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Vascular enumeration as a prognosticator for colorectal carcinoma

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Abstract

Vascular enumeration is thought to be an independent prognosticator for several human tumours, including breast, bladder and colorectal carcinomas. There have been 12 reports on the prognostic influence of vascular enumeration in colorectal carcinoma with different results. To test the prognostic influence of this factor in our patients, we have selected 126 patients with colorectal carcinoma Dukes' stages A to C treated only with curative surgery with no further adjuvant therapy. The minimal follow-up time was 5 years (60 months). After immunostaining with CD34, we performed a manual count of the vessels following Gasparini's criteria. In our series vascular enumeration showed significant association with the histological grade ($P=0.03$) with a cut-off point at 77 vessels/200×, but not with tumour staging and vascular and neural invasion ($P>0.05$). Vascular enumeration was a prognosticator for RFS (relapse-free survival) ($P=0.009$) and OS (overall survival)($P=0.01$) in all Dukes' stages in the univariate analysis, but this prognostic influence was lost in the multivariate analysis, in which only stage, histological differentiation, location and vascular and neural invasion behaved as significant independent prognosticators.

Keywords: Neovascularisation; Prognosticators; Colorectal; Carcinoma

1. Introduction

Neovascularisation has been known for a long time to be important for tumour development and metastasis in experimental models [1, 2]. However, only recently have several reports analysed the possible influence of vascularisation in human tumours. Weidner and colleagues [3] in 1991, reported the first study showing the prognostic influence of neovascularisation in breast carcinoma. The promising results reported by these authors led several groups to analyse the possible prognostic role of this factor in many kinds of solid tumours, such as breast cancer [4], prostatic carcinoma [5], squamous cell carcinoma of head and neck [6], malignant melanoma [7], and more recently, gastric or large bowel tumours [8, 9]. Different authors have employed different endothelial markers, mainly factor VIII, CD31 and CD34, and performed a manual count of the vessels with the light microscope. However, the results have not been homogeneous, a fact that we can attribute both to differences in study population and in experimental design.

To test the prognostic influence of vascularisation in patients with colorectal carcinoma in our area, we have performed vascular enumeration in 126 patients with this tumour.

2. Patients and methods

2.1. Study population

The study group of this retrospective study was based on 126 patients treated at Hospital General de Segovia, Spain between 1982 and 1991 by colectomy for colorectal carcinoma. None received postoperative chemotherapy or radiation therapy. These patients were a sample of all the patients with colorectal carcinoma in this hospital (420 patients in total) and fulfilled the criteria for inclusion in the study: classical colorectal carcinomas (excluding signet ring and mucinous variants) with the same therapeutic protocol patients with metastatic disease at the moment of diagnosis were excluded (Dukes' stage D), as in these cases surgery cannot be

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considered curative), and follow-up time of at least 5 years. Patients who died from intercurrent unrelated disease or who were lost within this interval were considered as censored cases.

The clinical records were obtained at admission and follow-up information obtained from the hospital's records or directly from the patients or their families. The relapse-free survival (RFS) and the overall survival (OS) were chosen as prognostic indicators. We defined RFS as the time elapsed between the initial treatment for the tumour and the reappearance of a tumour of the same histological type in the patient and OS as the time elapsed between initial diagnosis and death attributable to the carcinoma.

Histological sections of formaldehyde-fixed, paraffin-embedded material stained with haematoxylin-eosin were reviewed at the time of the study. The following variables were recorded: histological grade, neural invasion and vascular invasion.

2.2. Immunohistochemical staining

Immunostaining was performed on paraffin-embedded archival tissue, following the avidin–biotin peroxidase technique. In brief, sections measuring 4–6 μm thick were cut, air-dried for 15 min, heat-fixed at 42°C and then air-dried overnight at room temperature. The slides were stored at room temperature until use. After deparaffinisation with xylene, endogenous peroxidase activity was eliminated by treating the slides with $EtOH/H_2O_2$ for 30 min at room temperature. Then, the slides were incubated with the diluted primary antibody (CD34, Dako 1/100 dilution) at 4°C in a humidified chamber for 90 min. Biotinylated anti-mouse IgG and avidin–biotin peroxidase complex were added in sequence. The sections were then incubated with ethyl-amino-carbazol (EAC) for 7–10 min for visualisation of the peroxidase reaction. After washing in water for a few minutes, the sections were counterstained with Mayer's haematoxylin, dehydrated in alcohol, cleared in xylene and mounted.

2.3. Vascular counts

The vascular counts were performed following the criteria reported by Gasparini and associates [4]. First, we identified the vascular hot-spot with a 4× objective and then measured the number of vessels in this area with a 20× objective (0.74 mm^2) (Fig. 1). We did not choose as vascular hot-spots areas with necrosis, ulceration or intense inflammation, for vascularisation in these areas could be related to the inflammatory reaction and not to the presence of a tumour. Following the first experimental design reported by Weidner and colleagues [3], we obtained two values: the number of vessels in the most vascular field and the median value of three fields in the most vascular area. Both values were expressed as number of vessels per 200× field and as vascular density (vessels/mm^2).

2.4. Statistical analysis

Statistical tests were performed using the BMDP (Biomedical Data) Statistical Software Package run on a Convex C3210 computer. A comparison of the vessel counts obtained with the different methods employed in our study was made by means of the correlation coefficient (r). Student's t-test was used to determine the possible associations between vascular assessment (considered as a qualitative variable) and the following quantitative parameters (tumour size, age of the patient) and the chi-squared test for qualitative parameters, such as histological grade, stage, location and vascular and neural invasion.

We also analysed the possible influence of the different factors on survival (estimated with the RFS and the OS). The patterns of RFS and OS were estimated by means of the product-limit (Kaplan–Meier) method on the basis of a 5-year median follow-up period. The role of each of the prognostic factors (univariate analysis) was evaluated using the log-rank test (comparison of two Kaplan–Meier curves) and their joint effect (multivariate analysis) with Cox's stepwise regression.

3. Results

3.1. Patients and histopathology

The main clinicopathological characteristics of our patients are summarised in Table 1. All the patients had colorectal carcinomas of nonspecific type (NOS). All underwent colectomy and they received no further therapy. Most patients had well differentiated tumours (60 cases, 48%).

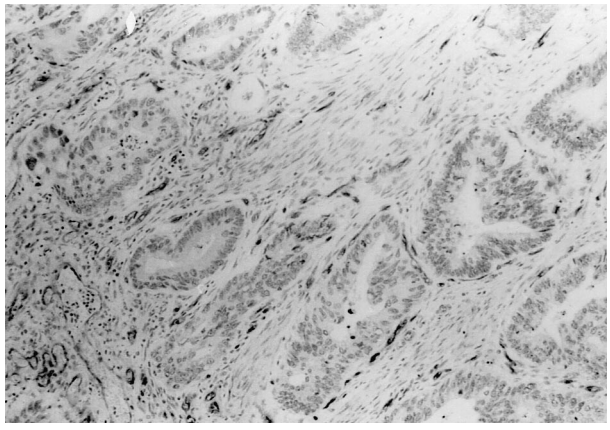

Fig. 1. Colon carcinoma stained for CD34, showing a moderate number of vessels (stained in black, immunohistochemistry for CD34).

3.2. Vascular counts

The values obtained in our study are summarised in Table 2. Initially we analysed the correlation between the two systems for manual count we had employed in our study. The correlation coefficient equalled 96%, therefore, allowing us to consider both values as interchangeable. For this reason, we have only employed the number of vessels per 200× field, as this measure was most widely employed by different authors in the literature rather than vessel density.

Table 3 summarises the association between vascularity and other pathological parameters estimated in our study. Vascular enumeration showed significant association only with histological differentiation (poorly differentiated tumours had a significantly higher number of vessels), but not with any other parameter analysed.

Table 1
Clinicopathological characteristics

Patients	
Total number	126
Mean age (95% confidence interval)	67.35 (32–87) years
	n (%)
Sex	
Male	70 (56)
Female	56 (44)
Dukes' stage	
A	15 (12)
B	61 (48)
C	50 (40)
Lymph node metastases	
Absent	76 (60)
Present	50 (40)
Tumour size, mean (range)	5.19 (1–15)
Histological grade (differentiation)	
Well	60 (48)
Moderate	57 (45)
Poor	9 (7)
Vascular invasion	
Absent	94 (75)
Present	32 (25)
Neural invasion	
Absent	113 (90)
Present	13 (10)

Table 2
Vascular counts

	Mean	95% Confidence interval
Absolute number of vessels 200×		
Most vascular field (MVF)	75.7	(25–128)
Median of three fields	72.2	(21.3–145.6)
Vascular density (vessels/mm^2)	97.7	(28.9–197.1)

3.3. Univariate analysis

63 patients (50%) experienced relapse in the follow-up time and 56 (44%) died of disease; therefore, 7 patients (6%) were alive with the disease 5 years after diagnosis. To analyse the possible influence of vascular density in prognosis we divided the patients in two groups, a low density and a high density group. As the cut-off value we selected the mean vessel count, considering the vascular density as a continuous variable and increasing the values at 5-vessel intervals until we found statistically significant differences. With this method, the cut-off point for the vascular count was 77 vessels per 200× field. Factors associated with shorter RFS and OS were age of the patient (>74 years) ($P=0.04$ for RFS and $P=0.02$ for OS), location (worst for rectal tumours) ($P=0.04$ for RFS and $P=0.01$ for OS), histological grade ($P=0.02$ for RFS and OS), lymph node metastases ($P=0.000$ for RFS and OS), Dukes' staging ($P=0.000$ for RFS and OS), vascular invasion ($P=0.01$ for RFS and OS), neural invasion ($P=0.001$ for RFS and 0.01 for OS) and vascular enumeration ($P=0.009$ for RFS and $P=0.01$ for OS; Fig. 2).

Table 3
Correlation between vascular counts and other histopathological characteristics

Parameter		⩽77 *n* (%)	>77 vessels/200× *n* (%)
Total number		66 (100)	60 (100)
Histological grade (differentiation)			
Well		35 (53)	25 (42)
Moderate		30 (45)	27 (45)
Poor		1 (2)	8 (13)
	$P=0.03$		
Mean tumour size		5.5 ± 2.03	4.85 ± 2.02
	NS $P=0.07$		
Lymph node status			
Negative		45 (68)	32 (53)
Positive		21 (32)	28 (47)
	NS $P=0.12$		
Dukes' stage			
A		9 (14)	6 (10)
B		36 (55)	25 (42)
C		21 (32)	29 (48)
	NS $P=0.16$		
Vascular invasion			
Present		12 (18)	20 (33)
Absent		54 (82)	40 (67)
	NS $P=0.08$		
Neural invasion			
Present		4 (6)	20 (33)
Absent		62 (94)	40 (67)
	NS $P=0.17$		

NS, non-significant.

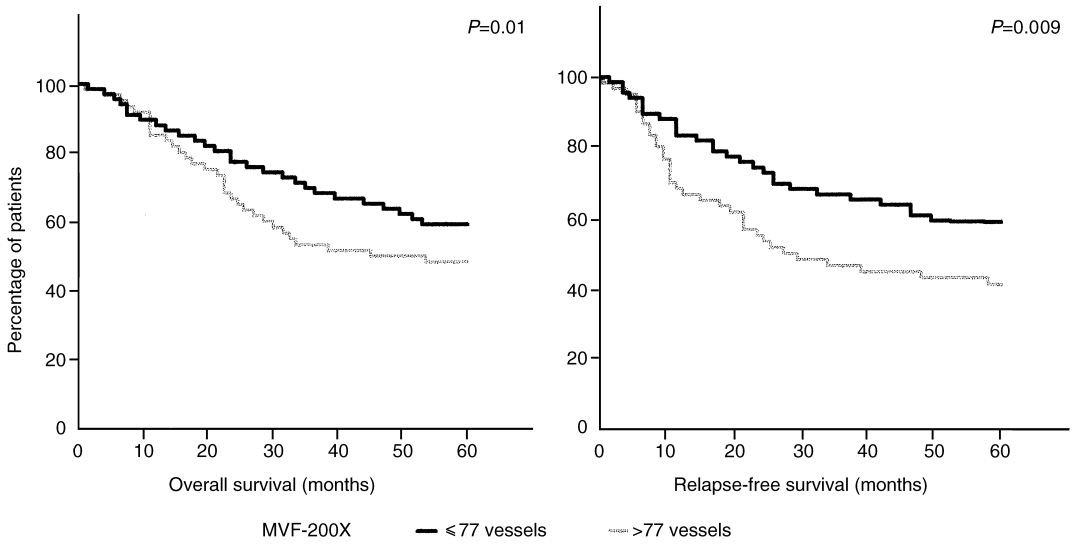

Fig. 2. Vascular enumeration versus relapse-free survival (RFS; right) and overall survival (OS; left).

3.4. Multivariate analysis of recurrences

Independent predictors of RFS in Cox regression analysis were Dukes' stage, $P=0.000$, location, $P=0.007$, histological grade, $P=0.06$, neural and vascular invasion $P=0.03$. Vascular enumeration lost significance in the multivariate analysis.

4. Discussion

Colorectal carcinoma is one of the most frequent tumours in industrialised countries (after breast carcinoma in women and lung carcinoma in men). Despite clear advances in early diagnosis and adjuvant therapy, this tumour is still associated with a high mortality [9, 10]. Tumour staging (either Dukes' or TNM) remains as the single most important prognosticator in these patients, but up to 20% of patients with tumours associated with a good prognosis (stage A) eventually die of disease. This group of patients might have benefited from adjuvant therapies, that are not widely used for early tumours; this fact has made several authors search for new prognostic factors that could define subsets of patients, mainly in early stages, and improve their survival with adjuvant therapies.

Several studies have shown a relationship between vascular enumeration employing manual counts and survival and/or relapse in different human tumours, mainly breast carcinoma [5]. Most authors have proved a negative prognostic influence of vascularisation in different tumours. To the best of our knowledge only 12 reports on the prognostic influence of vascularisation in colorectal carcinoma have been published in the literature (Table 4).

As in breast carcinoma and other tumours, there are many methodological differences between these studies that make their comparison difficult. First, some authors have employed factor VIII as the endothelial marker [11–19], whilst others have chosen either CD31 [14, 20, 21] or CD34 [13, 22]. In addition, some authors have chosen patients with tumours in all Dukes' stages [11, 13–16, 20–22], whilst others have only studied certain stages, mainly B [12, 17, 18]. The aforementioned facts make it extremely difficult to design a study about vascularisation that could be easily compared with other reports.

The aim of our study was to analyse the possible prognostic influence of vascularisation in patients with colorectal cancer in our area. Although the use of Chalkley graticles or image analysis systems [23] could reduce, at least partially, the subjectivity of the measures, we performed manual counts as all the other reports have employed this kind of measure. It is of extreme importance to adhere strictly to Gasparini's criteria for vascular counts [4], particularly if we wish to compare our results with other series. Moreover, although most authors have employed factor VIII, we chose CD34 as a vascular marker due to its increased sensitivity. According to Tanigawa and associates [22], it allows detection of three or four times more vessels than factor VIII. However, Tomisaki and associates [13] preferred factor VIII in his series as together with the increased sensitivity of CD34, unspecific immunostaining of leucocytes and other inflammatory cells can also occur, that can alter the counts, particularly if image analysis systems are used. Most reports have employed absolute number of vessels in 200× [11, 17, 20, 22], 100× [12] or 400× [15] fields, and only four authors have used vascular density for the counts [13, 14, 19, 20] and the cut-off values reported in the literature have ranged from 17.4 per 200× field [11] to 185 vessels/mm^2 [14]. In our series we estimated the best cut-off point with

Table 4
Summary of the results of the different reports on vascularisation in colorectal carcinoma

Author [Ref.]	No. patients	Stage (Dukes')	Lens	Field size (mm^2)	No. of fields measured	Material	Vascular marker	NV	MVD	Cut-off point with prognostic influence	Prognostic value of high NV or MVD
Saclarides [11]	48	A–D	200×	NS	NS	Paraffin	FVIII (PC-Dako)	17.4	NS		Negative
			100×	NS	NS			39.5	NS		
Bossi [20]	178	A–D	200×	0,636	3	Paraffin	CD31 (JC/70 Dako)	115	181	No	Not prognostic
Frank [12]	105	B2	100×	2.955	1	Paraffin	FVIII (PC-Dako)	28	NS	> 28	Negative
Tomisaki [13]	175	A–D	200×	0.739	1	Paraffin	FVIII (Nichirei Corp.)		33	32	Negative
							CD34 (Nichirei Corp.)		56		
Vermeulen [14]	34	A–D	250×	0.4	Several	Paraffin	CD31 (JC/70 Dako)	74	185		Negative
							FVIII (MC-Dako)	48	120		Negative
Takebayashi [15]	166		400×		1	Paraffin	FVIII (PC-Dako)	65.3	NS	> 65.3	Negative
Lindmark [16]	212	A–D	125×	NS	5	Frozen	FVIII (PC-Dako)	0–> 10	NS	> 10	Positive
Engel [21]	35	A–D	400×	0.152	3	Paraffin	CD31 (PC-Dako)	> 65	NS	> 65	Negative
Tanigawa [22]	133	A–D	200×		5	Paraffin	CD34	112	NS	> 105	Negative
Takahashi [17]	27	B	200×	0.739	1	Paraffin	FVIII (PC-Dako)	26	NS	> 25	Negative
Banner [18]	22	B	400×		10	Paraffin	FVIII (PC-Dako)	28	NS	No	Positive, non significant
Fox [19]	36	A–C	200×			Paraffin	FVIII	NS	26	No	Not prognostic

NV, number of vessels per 200× field; MVD, median vascular density; NS, not specified; PC, polyclonal; RFS, relapse-free survival; OS, overall survival; MC, monoclonal.

prognostic influence, which was 77 vessels per 200× field both for RFS and for OS (101 vessels/mm^2 if we chose vascular densities).

In our study we considered a vessel to be any structure stained with the chromogen, that could be separated from adjacent vessels and stromal cells, although it did not show a lumen, following the criteria set forth by Gasparini and other experts on vascularisation [4]. We chose the vascular hot-spot, that was usually located at the periphery of the tumours, to perform the measures. The vascular count only showed significant association with the histological grade; the poorly differentiated tumours had a significantly higher number of vessels than the well-differentiated ones. All the vascular parameters analysed in our study had prognostic influence in the univariate analysis. In the multivariate analysis only Dukes' stage, vascular and neural invasion, histological grade and location (rectal) had independent prognostic value. Therefore, in our series the prognostic influence of vascularisation was lost in the multivariate analysis. As shown in Table 4, not all the series have shown this prognostic influence; two series have even shown a 'protective' influence of vascularisation [16, 18], but one of these reports has been criticised by several specialists for using frozen material that can significantly alter the results of immunohistochemistry [16] and for several methodological errors. If vascularisation is to be considered a prognosticator in human cancer and if this consideration can open the way for the use of anti-angiogenic drugs in some subsets of patients, it will only be through the homogeneous design of the studies, according to Gasparini's or Weidner's criteria for vessel count.

In summary, our results seem to indicate the prognostic influence of vascularisation in colorectal carcinoma in our patients, but this influence is not independent of stage and other classical prognosticators. We feel that many more series with the same experimental design are needed before this factor can be applied to clinical practice in oncology or even used for selection of patients that could receive alternative therapies (anti-angiogenic) to improve survival.

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