

ORIGINAL ARTICLE

Tumour proliferation, angiogenesis, and ploidy status in human colon cancer

E Cristì, G Perrone, G Toscano, A Verzi, S Nori, D Santini, G Tonini, A Vetrani, A Fabiano, C Rabitti

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See end of article for authors' affiliations

Correspondence to:
Dr G Perrone, Campus Bio-Medico University, Via Emilio Longoni, 83 00155 Rome, Italy; g.perrone@unicampus.it

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Aims: Tumour angiogenesis is essential for carcinogenesis and facilitates the process of tumour development and metastasis. Vascular endothelial growth factor (VEGF) is a well characterised angiogenetic factor and is known to play a crucial role in new vessel development. To gain further insight into the effects of microvessel density and VEGF expression in colon cancer, their relation with tumour proliferation, ploidy status, and p53 expression was investigated in colon cancer.

Methods: Tissue samples of 50 archived colon cancers were analysed by immunohistochemistry for VEGF, p53, and the endothelial cell marker, von Willebrand factor (VWF), using specific antibodies. The same samples were re-cut for flow cytometric studies to obtain S phase fraction (SPF) and ploidy status.

Results: A positive significant correlation was found between SPF and angiogenesis. The median microvessel count in high SPF tumours was significantly higher than in low SPF ones. No association was found between VEGF expression and SPF. A positive correlation was found between ploidy status and p53 expression and microvessel count. Furthermore, a positive correlation was established between DNA ploidy, VEGF expression, and microvessel count.

Conclusion: This study provides evidence that in colon cancer, tumour growth may be stimulated by vascular supply, and the lack of a correlation between tumour cell proliferation and VEGF expression indicates that these two parameters may be regulated by separate mechanisms. Furthermore, the positive correlation between microvessel density, VEGF expression, and ploidy status provides more evidence that genetic alterations are involved in tumour angiogenesis.

Angiogenesis, the development and formation of new blood vessels, is important in a variety of physiological and pathological processes, such as growth and differentiation, ovulation, wound healing, and neoplasia.¹ Furthermore, increased vascular density has been shown to correlate with a higher incidence of metastasis and a worse prognosis in cancer.^{2,3}

In 1971, Folkman proposed a new view of the role of blood vessels in tumour growth in the form of a hypothesis that tumour growth is angiogenesis dependent.⁴ This hypothesis suggested that tumour cells and vascular endothelial cells within a neoplasm may constitute a highly integrated ecosystem, and that endothelial cells may be switched from a resting state to a rapid growth phase by a "diffusible" chemical signal produced by the tumour cells. The biological mechanisms underlying the angiogenetic process have not yet been completely clarified, but several positive regulators have been identified. Vascular endothelial growth factor (VEGF) is a well characterised angiogenetic factor and is known to play a crucial role in endothelial cell proliferation and migration and, consequently, new vessel development.

"Increased vascular density has been shown to correlate with a higher incidence of metastasis and a worse prognosis in cancer"

Because tumour growth has been associated with angiogenesis,⁵ studies on the relation between angiogenesis and cell proliferation parameters in different types of tumour have been performed, generating conflicting results.^{6–10}

The association between increased VEGF expression and in vivo tumour development has been attributed to VEGF involvement in the angiogenic process.¹¹ However, the direct impact of VEGF expression on tumour growth kinetics is not clearly defined.

Recent experimental results have shown that some oncogenes are involved in the regulation of angiogenesis,¹² and interesting data have been reported on the genetic inactivation of p53 in cancer cells: loss of wild-type p53 function contributes to activation of the angiogenic switch in tumours.¹³ In our recent study, in the same colon cancer series, we demonstrated a highly significant correlation between VEGF expression, microvessel count, and p53 tumour expression.¹⁴

To gain further insight into the effects of microvessel density and VEGF expression in colon cancer, we investigated their relation with tumour proliferation and ploidy status in a series of 50 colon cancers. In addition, we studied the possible association with p53 protein expression, which is known to regulate genes involved in the angiogenic process and has been linked to proliferation and apoptosis.^{14,15}

MATERIAL AND METHODS

Patients

Surgical specimens of colon carcinomas (n = 57), from patients with primary colon carcinoma, excluding those with multiple or metachronous cancer, diagnosed from January 1998 to August 2001, were consecutively selected from the archives of the service of pathology at Campus Bio-Medico, Rome, Italy.

The patients' ages ranged from 33 to 90 years (average, 65); 40 were male and 17 were female. No patient had received chemotherapy or radiotherapy before surgery.

The pathological features were classified using the UICC-TNM classification. Tumours were graded according to the

Abbreviations: DI, DNA index; IQR, interquartile range; SPF, S phase fraction; VEGF, vascular endothelial growth factor; VWF, von Willebrand factor

Table 1 Correlation between flow cytometric and clinicopathological parameters

Clinicopathological parameters	N	High SPF		p Value	Aneuploid		p Value
		N	%		N	%	
Age							
<50	4	2	50.0%	0.596	2	50.0%	1.000
>50	46	20	43.5%		28	60.9%	
T							
1	1	1	100%	0.994	1	100%	0.791
2	9	4	44.4%		6	66.7%	
3	36	16	44.4%		21	58.3%	
4	4	1	25.0%		2	50.0%	
N							
0	20	10	50.0%	0.567	12	60.0%	1.000
1, 2	30	12	40.0%		18	60.0%	
M							
0	37	17	45.9%	0.751	23	62.2%	0.744
1	13	5	38.5%		7	53.8%	
Grade							
1	2	1	50.0%	0.892	2	100.0%	0.496
2	38	16	42.1%		22	57.9%	
3	10	5	50.0%		6	60.0%	

p Values were assessed by Fisher's test.

World Health Organisation classification criteria as well, moderately, or poorly differentiated.

The specimens were fixed in 10% neutral buffered formaldehyde and embedded in paraffin wax. Based on the quality of the morphological preservation of all available haematoxylin and eosin stained slides of the surgical specimen sections, we selected one paraffin wax block for each case. Consecutive 3 µm sections were re-cut from each block for immunohistochemical studies. Furthermore, 50 µm sections, from the same paraffin wax blocks used for immunohistochemistry, were obtained for flow cytometric studies.

Immunohistochemical staining

Immunohistochemical staining was performed by the streptavidin-biotin method. Sections were dewaxed, and immunostaining was performed using a polyclonal rabbit antibody against the VEGF protein (A-20; Santa Cruz Biotechnology, Santa Cruz, California, USA), a monoclonal mouse antibody against the p53 protein (clone DO7; Dakocytomation A/S,

Glostrup, Denmark), and a rabbit antibody against von Willebrand factor (VWF; Dakocytomation A/S).

Slides were examined by two independent pathologists (GP, EC) blinded to each other's work and with no prior knowledge of the clinical and pathological parameters. For each colon carcinoma, staining was evaluated at the invasive edge of the tumour and at least 1000 cells were observed. The staining score was evaluated as the percentage of stained cells out of the total number of cell evaluated. For the purpose of our study and on the basis of previous experience, staining for p53 and VEGF was defined as positive when > 10% of tumour cells were stained and negative when none or ≤ 10% of tumour cells were stained. Furthermore, those tumours defined as VEGF positive on the basis of the percentage ratio of stained cells were re-graded according to the intensity of staining using a scale of 1–3, with 1 representing mild, 2 moderate, and 3 strong intensity of staining.¹⁶ Arbitrarily, tumour staining graded between 0 and 1 was defined as low intensity and between 2 and 3 as high.

Determination of the microvessel count was performed by hot spot analysis according to the Weidner method. Details of the immunohistochemical analysis and its interpretation are provided in our previous study.¹⁴

Flow cytometry

For flow cytometric studies, nuclear suspensions were prepared using a modified version of the method of Hedley *et al*,¹⁷ and were incubated for 30 minutes in propidium iodide solution. The S phase fraction (SPF) was evaluated in each tumour as the percentage of cells in S phase and cases were categorised at low or high SPF according to a 15% cutoff value. This value represents the mean SPF value in our series of colon carcinomas studied.

The DNA index (DI) was calculated as the ratio of the modal value of the DNA histogram of the tumour sample to that of the reference cells. By definition, diploid tumours have a DI of 1.0, and a single G0–G1 peak at 2n. Samples with a second peak distinct from the diploid 2n peak and with a corresponding 4n peak that show a DI > 1.0 were classified as aneuploid; those with a DI of 1.9–2.1 were tetraploid, although they were analysed as aneuploid.¹⁸ Cell kinetic measurements were performed on combined populations of diploid and aneuploid cells. Data were acquired with Flomax Software 2.0 and DNA histograms were analysed by multi-cycle for windows 3.0.

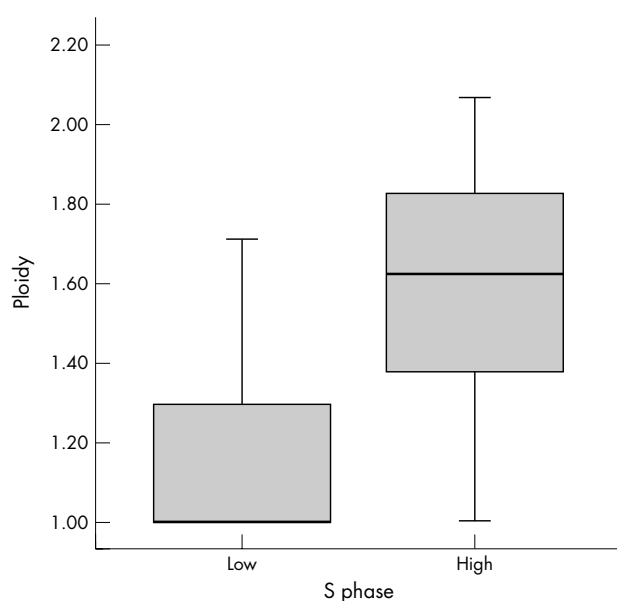


Figure 1 Relation between S phase (low, ≤ 15; high, > 15) and ploidy in colon cancer. Mann-Whitney test, $p < 0.0001$.

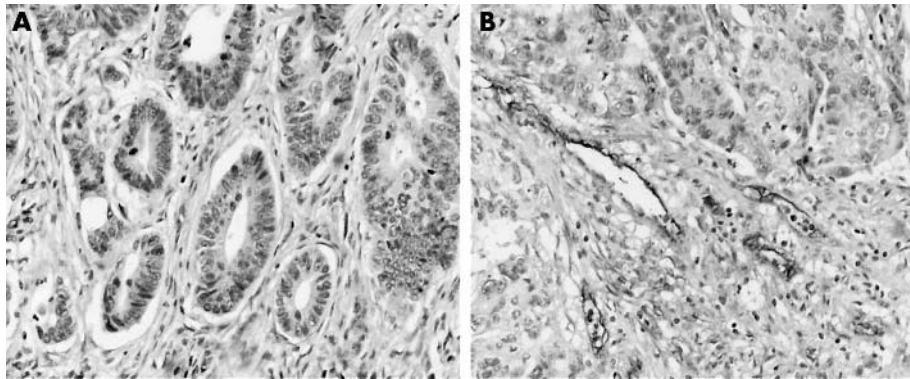


Figure 2 Representative immunohistochemical results from colon cancer. Immunostaining for von Willebrand factor (VWF) with rabbit anti-VWF antibody (Dakocytomation) of newly formed capillary vessels. (A) Tumour with low microvessel count, (B) tumour with high microvessel count (original magnification, $\times 200$).

Statistical analysis

Fisher's test was used to examine the relation of clinicopathological parameters and VEGF expression (positive ν negative) with SPF and ploidy status. In addition, the Mann Whitney U test for non-parametric independent variables was used to assess the association between SPF and ploidy status and VEGF expression (classified as high ν low), and to determine whether there was a significant difference between microvessel count, ploidy status, p53, and SPF. p Values < 0.05 were regarded as significant. SPSS software (version 10.00; SPSS, Chicago, Illinois, USA) was used for statistical analysis.

RESULTS

SPF, ploidy status, and clinicopathological parameters

An evaluable DNA histogram was obtained from 50 of the 57 samples studied. Seven tumour samples were not evaluated because of a paucity of lesions. The mean SPF was 15% (range, 3.5–37.7%). Twenty eight tumours had an SPF $\leq 15\%$ and 22 tumours had an SPF $> 15\%$ (table 1).

The DI was calculated as the ratio of the modal value of the DNA histogram of the tumour sample to that of the reference cells. By definition, tumours with DI = 1.0 were classified as diploid and tumours with a DI > 1.0 as aneuploid. Twenty tumours were diploid and 30 were aneuploid (table 1).

No association was found between flow cytometric data and the clinicopathological parameters considered (table 1).

A positive correlation was found between SPF and ploidy status. The DI was significantly higher in high SPF tumours than in low SPF ones (median, 1 (interquartile range (IQR), 1–1.32) ν 1.62 (1.38–1.81); Mann Whitney test, $p < 0.0001$; fig 1).

Correlation of SPF and ploidy with angiogenesis and p53 expression

SPF showed a positive correlation with the microvessel count. The microvessel count in high SPF tumours was significantly

higher than in low SPF ones (median, 142 (IQR, 122–159) ν 112 (87.8–142); Mann Whitney test, $p = 0.036$; table 2; fig 2).

There was no significant correlation between SPF and VEGF expression (Fisher's test, $p = 0.439$; table 2). Moreover, no correlation was found between SPF and VEGF, stratified according staining intensity (low ν high VEGF expression; Mann-Whitney test, $p = 0.072$; table 3).

In addition, the percentage of p53 positive cells in high SPF tumours was significantly higher than in low SPF tumours (median, 77.4 (IQR, 39.3–90.5) ν 47.8 (29.3–75.9); Mann Whitney test, $p = 0.041$; fig 3).

Ploidy status showed a positive correlation with microvessel count. The microvessel count in aneuploid tumours was significantly higher than in diploid tumours (median, 142 (IQR, 125–161) ν 115 (87.5–124); Mann Whitney test, $p = 0.003$).

There was no significant correlation between ploidy status and VEGF expression (Fisher's test, $p = 0.697$; table 2), although there was a positive correlation between ploidy status and VEGF, stratified according to intensity of staining (low ν high VEGF expression; Mann-Whitney test, $p = 0.027$; table 3).

In addition, the percentage staining for p53 in aneuploid tumours was significantly higher than in diploid ones (median, 75.2 (IQR, 45.6–88.7) ν 32 (27.6–63.9); Mann Whitney test, $p = 0.004$; fig 4).

DISCUSSION

In an attempt to evaluate the impact of angiogenesis and the angiogenetic factor VEGF on tumour growth, we investigated the relation between microvessel count, VEGF expression, proliferation, and ploidy status in a set of 50 colon cancers. Moreover, we also examined whether p53 expression correlated with tumour proliferation and ploidy status.

Angiogenesis was assessed by immunodetection of the endothelial cell marker VWF, which is more specific than CD31 or CD34 because CD31 showed inferior staining

Table 2 Correlation between angiogenesis and flow cytometric parameters

Flow cytometric parameters N (%)	MC		p Value*	VEGF staining		p Value†	
	Median	IQR		Positive/total	Positive (%)		
SPF							
≤ 15	28 (56%)	112	87.8–142	0.036	22/28	78.5%	0.439
> 15	22 (44%)	142	122–159		20/22	90.9%	
Ploidy							
Diploid	20 (40%)	115	87.5–124	0.003	16/20	80.0%	0.697
Aneuploid	30 (60%)	142	125–161		26/30	86.6%	

*Mann-Whitney test; †Fisher's test.

MC, microvessel count; SPF, S phase fraction; VEGF, vascular endothelial growth factor.

Table 3 Correlation of VEGF score with SPF and ploidy status

VEGF expression	N	SPF		p Value	Diploid	Aneuploid	p Value
		<15%	>15%				
Low	16	11 (69%)	5 (31%)	0.072	10 (62.5%)	6 (37.5%)	0.027
High	34	14 (41%)	20 (59%)		10 (29%)	24 (71%)	

*p Values were assessed by the Mann-Whitney test.
SPF, S phase fraction; VEGF, vascular endothelial growth factor.

performance compared with VWF¹⁹ and CD34 showed immunostaining of lymphatic vessels.²⁰ Furthermore, recently, it has been shown that the VWF positive microvessel count may be considered to be an independent prognostic marker for patients with colorectal cancer and may help to identify patients with an unfavourable prognosis.²¹ Hot spot analysis was performed using the Weidner method because when counted this way the microvessel count has been shown to correlate with patient outcome.^{22, 23}

To assess tumour proliferation status, we evaluated the S phase fraction by flow cytometry. The technical issues of validity and reproducibility related to prognostically useful methods of measuring tumour cell proliferation are a matter of controversy, but according to recent studies flow cytometric SPF is the most useful tumour cell proliferation marker to assess disease prognosis.^{24, 25}

Because VEGF exhibits angiogenic activity and increased VEGF has been related to in vivo tumour development,⁵ we examined the impact of VEGF expression and microvessel count on tumour growth.

In our series, we failed to demonstrate an association between VEGF expression and tumour proliferation, whereas a significant positive correlation was found between SPF and microvessel count. The median microvessel count in high SPF tumours was significantly higher than in low SPF ones ($p = 0.036$).

Our results are in agreement with a study by Zhang *et al*, in which transfection of a full length cDNA encoding the shortest isoform of VEGF (VEGF121) into MCF-7 cells showed that, in vitro, the growth rate of MCF-7 wild-type cells was indistinguishable from that of VEGF121 transfected

MCF-7, although subcutaneous implantation of transfected cells formed faster growing tumours in vivo because of intense neovascularisation.¹¹ Moreover, Takahashi *et al* showed that VEGF receptors were not present on tumour epithelial cells, so that VEGF probably has no direct mitogenic effect on colon cancer cells.²⁶

“The vascular endothelial growth factor protein may be an indirect mediator of tumour growth in colon cancer by microvessel proliferation induction”

These findings support the idea that tumour cell proliferation occurs in part because the growth of new tumour vessels allows for exchange of nutrients, oxygen, and waste products in a crowded cell population for which simple diffusion is inadequate. Moreover, the almost significant correlation ($p = 0.072$) between tumour cell proliferation and VEGF expression indicates that these two parameters are not directly correlated and any increase in S phase associated with VEGF expression is probably the result of its angiogenic activity.

In a recent study, we found an association between angiogenesis and p53 overexpression in colon cancer.¹⁴ In the same series, a significant association was found between p53 overexpression and aneuploidy ($p = 0.004$). This result is consistent with other reports,^{27, 28} suggesting that wild-type p53 function may be required to maintain diploidy.²⁹ Furthermore, wild-type p53 can prevent replication of damaged DNA and DNA re-replication that can lead to aneuploidy.³⁰

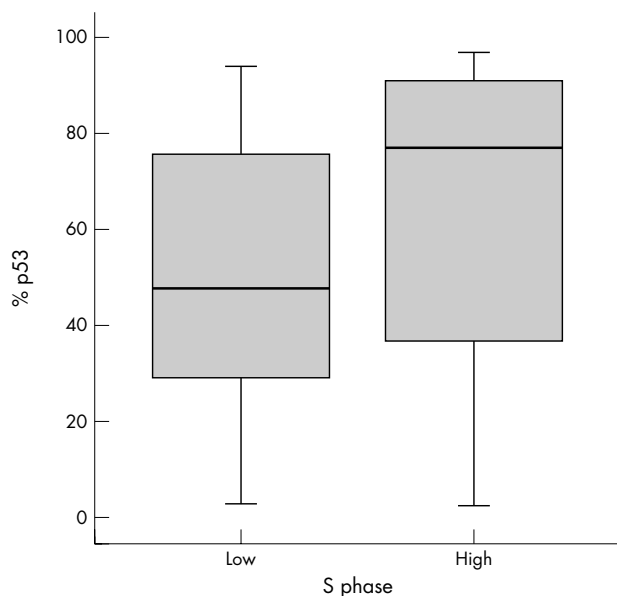


Figure 3 Relation between S phase (low, ≤ 15 ; high, > 15) and p53 expression. Mann-Whitney test, $p = 0.041$.

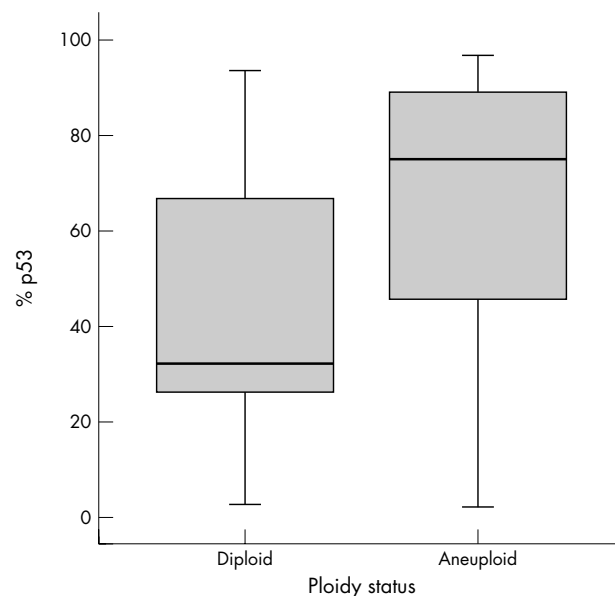


Figure 4 Relation between ploidy status and p53 expression. Mann-Whitney test, $p = 0.004$.

Take home messages

- Microvessel density was closely correlated with proliferation, suggesting that tumour growth may be stimulated by vascular supply in colon cancer
- The lack of a correlation between tumour cell proliferation and vascular endothelial growth factor (VEGF) expression indicates that they may be regulated by separate mechanisms
- The positive correlation between microvessel density, VEGF expression, and ploidy status provides more evidence that genetic alterations are involved in tumour angiogenesis

Taking into account the involvement of angiogenesis in tumour genetic alteration, we examined the relation between angiogenesis and DNA ploidy, because the tumour aneuploidy status reflects molecular genetic abnormalities.³¹⁻³² In our present study, a positive correlation was found between ploidy status and microvessel count ($p = 0.003$). Moreover, a positive correlation was found between DNA ploidy and VEGF expression. In fact, 24 of 34 colon cancers with high VEGF expression were aneuploid in flow cytometric studies, whereas only six of 16 colon cancers with low VEGF expression were aneuploid ($p = 0.027$). Mutated p53, which is associated with increased aneuploidy and increased VEGF, may form a common link to these findings.¹⁴⁻²⁷⁻²⁸

In conclusion, our present study provides evidence that microvessel density is closely correlated with S phase fraction in colon cancer, thereby showing that tumour growth may be stimulated by vascular supply. Therefore, the VEGF protein may be an indirect mediator of tumour growth in colon cancer by microvessel proliferation induction. Furthermore, the association between aneuploidy, high microvessel count, and VEGF expression is more evidence to suggest a direct relation between DNA alteration and the angiogenesis process.

Further prospective studies will be needed to confirm the clinical value of measuring the combination of angiogenesis with S phase fraction and ploidy status in colon cancer. Perhaps in the future, the combination of clinical and tumour related parameters in colon cancer will lead to a grading system with better prognostic and therapeutic value.

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Authors' affiliations

E Cristi, G Perrone, A Verzi, S Nori, C Rabitti, Surgical Pathology, Campus Bio-Medico University, Via Emilio Longoni, Rome 83 00155, Italy

G Toscano, A Fabiano, Surgical Pathology, Fatebenefratelli Hospital, Rome 00186, Italy

D Santini, G Tonini, Oncology Unit, Campus Bio-Medico University, Rome 00155, Italy

A Vetrani, Department of Anatomic Pathology and Cytopathology, University of Naples "Federico II", Naples 80131, Italy

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