PERGAMON

European Journal of Cancer 39 (2003) 1698–1703

European Journal of Cancer

www.ejconline.com

Co-expression of vascular endothelial growth factor C (VEGF-C) and c-erbB2 in human breast carcinoma☆

F.J. Hoar[a,*], S. Chaudhri[b], M.S. Wadley[a], P.S. Stonelake[a]

[a]*Department of Surgery, City Hospital, Birmingham B18 7QH, UK*
[b]*Department of Pathology, City Hospital, Birmingham B18 7QH, UK*

Received 9 December 2002; received in revised form 13 March 2003; accepted 22 April 2003

Abstract

Vascular endothelial growth factor C (VEGF-C) has angiogenic and lymphangiogenic properties and is associated with the development of lymphatic metastases in a number of epithelial malignancies. The aim of this study was to determine VEGF-C protein expression in a series of breast carcinomas and correlate this with axillary lymph node (LN) metastases, the presence of lympho-vascular invasion (LVI), bone marrow micro-metastases (BMM) and other clinico-pathological data including oestrogen receptor (ER) and c-erbB2 status. VEGF-C expression was determined by immunohistochemistry (IHC) in 51 tumours. ER and c-erbB2 were also assessed by IHC. Bone marrow analysis was performed using a combination of immunomagnetic separation and immunocytochemistry. Overall, 30/51 (59%) of the tumours were positive for VEGF-C. There was no significant correlation between VEGF-C expression and LN status, LVI, BMM, tumour size, grade or ER status. However, there was an association between c-erbB2 and VEGF-C expression ($P=0.013$). The correlation between VEGF-C and c-erbB2 suggests a functional relationship and may, in part, explain the aggressive phenotype associated with c-erbB2-positive tumours.

Keywords: Breast neoplasms; VEGF-C; c-erbB2; Lymphatic metastasis; Bone marrow

1. Introduction

Angiogenesis is well recognised as an essential process for tumour growth, invasion and metastatic spread in many human malignancies [1]. Vascular endothelial growth factor (VEGF) has been shown to play a major role in tumour angiogenesis in many studies [2,3]. Its biological functions are numerous and include vascular endothelial cell proliferation and migration, increased vascular permeability and upregulation of anti-apoptotic factors, urokinase-type serine proteases and matrix metalloproteinases [2]. Tumour VEGF expression and micro-vessel density (MVD) are closely related and both have been shown to be markers of tumour cell dissemination, including bone marrow micro-metastases (BMM) [4–6]. In breast cancer, a number of studies have shown tumour VEGF expression to have prognostic significance, in both node-positive and node-negative patients [7,8]. However, the role of the more recently discovered related factors, VEGF-B, VEGF-C and VEGF-D, in human malignancy is less well characterised.

VEGF-C was first identified as a ligand for the tyrosine kinase receptor VEGFR-3, which is associated with the lymphatic vasculature [9]. VEGF-C is also a ligand for VEGFR-2, which it shares with VEGF-A and VEGF-D. Structurally, VEGF-C most closely resembles VEGF-D and shares approximately 30% sequence homology to the central core of VEGF. Studies have shown VEGF-C to have both angiogenic and lymphangiogenic properties. VEGF-C stimulates endothelial cell proliferation and migration *in vitro*, but is less potent than VEGF [9]. It also increases vascular permeability. Both these functions appear to be mediated via the VEGFR-2 receptor [9]. *In vivo*, VEGF-C stimulates angiogenesis in the rabbit ischaemic hindlimb model, rabbit cornea assay and early chick chorioallantoic membrane [9].

☆ This work was presented to the Nottingham International Breast Cancer Conference, September 2001.

* Corresponding author at Cambridge Breast Unit, Box 97, Addenbrooke's Hospital, Cambridge CB2 2QQ, UK. Tel.: +44-1223-257-147.

E-mail address: fhoar@aol.com (F.J. Hoar).

0959-8049/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0959-8049(03)00382-4

VEGF-C overexpression in the skin of mice results in hyperplasia of lymphatic vessels whilst lymphangiogenesis is stimulated by VEGF-C in the differentiated avian chorio-allantoic membrane [9]. Recently, the *in vivo* role of VEGF-C in tumoral lymphangiogenesis and lymphatic metastasis has been examined. Breast cancer cells transfected to overexpress VEGF-C and injected into mice resulted in larger, more vascular tumours with increased regional lymph node and pulmonary metastases compared with controls [10]. In addition, using the lymphatic - specific antibody, LYVE - 1, intratumoral lymphangiogenesis was demonstrated in the VEGF-C overexpressing tumours, but not in the control tumours. Similar findings have been observed in other animal models [11,12].

A number of studies have investigated the role of VEGF-C in human malignancy [13–19] and many have shown a correlation with regional lymph node metastases and the presence of lympho-vascular invasion (LVI) [13–17]. Some have also demonstrated an association with survival [13,15–17]. However, few studies have investigated the role of VEGF-C in human breast cancers.

The aim of this study was to investigate whether VEGF-C expression in primary human invasive breast carcinoma correlated with standard clinico-pathological data and factors relating to angiogenesis and lymphangiogenic activity—in particular, axillary lymph node (LN) status, the presence of lympho-vascular invasion and c-erbB2 status. In addition, the bone marrow was analysed for the presence of tumour cells as an end marker of tumour angiogenesis to evaluate whether VEGF-C, like VEGF is associated with tumour cell dissemination.

2. Patients and methods

2.1. Patients and tissue samples

Fifty-one women with primary invasive breast cancer who were diagnosed and treated at the City Hospital in Birmingham between December 1997 and January 1999 were entered into this study. All women underwent surgery in the form of either mastectomy ($n=23$) or wide local excision ($n=28$) plus axillary node dissection together with intra-operative bone marrow aspiration. Local ethical committee approval was obtained and informed, written consent was given by all patients. Women with a previous history of malignancy were excluded from the study as a previous primary tumour or occult recurrent disease could potentially give rise to a positive bone marrow result unrelated to the breast cancer being treated. Other exclusion criteria included neo-adjuvant chemotherapy or hormonal therapy. The patients' age ranged from 30 to 87 years (median of 60 years). Data collected included tumour type, size and grade, the presence of lympho-vascular invasion and axillary lymph node status. VEGF-C, c-erbB2 and oestrogen receptor (ER) expression were assessed using immuno-histochemistry (IHC). Bone marrow was analysed for the presence of circulating tumour cells by a combined method of immunomagnetic cell separation and immunocytochemistry.

2.2. Immunohistochemical staining

Sections were cut from formalin-fixed paraffin blocks containing invasive primary tumour for each case and immunostaining performed using a standard streptavidin–biotin–horseradish peroxidase (HRP) technique. Sections were deparaffinised in xylene baths then rehydrated in alcohol. Endogenous peroxidase was blocked using hydrogen peroxide and non-specific binding inhibited with normal swine serum.

- *VEGF-C*: A rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA) that recognises amino-acids 230–419 of the carboxy terminus of human VEGF-C was used at a 1:125 dilution. Sections were incubated overnight at 4 °C.
- *C-erbB2*: A prediluted anti-c-erbB2 rabbit anti-human antibody was employed (DAKO, UK). Microwave antigen retrieval with 10-mM citrate buffer pH 6.0 was performed. Antibody was applied at room temperature for 10 min.
- *ER*: Oestrogen receptor status was assessed on the pre-operative core biopsy where available in the majority of cases. A rabbit anti-human ER antibody was used (DAKO, UK). Microwave antigen retrieval with 10 mM citrate buffer pH 6.0 was performed. Antibody was applied at room temperature for 1 h.

The secondary detection system used for all three antibodies was DAKO LSAB®2 kit followed by 3-3′-diaminobenzidine for colour development. Slides were lightly counterstained with haematoxylin. Positive controls included sections known to express the given antigen and were included for each staining run as were negative controls (buffer only for VEGF-C and ER staining, non-immune rabbit serum for c-erbB2 staining).

The immunohistochemical scoring systems for each antibody were as follows. Slides were scored by two independent observers and discordant results reviewed to reach a consensus. For c-erbB2, staining was scored on a scale of 0, 1+, 2+ or 3+. Positive c-erbB2 expression was defined by weak/moderate (2+) or moderate/strong (3+) complete membrane staining in more than 10% of the tumour cells. Scores of 0 or 1+ were deemed negative for c-erbB2. For the VEGF-C immunohistochemistry, slides were considered positive if >10% tumour cells were immunoreactive. ER scoring was performed using the Histo-score system.

2.3. Bone marrow analysis

Two 5-ml bone marrow aspirates were collected from each woman from the posterior iliac crest under general anaesthesia, one just before surgery and the other immediately after surgery. The bone marrow was subjected to Ficoll-Paque® (Pharmacia Biotech, UK) density gradient centrifugation. The interphase layer was removed, diluted in phosphate-buffered saline (PBS) and further centrifuged. The resultant pellet was re-suspended in PBS with 0.1% w/v bovine serum albumin (PBS/BSA). The cell concentration was determined and diluted to give a final cell count of 20×10^6 cells/ml. Magnetic beads coated with the anti-epithelial antibody BerEP4 (Dynabeads® Epithelial Enrich, Dynal, UK) were then added to 0.5 ml of cell suspension and incubated for 30 min at 4 °C. The tube was then placed in a magnet and the supernatant discarded. The remaining cells attached to the beads were re-suspended in PBS/BSA and cytospun on to poly-L-lysine-coated slides. Slides underwent immunocytochemistry using the EPiMET Epithelial Cell Detection Kit (Dynal®, UK) which uses the anti-cytokeratin antibody A45-B/B3. Slides were screened by two observers for the presence of tumour cells. Samples were scored positive if one or more cells were present with intense red cytoplasmic staining and the morphology was consistent with malignant epithelial cells. If either sample (pre-operative or postoperative) contained tumour cells, the result was recorded as positive for the purpose of this study [20].

2.4. Statistical analysis

Correlation between VEGF-C expression and clinico-pathological factors was analysed using the Mann–Whitney U test, Fisher's exact probability test and Chi-square test. Statistical analyses were performed using GraphPad Prism 3.00 for Windows, GraphPad Software, San Diego, CA, USA. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Immunohistochemistry and bone marrow

Overall, 30/51 (59%) of the tumours were positive for VEGF-C by immunohistochemistry. Generally, there was diffuse staining of the tumour cell cytoplasm in positive cases (Fig. 1), but a few tumours exhibited stronger staining at the invasive edge. In addition, occasional immunopositivity was observed in adjacent normal breast tissue, areas of ductal carcinoma *in-situ* and in peritumoral stroma, with some blood vessels exhibiting distinct positive immuno-reactivity. In 15/51 cases (29%), the tumour exhibited positive membrane immunoreactivity for c-erbB2. Bone marrow analysis demonstrated cytokeratin-positive tumour cells in 12 cases (24%). The number of tumour cells per positive sample ranged from 1 to 11 (median 2.5, 95% Confidence Interval (CI) 2.1–4.9).

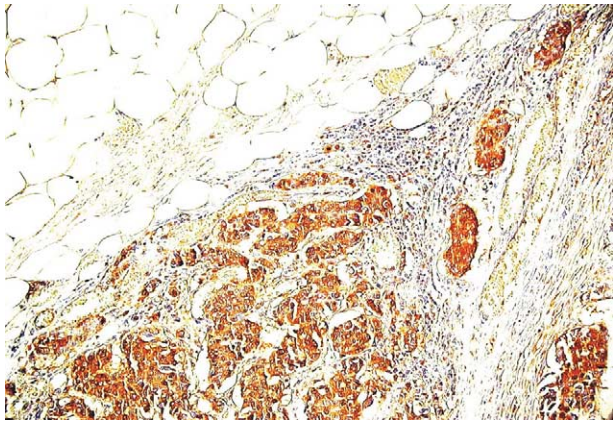

Fig. 1. Example of immuno-histochemical expression of VEGF-C in breast carcinoma.

3.2. Associations with clinico-pathological factors

Correlation between VEGF-C expression and standard clinico-pathological data, c-erbB2 expression and bone marrow status are shown in Table 1. No association was observed between VEGF-C expression and the patient's age, tumour type, ER status and the presence of axillary lymph node metastases There was no association between VEGF-C and tumour size using either categorical data (Table 1) or continuous data (Mann–Whitney, $P=0.93$). However, there was a statistically significant relationship between VEGF-C and c-erbB2 expression ($P=0.012$), with 87% of c-erbB2-positive tumours co-expressing VEGF-C. In addition, there was a trend for VEGF-C positivity with increasing tumour grade and the presence of lympho-vascular invasion, but neither reached statistical significance.

An association between c-erbB2 expression and tumour type was significant (χ^2 test, $P=0.046$), with 43% of ductal tumours, but none of the lobular or special types being positive. No association was observed between c-erbB2 expression and tumour size, LN status, LVI or ER status. There was a strong association between the presence of BMM and tumour size, with a median tumour size of 35 (interquartile range: 25.5–67 mm) and 20 mm (interquartile range: 15–27 mm) for BMM-positive and -negative groups, respectively (Mann–Whitney U test, $P=0.001$). No correlation between BMM and the other clinico-pathological parameters was observed.

4. Discussion

In this series of breast carcinomas, 59% tumours expressed VEGF-C protein, as determined by immuno-

Table 1
Correlation between tumour VEGF-C expression and variables

	N	VEGF-C (+)	VEGF-C (−)	*P* value
Age (median, yrs)	51	59	60	0.92[a]
Tumour size				
⩽2 cm	23 (45)	16 (70)	7 (30)	0.81[b]
>2–⩽5 cm	21 (41)	8 (38)	13 (62)	($\chi^2 = 0.06$)
>5 cm	7 (14)	6 (86)	1 (14)	
Grade				
1	14 (28)	6 (43)	8 (57)	0.14[b]
2	18 (35)	11 (61)	7 (39)	($\chi^2 = 2.09$)
3	19 (37)	13 (68)	6 (32)	
Tumour type				
Ductal	30 (59)	19 (63)	11 (37)	
Lobular	8 (16)	4 (50)	4 (50)	0.45[c]
Mixed	9 (17)	6 (67)	3 (33)	($\chi^2 = 2.63$)
Other	4 (8)	1 (25)	3 (75)	
LN status				
Negative	27 (53)	16 (59)	11 (41)	1[d]
Positive	24 (47)	14 (58)	10 (42)	
LVI				
Absent	36 (71)	19 (53)	17 (47)	0.22[d]
Present	15 (29)	11 (73)	4 (27)	
Oestrogen receptor				
Positive	34 (67)	21 (62)	13 (38)	1[d]
Negative	15 (29)	9 (60)	6 (40)	
NK	2 (4)			
c-erbB2				
Positive	15 (29)	13 (87)	2 (13)	0.012[d]
Negative	36 (71)	17 (47)	19 (53)	
BMM				
Positive	12 (24)	6 (50)	6 (50)	0.52[d]
Negative	39 (76)	24 (62)	15 (38)	

BMM, bone marrow micrometastases; LN, lymph node; LVI, lympho-vascular invasion; NK, known. % values in parentheses.
[a] Mann–Whitney U test.
[b] Chi square for trend test.
[c] Chi square test.
[d] Fisher's exact test.

histochemistry. Although tumour cell immuno-reactivity was generally homogeneous throughout most samples, some did display increased staining at the invasive edge. This is in keeping with previous studies that have found VEGF-C is expressed in 40–60% of invasive breast cancers [21–23]. Increased expression at the invasive tumour edge has previously been observed [22,23]. A recent study examining VEGF-C expression in colorectal carcinoma demonstrated VEGF-C expression at the deepest edge of the tumour was associated with lymphatic and vascular invasion, the presence of nodal metastases and survival, whilst no such correlations existed for expression in the central part of the tumour [17].

A number of studies have demonstrated an association between VEGF-C expression and regional nodal metastases in a variety of human malignancies, including colorectal, gastric and cervical carcinomas [13–17]. We did not, however, find such an association in our study of breast carcinomas. Kurebayashi and colleagues investigated VEGF-C mRNA expression in a series of 20 breast cancers and found a significant association with axillary nodal metastases [21]. However, three subsequent studies of breast cancer have failed to demonstrate any relationship between VEGF-C and lymph node status [22–24]. Kinoshita and colleagues suggested that VEGF-C may be responsible for early events in the process of lymphatic metastasis, particularly as *in-situ* carcinoma often stained positive for VEGF-C [23], a finding also observed in our study. Another explanation for the lack of correlation between VEGF-C and axillary lymph node spread may be the technique employed to examine for lymph node metastases. Ohta examined VEGF-C expression in node-negative lung cancer and found a significant association between high VEGF-C expression and the presence of cytokeratin-positive lymph node micro-metastases [25].

We found no significant relationship between VEGF-C expression and tumour size, tumour grade and ER status, in keeping with previous reports [22–24]. In addition, no significant association was observed with lympho-vascular invasion, although a larger study size may have confirmed the observed trend. Whilst Gunningham and colleagues found no association with vascular invasion [22], Kinoshita found a positive correlation between VEGF-C expression and lymphatic vessel invasion, but not with venous invasion, although they did not clarify how the vessels were distinguished [23]. Recently described lymphatic endothelial-specific markers, such as LYVE-1, should allow differentiation of vascular from lymphatic vessel invasion in future studies [26].

Increased tumour VEGF-C expression has been reported to be associated with a worse prognosis in breast cancer and other malignancies [13,15–17,23] but other studies have not found such an association. Survival data were not examined in our study.

The presence of tumour cells in the bone marrow has been reported in a variety of human malignancies, including breast cancer, and a number of studies have shown BMM to be associated with a worse prognosis [27,28]. The presence of tumour cells in the bone marrow requires a complex series of events including tumour cell shedding and gaining access to the vascular system, in which VEGF-induced angiogenesis certainly plays a critical role [29]. Many studies have demonstrated the relationship between VEGF expression and tumour microvessel density (MVD) and a significant correlation between MVD and the presence of BMM has been reported in breast cancer [4]. In addition, high tumour VEGF expression has been shown to be associated with the presence of BMM in gastric and non-small cell lung cancers [5,6]. Since VEGF-C has been shown to

have angiogenic properties *in vitro* and *in vivo*, we investigated whether a similar relationship between BMM and VEGF-C existed, but found no such association.

C-erbB2 (HER2/neu) plays an important role in oncogenic transformation and tumorigenesis. Although no specific ligand has yet been identified for c-erbB2, it appears its primary role may be as a co-receptor with the other erbB receptors. Amplification and/or overexpression of c-erbB2 occurs in 20–30% of breast cancers and is associated with a worse prognosis compared with c-erbB2-negative tumours [30]. Our study found a statistically significant association between VEGF-C and c-erbB2 expression in human breast cancers, confirming the recent findings of Yang and colleagues [24].

Despite their structural similarities, the VEGF family members all have differing regulatory mechanisms. *VEGF-C* gene expression is induced by several growth factors including EGF and pro-inflammatory cytokines, but unlike VEGF-A, VEGF-C is not regulated by hypoxia [9]. Increased VEGF expression and angiogenesis are associated with the overexpression of c-erbB1 (EGFR) and c-erbB2, whilst specific neutralising antibody to either receptor reduced VEGF levels [31]. Recent *in vitro* studies have shown a regulatory link between VEGF-C and c-erbB receptors and ligands. Transforming growth factor α (TGFα), betacellulin and heregulin-β1 (HRG- β1) upregulate both VEGF-C and VEGF-A mRNA in head and neck squamous cell carcinoma cell lines [32]. HRG- β1, a ligand for c-erbB3 and c-erbB4, has also recently been shown to stimulate the upregulation of VEGF-C mRNA and protein levels in breast cancer cell lines. This effect is inhibited by the addition of the c-erbB2 blocking antibody Herceptin [33].

Our study provides further evidence for a link between c-erbB2 and VEGF-C in malignant disease. As VEGF-C has been shown to increase tumour angiogenesis, lymphangiogenesis and nodal metastasis in animal models, the upregulation of this novel factor by c-erbB2 explains, in part, the aggressive phenotype associated with c-erbB2-positive tumours.

References

1. Folkman J. Tumour angiogenesis: therapeutic implications. *N Eng J Med* 1971, **285**, 1182–1186.
2. Ferrara N. Vascular endothelial growth factor and the regulation of angiogenesis. *Rec Prog Horm Res* 2000, **55**, 15–36.
3. Toi M, Matsumoto T, Bando H. Vascular endothelial growth factor: its prognostic, predictive, and therapeutic implications. *Lancet Oncol* 2001, **2**, 667–673.
4. Fox SB, Leek RD, Bliss J, *et al*. Association of tumor angiogenesis with bone marrow micrometastases in breast cancer patients. *J Natl Cancer Inst* 1997, **89**, 1044–1049.
5. Kakeji Y, Maehara Y, Shibahara K, *et al*. Clinical significance of micrometastasis in bone marrow of patients with gastric cancer and its relation to angiogenesis. *Gastric Cancer* 1999, **2**, 46–51.
6. Ohta Y, Nozaki Z, Nozawa H, *et al*. The predictive value of vascular endothelial growth factor and Nm23 for the diagnosis of occult metastasis in non-small cell lung cancer. *Jpn J Cancer Res* 2001, **92**, 361–366.
7. Linderholm B, Lindh B, Tavelin B, Grankvist K, Henriksson R. p53 and vascular endothelial growth factor (VEGF) expression predicts outcome in 833 patients with primary breast cancer. *Int J Cancer* 2000, **89**, 51–62.
8. Gasparini G, Toi M, Gion M, *et al*. Prognostic significance of vascular endothelial growth factor protein in node negative breast carcinoma. *J Natl Cancer Inst* 1997, **89**, 139–147.
9. Olofsson B, Jeltsch M, Eriksson U, Alitalo K. Current Biology of VEGF-B and VEGF-C. *Curr Opin Biotechnol* 1999, **10**, 528–535.
10. Skobe M, Hawighorst T, Jackson DG, *et al*. Induction of tumour lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* 2001, **7**, 151–152.
11. Mandriota SJ, Jussila L, Jeltsch M, *et al*. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J* 2001, **20**, 672–682.
12. He Y, Kozaki K, Karpanen T, *et al*. Suppression of tumour lymphangiogenesis and lymph node metastasis by blocking endothelial growth factor receptor 3 signalling. *J Natl Cancer Inst* 2002, **94**, 819–825.
13. Yonemura Y, Endo Y, Fujita H, *et al*. Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. *Clin Cancer Res* 1999, **5**, 1823–1829.
14. Akagi K, Ikeda Y, Miyazaki M, *et al*. Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. *Br J Cancer* 2000, **83**, 887–891.
15. Hirai M, Nakagawara A, Oosaki T, Hayashi Y, Hirono M, Yoshihara T. Expression of vascular endothelial growth factors (VEGF-A/VEGF-1 and VEGF-C/VEGF-2) in post-menopausal uterine endometrial carcinoma. *Gynae Oncol* 2001, **80**, 181–188.
16. Ueda M, Terai Y, Yamashita Y, *et al*. Correlation between vascular endothelial growth factor-C expression and invasion phenotype in cervical carcinomas. *Int J Cancer* 2002, **98**, 335–343.
17. 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**, 157–166.
18. Noguchi T, Takeno S, Shibata T, *et al*. VEGF-C expression correlates with histological differentiation and metastasis in squamous cell carcinoma of the esophagus. *Oncol Rep* 2002, **9**, 995–999.
19. Niki T, Iba S, Tokunou M, *et al*. Expression of vascular endothelial growth factors A, B, C and D and their relationships to lymph node status in lung adenocarcinoma. *Clin Cancer Res* 2000, **6**, 2431–2439.
20. Wadley MS, Jones CE, Bramhall SR, Lee MJR, Stonelake PS. Detection of bone marrow micro-metastases in primary breast cancer: pre-or post-operative sampling? *Br J Surg* 1999, **86**(Suppl. 1), 54.
21. Kurebayashi J, Otsuki T, Kunisue H, *et al*. Expression of vascular endothelial growth factor family members in breast cancer. *Jpn J Cancer Res* 1999, **90**, 977–981.
22. Gunningham SP, Currie MJ, Han C, *et al*. The short form of the alternatively spliced Flt-4 but not its ligand vascular endothelial growth factor is related to lymph node status in human breast cancers. *Clin Cancer Res* 2000, **6**, 4278–4286.
23. Kinoshita J, Kitamura K, Kabashima A, Saeki H, Tanaka S, Sugimachi K. Clinical significance of vascular endothelial growth factor-C (VEGF-C) in breast cancer. *Breast Cancer Res Treat* 2001, **66**, 159–164.
24. Yang W, Klos K, Yang Y, Smith TL, Shi D, Yu D. ErbB2 Overexpression correlates with increased expression of vascular

endothelial growth factors A, C and D in human breast carcinoma. *Cancer* 2002, **94**, 2855–2861.
25. Ohta Y, Nozawa H, Tanaka Y, Oda M, Watanabe Y. Increased vascular endothelial growth factor and vascular endothelial growth factor-c and decreased nm23 expression associated with microdissemination in the lymph nodes in stage I non-small cell lung cancer. *J Thorac Cardiovasc Surg* 2000, **119**, 804–813.
26. Banerji S, Ni J, Wang SX, *et al*. LYVE1, a new homologue of the CD44 glycoprotein, is a lymph specific receptor for hyaluronan. *J Cell Biol* 1999, **144**, 789–801.
27. Diel IJ, Kaufmann M, Costa SD, *et al*. Micrometastatic breast cancer cells in bone marrow at primary surgery: prognostic value in comparison with nodal status. *J Natl Cancer Inst* 1996, **88**, 1652–1658.
28. Braun S, Pantel K, Muller P, *et al*. Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II or III breast cancer. *N Eng J Med* 2000, **342**, 525–533.
29. McDonnell CO, Hill ADK, McNamara DA, *et al*. Tumour micrometastases: the influence of angiogenesis. *Eur J Surg Oncol* 2000, **26**, 105–115.
30. Slamon DJ, Clark GM, Wong SJ, *et al*. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987, **235**, 177–182.
31. Petit AM, Rak J, Hung MC, *et al*. Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* 1997, **151**, 1523–1530.
32. O-charoenrat P, Rhys-Evans P, Modjtahedi H, Eccles SA. Vascular endothelial growth factor family members are differentially regulated by c-erbB signalling in head and neck squamous carcinoma cells. *Clin Exp Metastasis* 2000, **18**, 155–161.
33. Tsai P, Shiah S, Lin M, Wu C, Kuo M. Up-regulation of vascular endothelial growth factor C in breast cancer cells by heregulin-β1. *J Biol Chem* 2003, **278**, 5750–5759.