Research Paper

Prognostic Significance of Vascular Endothelial Growth Factor-A Expression in Colorectal Cancer

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AIM: **To study the expression status and clinical relevance of vascular endothelial growth factor-A (VEGF-A) in colorectal cancer (CRC) tissues.**

METHODS: **VEGF-A expression was investigated by immunohistochemistry in 89 cases with CRC. Some demographic and histopathologic variables were compared with VEGF-A expression to determine the prognostic significance in CRC.**

RESULTS: **VEGF-A (–) was found in 24 cases; (+), (++) and (+++) stainings were detected in 24, 35 and 6 cases, respectively. VEGF-A (–) was found in 20 of 58 cases with left colon cancer, while only 4 of 31 cases with right colon cancer were VEGF-A (–)(P = 0.024). There was a trend for lower tumor grade and lesser serosal invasion in cases with VEGF-A (–) samples (P = 0.07 and P = 0.079, respectively). Although the correlation was not statistically significant, there was a trend for lower death rate in cases with VEGF-A (–) tumor (P = 0.087). The longest survival was found in cases with VEGF-A (–) tumor and the shortest survival was found in cases with VEGF-A (+++) tumor. Median survival for patients with VEGF-A (–), (+), (++) and (+++) tumors was 59, 47, 35 and 11 months, respectively (P = 0.02). The Cox proportional hazards model identified stage IV disease and VEGF-A (+++) tumor as having the most important influences upon overall survival (odds ratio: 5.1, 95% confidence interval: 2.0-13.0 and odds ratio: 3.6, 95% confidence interval: 1.0-12.7, respectively), followed by serosal invasion (odds ratio: 2.4, 95% confidence interval: 1.0-5.9).**

CONCLUSION: **This study shows that VEGF-A is a poor prognostic factor in cases with CRC but the relatively small size of the study group precluded the correlation with all the known prognostic indicators.**

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Keywords:

VEGF

angiogenesis

colon cancer

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Introduction

The formation of new blood vessels is known as angiogenesis. This process has an important role in various pathologic conditions including inflammatory disorders, retinal vascular disorders, psoriasis, and cancer as well as physiologic conditions [1]. Increased vascularity causes both the growth of the tumor and also to the increased probability of the metastasis. There are many growth factors in the body regulating angiogenesis and these are named as angiogenic factors. On the other hand some inhibitory factors inhibit angiogenesis and there is a fine balance between inhibitory and stimulatory factors in the maintenance of angiogenic factors [2]. Vascular endothelial growth factor (VEGF[2]) is a very well known angiogenic factor and is known to play an essential role in neovascularization [3,4]. VEGF is a diffusible homodimeric glycoprotein produced by healthy and neoplastic cells and VEGF family consists of 6 molecules: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor. VEGF-A variant is the predominant and most critical regulator of the development of the vascular system. VEGF-A stimulates endothelial cell proliferation and act as an anti-apoptotic factor [3-8]. In human cancers, angiogenesis has been found to be associated with increased growth and metastatic potential of tumors. VEGF-A expression is increased in most tumors and is the most commonly studied angiogenic factor in various solid tumors as well as hemopoietic malignancies [9-16]. Generally, VEGF levels have been found to correlate with increased microvessel density, decrease in apoptotic index, increase in the incidence of metastasis, decrease in overall survival, and poor prognosis [9,10,12,14,17-19]. For this reason, anti-angiogenic strategies are logic and helpful approach in the management of the malignant tumors [2].

In this study, VEGF-A expression was studied in 89 cases with colorectal cancer (CRC) and some demographic and histopathologic variables were compared with VEGF-A expression to determine the prognostic significance of this expression.

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[2]**Abbreviations:** VEGF, vascular endothelial growth factor; CRC, colorectal cancer; LVI, lymphovascular invasion; SI, serosal invasion.

Materials and Methods

Patients

Eighty nine samples taken from cases with CRC operated and followed by the Departement of Surgery, Faculty of Medicine of Cukurova University were included in this study. Biopsy samples were re-evaluated and re-graded (SZ). Immunohistochemical staining for VEGF-A was performed as shown below.

Immunostaining

Immunohistochemical staining was performed by the streptavidine-biotin method. Sections with a 5-μm thickness were deparaffinized and incubated with 0.3% hydrogen peroxide for 20 min to block the endogenous peroxidase activity. The slides were incubated with trypsin for 30 min at 37°C. After being washed with PBS, sections were incubated with anti-VEGF-A (#6F25-100V6, Oncogene Research Products, La Jolla, CA, USA) for 1 h at room temperature. Followed by two washes with PBS, sections were incubated with biotinylated secondary antibody (#85-9143, Zymed Laboratories, San Francisco, CA, USA) for 10 min at room temperature; followed by washes and were treated with streptavidine peroxidase reagent for 10 min at room temperature. Sections were washed again twice and were incubated with diaminobenzidine solution for 5 min. Finally the slides were counterstaining with Mayers' hematoxylin and mounted.

Interpretation of immunohistochemical staining

Immunostaining for VEGF-A was considered as positive when unequvocal staining of membrane or cytoplasm was seen in tumor cells. Evaluation of the density of staining for VEGF-A was made semiquantitatively as fallows:

- Very scatered or no staining or less than 10% staining was scored as (–)
- More than 10%, less than 25% staining was scored as (+)
- More than 25%, less than 75% staining was scored as (++)
- More than 75% staining was scored as (+++)

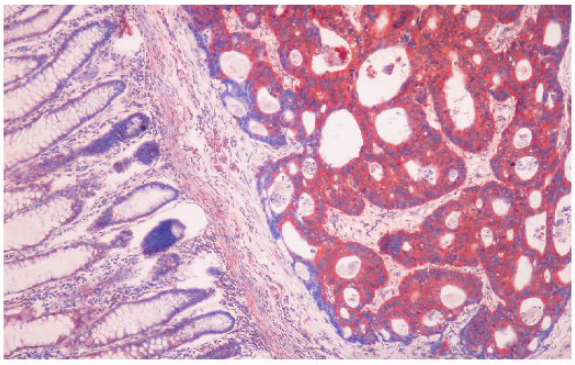

Figure 1: An example of VEGF-A (+++) staining in tumor tissue and negative staining in mucosa

Table 1: Demographic and histopathological data of the studied colorectal cancer patients

	Age	*P* value *value*	Involved lymph node	*P* value
Sex				
Male (47)	58.5 ± 11.8	0.892	2.4 ± 5.2	0.216
Female (42)	58.8 ± 15.0		1.27 ± 2.9	
Histological type				
Adeno (72)	57.9 ± 13.7	0.302	1.9 ± 4.5	0.866
Mucinous (17)	61.7 ± 11.7		2.1 ± 3.6	
Stage				
II (48)	57.0 ± 15.3	0.223	0.0 ± 0.0	< 0.001
III (27)	59.2 ± 10.3		4.4 ± 3.7	
IV (13)	64.2 ± 10.1		3.8 ± 8.7	
Grade				
I (22)	55.3 ± 15.5	0.274	0.3 ± 0.8	0.091
II (52)	59.2 ± 13.5		2.2 ± 5.0	
III (14)	62.5 ± 7.8		3.4 ± 4.3	
Metastasis				
No (76)	57.8 ± 13.6	0.078	1.6 ± 3.1	0.098
Ovary (2)	78.0 ± 9.9		0.5 ± 0.7	
Liver (10)	61.9 ± 8.6		4.8 ± 10.0	
Serosal invasion				
No (19)	58.2 ± 12.9	0.869	0.4 ± 1.0	0.065
Yes (57)	58.8 ± 14.4		2.7 ± 5.1	
Lymphovascular invasion				
No (56)	57.1 ± 13.8	0.111	1.8 ± 4.7	0.330
Yes (19)	63.0 ± 14.2		3.0 ± 4.2	
Relapse				
No (35)	57.7 ± 13.5	0.603	0.3 ± 0.8	0.020
Yes (44)	59.3 ± 13.8		3.2 ± 5.5	
VEGF-A				
(–) (24)	56.3 ± 13.5	0.769	1.4 ± 3.0	0.886
(+) (24)	59.8 ± 8.5		1.9 ± 2.8	
(++) (35)	59.7 ± 16.6		2.1 ± 5.8	
(+++) (6)	57.5 ± 7.5		2.8 ± 3.1	
VEGF-A				
(–)	56.3 ± 56.3	0.318	1.4 ± 3.0	0.488
(+) ~ (+++)	59.5 ± 13.3		2.1 ± 4.7	

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Table 2: Histopathological and demographic data of colorectal cancer patients with or withou VEGF-A expression in tumor tissues

	VEGF-A (–)	VEGF-A (+) ~ (+++)	*P* value
Sex			
Female	14	28	0.149
Male	10	37	
Localization			
Left	20	38	0.024
Right	4	27	
Histological type			
Adeno	19	53	0.508
Mucinous	5	12	
Grade			
I	10	12	
II	11	41	0.070
III	3	11	
Stage			
II	15	33	
III	8	19	0.226
IV	1	12	
Serosal invasion			
No	9	10	0.079
Yes	15	42	
Lymphovascular invasion			
No	20	36	0.185
Yes	4	15	
Relapse			
No	12	23	0.134
Yes	9	35	
Metastasis			
No	23	53	
Ovary	0	2	0.273
Liver	1	9	
Status			
Exitus	11	39	0.087
Alive	11	17	

Statistical analyses

The software package SPSS 12.0 (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analyses. Comparisons were applied using the student *t* test or one way ANOVA. Mann-Whitney U test or Kruskal-Wallis test was used when variables were not normally distributed. The categorical variables between the groups were analyzed by using the χ^2 test or Fisher's exact test. The rates of mean or median survival were estimated with the use of the Kaplan-Meier method and the curves were compared by the log-rank test. Cox proportional hazard regression model was applied to identify multivariate predictors (forward procedure, Wald method). Results were presented as mean ± SD and median plus range (from minimum to maximum). All reported *P* values are two-tailed.

Results

Eighty-nine cases with CRC were included in this study. Female/male ratio was 42/47, and the mean age was 58.6 ± 13.3. Histopathologically, 72 cases had adeno cancer and 17 cases had mucinous cancer. The tumor grade was defined in 88 cases: grade II, III and IV disease were identified in 22, 52 and 14 cases, respectively. Lymphovascular invasion (LVI) and serosal invasion (SI) were detected in 75 and 76 cases, respectively. There was no stage I disease; 48, 27 and 13 cases had stage II, III and IV diseases, respectively. Twelve cases had distant metastasis during the first presentation and there was no evidence of metastasis in 76 cases. Ten cases lost to follow up. Tumor relapse occurred in 44 cases. An example of VEGF-A (+++) staining was shown in Figure 1. The demographic and histopathological findings of the patients were shown in Table 1.

VEGF-A was studied by immunohistochemical staining and scored according to the stained tumor cell percentage as defined above. VEGF-A (–) was found in 24 cases; and there was (+), (++) and (+++) staining in 24, 35 and 6 cases, respectively. Totally 24 cases were evaluated as VEGF (–) and 65 cases (73%) evaluated to exhibit VEGF-A expression. VEGF-A (–) was found in 20 of 58 cases with left colon cancer, while only 4 of 31 cases with right colon cancer were VEGF-A (–)(P = 0.024). VEGF-A (–) tumors tended to have lower tumor grade (P = 0.07). Grade III disease was detected in 14 cases and 11 of them showed VEGF-A expression, while only 3 of the 24 cases with VEGF-A (–) tumors had grade III tumor. SI was detected in 15 of 24 cases with VEGF-A (–) tumors, however 42 of 52 VEGF-A-expressing tumors showed SI (P = 0.079). There was a trend for the higher rate of death in VEGF-A-expressing cases as compared with VEGF-A (–) cases (P = 0.087). Table 2 shows the histopahological and demographic findings in cases with *vs.* without VEGF-A expression and Table 3 shows these parameters according to the VEGF-A expression score.

Survival rates were calculated by the Kaplan-Meier method. The longest survival was found in cases with VEGF-A (–) tumor and the shortest survival was found in cases with VEGF-A (+++) tumor. The median survival rates for patients with VEGF-A (–), (+), (++), and (+++) tumors were 59, 47, 35, and 11 months, respectively (Table 4). Survival curves are shown in Figure 2. The survival rates according to the tumor stage and VEGF-A expression status have been shown in Table 5. Interestingly, the stage II and VEGF-A (–) cases lived more than 75 months. By Cox proportional hazards

Table 3: Demographic and histopathological variables of the colorectal cancer patients with different VEGF-A expression scores

	VEGF-A (–) (n = 24)	VEGF-A (+) (n = 24)	VEGF-A (++) (n = 34)	VEGF-A (+++) (n = 6)	*P* value
Age					
Mean ± SD	56.3 ± 13.5	59.8 ± 8.5	59.4 ± 16.7	57.5 ± 7.5	
Range	26-82	45-72	20-85	48-70	0.504
Median	54.5	62.5	62	56	
Sex					
Male	10	14	20	3	0.621
Female	14	10	15	3	
Involved lymph node					
Mean ± SD	1.4 ± 3.1	1.9 ± 2.8	2.6 ± 6.4	2.8 ± 3.1	0.480
Range	0-11	0-8	0-31	0-7	
Median	0	0	0	2	
Stage					
II	15	12	19	2	0.193
III	8	9	7	3	
IV	1	2	9	1	
Histological type					
Adeno	19	18	30	5	0.769
Mucinous	5	6	5	1	
Grade					
I	10	4	8	0	0.012
II	11	18	20	3	
III	3	1	7	3	
Serosal invasion					
Yes	15	15	23	4	0.079
No	9	4	6	0	
Lymphovascular invasion					
Yes	4	4	9	2	0.111
No	20	14	20	2	
Localization					
Left	20	12	24	2	0.030
Right	4	12	11	4	
Relapse					
No	12	7	14	2	0.538
Yes	9	12	19	4	

model, the stage IV disease and VEGF-A (+++) tumor were identified as having the most important influences upon overall survival (odds ratio: 5.1, 95% confidence interval: 2.0-13.0 and odds ratio: 3.6, 95% confidence interval: 1.0-12.7, respectively), followed by SI (odds ratio: 2.4, 95% confidence interval: 1.0-5.9)(Table 6).

Discussion

VEGF-A is a well characterised angiogenic factor and is known to play an essential role in the development of new vessels both in benign and malignant conditions. VEGF-A has been identified at the beginning of 1980s and has been recognized as an essential regulator of normal and abnormal blood vessel growth. In most of the tumors, VEGF-A has been found to be an independent poor prognostic indicator for disease-free-survival and overall survival. In 1990s, the monoclonal antibody targeting VEGF-A, which is named as bevacizumab, has been developed. Dramatic tumor suppression has been reported by this agent [20]. After this good response, studies about VEGF-A in CRC as well as other tumors have been increasing dramatically. In conclusion, VEGF-A is a key mediator of angiogenesis and blockage of angiogenesis is an effective and important strategy in treatment of human cancers. Therefore, to know the VEGF-A expression status of a tumor is important for two reasons: firstly to determine the prognosis of the tumor and secondly to incorporate the treatment targeting VEGF-A if the tumor expresses this factor.

Angiogenesis has been studied in gastrointestinal tumors, especially in CRC. Angiogenesis is an early event in colorectal carcinogenesis. VEGF-A has been found to be upregulated in polyposis of *APC* (*Adenomatosis Polyposis Coli* gene) knockout mice, and it has also been shown that angiogenic switch may occur between Tis and T_1, simultaneous to initiation of invasion, in the early development of colon cancer [21,22].

Microvessel density, CD31, platelet-derived endothelial cell growth factor (PDECGF), and VEGF-A are the most commonly used parameters to determine the tumor vascularity. Methodologically, immunohistochemical staining has been used in most of the studies, less frequently PCR and ELISA have been used for this aim. Pre and/or postoperative serum/plasma levels of VEGF using ELISA have been found to be poor prognostic indicator in some studies [23-27]. Generally, 10% or more staining with VEGF-A has been evaluated as positive expression like our study. In addition, the quantitative intensity of staining for VEGF-A has been assessed using scale of (+) to (+++)[19,28-32]. We also used this scoring method and found that 73 % of our cases showed VEGF-A expression; among these 27% showed poorer staining and 46% showed strong staining. VEGF-A has been found to be upregulated in higher than 60-70% of the colorectal tumors [32-34]. In most of the studies, it has been shown that higher VEGF-A expression is associated with higher probability of local and systemic metastasis and also shorter survival. However, there are some uncertainties about the prognostic significance of VEGF-A in some of these studies. VEGF-A has been found to be correlated with overall survival, lymph node status,

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Table 4: Survival of CRC patients according to clinicopathological variables

	Survival time (month)		Death/Total (n)	*P* value
	Mean	Median		
Histological type				
Adeno	52	36	39/65	0.2
Mucinous	28	24	10/12	
Stage				
II	69	60	18/40	< 0.001
III	24	24	22/26	
IV	17	17	9/11	
Metastasis				
Yes	18	6	8/10	< 0.001
No	52	36	41/67	
Serosal invasion				
Yes	35	24	38/49	0.005
No	74	–	8/19	
Lymphovascular invasion				
Yes	19	12	15/17	0.002
No	56	36	30/50	
Localization				
Left	45	24	35/52	0.9
Right	50	24	16/25	
Relapse				
No	89	–	8/34	< 0.001
Yes	21	12	39/41	
VEGF-A				
(–)	59	36	11/22	
(+)	47	36	13/19	0.02
(++)	35	24	20/30	
(+++)	11	12	5/6	
Total	49	24		

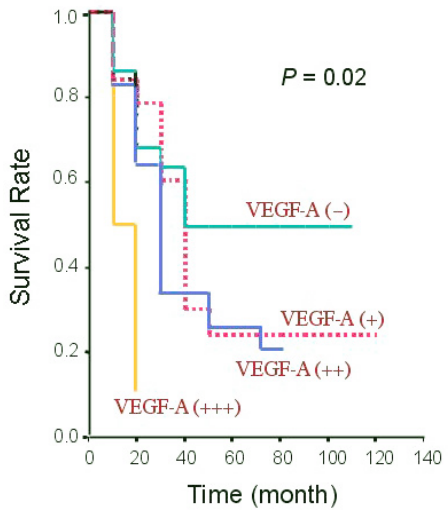

Figure 2: Comparision of survival curves according to VEGF-A expression status

Table 5: Survival time of patients according to tumor stage and VEGF-A expression

	Survival time (mean/median, month)		
	Stage II	Stage III	Stage IV
VEGF-A			
(–)	75/–	30/12	6/6
(+)	62/48	26/24	36/6
(++)	50/48	21/24	11/6
(+++)	11/6	11/12	6/6
Death/total	18/40	22/26	9/11
P value	0.3	0.07	0.5

relapse risk, hematogenous and/or liver metastasis, and higher SPF, but not with stage, time to recurrence, TNM, grade of the tumor, etc [17-19,30-32,35-42]. In our cases, we did not find the association between age and sex, histological type and grade of the tumor, and SI and LVI. There was an important association of involved lymph node number with tumor stage and tumor relapse. Although the correlation was not statistically significant, there was a trend for higher grade of tumors to exhibit more LVI potential and more distant metastasis. Higher number of involved lymph nodes was also associated with higher tumor grade, higher metastatic potential and more SI. When we evaluated the VEGF-A expression status, there was not an association of VEGF-A with age and sex. However, left colon cancer was significantly associated with less VEGF expression when compared with right colon cancer. In addition, there was a trend for higher VEGF-A expression with higher grade of tumor, higher SI, and more death. Most importantly in this study it has been found that survival times were the shortest in cases with strong VEGF-A expression and the difference was very obvious according to the VEGF-A expression status. No correlation between the VEGF-A expression level and all known prognostic factors may be due to two possibilities: (i) relatively small sample size in our study, and (ii) as seen in world literature, VEGF-A expression is an important poor prognostic indicator but there are no

Table 6: Results of Cox proportional hazards model

	Regression coefficient (B)	Odds ratio	95% Confidence interval	*P* value
Age	0.001	1.0	0.9-1.0	0.912
Sex	0.273	1.3	0.7-2.4	0.396
Localization	0.198	1.2	0.6-2.5	0.601
Lymphovascular invasion	0.437	1.5	0.7-3.1	0.225
Serosal invasion	0.897	2.4	1.0-5.9	0.047
VEGF-A (–)				Ref.
VEGF-A (+)	0.061	1.1	0.4-2.6	0.898
VEGF-A (++)	0.401	1.5	0.6-3.4	0.351
VEGF-A (+++)	1.283	3.6	1.0-12.7	0.046
Stage II				Ref.
Stage III	0.720	2.1	0.9-4.5	0.072
Stage IV	1.636	5.1	2.0-13.0	0.001

significant correlations between VEGF-A and all the poor prognostic indicators defined for colon cancer.

It is a challenge for the therapeutic approach to cope with the lymph node (–) or stage II CRC disease. It is known that the adjuvant chemotherapy is not standard and the necessity of adjuvant chemotherapy in these cases is controversial. However, 20% of these cases die from CRC metastasis and/or recurrence. Only some factors such as perforation, obstruction or some chromosomal changes have been suggested as high risk category among cases with the stage II disease. A retrospective analysis suggested that patients with poor prognostic indicators might benefit from adjuvant therapy, however there is no prospective data about this matter [43,44]. High/strong microvessel density and/or VEGF-A expression have been found to be associated with higher risk of relapse and shorter survival in patients with lymph node (–) or stage II CRC cancer [30,31]. According to these findings, VEGF-A may be used to identify the patients at high risk of relapse who may benefit from adjuvant strategies such as chemotherapy and/or anti-angiogenic treatment. In our study, there were 48 cases with the stage II disease and two thirds of these showed VEGF-A expression. As suggested by some other studies, VEGF-A may be predictive to determine the population requiring adjuvant chemotherapy in stage II disease.

Anti-angiogenic treatment is a novel approach in various malignant tumors. Targeting the tumor vasculature has been found to be useful both *in vitro* and *in vivo* studies. CRC is an important model to target the VEGF-A. It is known that the antibody targeting the VEGF-A (bevacizumab) and small molecule inhibitors specific for the receptor tyrosine kinase for VEGF (SU5416 and ZD6474) are the important drugs for tailored treatment in clinical setting. These agents have been used with or without chemotherapy and have been found to be effective and clinical studies are ongoing [42,45-52]. For these reasons, to determine the tumor angiogenesis by VEGF or other parameters will be informative for tumor biology/prognosis and also tailored treatment in cases with CRC as seen in other tumors.

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