available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.ejconline.com

Vascular endothelial growth factors and receptors in colorectal cancer: Implications for anti-angiogenic therapy

Sarah E. Duff[a], *Maria Jeziorska*[b], *Daniela D. Rosa*[c,*], *Shant Kumar*[b], *Najib Haboubi*[d], *David Sherlock*[e], *Sarah T. O'Dwyer*[a], *Gordon C. Jayson*[c]

[a]*Department of Surgery, Christie Hospital NHS Trust, Wilmslow Road, Manchester M20 4BX, United Kingdom*
[b]*Laboratory Medicine Academic Group, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, United Kingdom*
[c]*Cancer Research UK, Department of Medical Oncology, Christie Hospital NHS Trust, Wilmslow Road, Manchester M20 4BX, United Kingdom*
[d]*Department of Pathology, Trafford General Hospital NHS Trust, Moorside Road, Davyhulme, Manchester M41 5SL, United Kingdom*
[e]*Department of Surgery, North Manchester General Hospital, Delauneys Road, Manchester M8 5RB, United Kingdom*

ARTICLE INFO

Article history:
Received 9 August 2005
Received in revised form
12 September 2005
Accepted 14 September 2005
Available online 29 November 2005

Keywords:
VEGF
VEGFR
Colorectal cancer
Liver metastasis
Lymph nodes metastasis
Anti-VEGF antibodies

ABSTRACT

There are conflicting associations between growth factor expression and clinicopathological variables in colorectal cancer. This study aimed to define the expression of members of the VEGF family and the receptor, VEGFR2, in primary and metastatic sites of colorectal cancer and their relationship to metastatic potential. Thirty colorectal cancers, 12 lymph node metastases and 9 liver metastases were immunostained for VEGF-A, VEGF-C, VEGF-D and VEGFR2. VEGFR2 was expressed by endothelial cells and by the malignant epithelium. VEGF-C and VEGFR2 were co-expressed in the same territory and correlated throughout the primary tumour and in metastatic lymph nodes, but not in liver metastases. Their expression at the invasive tumour edge correlated with expression in metastatic nodes. The benefit of anti-VEGF antibodies might be increased by directing additional therapies against VEGF-C or against the kinase receptors to target redundancy in the system. A component of the therapeutic benefit might be due to a direct anti-tumour effect as well as an anti-angiogenic effect.

1. Introduction

The Vascular Endothelial Growth Factor (VEGF) family is important for the process of angiogenesis which is central to the growth and metastasis of cancer [1]. Indeed, recent data have shown that the addition of an anti-VEGF antibody to conventional cytotoxic chemotherapy confers a survival advantage to patients with metastatic colorectal cancer [2]. The most studied member of the VEGF family is VEGF (VEGF-A) [3], which is vital at all stages of human development. Newer members of the VEGF family include VEGF-C and VEGF-D [4,5], which possess lymphangiogenic effects in addition to their angiogenic action. The VEGF family exert their effects through activation of one or more of three related VEGF tyrosine kinase receptors (VEGFRs) [6].

The principal lineage that expresses VEGFRs is the endothelial cell, but increasingly, it is becoming apparent that the receptors can be expressed on malignant cell types, both on human cancer cell lines in vitro [7,8] and in human tissues including ovarian cancer [9], renal cell carcinoma [10], squamous cell carcinomas of the head and neck [11] and pancreatic cancer [12]. Co-expression of functional VEGFRs with their corresponding ligands in tumours raises the possibility of autocrine loops, whereby a tumour is capable

* *Corresponding author*: Tel.: +44 161 446 3606; fax: +44 161 446 3461.
E-mail address: dornellesrosa@hotmail.com (D.D. Rosa).
0959-8049/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2005.09.018

of stimulating its own growth, progression and survival. The existence of autocrine loops has important implications for the development of novel anti-VEGF and anti-VEGFR compounds that could have potential anti-angiogenic and direct anti-tumour effects in human malignancy [13].

The association between tumour growth factor expression and clinicopathological variables, including outcome, is not straightforward. Various authors have examined expression of VEGF-A in colorectal cancer and concluded either that increased expression is associated with negative clinicopathological variables [14] or alternatively, that no such association could be demonstrated [15]. Similarly, expression of VEGF-C and VEGF-D is noted in a variety of human malignancies and tends to correlate with negative clinicopathological variables, in particular, lymph node involvement and lymphatic invasion, although the relationships observed are not always consistent [16]. The reported inconsistencies may be due to the balance between the different members of the VEGF family and their relative levels of expression together with receptor availability.

The aim of this study was to define the expression pattern of the VEGF family members and VEGFR2 in primary and secondary sites of colorectal cancer and examine their influence on metastatic spread.

2. Materials and methods

Thirty primary colorectal cancer specimens, 12 associated lymph node metastases and 9 subsequent liver metastases were examined for growth factor and receptor expression using immunohistochemical techniques. The clinicopathological details of patients studied are shown in Table 1.

Tissue sections were mounted on 3-aminopropyl-triethoxysilane coated slides and immunostained for the antigens VEGF-A, VEGF-C, VEGF-D and VEGFR2 using a standard immunoperoxidase technique. Briefly, the sections were dewaxed in xylene and rehydrated through a graded ethanol series. Subsequently, endogenous peroxidase was blocked by immersion in 1% hydrogen peroxide for 30 min at room temperature. Following serum blocking, the primary antibody was applied and incubated overnight at 4 °C. A biotinylated secondary antibody was applied and incubated at room temperature for 45 min, followed by horse-radish peroxidase conjugated streptavidin for 45 min. Finally, the chromogenic substrate 3,3-diaminobenzidine tetrahydrochoride (DAB) was added. The primary antibodies and dilutions used were: VEGF-A: rabbit anti-human VEGF-A IgG (Santa Cruz, CA, USA), 1:400; VEGF-C: rabbit anti-human VEGF-C IgG (Zymed, San Francisco, USA), 1:50; VEGF-D: mouse monoclonal anti-human VEGF-D IgG (R&D Systems, Abingdon, UK), 1:500; VEGFR2: mouse monoclonal anti-VEGFR2 IgG (Santa Cruz, CA, USA), 1:50; and rabbit anti-VEGFR2 IgG (Abcam, Cambridge, UK), 1:50 to verify the findings.

The slides were reviewed independently by two observers (SED, MJ) blinded to clinical details and to which lymph nodes and/or liver metastases belonged to which primary tumour. The intensity of tissue staining was scored on a semi-quantitative scale from 0 to 5 (0: no stain; 5: strongest stain). Assessment was made at different areas of the tissue, including the normal colonic mucosa (N), mucosa at the junction between normal and malignant tissue (J), superficial part of the tumour (TS), central tumour (TC) and tumour at the invading edge (TI). In the event of discrepancies between the scorers, the slides were reviewed and scoring agreed by consensus.

Table 1 – Clinicopathological details of patients studied

Parameter	Group 1 (patients with non-metastatic tumours or with lymph node metastasis)	Group 2 (patients with liver metastasis)
Number of patients	21	9
Age (years)[a]	71 (49–86)	53 (39–76)
Gender (male:female)	10:11	7:2
T stage		
T1	1	0
T2	4	0
T3	13	8
T4	3	1
N stage		
N0	9	5
N1	7	3
N2	5	1
Differentiation		
Well differentiated	0	0
Moderately differentiated	13	9
Poorly differentiated	8	0
Dukes' stage		
A	1	0
B	7	4
C	11	4
D	2	1

a Median (range).

The study was approved by the local research ethics committee and informed consent sought in accordance with the recommendations of the committee.

Non-parametric tests were used for statistical analysis. Comparisons between medians of related variables were made with the Wilcoxon signed rank test for 2 variables and the Friedman test for greater than 2 related variables. Correlations between variables were examined with Spearman's rank correlation coefficients. All statistical tests were two-sided and $P < 0.05$ was taken as statistically significant.

3. Results

Primary tumours were stained for VEGFR2 using a monoclonal anti-VEGFR2 antibody. Weak staining was identified on vascular endothelial cells (Fig. 1a) and strong immunostaining was seen on malignant colorectal epithelium (Fig. 1b). Such intense expression in colorectal epithelial cells was unexpected, so in order to confirm this finding immunostain-

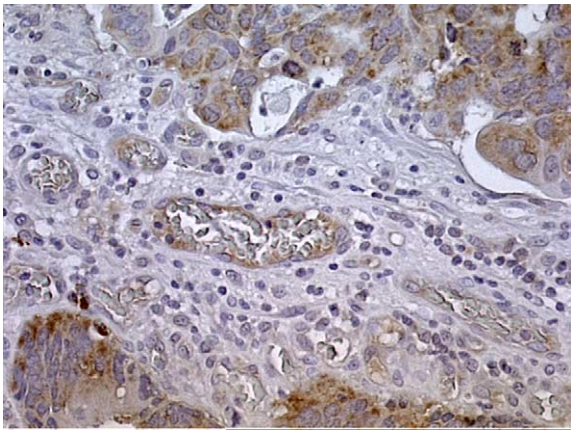

Fig. 1a – VEGFR2 immunostaining in vascular endothelial cells. A moderately differentiated colonic adenocarcinoma immunostained for VEGFR2 with mouse monoclonal anti-VEGFR2 antibody, counterstained with haematoxylin at magnification 200×. Positive immunostaining is seen on endothelial cells (arrowheads) and on tumour epithelial cells (arrows).

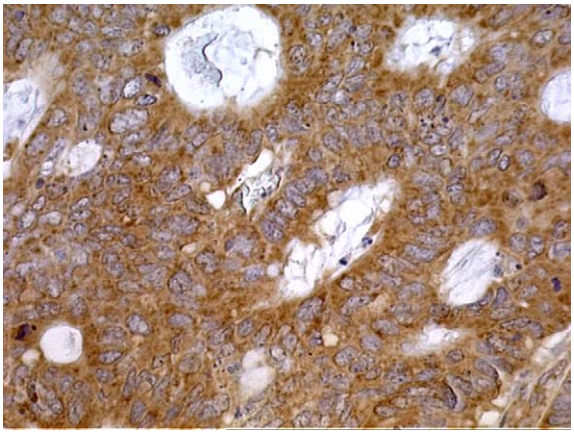

Fig. 1b – VEGFR2 immunostaining in colorectal cancer cells. The invasive edge of a moderately differentiated colonic adenocarcinoma immunostained with mouse monoclonal anti-VEGFR2 antibody. The section is counterstained with haematoxylin and magnification is 100×.

ing was repeated using the different rabbit polyclonal anti-VEGFR2 antibody. Results confirmed the initial findings (Fig. 1c).

The median intensity of immunostaining for VEGF-A, VEGF-C, VEGF-D and VEGFR2 increased from normal mucosa, through the junctional mucosa and throughout the tumour to a maximum at the invasive tumour edge ($P < 0.001$ for all antigens, Table 2). There were no differences in median expression intensities of VEGF-C, VEGF-D, VEGF-A and VEGFR2 at the TI and in lymph node metastases, nor were any differences identified between VEGF-C, VEGF-D and VEGF-A TI expression and expression in liver metastases. However, VEGFR2 expression intensity was reduced in liver metastases in comparison to TI expression (Wilcoxon signed rank test, $P = 0.007$; Table 2).

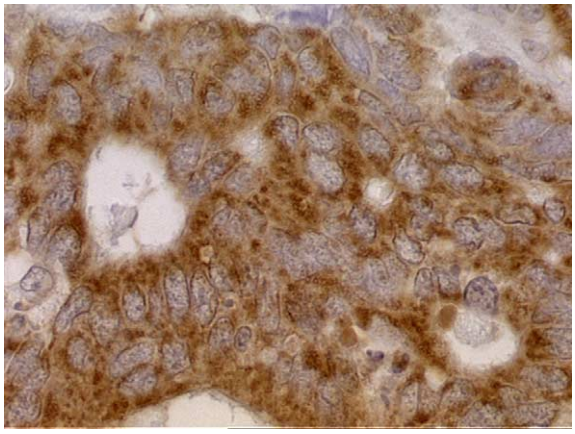

Fig. 1c – Immunostaining for VEGFR2 in colorectal cancer. Serial sections of a moderately differentiated colonic adenocarcinoma were immunostained with mouse monoclonal anti-VEGFR2 antibody. Counterstaining was with haematoxylin and magnification is 200×.

The expression intensity of VEGF-C and VEGFR2 correlated at four of the five locations assessed in the primary tumour (Spearman's rank correlation coefficients for J, TS, TC, and TI were 0.50, 0.41, 0.58 and 0.42, with corresponding *P*-values of 0.03, 0.028, 0.001 and 0.019, respectively). Serial staining of sections demonstrated that VEGF-C and VEGFR2 were co-expressed in the same territory, raising the possibility that the former might be capable of activating the latter (Figs. 1d and 1e). The intensity of epithelial cell expression of VEGF-C, VEGF-D and VEGF-A did not correlate with one another, nor did epithelial cell expression intensity of VEGF-D or VEGF-A correlate with that of VEGFR2 within the tumour sites examined. A similar pattern for VEGF-C/VEGFR2 was seen in metastatic lymph nodes, whereby the expression of VEGF-C and VEGFR2 correlated (Spearman's rank correlation coefficient, 0.72, $P = 0.009$), but no correlation was seen in liver metastases. VEGF-C and VEGFR2 expression at TI also correlated with expression in the corresponding lymph node metastases (Spearman's rank correlation coefficients 0.72, $P = 0.009$ and 0.66, $P = 0.02$, respectively).

4. Discussion

Malignant epithelial cell expression of VEGFRs has been recognised in various tumours, along with the identification of autocrine loops between the tumour-produced ligand and receptor. In the context of colorectal cancer hitherto, there has been little published evidence of tumour epithelial VEGFR expression. Up-regulation of VEGFR mRNA expression has been reported in endothelial but not neoplastic cells, in animal tumour models of colorectal cancer liver metastasis [17]. Similar findings have been described in human primary tumours, with up-regulated VEGFR1 and VEGFR2 mRNA in endothelial cells but not in tumour epithelium [18]. However, others have reported increased levels of VEGFR1 and VEGFR2 mRNA and reduced VEGFR3 mRNA in adenomas and carcinomas in comparison to normal tissues [19]. Using immunohistochemical techniques, VEGFR2 protein expression was

Table 2 – Immunostaining intensities in primary and secondary sites of colorectal cancer

Growth factor	Tumour site examined for immunostaining intensity						
	N	J	TS	TC	TI	LN	Liver
VEGF-C							
Number	25	23	29	30	30	12	9
Median (range)	0 (0–2)	1 (0–4)	2 (0–4)	3 (0–5)	4 (0.5–5)[b]	3 (0.75–5)	2 (0.25–4)
VEGF-D							
Number	23	23	29	30	30	12	9
Median (range)	0 (0–2)	1 (0–3)	0.5 (0–2.5)	1 (0–3)	1.75 (0–4)[b]	1.5 (0.25–4)	1 (0–3)
VEGF-A							
Number	25	22	30	30	30	12	9
Median (range)	0 (0–0.5)	0 (0–1.5)	0 (0–1)	0.5 (0–3)	0.5 (0–3)[b]	1.25 (0.25–3.5)	0.5 (0–1.5)
VEGFR2							
Number	26	20	30	30	30	12	9
Median (range)	0 (0–3)	0.75 (0–4)	0.5 (0–4)	3 (0–5)	4 (1–5)[b]	3.5 (0–5)	0 (0–1.5)[a]

N, normal mucosa; J, junctional mucosa; TS, superficial tumour; TC, central tumour; TI, invasive tumour edge; LN, lymph node metastasis; liver, liver metastasis. The number of cases available for assessment at each tumour site ranged from 20 to 30, the full set of 30 primary tumours could not be assessed at all sites in all cases, due to the limitations of tissue availability.

a $P = 0.007$, TI vs. liver met (Wilcoxon signed rank test).

b $P < 0.001$ (Friedman test) for difference in median staining intensity across the primary tumour sites examined.

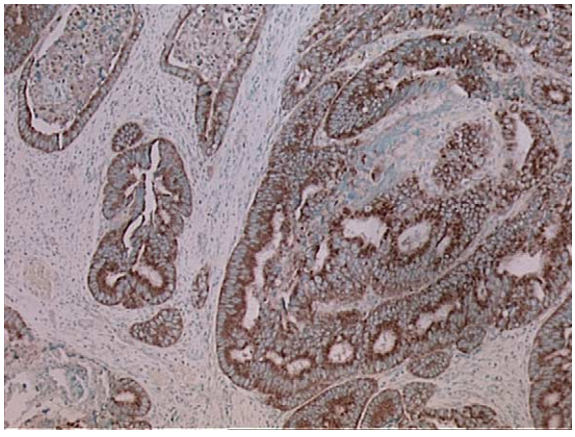

Fig. 1d – Immunostaining for VEGFR2 in colorectal cancer. Serial sections of a moderately differentiated colonic adenocarcinoma were immunostained with rabbit polyclonal anti-VEGFR2 antibody. Counterstaining was with haematoxylin and magnification is 200×.

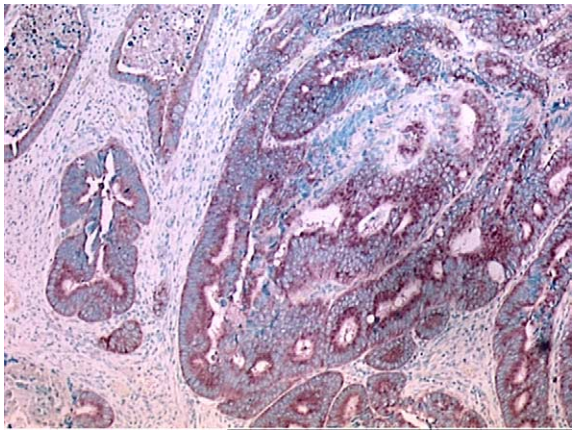

Fig. 1e – Co-localisation of VEGF-C and VEGF-FR2 in colorectal cancer. Serial sections of a moderately differentiated rectal adenocarcinoma were immunostained for VEGF-C and VEGF-FR2 and counterstained with methyl green. The chromogen was DAB and VIP for VEGF-C and VEGF-FR2, respectively. Immunolocalisation of antigens is to the malignant colonic epithelial cells and the magnification is 40×.

identified in both vascular endothelial and colorectal tumour cells in 65% of 136 colorectal cancer specimens [20]. Increased VEGFR2 expression was observed on malignant endocrine cells in the gastrointestinal tract [21] and VEGFR3 expression in colorectal cancer has previously been identified by two separate groups [22,23]. The present study has shown that VEGFR2 is expressed consistently in colorectal cancer. The presence of tumour-expressed VEGFR2 allows for the existence of autocrine and paracrine circuits between the tumour-expressed ligands VEGF-A, VEGF-C and VEGF-D and the receptor.

The pattern of immunostaining throughout the primary tumours was similar for the three growth factors and VEGFR2. In all cases, expression increased from the normal mucosa, through the junctional mucosa and throughout the tumour to a maximum at the invasive tumour edge. Most studies addressing the relationship between tumour-expressed growth factors and prognosis in colorectal cancer fail to assess the intra-tumoral heterogeneity of growth factor expression, which may explain the conflicting associations reported with clinicopathological variables and outcome. The invasive tumour edge is considered to have the highest malignant potential in comparison to other parts of the tumour [24]. Studies from Japan have also examined the topographical distribution of VEGF-A and VEGF-C throughout primary colorectal cancers [14,25] and shown that growth factor expression increased throughout the tumour and that TI

expression correlated with microvessel density and poorer outcome.

This report is the first to show that the liver metastases of colorectal cancer express VEGF-C and VEGF-D. Expression of the growth factors was not significantly different from their expression at TI in the primary site. However, VEGFR2 expression was reduced in comparison to the primary site. This is likely to be due to the microenvironmental conditions found in the liver, particularly the oxygen tension. The relatively high levels of oxygen in hepatic parenchyma mean that VEGF-A is less of an angiogenic driving force at this site than in the primary tumour. This is corroborated by the lower levels of VEGF-A seen in liver metastases in comparison to metastases at other intra-abdominal sites [26]. The clinical relevance of this finding is that new approaches to cancer therapeutics involving tyrosine kinase inhibitors may have limited effectiveness in the treatment of colorectal hepatic metastases in comparison to other metastatic sites.

Co-localisation between VEGF-C and VEGFR2 was demonstrated by staining serial tissue sections. VEGF-C and VEGFR2 correlated in their expression at multiple sites within the primary tumour and in lymph node metastases. TI expression of the growth factor and receptor further correlated with corresponding expression in lymph node metastases. The close relationships observed suggest that VEGF-C/VEGFR2 interaction is important in loco-regional nodal spread in colorectal cancer. This proposal is supported by the correlation noted between levels of colorectal tumour VEGFR2 mRNA and lymph node metastasis by Hanrahan and colleagues [19].

Within colorectal cancer, multiple members of the VEGF family are expressed in addition to their receptor, VEGFR2. This redundancy within the VEGF pathways implies that targeting a single ligand e.g. VEGF-A with an antibody, bevacizumab (Avastin®), might have limited effect as the other tumour-expressed ligands are still able to interact with tumour-expressed VEGFR2, thus allowing a tumour to overcome the effects of a single agent anti-VEGF-A antibody. Despite this, combination chemotherapy and bevacizumab regimens are associated with a survival advantage in comparison with chemotherapy alone for patients with advanced colorectal carcinoma [2]. Thus it may be possible to increase the benefit already seen by targeting VEGF-C either by inhibiting the growth factor in addition to VEGF-A or through the use of receptor tyrosine kinase inhibitors. Furthermore, the presence of VEGFR2 on the malignant colorectal cancer cell also permits anti-VEGF-A therapies to possess both anti-angiogenic and direct anti-tumour actions, as they are able to disrupt the autocrine/paracrine circuits that tumour-expressed ligand-receptor pairs create.

In summary, our data suggest that VEGF inhibitors may have an anti-tumour and anti-angiogenic effect in colorectal cancer. These dual-lineage targets might be critical for the development of effective signal transduction inhibitors. Furthermore, if we are to optimise the effectiveness of VEGF inhibitors we may also need to target VEGF-C in addition to VEGF-A.

Conflict of interest statement

None declared.

REFERENCES

1. Veikkola T, Alitalo K. VEGFs, receptors and angiogenesis. *Semin Cancer Biol* 1999;**9**:211–20.
2. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004;**350**:2335–42.
3. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol* 2002;**20**:4368–80.
4. Joukov V, Sorsa T, Kumar V, et al. Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J* 1997;**16**:3898–911.
5. Achen MG, Jeltsch M, Kukk E, et al. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci USA* 1998;**95**:548–53.
6. Karkkainen MJ, Petrova TV. Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. *Oncogene* 2000;**19**:5598–605.
7. Wu W, Shu X, Hovsepyan H, et al. VEGF receptor expression and signaling in human bladder tumors. *Oncogene* 2003;**22**:3361–70.
8. Soker S, Kaefer M, Johnson M, et al. Vascular endothelial growth factor-mediated autocrine stimulation of prostate tumor cells coincides with progression to a malignant phenotype. *Am J Pathol* 2001;**159**:651–9.
9. Boocock CA, Charnock-Jones DS, Sharkey AM, et al. Expression of vascular endothelial growth factor and its receptors flt and KDR in ovarian carcinoma. *J Natl Cancer Inst* 1995;**87**:506–16.
10. Tsuchiya N, Sato K, Akao T, et al. Quantitative analysis of gene expressions of vascular endothelial growth factor-related factors and their receptors in renal cell carcinoma. *Tohoku J Exp Med* 2001;**195**:101–13.
11. Neuchrist C, Erovic BM, Handisurya A, et al. Vascular endothelial growth factor C and vascular endothelial growth factor receptor 3 expression in squamous cell carcinomas of the head and neck. *Head Neck* 2003;**25**:464–74.
12. von Marschall Z, Cramer T, Hocker M, et al. De novo expression of vascular endothelial growth factor in human pancreatic cancer: evidence for an autocrine mitogenic loop. *Gastroenterology* 2000;**119**:1358–72.
13. Harmey JH, Bouchier-Hayes D. Vascular endothelial growth factor (VEGF), a survival factor for tumour cells: implications for anti-angiogenic therapy. *Bioessays* 2002;**24**:280–3.
14. Kaio E, Tanaka S, Kitadai Y, et al. Clinical significance of angiogenic factor expression at the deepest invasive site of advanced colorectal carcinoma. *Oncology* 2003;**64**:61–73.
15. Nanni O, Volpi A, Frassineti GL, et al. Role of biological markers in the clinical outcome of colon cancer. *Br J Cancer* 2002;**87**:868–75.
16. Duff SE, Li C, Jeziorska M, et al. Vascular endothelial growth factors C and D and lymphangiogenesis in gastrointestinal tract malignancy. *Br J Cancer* 2003;**89**:426–30.
17. Warren RS, Yuan H, Matli MR, et al. Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest* 1995;**95**:1789–97.
18. Brown LF, Berse B, Jackman RW, et al. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. *Cancer Res* 1993;**53**:4727–35.
19. Hanrahan V, Currie MJ, Gunningham SP, et al. The angiogenic switch for vascular endothelial growth factor (VEGF)-A, VEGF-B, VEGF-C, and VEGF-D in the adenoma-carcinoma

sequence during colorectal cancer progression. *J Pathol* 2003;**200**:183–94.
20. Tanigawa N, Amaya H, Matsumura M, et al. Correlation between expression of vascular endothelial growth factor and tumor vascularity, and patient outcome in human gastric carcinoma. *J Clin Oncol* 1997;**15**:826–32.
21. La Rosa S, Uccella S, Finzi G, et al. Localization of vascular endothelial growth factor and its receptors in digestive endocrine tumors: correlation with microvessel density and clinicopathologic features. *Hum Pathol* 2003;**34**:18–27.
22. Witte D, Thomas A, Ali N, et al. Expression of the vascular endothelial growth factor receptor-3 (VEGFR-3) and its ligand VEGF-C in human colorectal adenocarcinoma. *Anticancer Res* 2002;**22**:1463–6.
23. Kawakami M, Furuhata T, Kimura Y, et al. Quantification of vascular endothelial growth factor-C and its receptor-3 messenger RNA with real-time quantitative polymerase chain reaction as a predictor of lymph node metastasis in human colorectal cancer. *Surgery* 2003;**133**:300–8.
24. Oh-e H, Tanaka S, Kitadai Y, et al. Angiogenesis at the site of deepest penetration predicts lymph node metastasis of submucosal colorectal cancer. *Dis Colon Rectum* 2001;**44**:1129–36.
25. Furudoi A, Tanaka S, Haruma K, et al. Clinical significance of vascular endothelial growth factor C expression and angiogenesis at the deepest invasive site of advanced colorectal carcinoma. *Oncology* 2002;**62**:157–66.
26. Cascinu S, Graziano F, Catalano V, et al. Differences of vascular endothelial growth factor (VEGF) expression between liver and abdominal metastases from colon cancer. Implications for the treatment with VEGF inhibitors. *Clin Exp Metastasis* 2000;**18**:651–5.