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Original Research Article

Analysis of Histological Prognostic Factors in Colorectal Carcinoma and Relationship of VEGF and E-Cadherin Expression in Prognostic Assessment

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Abstract

Colorectal carcinomas (CRC) are associated with several histopathological and molecular factors of prognostic and hence therapeutic implications, including for epithelial mesenchymal transition (EMT) and angiogenesis. Downregulation of E-cadherin is a crucial step in EMT, directly related to tumor progression, invasion, and metastasis. Vascular endothelial growth factor (VEGF) induces angiogenesis and thereby tumour progression and metastases. Our objective was to analyse VEGF and E-cadherin expression in patients with CRC and to effectively assess the relationship of these with histopathological factors. 50 cases of CRC obtained over eighteen months duration, were included in the study. Haematoxylin and Eosin-stained sections were studied for histopathological factors. Immunohistochemistry with E-Cadherin and VEGF were performed on representative sections and evaluated. Histopathological features of importance noted were low grade tumour budding (55.5%), Infiltrative invasive front (83%), lympho-vascular invasion (50%). Preserved expression of E-Cadherin was seen in 66% of the cases, and 34% reduced expression, predominantly noted in patients above the age of 50, increased tumour size, mucinous variants, advanced TNM stage, lymph node involvement and high-grade tumour budding. VEGF staining was seen in 64% of the cases with varying intensity. Increased VEGF expression was noted in poorly differentiated tumours, advanced Duke's stage and with lymph node metastasis. Colorectal carcinomas with high grade tumour budding were associated with lymph vascular invasion, infiltrative invasive front and significantly correlated with TNM stage. High grade tumours and adverse histopathological prognostic factors revealed a reduced E-cadherin expression and increased VEGF expression.

Keywords: Colorectal carcinoma, E-cadherin, Vascular endothelial growth factor, Immunohistochemistry, Histopathology, oncopathology.

INTRODUCTION

Colorectal carcinoma is the third most common cancer worldwide [1]. Colorectal carcinoma is morphologically diverse with several histological types; adenocarcinoma being the most common. Several clinicopathological indicators and biological markers have been identified at the cellular level in colorectal carcinomas with an impact on prognosis, few of the biological markers being used are Cytokeratin, Beta-catenin, MUC2, MUC 4, VEGF, E-cadherin, etc. [2]. E cadherin belongs to the 'classical'/ type-I cadherin subfamily, encoded by the CDH1 gene: a calcium-dependent transmembrane glycoprotein. It is present in the adherens junctions at the basolateral aspect of epithelial cells and contributes to cell-cell interactions [3]. E-cadherin downregulation is regarded as an essential step in the epithelial-mesenchymal transition and is related to tumor progression, loss of differentiation, invasion, and metastasis [4]. Vascular endothelial growth factor (VEGF) belongs to VEGF platelet-derived growth factor (PDGF) family of structurally related mitogens. VEGF is a homodimeric glycoprotein with a molecular weight of about 45 kDa. It induces angiogenesis through a direct effect on

endothelial cells [5]. VEGF in addition to being a key factor in angiogenic switch and vascularization of the cancer growth, enhances the secretion and activity of certain enzymes that can degrade the ECM and encourages tumor growth by protecting neo-vasculature from apoptosis. Thus, VEGF is not only considered an essential component of tumor angiogenesis but also a critical factor in tumor development [5]. We conducted this study intending to analyze VEGF and E-cadherin expression by immunohistochemistry in colorectal carcinoma. Also, to evaluate the correlation of E-cadherin and VEGF expression with laboratory and histopathological prognostic factors.

METHODOLOGY

This study was conducted on a total of 50 cases of colorectal carcinoma of which 14 were biopsies and 36 were resected specimens. The cases were collected over a period of 18 months. The relevant clinical and radiological details were collected after obtaining clearance from the Institutional Ethical Committee. After adequate fixation of the specimens in neutral buffered formalin, the representative sections were then processed for routine paraffin embedding, stained with hematoxylin and eosin, and studied for histopathological features. Representative sections were then selected for immunohistochemical analysis with VEGF and E- Cadherin antibodies.

Interpretation of E Cadherin staining

The quantity of E-cadherin positive cells is scored as 0 to 4 by counting at least 100 tumor cells in areas of heterogeneous E-cadherin expression. The intensity of E-cadherin expression is also assessed and a composite score is given. The scoring of tumor immunostaining was based on the percentage of immunopositive cells. Thereby, no positive cells were given a score of 0, <10% positive cells scored as 1, 10-50% as 2, 50-80% as 3, and >80% were assigned a score of 4. This score was further multiplied by the staining intensity score i.e. 0- negative; while weak, intermediate, and strong were given a score of 1-3, yielding total scores of 0–12, and a score of more than 4 was considered positive [6].

Interpretation of VEGF Immunohistochemistry

VEGF expression in colorectal carcinoma was scored semi-quantitatively. The absence of immunoreactive cells was given a score of 0, <5% as 1, 5- 50% as 2, and >50% of immunoreactive cells as 3. The intensity of staining was further scored semi-qualitatively as 0 for negative while weak, intermediate, and strong as 1, 2, and 3 respectively. The final score was then given as the sum of the two scores obtained and grouped as negative: 0, weak: 2, moderate: 3, and strong: 4-6. However, moderate, and strong immunoreaction were only considered positive for statistical evaluation [7]. The tumors were classified according to the classification proposed by the World Health Organization (WHO). Histological grading of the tumors was also done as per the guidelines proposed by WHO and American Joint Committee on Cancer (AJCC). Tumor budding was assessed and grouped into a three-tier system as used by the Japanese Society for Cancer of the Colon and Rectum; counts of 0–4 was classified as low-grade, 5-9 buds as intermediate grade and counts of 10 or more foci were classified as high-grade budding8. Inflammatory reaction was assessed using the four-degree scale introduced by Klintrup *et al.,* (2005) wherein a score of 0 indicated no increase in inflammatory cells. Score 1 was assigned for a mild and patchy increase of inflammatory cells at the invasive margin, but no destruction of invading cancer cell islets by the inflammatory cells, score of 2 was given when inflammatory cells formed a band-like infiltrate at the invasive margin with some destruction of cancer cell islets by inflammatory cells and a score of 3 indicated a prominent inflammatory reaction, with the formation of a cup-like zone at the invasive margin, with the destruction of cancer cell islets often being present [9]. Lymphovascular invasion, perineural invasion, tumor necrosis, the depth of invasion, the nature of the invasive front, the number of lymph nodes involved, and lymphatic emboli were the other histopathological factors recorded.

STATISTICAL ANALYSIS

The data obtained were entered into an excel sheet and analyzed using the Statistical Package for the Social Sciences version 23 statistical program (SPSS Inc, Chicago). Frequencies and percentages of all the variables were computed. The data thus analyzed, were correlated for relevant findings. Correlation of the expression of E-cadherin and VEGF positive cases along with the histopathological characteristics of the tumours were determined using the Pearsons Chi-square test and Fischer exact test (when n <5). P values less than 0.05 were considered to be statistically significant.

RESULTS

A total of 50 cases of colorectal carcinoma were included in the study. Age distribution in colorectal carcinomas showed a predominance in the 5th decade (28%), with a male-to-female ratio being 1.5:1. Rectum was the commonest site (28%), and adenocarcinoma was the most common histological subtype accounting for 80% (n=40) of the cases in our study. Mucinous carcinoma was seen in 14% and one case each of signet ring cell carcinoma, adenocarcinoma with areas of neuroendocrine carcinoma, and mucinous carcinoma with areas of neuroendocrine carcinoma respectively were encountered in this study. Most of the tumors were well differentiated (66%). Low-grade tumor budding was seen in 56% of the cases (n=20), high-grade tumor budding in 14% (n=5) and one showed intermediate-grade tumor budding. A majority, i.e., 44% of cases in

our study displayed a mild inflammatory infiltrate (score 1) and 22% of cases showed severe inflammatory infiltrate (score 3). The cases were staged according to the tumor-node-metastasis staging system. Of the 50 cases, 22 cases (61%) were grouped under the pT3 group, followed by pT2 (17%). Cases in pT4a, pT4b, and pT1 were 4 (11%), 3 (8%), and 1 (3%) respectively. Many cases had a nodal stage of pN0, with pN1a being the most common node positive stage. Of the 50 cases, only one case had proven metastasis. Lymphovascular invasion (LVI) was seen in 50% of the cases (n=18) while perineural invasion was seen in 12 cases (33%).

E- cadherin expression

In this study, based on the intensity of expression and proportion of cells expressing E cadherin, the cases were grouped into 2 categories cases showing preserved E-cadherin expression and the ones showing reduced expression. It was noted that 31 (62%) of patients had a reduced E- Cadherin expression and the remaining 19 (38%) cases. Age was not a defining factor for E-Cadherin expression, taking a cut-off of 50years. When the histopathological type was correlated, it is seen that a severe degree of reduction was noted more in mucinous carcinoma, thus explaining the higher and faster rate of invasion associated with it. Tumor differentiation and grade had no significant association with E-cadherin expression. When the surrounding inflammation was seen as severe, a corresponding marked loss of E-Cadherin expression was seen (Table 1). There was no significant correlation between the reduced expression of E-cadherin and age, gender, or tumour differentiation.

Table 1: Correlation of E-Cadherin expression with clinicopathological variables in colorectal carcinoma (n=50)

Clinicopathological variables	E-Cadherin expression		
	Preserved expression (%)	Reduced expression (%)	p-value
Age			
<50 (n=23)	9 (18)	14(28)	0.9
>50 (n=27)	10 (20)	17(34)	
Gender			
Male (n=30)	12 (24)	18 (36)	0.7
Female (n= 20)	7 (14)	13 (26)	
WHO grading.			
Well differentiated (n= 23)	7 (14)	16 (32)	0.4
Moderately differentiated (n= 22)	10 (20)	12 (24)	
Poorly differentiated (n= 5)	2 (4)	3 (6)	
AJCC grading			
High grade (n= 6)	3 (6)	3 (6)	1
Low grade (n= 44)	16 (32)	28 (56)	
Inflammation			
Mild (n= 22)	11 (22)	11 (22)	0.07
Moderate (n= 17)	6 (12)	11 (22)	
Severe (n= 11)	2 (4)	9 (18)	

Few histopathological factors could be assessed only on resected specimens. These factors were also correlated with E-Cadherin and VEGF expression. TNM stage IV tumors and 59% (n=10) of stage 3 tumors showed a reduced expression of E-Cadherin. Statistically significant correlation could not be established between tumor budding and E-cadherin expression in this study, but it was noted that 80% (4 of 5) of the cases with high grade tumor budding had reduced E- Cadherin expression. This could effectively mean that E-Cadherin expression would be reduced with high grade tumor budding (Table 2). Reduced E-Cadherin expression was seen in 63% cases which had lymph node involvement. However, there was no correlation with lymph vascular invasion, lymphatic emboli, lymph node ratio, necrosis and perineural invasion. The reduced expression of E-cadherin showed a statistical correlation with maximum diameter of the tumor (p- 0.03). Tumors of larger size show a severe reduction in expression of E-cadherin.

Table 2: Carcinoma colon: E- Cadherin expression v/s pathological factors in resected specimens (n=36)

Histopathological factors	Total (%)	Preserved Expression	Reduced Expression	P- value
Dukes stage				
Stage A	3 (8)	1	2	0.7
Stage B	14 (39)	7	7	
Stage C	19 (53)	7	12	
Maximum diameter of the tumor				
<4	8 (22)	6	2	0.03

Histopathological factors	Total (%)	Preserved Expression	Reduced Expression	P- value
>4	28 (78)	9	19	
Tumor budding				
Low grade	20 (55)	9	11	
Intermediate grade	1 (3)	1	0	0.9
High grade	5 (14)	1	4	
Absent	10 (28)	4	6	
Lymphovascular invasion				
Present	18 (50)	10	8	0.09
Absent	18 (50)	5	13	
Lymph node involvement				
Present	19 (53)	7	12	0.5
Absent	17 (47)	8	9	
Lymphatic emboli				
Present	6 (17)	2	4	0.6
Absent	30 (83)	13	17	
Perineural invasion				
Present	12 (33)	5	7	1.0
Absent	24 (67)	10	14	
Invasive front				
Infiltrative	30 (83)	12	18	
Expansive	4 (12)	2	2	0.7
Pushing	2 (5)	1	1	
Lymph node ratio				
<0.2	22 (61)	9	13	0.9
>0.2	14 (39)	6	8	
Desmoplasia				
Mild	20 (56)	9	11	
Moderate	7 (19.5)	3	4	0.5
Severe	2 (5)	1	1	
Absent	7 (19.5)	2	5	
Necrosis				
Absent	12 (33)	5	7	
Luminal	4 (11)	2	2	
Focal	9 (25)	3	6	0.9
Moderate	5 (14)	3	2	
Extensive	6 (17)	2	4	

Vascular Endothelial Growth Factor (VEGF) expression:

Expression of VEGF based on the proportion of tumor cells expressing VEGF as well as the intensity of expression, was divided into four categories as described earlier. However, moderate and strong immunoreaction were only considered as positive for statistical evaluation. 52% (n=26) cases displayed a strong VEGF expression while 36% (n=18) showed a negative expression. A weak and moderate expression of VEGF was seen in the other 12% (n=6) of cases each. A strong VEGF expression (57%) was noted in more cases with advancing Duke's stage in the presence of lymphovascular invasion (75%) and nodal metastasis (Table 3).

Table 3: Carcinoma colon- VEGF expression v/s clinicopathological parameters (n=50)

Clinicopathological parameters	Negative (%)	Positive (%)	p value
Age			
<50 (n= 23)	9(18)	14(28)	0.4
>50 (n= 27)	12 (24)	15(3)	
Gender			
Male (n= 30)	15 (30)	15(30)	0.1
Female (n= 20)	6 (12)	14(28)	
Inflammation			
Mild(n=22)	8(16)	14 (28)	

Clinicopathological parameters	Negative (%)	Positive (%)	p value
Moderate (n=17)	8 (16)	9(18)	0.7
Severe(n=11)	5 (10)	6 (12)	

Table 4: Carcinoma colon: VEGF expression V/s Histopathological parameters in resected specimens (n=36).

Histopathological factors	Negative	Positive	p-value
Stage			0.3
Stage I(n=6)	4	2	
Stage II(n=10)	5	5	
Stage III(n=17)	5	12	
Stage IV(n=2)	1	1	
Lymphovascular invasion			
Present (n=1)	5	12	0.1
Absent (n=18)	9	7	
Lymph node involvement			
Present (n=19)	10	9	0.1
Absent (n=17)	6	11	
Lymphatic emboli			
Present (n=6)	2	4	0.4
Absent (n=30)	14	16	
Perineural invasion			
Present (n=12)	3	9	0.09
Absent (n=24)	13	11	
Lymph node ratio			
<0.2 (n=22)	12	10	0.1
>0.2 (n=14)	4	10	
Necrosis			
Absent (n=12)	7	5	
Luminal(n=4)	1	3	0.75
Focal(n=9)	4	5	
Moderate(n=5)	2	3	
Extensive(n=6)	2	4	

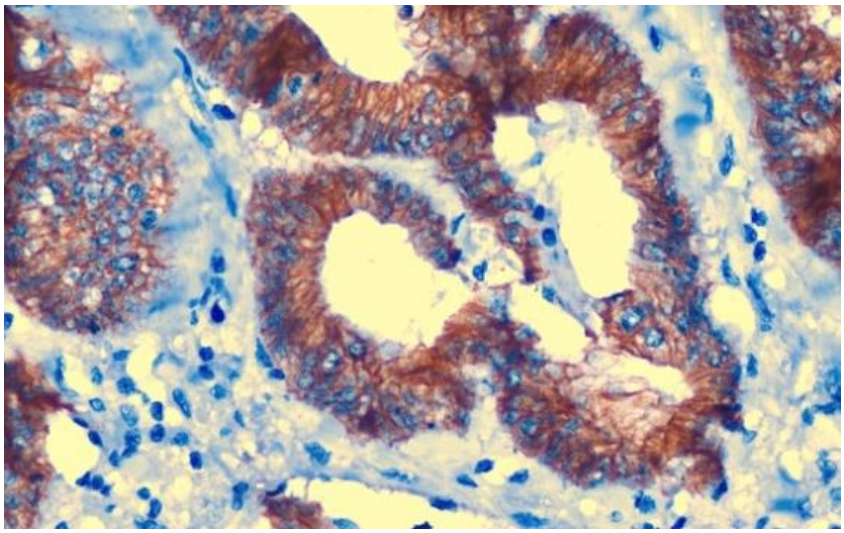

Figure 1: Photomicrograph: Immunohistochemistry with E-cadherin showing strong (Score7) membranous expression (40x)

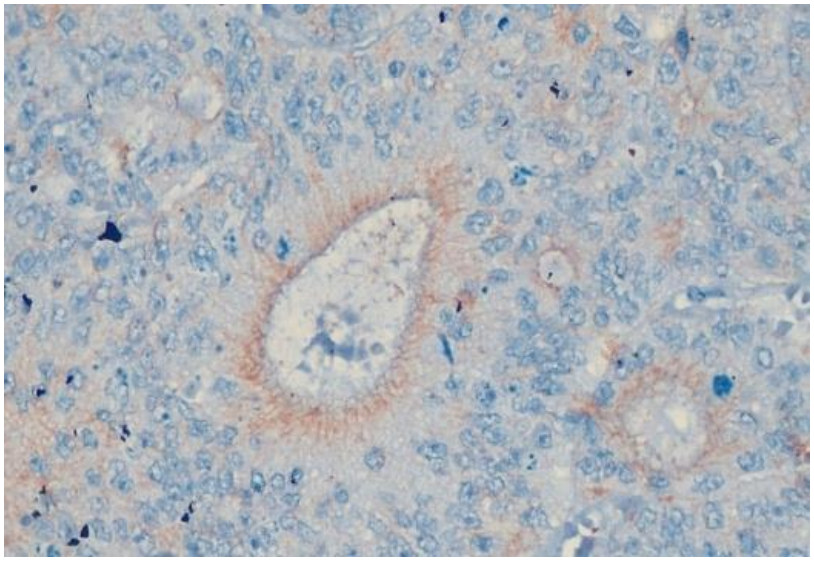

Figure 2: Photomicrograph-Immunohistochemistry with E-Cadherin showing a reduced expression (score2) (40x)

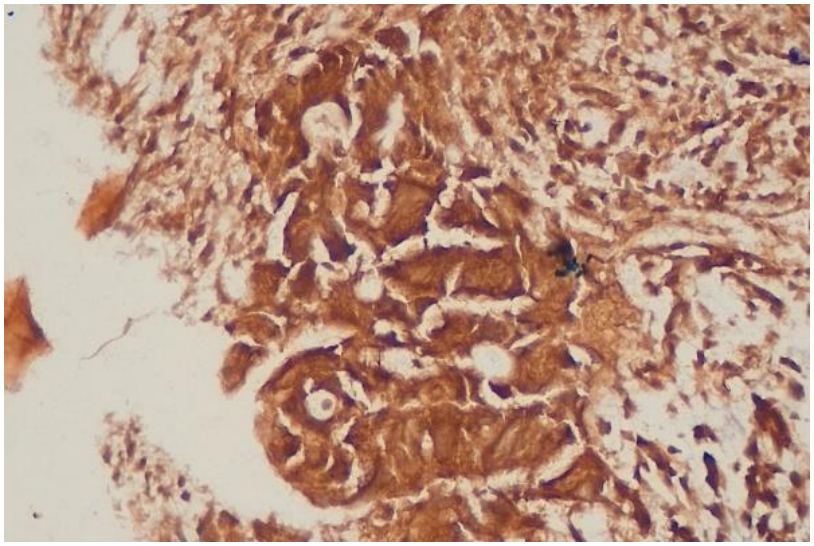

Figure 3: Photomicrograph:Immunohistochemistry of a case of colorectal carcinoma showing strong VEGF expression (40x).

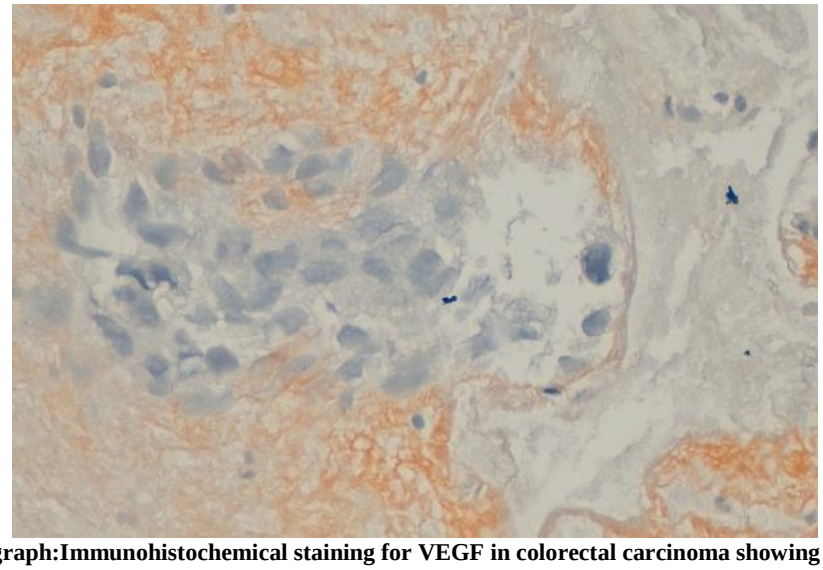

Figure 4: Photomicrograph:Immunohistochemical staining for VEGF in colorectal carcinoma showing weak expression (40x).

DISCUSSION

In this study, of 50 cases of colorectal carcinoma, we have attempted immunohistochemical analysis of E-cadherin and VEGF expression, besides correlating them with other histological parameters. In the current study, the age of presentation ranged from 24-74 years, with a predominance of the 5th decade (n=17) and with a male predominance M: F ratio was 1.5:1, both factors similar to observations by Patra *et al.,* [10] and Sudharshan *et al.,* [11]. Rectum was the most common site of tumor location (n=14; 28%), comparable with the studies done by Sudharshan *et al.,* and Gupta *et al.,* [11,12]. Adenocarcinoma was the most common histological subtype amounting to 80% (n=40) of the cases, followed by mucinous carcinoma seen in 14% (n=7) cases. This was similar to the study done by Gryfe *et al.,* with 90% of adenocarcinoma, with only 5% of mucinous adenocarcinoma but Patra *et al.,* found a slightly higher rate (23.6%) of mucinous carcinoma [13,10]. Many of these tumors (46%, n=23) were Well differentiated carcinomas similar to reports published by Poomakala *et al.,* [14] but Mehta *et al.,* [15] (76%) found a predominance of moderately differentiated, and Patra *et al.,* found poorly differentiated carcinoma as the most common in their study [10]. A predominance of Stage III tumors (47%) was seen in our study. This was in concordance with the study done by Ozguven *et al.,* [16]. Low-grade tumor budding was seen in 55.5% of cases and 14% exhibited high-grade tumor budding. High-grade budding was more frequently associated with lymphovascular invasion and an infiltrative invasive front, similar to the reports of Karamitopoulou *et al.,* [17].

Lymphovascular invasion was seen in 50% of the cases in this study. A significant statistical correlation was found between LVI and the TNM stage with a p-value of 0.027. Lim *et al.,* also have demonstrated that tumors that display LVI are likely to be in the advanced stages in terms of T and N [18]. Perineural invasion was seen in 33% (n=12) of the cases. The frequency of PNI-positive cases was assessed based on the worsening tumor grade in resected specimens. Twenty-six percent of well-differentiated tumors displayed PNI, compared with 31% and 60% of moderately and poorly differentiated tumors, respectively. Leibig *et al.,* have found a similar association between perineural invasion and poor tumor differentiation [19]. In our study, lymph node ratio (LNR) was assessed and was found to have an inverse relation with age with a lower number of retrieved lymph nodes with advancing age (p = 0.03). This agreed with the findings of Edler *et al.* They concluded that the reason for this inverse relationship was not understandable but could be because extensive lymphadenectomy is more generally performed in younger patients. An added assumption that was reported in the same study was that with increasing age lymph nodes possibly undergo a process of involution [20]. A significant correlation was also noted between LNR and the histologic type (p=0.018), with 6 of 7 mucinous tumors showing LNR >0.2. Additionally, our study also found a significant correlation between lymph node ratio and perineural invasion (p=0.015) and tumor grade (p=0.015).

Tumor necrosis was also studied meticulously to find its association with other clinic-pathological factors. Moderate to extensive necrosis was seen predominantly in tumors >4cm in size. In this study, we could not find an association of desmoplasia with any of the histopathological factors. This was in discordance with the findings of Ueno *et al.,* who reported an association of desmoplastic reaction and histopathological variables like pT and pN stages, tumor grade, venous invasion, and tumor budding [21]. The majority of the cases in our study displayed a mild chronic inflammatory infiltrate. However, no correlation was seen between tumor lymphocytic infiltration and grading or staging. E-cadherin expression was evaluated for all 50 cases included in this study. Preserved membranous E-cadherin staining, with an intensity equal to that of the adjacent normal epithelium, was seen in 38% (19 of 50) of cases. This figure was lower in comparison to 95.7% of cases expressing positive expression in the study conducted by Kang *et al.,* [22]. The reduced expression of E-cadherin did show a significant correlation with the maximum diameter of the tumour (p=0.03), similar to the observations made by Miladi-Abdennadher depicting that with an increase in the size of the tumors the expression of E-cadherin is reduced [23]. A reduced E-cadherin expression was observed in 6 of the 7 cases of mucinous adenocarcinomas, this could explain the rapidly infiltrating nature of mucinous tumors. Stage IV cancer and a majority of Stage III cancers showed reduced E-cadherin expression, comparable to the study of Elzagheid *et al.,* [24]. A reduced expression of E-cadherin was seen in 80% of the tumors with high-grade tumor budding, similar to the conclusion of Zlobec *et al.,* [25]. Our study could not establish a significant correlation between E-cadherin expression and tumor site, grade, differentiation, lymphovascular invasion, lymph node ratio, necrosis, desmoplasia, perineural invasion and the nature of invasive front.

VEGF expression, in this study, was assessed by the cytoplasmic staining on immunohistochemistry. While 36% (n=18) cases were negative for VEGF expression, the rest of the cases did show varying intensity and proportion of cytoplasmic staining with VEGF. All of the poorly differentiated tumors and the high-grade tumors had a strong expression of VEGF, also the intensity of VEGF staining in stage III tumors (64% of cases) was higher compared to cases in Stage I and Stage II, comparable to the results of Vahedi *et al.,* [26]. The incidence of cases with strong VEGF expression was found to increase with the advancement

of Dukes stage. A majority (63% cases) in which lymph node metastasis was seen displayed a strong VEGF expression, comparable with the reports of Wen *et al.*, [27]. VEGF staining did not have any statistical correlation with clinicopathological factors like age, gender, tumor location, histological type, differentiation or grade, lymphovascular invasion, perineural invasion, tumor budding, peritumoral inflammation, necrosis, and desmoplasia.

CONCLUSION

The current study revealed that loss of E-Cadherin expression was seen predominantly in patients above the age of 50, increase in tumor size, mucinous variants, advanced TNM stage, cases with lymph node involvement, increase in peritumoral inflammation and high-grade tumor budding. Increase in VEGF expression was noted in poorly differentiated tumors, advanced Duke's stage, and in cases with lymph node metastasis. VEGF also has been considered an important therapeutic target in treatment as it could result in the remodeling of the tumor vasculature leading to a decrease in tumor perfusion. A few preclinical trials have demonstrated that anti-VEGF therapy together with chemotherapy can result in inhibition of tumor growth. However, further studies with a larger sample size may be required to ascertain these findings.

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Conflict of interest

The authors declare they have no conflict of interest.

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