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Prognostic value of vascular endothelial growth factor expression in colorectal cancer patients

J.-C. Lee [a,*], N.-H. Chow [b], S.-T. Wang [c], S.-M. Huang [d]

[a] *Department of Surgery, National Cheng Kung University Hospital, 138 Sheng-Li Road, Tainan, Taiwan*
[b] *Department of Pathology, National Cheng Kung University Hospital, 138 Sheng-Li Road, Tainan, Taiwan*
[c] *Department of Public Health, National Cheng Kung University Hospital, 138 Sheng-Li Road, Tainan, Taiwan*
[d] *Department of Surgery, Buddhist Tzu Chi General Hospital, Hualien, Taiwan*

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Abstract

Solid tumours require neovascularisation for growth and metastasis. Vascular endothelial growth factor (VEGF) has been shown to be an important regulator of tumour angiogenesis. To examine the relevance of VEGF in the neoplastic transformation of human colon, we analysed protein expression in a total 30 polyps and 145 colorectal carcinomas by immunohistochemistry. All adenoma specimens, regardless of histological differentiation, and normal colonic mucosa did not express VEGF. Amongst 90 patients with non-metastatic colorectal cancer, VEGF expression was observed in 43 (48%) cases, whilst 29 of the 55 patients (53%) with metastases expressed the angiogenic factor. Both the proportion and intensity of VEGF expression were positively associated with the progression of colon carcinogenesis. Tumours with the highest VEGF expression tended to correlate with patients' survival, although VEGF expression did not emerge as an independent risk factor in a multivariate analysis. After exclusion of the patients with distant metastases, both univariate and multivariate analysis did not indicate any prognostic value for the tissues with the highest VEGF expression. Our results suggest that VEGF may play a role in the progression of colon cancer, although evaluation of this angiogenic phenotype did not provide additional prognostic information compared with that obtained from Dukes' staging of the tumours.

Keywords: Colorectal cancer; Adenoma; Colorectal carcinogenesis; Angiogenesis; Metastasis; Vascular endothelial growth factor; Immunohistochemistry

1. Introduction

Traditionally, the clinical outcome of colorectal cancer patients may be predicted by pathological staging, by either Dukes' staging or the UICC-TNM system. However, some of Dukes' stage A (approximately 10%) and Dukes' stage B patients (30–40%) will develop local recurrence or distant metastasis years after receiving standard surgical treatments. Therefore, it is important to find some other indicators that can predict for recurrence or the development of metastases so that we can screen for high-risk early-stage patients who may need preventive chemotherapy or other adjuvant therapy.

Angiogenesis, or the development of a vascularised stroma, is essential for tumours to grow beyond a minimal size [1,2] and metastasise [3–5]. Growth factors secreted by tumour cells regulate angiogenesis by acting via members of a family of endothelial-specific receptors. Although various factors may be capable of either positively or negatively regulating endothelial cell growth and neovascularisation, vascular endothelial growth factor (VEGF), also known as vascular permeability factor, has been shown to be definitely associated with angiogenesis [6,7]. VEGF is a 34–42 kDa multifunctional glycosylated dimeric protein with structure homology to platelet-derived growth factor [8] and is expressed in four isoforms derived by alternative mRNA splicing [9–11], i.e. $VEGF_{206}$, $VEGF_{189}$, $VEGF_{165}$ and $VEGF_{121}$. Of these, $VEGF_{165}$ is known to be the most abundant isoform. VEGF may contribute to angiogenesis by stimulating endothelial cell mitogenesis and

* Corresponding author. Tel.: +886-6-235-3535, ext. 5183; fax: +886-6-276-6676.
E-mail address: leejc@mail.ncku.edu.tw (J.-C. Lee).

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inducing microvessel permeability [12,13]. VEGF has been detected in a variety of human neoplasms [14–22]. The expression of VEGF in colorectal cancer, either at the mRNA or protein level, is reported to be higher than that in normal colonic mucosa. In addition, VEGF levels are higher in metastatic cell lines and colorectal cancer tissues than in non-metastatic cells or tissues [23].

In contrast to the number of studies reporting an association between high microvessel density and a greater incidence of metastases and decreased survival [24–28], few studies on the role of VEGF expression in predicting the prognosis of the patients with cancers, especially colorectal cancers, have been published. Amongst those studies, some have indicated VEGF expression as an independent factor in predicting patient prognosis [29–31], whilst others reported no such association [32–34]. This retrospective cohort study was performed to clarify the relationship between VEGF expression and the long-term survival of colorectal cancer patients.

2. Patients and methods

2.1. Patients and tumour specimens

145 paraffin-embedded colorectal cancer specimens resected at the Department of Surgery, National Cheng Kung University Hospital from 1989 to 1993 were studied. These specimens belonged to consecutive patients collected from chronological hospital records both retrospectively and non-selectively. None of the patients had received chemotherapy or radiation therapy before surgery. 41 patients were recruited in 1989, 29 in 1990, 40 in 1991, 25 in 1992 and 10 in the first 3 months of 1993. There were several reasons for the low numbers of patients studied; the hospital was newly set up in 1988 and thus was not well known to the general public; the incidence of colorectal cancer was comparatively low at these times. However, the number of operations for colorectal cancer in our hospital has gradually increased in the last 2 years up to 60–80 patients/year. The patients were staged with a modified Dukes' system (Astler-Coller modification) [35] as follows: 90 patients, non-metastatic cancers (6 patients (4%), Dukes' class A, T_1NoMo (UICC-TNM classification) [36,37]; 84 patients (58%), Dukes' class B, T_{2-4} NoMo); and 55 patients, metastatic cancers (35 patients (24%), Dukes' C, TxN_{1-3} Mo; 20 patients (14%), Dukes' D, $TxNxM_1$). Postoperative adjuvant chemotherapy with 5-fluorouracil (5-FU) plus leucovorin (LV) was routinely administered to the patients with Dukes' stage C or D. All patients were regularly followed-up at the outpatient clinic after operation and survival data as of September 1998 were ascertained through hospital records. The median follow-up length was 53 months (range: 1–91 months).

Another 30 polypectomised specimens were also recruited for this study. They were classified according to histological differentiation: 10 were mild dysplastic, 10 were moderate dysplastic and another 10 were severe dysplastic.

2.2. Immunohistochemical staining

After an initial review of all the available haematoxylin and eosin-stained slides of surgical specimens, representative paraffin blocks for each case were selected for immunohistochemical study. Polyclonal anti-VEGF antibody generated by immunising rabbits with peptide of the N-terminal region of $VEGF_{165}$ (Calbiochem, Cambridge, MA, USA) was used as previously described [38]. Optimal staining conditions were carefully determined using non-neoplastic human kidneys as a positive control [39]. The best staining results were obtained after pretreatment of sections with 0.2% trypsin (Sigma Chemical Co., St Louis, MO, USA) for 10 min (data not shown). Briefly, sections were washed for 5 min with phosphate-buffered saline and blocked with 3% hydrogen peroxide in ethanol at room temperature. They were then incubated with 3% normal horse serum for 15 min at room temperature to block non-specific binding. Primary antibody diluted 1/40 was added and the slides incubated overnight at 4°C. The LSAB® 2 kit (Dako Co., Carpinteria, CA, USA) was used to detect the resulting immune complex and the activity was visualised using the aminoethyl carbazole substrate kit (Zymed Laboratory, San Francisco, CA, USA). Finally, sections were counterstained with haematoxylin. Negative controls were obtained by incubating the slides with non-immune rabbit IgG instead of the primary antibody. Slides were examined by two investigators without prior knowledge of the corresponding clinicopathological data.

The immunoreactivity for VEGF in tumour cells was graded as either negative or positive according to a four-value classification scale as follows: those without any staining in any of the tumour cells were graded as negative; those with positive staining in less than 5% of tumour cells were graded as '+', whilst those with between 5 and 25% staining were graded as '++' and those with greater than 25% staining were graded as '+++'.

2.3. Statistical analysis

Association of VEGF staining with clinicopathological parameters was analysed using the Chi-square test. A P value of 0.05 or less was considered significant.

The survival parameters of time to recurrence and time to death were computed using the method of Kaplan and Meier. The log-rank test was used to test for differences in the time to recurrence and survival

amongst patient subgroups (univariate analysis). Patients were dichotomised at '+++' versus '++ or less' for VEGF staining, 'A or B' versus 'C or D' for Dukes' staging, 'pT1, pT2 or pT3' versus 'pT4' for pT stage and 'pN0' versus 'pN1, pN2, or pN3' for pN stage.

A multivariate analysis using the Cox proportional hazards model was performed to investigate the independence of the risk factors identified as significant in the univariate analysis.

3. Results

The demographic and tumour characteristics of the 145 patients are summarised in Table 1. Our series had a majority of locally advanced cases with 66% of patients with T4 tumours. In the 84 patients with Dukes' stage B tumours, 53 (63%) had T4 tumours; in the 35 patients of Dukes' stage C, 22 (66%) had T4 tumours. 38 (75%) of the 51 patients with sigmoid colon cancer, 42 (82%) of the 51 patients with proximal colon cancer, and 15 (35%) of the 43 patients with rectal cancer had T4 tumours.

Table 1
Frequency of prognostic factors investigated in 145 colorectal carcinomas

Prognostic factors	No. of patients (%)
Dukes' category	
A	6 (4)
B	84 (58)
C	35 (24)
D	20 (14)
pT stage	
pT1	6 (4)
pT2	27 (19)
pT3	17 (12)
pT4	95 (66)
pN stage	
pN0	96 (66)
pN1	28 (19)
pN2, or pN3	21 (14)
M stage	
M0	125 (86)
M1	20 (14)
Differentiation	
Moderate or well	131 (90)
Poor	14 (10)
Location	
Proximal colon	51 (35)
Sigmoid colon	51 (35)
Rectum	43 (30)
Sex	
Male	80 (55)
Female	65 (45)
Age (years)	
<65	68 (47)
$\geqslant 65$	77 (53)

Table 2
VEGF expression of 145 colorectal carcinomas with regard to staging, and VEGF expression of 30 adenomas

VEGF	Adenomas (%)	Non-metastatic tumours (%)	Metastatic tumours (%)
Negative	30 (100)	47 (52)	26 (47)
+	0 (0)	26 (29)	9 (16)
++	0 (0)	10 (11)	6 (11)
+++	0 (0)	7 (8)	14 (25)

Chi square = 37.0, degrees of freedom, 6; $P < 0.0001$.

Neither normal colonic mucosa nor adenomas of different histological differentiation were immunoreactive with the anti-VEGF antibody (Table 2). VEGF was mainly expressed in the cytoplasm or the membrane of the cancer cells (Fig. 1). Amongst non-metastatic colorectal cancer patients ($n = 90$), 47 (52%) were negative; 26 (29%) were scored as '+', 10 (11%) as '++', and 7 (8%) as '+++'. Amongst metastatic colorectal cancer patients ($n = 55$), 26 (47%) were negative; 9 (16%) were classified as '+', 6 (11%) as '++' and 14 (25%) as '+++'. Expression of VEGF was positively correlated with the progression of colorectal cancers ($P < 0.0001$, Table 2). With regard to clinicopathological indicators such as subdivisions of TNM system, VEGF expression was significantly higher in patients with lymph node metastasis (Table 3a, $P = 0.05$) and distant metastasis (Table 3b, $P = 0.03$) but not in patients with depth of invasion (Table 3c, $P = 0.15$). However, no apparent relationship was observed between VEGF expression and tumour location, differentiation, age or sex.

Fig. 2 shows the Kaplan–Meier survival curve for the patients with '+++' VEGF expression, and those with '++' or less expression. In univariate analysis (Table 4),

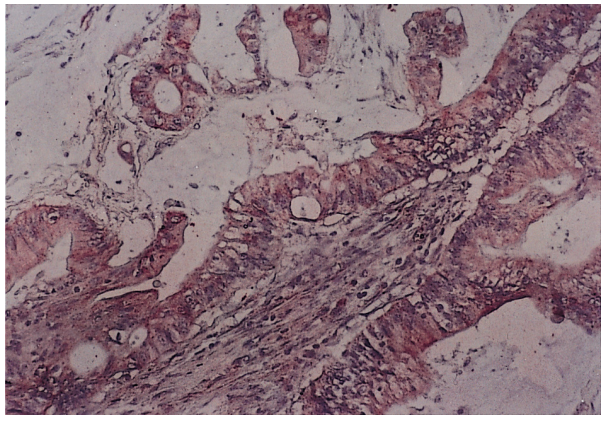

Fig. 1. All of the tumour cells show cytoplasmic staining for VEGF with variable expression intensity in different areas. Biotin–streptavidin stain/aminoethyl carbazole chromogen/Meyer's haematoxylin, original magnification ×300.

Table 3a
VEGF expression of 145 colorectal carcinomas with regard to pN stage

VEGF	pN0 (%)	pN1 (%)	pN2, or pN3 (%)
Negative	48 (50)	13 (46)	12 (57)
+	27 (28)	6 (21)	2 (10)
++	11 (11)	5 (18)	0 (0)
+++	10 (10)	4 (14)	7 (33)

Chi-square = 12.6, degrees of freedom, 6; $P = 0.05$.

Table 3b
VEGF expression of 145 colorectal carcinomas with regard to M stage

VEGF	M0 (%)	M1 (%)
Negative	67 (54)	6 (30)
+	31 (25)	4 (20)
++	13 (10)	3 (15)
+++	14 (11)	7 (35)

Chi-square = 9.1, degrees of freedom, 3; $P = 0.03$.

Table 3c
VEGF expression of 145 colorectal carcinomas with regard to pT stage

VEGF	pT1 (%)	pT2 (%)	pT3 (%)	pT4 (%)
Negative	4 (67)	15 (56)	13 (76)	41 (43)
+	1 (17)	9 (33)	2 (12)	23 (24)
++	1 (17)	2 (7)	0 (0)	13 (14)
+++	0 (0)	1 (4)	2 (12)	18 (19)

Chi-square = 13.3, degrees of freedom, 9; $P = 0.15$.

the survival of the patients with '+++' VEGF staining was significantly worse than that of the patients with negative, '+', or '++' VEGF staining ($P < 0.01$). There was no difference, however, amongst the patients with negative, '+' or '++' VEGF staining (data not shown). For those of '+++' VEGF staining ($n = 21$), 14 died as of September 1998. Amongst them, 7 manifested liver metastases, 5 lung metastases, 2 had distant lymph node metastases, 3 had peritoneal carcinomatosis and one bone metastases. The levels of VEGF expression (+++) were not correlated with specific metastatic sites.

The difference in survival between the patients with '+++' VEGF staining and the remaining patients was insignificant in a multivariate analysis using the Cox model stratified by Dukes' stage. Thus, VEGF expression '+++' appears to be an important prognostic factor for patients with colorectal cancer. However, it is not an independent prognostic factor and was correlated with Dukes' stage, lymph node metastasis and distant metastasis. After exclusion of patients with distant metastases, both univariate and multivariate analysis did not indicate a prognostic value for survival for patients with the highest VEGF expression. VEGF

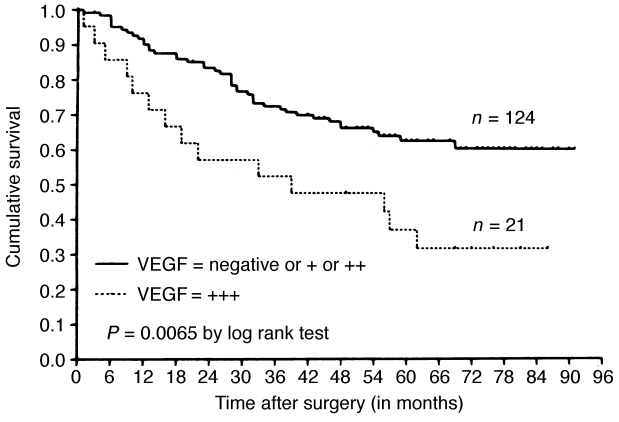

Fig. 2. Kaplan–Meier survival curves of 145 colorectal carcinomas with regard to VEGF expression. Chi-square for the log-rank test (Mantel–Cox method), 7.4; degrees of freedom, 1; $P < 0.0065$.

Table 4
The mean survival time of the patients in different groups of various risk factors

Risk factors	Mean ± SEM[a]	P value[b]
Dukes' stage		
A or B	62.9 ± 1.7	< 0.0001
C or D	42.6 ± 2.4	
pT stage		
pT1, pT2 or pT3	57.7 ± 1.6	< 0.0001
pT4	44.4 ± 2.7	
pN stage		
pN0	60.0 ± 2.1	< 0.0001
pN1, pN2 or pN3	33.1 ± 3.0	
M stage		
M0	57.0 ± 1.8	< 0.0001
M1	14.7 ± 3.3	
VEGF expression		
Negative or + or ++	53.1 ± 2.1	< 0.01
+++	37.0 ± 2.1	
Differentiation		
Moderate or well	51.7 ± 2.1	NS
Poor	35.9 ± 4.3	
Location		
Proximal colon	43.0 ± 2.6	NS
Sigmoid colon	45.1 ± 3.0	
Rectum	50.9 ± 3.7	
Sex		
Female	42.6 ± 2.4	NS
Male	52.8 ± 2.7	
Age (in years)		
< 65	42.6 ± 2.0	NS
⩾ 65	51.0 ± 3.1	

NS, non-significant.

[a] The mean survival time, in months, was calculated by the Kaplan–Meier estimates of survival functions.

[b] The P values were based on the log rank test. $P < 0.05$ was considered statistically significant.

expression was also not a prognostic factor for time to recurrence ($\pm$SEM was 43.1 ± 5.7 months for '+++' and 45.7 ± 1.9 months for '++' or less).

Local recurrence only was noted in 16 patients amongst 102 proximal and sigmoid colon cancers (16%), distant metastases only in 13 patients (13%), and combined local recurrence and distant metastases in 10 patients (10%). In 43 rectal cancer patients, 7 patients were noted to have local recurrence only (16%), 11 patients with distant metastases only (26%) and 2 patients with both local recurrence and distant metastases (5%).

4. Discussion

Angiogenesis, or the development of a vascularised stroma, is essential for tumours to grow beyond a minimal size [1,2] and metastasise [3,4]. It has been demonstrated that increased vessel counts in solid tumours are associated with a higher risk of metastasis of various types of cancers [3,25–27], including colon cancer [28]. In support of this hypothesis, expression of VEGF has been shown to correlate positively with microvessel count and metastasis [24]. This study was performed to investigate the role of VEGF expression in the progression of colorectal cancers and its value in predicting the prognosis of patients.

In our study, VEGF could not be detected in normal colonic mucosa or adenomatous polyps of different differentiations. In contrast, VEGF expression was obtained in approximately half of colorectal cancers (50%). Both the incidence and proportion of VEGF expression increased with the progression of colorectal carcinogenesis classified by either TNM system or Dukes' stage, consistent with prior studies reported by Takahashi and colleagues [24] and Kang and associates [31].

There are few studies dealing with the role of VEGF expression in predicting the prognosis of the patients with cancers, especially colorectal cancers and results published to date remain controversial. In our series in the univariate analysis, patients with the highest VEGF expression had significantly poorer prognosis with earlier recurrence and death than those with intermediate or low expression levels. However, this difference was not observed after stratification by pathological staging suggesting that the increased expression of VEGF occurs as colorectal cancers progress. This result is consistent with that of Takahashi and colleagues [32]. In their series, 8 of 27 Dukes' stage B cancer patients developed liver, lung or lymph node metastases at a mean follow-up time of 24 months. Although perineural invasion, vessel count and VEGF correlated with time to recurrence in the univariate analysis, VEGF and perineural invasion did not emerge as independent risk factors in a multivariate analysis. Similar findings have been reported by Obermair and colleagues, who showed a positive relationship between VEGF protein levels and microvessel counts in breast cancers. However, VEGF levels did not significantly affect disease-free survival [33]. A cohort study in squamous cell lung carcinoma also failed to demonstrate VEGF expression as an independent prognostic factor [34].

In contrast, Kang and associates [31] found that immunohistochemical expression of VEGF increased at advanced stages, and was an independent prognostic factor for colorectal cancer patients. Studies of a large series of gastric cancer specimens showed that VEGF was a significant prognostic factor and may contribute to disease progression [29,30].

These differing reports may result from the sparcity of reports and the limited numbers of patients studied. More research is needed to elucidate the role of VEGF expression in predicting the prognosis of colorectal cancer patients.

Our study series had a high percentage of T4 tumours in both the early and advanced colorectal cancers. Between 1989 and 1993, the public was not well informed about the early symptoms of the cancers due to its low incidence. As a result, patients were generally unaware of their condition until the tumour had reached an advanced invasive stage and thus many patients were late in seeking medical attention. Rectal cancers have early indicators such as a bloody stool, whereas the signs and symptoms of proximal or sigmoid colon cancers are not as clear. This may have been a factor in the lower percentage of T4 tumours that was observed in rectal cancers compared with the proximal or sigmoid colon cancers.

In conclusion, we found that VEGF expression was positively correlated with the progression of colorectal cancer as reported in studies on gastrointestinal cancers. However, evaluation of VEGF phenotypes in colorectal cancer patients does not provide additional prognostic value compared with conventional prognostic indicators.

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