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Evaluation of VEGF Immunohistochemical Expression and Correlation with Clinicopathologic Features in Colorectal Cancer

Original Article

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ABSTRACT

Background:
Vascular endothelial growth factor (VEGF) is a signal protein produced by cells that stimulates angiogenesis and increased vascular permeability. The prognostic value of VEGF expression in colorectal cancer is unclear as shown by the discordant results still reported in the literature. This study was designed for the evaluation of VEGF expression and correlation with clinicopathologic features in colorectal cancer.

Materials and Methods:
In this study, 60 paraffin – embedded blocks including colorectal cancer specimens and adjacent non-neoplastic tissues were evaluated for VEGF immunohistochemical expression and their correlation with clinicopathologic features among the patients who admitted in Sari Imam Khomeini hospital.

Results:
Among the 60 patients (30 males,30 females) 51.7% (31 cases) showed VEGF overexpression (High staining) , while it was 1.7% (1 case) in adjacent normal tissue (p=0.004). VEGF expression was correlated with stage(p-value<0.001),grade(p-value<0.001), lymph node metastasis(p-value<0.001), recurrence(p-value<0.001) and tumor size (p-value<0.001). In other clinicopathologic parameters, no relationship with VEGF expression was seen.

Conclusion:
These findings provide further evidence for the role of VEGF in colorectal carcinogenesis. Some previous studies showed discordant results, consequently more studies with larger sample size to confirm results are recommended.

Keywords: Colorectal cancer; VEGF; Clinicopathology; Immunohistochemistry

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INTRODUCTION

Colorectal carcinoma is common in Northwest Europe, North America and other parts of Anglo saxon but relatively low in Africa, Asia and some parts of South America(1-3). Colorectal cancer is one of the deadliest cancers and responsible for more than half a million deaths globally each year(4).Also, cancer in Iran has increased at a significant rate over the past years (5). Men and women are equally affected, and the mean age of the colorectal carcinoma diagnosis was 62 years. Colorectal cancer is highly curable form of the gastrointestinal cancer. Both the environmental and genetic factors in etiology and pathogenesis of colorectal cancer are important(6,7).

In recent years, several studies have been

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conducted pertaining to the relationship between the expression of various markers with clinicopathologic features and survival of patients in colorectal cancer, and through these studies, new techniques based on medical treatment for prevention and treatment of colorectal cancer have been used.

The pathologic stage is considered as an important factor in association with prognosis in colorectal cancer, however, more accurate indicators about the biological behavior of colorectal cancer are required to improve the specificity and sensitivity of medical treatment. Angiogenesis plays an important role in the growth and spread of cancer, but its role as a prognostic indicator remains controversial. Perhaps the most important clinical application of tumor angiogenesis is the development of antiangiogenic therapy that could be used as a practical treatment(8).

VEGF is a signal protein produced by cells that stimulates the growth of blood vessels (angiogenesis). This protein marker increased vascular permeability and has the highest potential of angiogenesis(9-13). VEGF signal transduction pathway is composed of 5 glycoprotein belonging to the VEGF family including VEGFA VEGFC, VEGFB, VEGFD, and placental growth factor (PLGF); 3 receptors (VEGF receptor-1 [FTL-1], VEGF receptor-2 [FLK-1 / KDR]and VEGF receptor-3 [FLT-4]) and 2 co- receptors (NRP-1, NRP-2) (14-16) .The receptors related with various VEGF ligands are tyrosine kinase and there are in vascular endothelial cells(15,16).

Angiogenesis plays an important role in the development of colorectal cancer. Evidence-based clinical and preclinical studies indicated that VEGF is an important angiogenic factor in colorectal cancer. In some studies, to evaluate the relationship between VEGF expression with clinicopathologic features there were significant correlations between the incidence of this tumor marker with stage, anatomic location and specific long-term survival of the patients(17). In few studies, there was a significant correlation with tumor grade(18). The prognostic value of vascular endothelial growth factor (VEGF) in colorectal cancer is unclear and in the articles that were published, the controversy could be seen(19).

Based on the importance and prevalence of colorectal cancer in Iran and controversies about VEGF, we decided to investigate the relationship between the VEGF expression with clinicopathologic features in colorectal cancer by immune histochemical method to provide better conditions for further research and new treatments in this area.

MATERIALS AND METHODS

Our study was a case-control method. This study aimed to investigate the relationship between the expression of VEGF marker with clinicopathologic features in colorectal cancer, on paraffin blocks in Sari Imam Khomeini Hospital Archives of Pathology between 2008 until 2014.

The study included patients who previously underwent colon and rectum surgery. Also in the control group, paraffin blocks of adjacent normal looking tissue were used. The sample size in this study was compared with previous studies and was calculated using the formula. During the study, 60 patients with colorectal cancer were in the case group and the control group received the same number. The clinicopathologic parameters that were evaluated in this study included: age, gender, tumor type, tumor location, tumor size, histological grade, lymph node metastasis (N), distant metastasis (M), stage and recurrence. The patients' age were divided into two groups (under 50 and above 50 years). Tumor size and lymph node metastasis were divided into three groups (less than 2 cm, 2 to 5 cm and above 5 cm) and (Lack of involvement- 1 to 3, involvement-4 and more than 4 involvement) respectively.

The patients who had preoperative chemotherapy or radiotherapy or family history of IBD or polyposis were excluded. All samples were fixed in formalin 10% and paraffin blocks were used. The patient data were entered in the questionnaire using their dossier and for follow-up, their addresses and telephone numbers were used.

The following archival paraffin blocks were removed and from the areas of tumor and adjacent normal looking tissue several slides were prepared with hematoxylin – eosin staining.

During the investigation of slides in addition to detecting tumor, other microscopic parameters such as tumor type and differentiation (histological grade) were also evaluated.

Immunohistochemical staining was performed on the samples with a diameter of 4 mm, which were cut by a device. At first, the cut samples were placed on specific slides for a period of one hour at 60 ° C in incubator. For the removal of paraffin, xylene solution and 96% ethanol were used and the slides were then rinsed under running water. After drying, the slides were immersed into a container containing 1% hydrogen peroxide and methanol. After 10 minutes, they were transferred to reach boiling point by autoclaving at a pressure 1.5 atm. Then the samples were ejected until solution temperature increases to a value equal to the

room temperature. After washing in running water and wash buffer Dako Pen was used to determine the border of tissues and diagnostic kits VEGF antibody was used to completely cover the surface of the tissues. In the next step, the samples were exposed for 60 min within Envision at room temperature. After 2 washes in wash buffer, DAB solution was poured onto glass slides and In case of a change in color after 1 to 2 minutes, the slides were placed in wash buffer for 2 minutes again. Then the slides were washed in distilled water and counterstained in Mayer's Hematoxylin Solution. Afterwards, the slides were fixed with xylene and finally mounted with entellan glue. Eventually the slides were studied by two expert pathologists who had no clinicopathologic knowledge of the patients' data and VEGF expression and intensity of staining were reported.

To improve the accuracy of diagnosis and to determine the staining of cells, multiple microscopic fields in low and high-power(X100-X400) were examined and the percentage of the stained tumor cells was estimated.

Staining intensity of VEGF was graded on a scale with four grades:

0: positive staining in less than 5% of tumor cells.

1+: positive staining between 5% untill 25% of tumor cells.

2+: positive staining between 26% untill50% of tumor cells.

3+: positive staining Over 50% of the tumor cells.

0 to 1+ grade as low staining and +2 to 3+ grade as high staining were considered for VEGF. VEGF protein was a cytoplasmic marker and cell cytoplasm appeared brown in color in the positive cases.

Finally, the obtained results were analyzed by statistical software SPSS (IBM SPSS Statistics 20.0.1). Chi –square and Kendall and Fisher's exact tests were used to analyze the relationship between the expression of VEGF in colorectal cancer with clinicopathologic features. A p-value less than 0.05 was considered statistically significant.

RESULT

A total of 60 patients (30 men and 30 women) were enrolled and evaluated in this study. The clinicopathologic findings in patients under study were summarized in Table 1. Patients mean age was 58.27 ± 12 years, regardless of gender (57.14 to 61.39 with 95% CI). The significant difference between the average age of men (57.67±12.1) and women (58.87±12.3) was not observed (*p*-value>0.05). After immunohistochemical staining, VEGF expression and intensity of staining in the two groups (case & control) were compared (Table 2).

By definition (see Materials and Methods) in case group 51.7% (n=31) samples showed high staining(2+ , 3+) and 48.3% (n=29) revealed low staining(0 , 1+) but in control group only 1.7%(n=1) showed high staining (figures 1 & 2).

By comparing VEGF expression with clinicopathologic features in colorectal cancer and the statistical analysis of data, a significant association between the expression of tumor marker with tumor size, histologic grade, number of involved lymph nodes, and the stage of the disease was observed.

In addition, after treatment initiation and early follow-up of the patients, there was a significant relationship between VEGF expressions with recurrence of disease.

None of the patients were observed to have distant metastases at the time of initial diagnosis. However, to pursue further follow-up, 7 patients showed distant metastases. Despite the greater number of metastases in patients with high staining no significant association between distant metastases with the expression of marker was seen.

A significant association between the histological grade, number of involved lymph nodes, and the stage of disease with the VEGF staining intensity in tumor cells was identified. Significant differences in VEGF staining intensity was observed between stage III compared with stage I and II. The number of involved lymph nodes was correlated with increasing marker staining intensity. The comparison of clinicopathologic parameters of patients with VEGF expression are listed in Table 3.

In our study, tumor-adjacent normal tissues were used as control group, then VEGF expression and intensity of staining in these tissues were evaluated. Since the control group was selected from the adjacent normal tissues, thus both groups were quite matched for age and sex, the reason to analyze and compare VEGF expression in the two groups McNemar test was used and the results showed significant differences between the groups (*p*-value=0.004).

DISCUSSION

Colorectal cancer still is responsible of cancer-associated high morbidity and mortality in both men and women worldwide(20). Several studies in different countries on the relationship between VEGF expression with clinicopathologic parameters were conducted regarding colorectal cancer.

Riyad Bendardaf et al., studied the relationship

Table 1: Clinicopathologic findings in patients with colorectal cancer

Recurrence	Stage of Tumor	Distant metastasis	Lymph node metastasis	Tumor grade	Tumor size	Natomic location	Tumor type	Gender	Age
16.7% (n=10) Had recurrence	23.3%(n=14) in stage I	11.7% (n = 7) with distant metastasis	55% (n=33) Lack of involvement	46.7% (n=28) well-differentiated	20% (n=12), under 2 cm	11.7% (n=7): ascending colon	90% (54 cases), adenocarcinoma (NOS)	50% (n=30) male	35%(n=21) under 50 years
83.3%(n=50) N0 recurrence	40%(n = 24) in stage II A and II B	88.3% (53 cases) without distant metastases	31.7%(n=19) from 1 to 3	30% (n = 18), moderately differentiated	65% (n = 39) from 2 to 5 cm	13.3% (n=8), descending colon	10% (6 cases), mucinous	50% (n = 30) female	65%(n= 39) above 50 years
	36.7%(n=22) in stage III A, III B and III C		13.3%(n=8), 4 and more than 4	23.3% (n=14) Poorly differentiated	15%(n=9) above 5 cm	21.7% (n=13): Sigmoid 33.3% (n=20): the cecum 20% (n=12): rectal			

Table 2: Comparison of the expression and VEGF staining intensity in both groups (case & control)

Groups \ Staining intensity	Low		High	
	0	+1	+2	+3
Case	35%(n=21)	13.3%(n=8)	30.0%(n=18)	21.7%(n=13)
Control	78.3%(n=47)	20%(n=12)	1.7%(n=1)	0

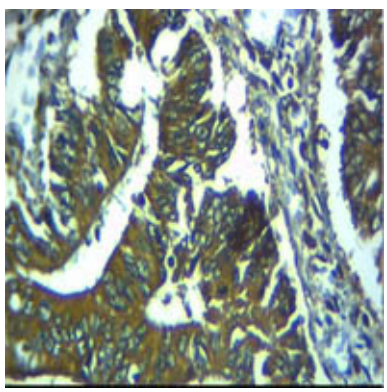

Fig.1: 3+ cytoplasmic staining (High) in the tumoral cells with VEGF marker in IHC staining (magnification 400X).

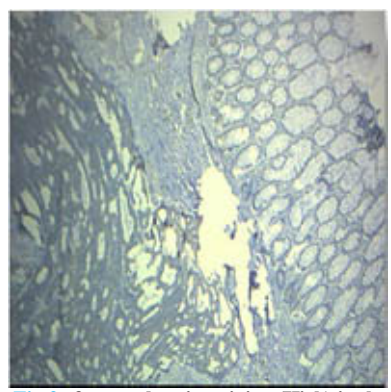

Fig.2: 3+ cytoplasmic staining (High) in the tumoral cells with VEGF marker (left) - Non-staining (Low) in tumor- adjacent normal looking tissue (right) (magnification 100X).

between the expression of VEGF marker in colorectal cancer with tumor location and stage of disease. Finally, a strong correlation was observed between the expression of markers with the stage of disease (p=0.005) and the VEGF expression in left colon and rectum compared with the right colon was significantly higher (61% vs. 45%)(17). In our study, VEGF expression had a correlation with disease stage, but no correlation was seen between expression marker and tumor location.

In a study conducted by Ali F. Hashim et al., the VEGF expression in 51.9% of colorectal cancer patients was observed and compared with 18.2% in the control group which was considerably higher. IHC positivity for VEGF was associated with malignancy grade but there was no significant difference observed between the 3-stages of colorectal cancer(I, II, III) with VEGF expression (18). Our study confirmed the relationship between VEGF expression with the grade of malignancy. Furthermore, a statistically significant correlation with disease stage was seen,

Table 3: The relationship between VEGF expression with clinicopathologic parameters in colorectal cancer

Clinicopathologic parameters	Relationship with VEGF expression	*p*-value	Type of test
Age	No	0.287	Chi- square
Gender	No	0.12	Chi -square
Tumor type	No	0.196	Fisher's exact test
Anatomic location	No	0.211	Fisher's exact test
Tumor size	Yes	<0.001	Kendall test (Kendall=0.519)
Tumor grade	Yes	<0.001	Kendall test (Kendall=0.669)
Lymph node metastasis	Yes	<0.001	Kendall test (Kendall=0.486)
Distant metastasis	No	0.1	Fisher's exact test
Stage of tumor	Yes	<0.001	Kendall test (Kendall=0.573)
Recurrence	Yes	<0.001	Fisher's exact test

not to mention an increase in staining intensity of VEGF was correlated with disease stage.

In another study by Kyriakos Zafirellis et al., they evaluated the prognostic significance of VEGF expression in patients with colorectal cancer by immunohistochemical analysis. In this study, the intensity of staining in tumors with lymph node metastasis was higher than tumors without lymph node involvement (p<0.0001). Additionally, the intensity of staining in stage III compared with stage I and II was higher (p<0.0001). The number of involved lymph nodes in tumors with high staining was significantly more than those tumors with low staining (p-value=0.031)(19). The results of this study were similar to our study, a significant relationship between the VEGF expression with lymph node metastasis was seen and the number of involved lymph nodes was correlated with increasing marker staining intensity. Likewise, the increase in VEGF staining intensity was correlated with the stage of the disease.

In the study of Yalcin Kekec et al., patients who did not show expression of VEGF in tumor cells, histologic grade were significantly lower(20).

Dan Cano et al. examined the expression of HIF-1alpha and VEGF in colorectal carcinoma and its correlation with clinical and prognostic implications. In the end, it was found that the expression of HIF-1alpha and VEGF in patients was 54.93% and 56.34 %, respectively, and the expression of both markers significantly was associated with tumor stage, lymph node and liver metastasis (p<0.05) (21). In our study, unlike the results of this study, no significant correlation was found between the expression of VEGF with distant metastasis.

In another study by Qingguoli et al., they evaluated the clinicopathologic and prognostic value of VEGF and HER-2 / neu markers expression in colon carcinoma. In this study, the expression of VEGF and HER-2 / neu in tumor cells was 55.5 and 15.5 respectively. VEGF expression significantly was correlated with tumor size, stage, lymph node metastasis, distant metastasis (p<0.05)(22). The results of this study in 4 mentioned variables except for distant metastases in other cases were confirmed by our study.

Also Mohammed S. Hedaya and et al., in their study showed COX-2 & VEGF were over expresed intensly in the advanced stage and grade of the CRC samples obtaind during surgery(23).

In the study of Linchun Wen and et al., revealed that improved overall survival rates were significantly associated with the absence of VEGF expression. VEGF expression related to lymph node metastasis, histological grade and the stage of the tumor that similar to our study(24).

Overall, our results demonstrate a strong correlation between the expression in tumoral cells with several clinicopathologic parameters. These parameters included: stage of disease, histologic grade, lymph node metastasis, recurrence and tumor size. But no significant correlation was found between the VEGF expression with age, gender, tumor type, location and distant metastasis.

CONCLUSION

This study showed a strong relationship between marker VEGF expression with some clinicopathologic features in colorectal cancer that the most important of them can be noted to the histologic grade and the stage. Both mentioned variables are correlated with VEGF expression and marker staining intensity. These findings provide further evidence for the role of VEGF in colorectal carcinogenesis. Given the importance of

colorectal cancer and discordant results in the some previous literature, more studies on VEGF markers seem necessary to better understand its relationship the prognosis of the disease and the development of new medical treatments (anti-VEGF drugs).

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