J Pathol, 1994. **141**: p. 673-683.
5. Zanetta, L., et al., . Int J Cancer, 2000. **85**: p. 281-288.
6. Carmeliet, P. and R.K. Jain,. Nature, 2000. **407**: p. 249-257.
7. Zhou, Z., et al., . Angiogenesis, 1999. **3**: p. 181-188.
8. Kim, C.S., T. Wang, and J.A. Madri,. Lab Invest, 1998. **78**: p. 583-590.
9. Yang, S., et al., . Am J Pathol, 1999. **155**: p. 887-895.
10. Halama, T., et al.,. J Invest Dermatol, 2001. **116**: p. 110-117.
11. Albelda, S.M., et al.,. J Cell Bio, 1991. **114**: p. 1059.
12. Deaglio, S., et al., . J Immunol, 1996. **156**: p. 727-734.
13. Fulzele, S.V., et al., . Pharm Res, 2006. **23**: p. 2094-106.
14. Muruganandham, M., et al.,. Mol Cancer Ther 2006. **5**: p. 1950–7.
15. Ragel, B.T., et al., . Cancer 2007. **109**: p. 588-97.
16. Martin, L., et al., . Br J Cancer, 1997. **76**: p. 1046-54.
17. Horak, E.R., et al., . Lancet, 1992. **340**: p. 1120-24.
18. Engel, C.J., et al., . Am J Surg Pathol, 1996. **20**: p. 1260.
19. Vermeulen, P.B., et al., . Br J Cancer, 1999. **79**: p. 316.
20. Giatromanolaki, A., et al., . Cancer, 1999. **86**: p. 1421.
21. Maeda, K., et al.,. J Clin. Oncol, 1995. **13**: p. 477-481.
22. Tarta, C., et al., . Arq Gastroenterol, 2002. **39**: p. 32-38.
23. Scoazec, J.Y.,. Electronic J Oncol, 2000. **2**: p. 23-32.
24. Garlanda, C. and E. Dejana, *Heterogeneity of endothelial cells specific markers.* . Arterioscler Thromb Vasc Biol, 1997. **17**: p. 1193-1202.
25. Grafe, M., et al.,. Am J Physiol, 1994. **267**: p. 2138-2148.
26. Scholz, D. and J. Schaper,. Cell Tissue Res, 1997. **290**: p. 623-631.
27. Arid, W.C., et al.,. J cell Biol, 1997. **138**: p. 1117-1124.
28. Ioffreda, M.D., et al., . Endothelium, 1993. **1**: p. 47-54.
29. DeLisser, H.M., et al.,. Am J Pathol, 1997. **151**: p. 671-677.
30. Mantzaris, N.V., S. Webb, and H.G. Othmer, . J Math Biol., 2004.
31. Gupta, M.K. and R.Y. Qin,. . World J Gastroenterol, 2003. **9**: p. 1144-1155.
32. Liekens, S., E. DeClercq, and J. Neyts, . Biochemical Pharmacology, 2001. **61**: p. 253-270.
33. Folkman, J.,. J Natl Cancer Inst, 1990. **82**: p. 4-6.
34. Nagyl, J.A., et al., . Biochem Biophysio Acta, 1989. **948**: p. 305-326.
35. Folberg, R., M.J.C. Hendrix, and A.J. Maniotis, . Am J Pathol, 2000. **156**: p. 361-381.
36. Folkman, J., . New Engl J Med, 1995. **333**: p. 1757-1763.
37. Bergers, G., et al., Science, 1999. **284**: p. 808-812.
38. Bissell, M.J., Am J Pathol, 1999. **155**: p. 675-678.