Vasohibin-1 in human breast carcinoma: A potential negative feedback regulator of angiogenesis

Kentaro Tamaki,[1,4,6] Takuya Moriya,[2] Yasufumi Sato,[3] Takanori Ishida,[1] Yohei Maruo,[4] Kousuke Yoshinaga,[5] Noriaki Ohuchi[1] and Hironobu Sasano[4]

[1]Department of Surgical Oncology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574; [2]Department of Pathology 2, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192; [3]Department of Vascular Biology, Institute of Department, Aging, and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574; [4]Department of Pathology, Tohoku University Hospital, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574; [5]Department of Gynecology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574

(Received August 11, 2008/Revised September 21, 2008/Accepted September 22, 2008/Online publication November 25, 2008)

Vasohibin-1 is a recently identified negative feedback inhibitor or suppressor of angiogenesis induced by vascular endothelial growth factor (VEGF)-A. The status of vasohibin-1 in human breast carcinoma has not been examined. We examined 151 breast specimens including 98 cases of invasive ductal carcinoma (IDC), 12 of ductal carcinoma *in situ* (DCIS), 16 of fibroadenoma (FA), six of inflammatory lesion, nine of fibrocystic change and seven of non-pathological breast tissue. We immunolocalized vasohibin-1 and compared its immunoreactivity to that of VEGF-A, basic fibroblastic growth factor (bFGF), VEGF receptor 2 (Flk-1), CD31, CD34 and Ki-67/MIB-1. The correlation of vasohibin-1 immunoreactivity with overall survival (OS), and disease-free survival (DFS) of the patients with breast carcinoma was also evaluated. In addition, we evaluated Ki-67 and CD31, and Ki-67 and vasohibin-1 double-immunostaining for further characterization of neovascularization. Vasohibin-1 was detected in endothelial cells of human breast and its immunodensity was significantly higher in IDC and inflammatory lesions than the other types ($P < 0.001$). In addition, a significant positive correlation was detected between vasohibin-1 and VEGF-A, bFGF or Flk-1 ($P < 0.001$). There was also positive associations between vasohibin-1 and OS ($P = 0.004$) and between vasohibin-1 and DFS ($P \leq 0.001$) in carcinoma cases. Results of double-immunostaining demonstrated the ratio of Ki-67-positive cells among vasohibin-1-positive endothelial cells (46.5%) was significantly higher than those among CD31-positive cells (23.5%). This is the first study demonstrating the status of vasohibin-1 in human breast lesions, which indicates that vasohibin-1 is associated with neovascularization and may especially play important roles in the regulation of intratumoral angiogenesis in human breast cancer. (*Cancer Sci* 2009; 100: 88–94)

Angiogenesis or the formation of new blood vessel networks, not only plays a pivotal role in human normal development, but also in pathophysiological conditions such as inflammatory diseases and neoplasms. Angiogenesis is generally regulated by an *in situ* balance between stimulatory and inhibitory factors of angiogenesis.[1,2] However, this "angiogenic homeostasis" may be disrupted in pathological conditions such as cancer and dysregulated or excessive production and/or secretion of angiogenesis inducers result in excessive formation of abnormal blood vessels. In general, various biological phenomena in physiological conditions are under stringent control by numerous negative feedback systems as seen in endocrine mechanisms the including hypothalamic–pituitary–adrenal system to maintain their homeostasis. However, little has been known about such negative feedback mechanisms of angiogenesis in both physiological and pathological conditions.

Vasohibin-1 has been very recently identified as one of the first established negative feedback regulators of angiogenesis.[2–5] This interesting factor was identified as one of vascular endothelial growth factor (VEGF)-induced genes with anti-angiogenic properties in endothelial cells (EC) using cDNA microarray analysis.[3,4,6] Vasohibin-1 was subsequently demonstrated to be specifically expressed in EC in response to angiogenic stimulators such as VEGF and basic fibroblastic growth factor (bFGF).[3,6] Vasohibin-1 is also abundantly present in human placenta and fetus[2,3,5] in which angiogenic events markedly occur *in vivo*. VEGF-A is the most potent factor for angiogenesis among known VEGF family members, stimulating protease synthesis, migration and proliferation of EC.[7] In addition, the great majority of VEGF-A-mediated signals are transduced via VEGF receptor 2 (Flk-1)[8] and protein kinase Cδ (PKCδ), one of the signals located in important downstream intrasignaling pathway of Flk-1, and they also induced vasohibin-1 expression markedly.[4] Yoshinaga *et al.* demonstrated that the VEGF-A-mediated induction of vasohibin-1 was preferentially mediated via the Flk-1 signaling pathway in human endometrial carcinoma.[9] However, the status of vasohibin-1 in other human malignancies has not been examined in detail.

Therefore, in this study, we first immunolocalized vasohibin-1 in human breast disorders including breast cancer in order to examine whether this factor is expressed in endothelial cells or not in human breast tissues. We then correlated the findings with various clinicopathological factors of the cases including microvessel density (MVD)[10,11] in order to correlate the status of vasohibin-1 with vascularity of the lesions. We also correlated vasohibin-1 immunoreactivity with neovascularization or proliferating endothelial cells using double immunostaining of Ki-67 in order to further characterize vasohibin-1 expression and its clinical and/or biological significance in human breast disorders.

Materials and Methods

Breast tissue specimens. We retrieved 151 Japanese female cases of breast tissues from surgical pathology files of Tohoku University Hospital (Sendai, Japan). These subjects were operated on between 1995 and 1998 at the Department of Surgery, Tohoku University Hospital. The median age of the patients was 48 years (range, 15–81). The protocol for this study was approved by the Ethics Committee at Tohoku University School of Medicine (Sendai, Japan). The relevant clinicopathological information including age, histological type, stage classification, histological grade for invasive ductal carcinoma (IDC), grading scheme for ductal carcinoma *in situ* (DCIS) (van Nuys classifications[12] for DCIS and T1mic) are summarized in Table 1. Histological findings were 98 cases of IDC including eight cases of T1mic, 12 of DCIS, 16 fibroadenoma (FA), six of

[6]To whom correspondence should be addressed.
E-mail: nahanisikenta@yahoo.co.jp

doi: 10.1111/j.1349-7006.2008.01015.x

Table 1. Clinicopathological characters of examined cases

Histological type	
IDC	98
(T1mic)	8
DCIS	12
FA	16
Inflammatory lesion	6
Fibrocystic change	9
Non-pathological breast tissue	7
Age, years (range)	
All cases	48 years (15–81)
IDC	53 years (28–81)
DCIS	47 years (40–81)
FA	39 years (15–52)
Inflammatory lesion	49 years (35–70)
Fibrocystic change	47 years (39–48)
Non-pathological breast tissue	40 years (34–48)
UICC stage grouping	
Stage 0	12
Stage I	38
Stage II	36
II A	23
II B	13
Stage III	18
III A	8
III B	6
III C	4
Stage IV	6
Histological grade (for IDC)	
G 1	34
G 2	42
G 3	14
Van Nuys scheme (for DCIS and T1mic)	
Group 1	8
Group 2	12

DCIS, ductal carcinoma *in situ*; FA, fibroadenoma; IDC, invasive ductal carcinoma; UICC, International Union Against Cancer staging.

inflammatory lesion, nine of fibrocystic change and seven of non-pathological breast tissue taken from the lumpectomy specimen for breast cancer operation. In the nine cases of fibrocystic change, we evaluated vessels in the areas adjacent to adenosis or ductal hyperplasia. Stage grouping was based on *TNM Classification of Malignant Tumors Sixth Edition* by the International Union Against Cancer (UICC).[13] The tumor grade was determined according to the criteria of Elston and Ellis.[14]

Immunohistochemistry. We performed immunohistochemical staining for vasohibin-1, Flk-1, CD31, Ki-67, VEGF-A and FGF-2. The specimens had been fixed in 10% formalin, embedded in paraffin, cut into 4-μm thick sections and placed on glue-coated glass slides. Sections were deparaffinized in xylene, and hydrated with graded alcohols and distilled water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxidase for 10 min at room temperature. Antigen retrieval was performed using Autoclave (TOMY SX-500 HIGH PRESSURE STEAM STERILIZER, TOMY SEIKO CO., LTD, Tokyo, Japan) in 10 nmol ethylene diamine tetra acetate (EDTA; pH 8) for vasohibin-1 and in citrate buffer for Flk-1, CD31, Ki-67 and FGF-2, heated at 121°C for 5 min, and for VEGF-A using microwave in citrate buffer for 15 min. Sections were subsequently incubated for 30 min at room temperature (RT) in a blocking solution of 10% rabbit serum (Nichirei Biosciences, Tokyo, Japan) for vasohibin-1, Flk-1, CD31, CD34 and Ki-67, and a blocking solution of 10% goat serum (Nichirei Bioscience) for VEGF-A and FGF-2, and then immunostained for 16 h at 4°C with primary antibodies. The primary antibodies of vasohibin-1, Flk-1, CD31, Ki-67, VEGF-1 were mouse monoclonal antibodies, whereas the primary antibody against FGF-2 was a rabbit polyclonal antibody, and were used as follows: antihuman vasohibin-1 monoclonal antibody[9,15] diluted at 1:3200; anti-VEGFR-2 (Flk-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:3200; anti-CD31 (Dako, Copenhagen, Denmark) diluted at 1:40; anti-CD34 (Nichirei Bioscience) diluted at 1:100, Ki-67 (Dako) diluted 1:300; anti-VEGF-A (Laboratory Vision, Fremont, CA, USA) diluted at 1:50; and anti-FGF-2 (Santa Cruz Biotechnology) diluted at 1:100. Antihuman vasohibin-1 monoclonal antibody (mAb) was raised against the synthetic fragment (Gly286-Arg299) of human vasohibin-1 as described by Watanabe *et al*.[3] The specificity and sensitivity of this mAb was confirmed by both western blotting and immunohistochemical analysis.[3] For vasohibin-1, Flk-1, CD31, CD34 and Ki-67 immunohistochemistry, secondary antibody reactions were performed using biotinylated rabbit antimouse antibody (Nichirei Bioscience) at a dilution of 1:100 for 30 min at RT and peroxidase-conjugated avidin (Nichirei Bioscience) was used according to the manufacturer's instructions. Envision (Dako) was used for immunostaining of VEGF-A and FGF-2. Reacted sections were visualized using 3,3′-diaminobenzidine-tetrachloride (DAB)/30% H_2O_2 in 0.05 mol/L Tris buffer (pH 7.6) and counterstained with hematoxylin–eosin (HE) for nuclear staining.

Double staining procedure. For the quantification of proliferating endothelial cells, Ki-67/CD31 and Ki-67/vasohibin-1 double-labeling immunohistochemical staining was performed. A mAb directed against Ki-67 (Dako) was diluted at 1:300 following antigen retrieval using Autoclave in a citrate buffer, and incubated for 30 min at RT in a blocking solution of 10% rabbit serum (Nichirei Bioscience). A secondary antibody reaction was performed using biotinylated rabbit antimouse antibody (Nichirei Bioscience) at a distribution of 1:100 for 30 min at RT. Peroxidase-conjugated avidin (Nichirei Bioscience) was subsequently used in this study. DAB was used to visualize the binding of the first antibody. Antigen retrieval was then performed using a microwave for 15 min in 10 nmol EDTA (pH 8) for vasohibin-1 and in a citrate buffer for CD31. The reacted sections were then incubated for 30 min with antibodies against vasohibin-1 diluted at 1:3200 and CD31 (Dako) diluted at 1:40. Following the reaction with biotinylated rabbit antimouse antibody (Nichirei Bioscience) diluted at 1:100 as a secondary antibody and alkaline phosphatase-conjugated avidin (Nichirei Bioscience), an alkaline phosphatase substrate kit III (Vector Laboratories, Burlingame, CA, USA) was employed.[16,17]

Immunohistochemical analysis. Two of the authors (K. T. and Y. M.) independently evaluated the immunohistochemical staining of the tissue sections. They were blinded to the clinical course of the patients and the average of numbers counted by the two investigators was used for subsequent analysis. We used Olympus (Tokyo, Japan) BX50 and 20X objectives for the analysis.

The number of microvessels was counted within the tumor of IDC and FA, whereas in DCIS, the number of vessels in the stroma among intraductal components was evaluated. In inflammatory lesions, fibrocystic change and non-pathological breast tissues, the greatest number of vessels in the tissue sections was determined as MVD.[10,11,18–20] Microvessels were identified based on the architecture, lumen lined by endothelial cells, complemented by positivity of the endothelial cells with anti-CD31 after scanning the immunostained section at low magnification (×40 and ×100).[10,11] The areas with the greatest number of distinctly highlighted microvessels were selected, and counted at one higher power (×200).[10,11] Any immunostained endothelial cells or clusters separated from adjacent vessels were counted as a single microvessel, even in the absence of vessel lumen. Each single count was defined as the highest number of microvessels identified at the "hot spot". Vasohibin-1- and Flk-1-positive signals

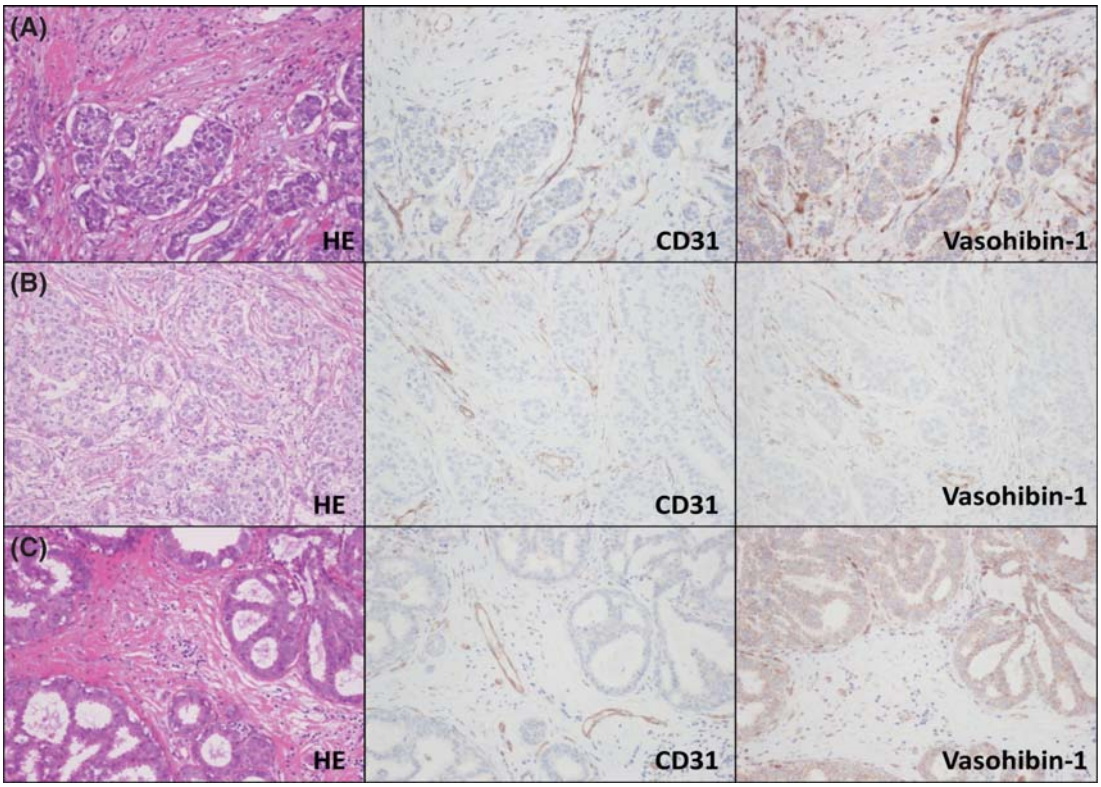


Fig. 1. Representative illustrations of histological and immunohistochemical findings of breast carcinoma cases examined. (A,B) Two invasive ductal carcinoma (IDC) cases stained positively for CD31 and vasohibin-1, whereas (C) a ductal carcinoma *in situ* (DCIS) case stained positive only for CD31 and not for vasohibin-1. (Original magnification, ×200.)

were counted in the hot spot in which the highest number of anti-CD31-positive vessels was identified. We also counted the average of vasohibin-1-positive vessels in 10 representative fields per case (×200). We defined vasohibin-1-positive ratio as the number divided by the number of vasohibin-1-positive vessels by that of CD31-positive vessels in the hot spot. An evaluation of Ki-67 immunoreactivity was performed at high power field (×400) and used as a marker of cell proliferation. More than 500 tumor cells from each of three different representative fields were evaluated and the labeling index was subsequently obtained. VEGF-A immunoreactivity was evaluated using grading, interpreting both relative immunointensity and the proportion of tumor cells associated with an unequivocal positive reaction.[21,22] Relative immunointensity was graded 0 (no staining) to 3 (strong staining), percentage of cells staining positive as 0 (no tumor cells positive), 1 (positive staining in <10% of the tumor cells), 2 (positive staining in 10–50% of the tumor cells) and 3 (positive staining in >50% of the tumor cells).[20,21] A semiquantitative method was used to evaluate the degrees of FGF-2 immunostaining ranging from 0 (no expression), 1 (weak), 2 (moderate) to 3 (highest level of expression).[23] The proportion of proliferating endothelial cells (CD31 and vasohibin-1-positive vessels) was defined as the number of endothelial cells with Ki-67-stained nuclei divided by the total number of endothelial cells.

Analyzes of OS and disease-free survival (DFS) curves were performed by employing the Kaplan–Meyer method. The segregation point of the parameter at 21 for vasohibin-1-positive vessels was determined by the Cox proportional hazards regression model. The values of survival rates represented estimated survival rates. Factors independently associated with OS and DFS – vasohibin-1, MVD, VEGF-A and Ki-67 – were identified by multivariate analyses using multiple regression analysis.

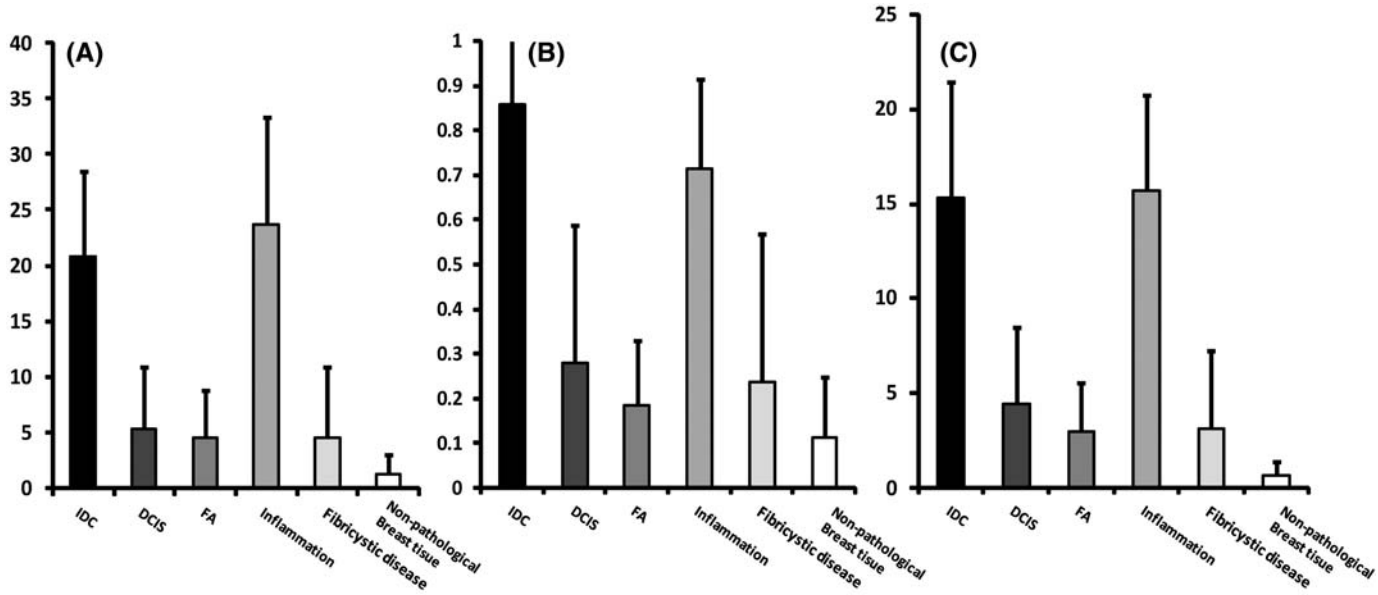
Statistical analysis, such as the one-factor ANOVA and simple regression analysis, were performed using StatMate III for Windows ver. 3.18 (ATMS, Tokyo, Japan). The results were considered significant at $P < 0.05$.

Results

MVD. The representative findings of immunostaining for HE, CD31 and vasohibin-1 are illustrated in Fig. 1. The average number of microvessels detected by CD31 was 24.6 ± 8.3 in IDC, 21.7 ± 11.7 in DCIS, 26.3 ± 15.7 in FA, 34.2 ± 15.4 in inflammatory lesions, 20.6 ± 14.4 in fibrocystic change and 13.6 ± 10.3 in non-pathological breast tissue, respectively. Statistically significant differences of MVD among the lesions were detected only between IDC and non-pathological breast tissue ($P = 0.001$).

Vasohibin-1 immunohistochemistry. Vasohibin-1 immunoreactivity was detected only in endothelial cells (Fig. 1). Vasohibin-1-positive microvessels in the hot spot were 20.9 ± 7.7 in IDC, 5.3 ± 5.5 in DCIS, 4.6 ± 4.1 in FA, 23.7 ± 9.7 in inflammatory lesions, 4.6 ± 6.3 in fibrocystic change and 1.3 ± 1.8 in non-pathological breast tissue. There were statistically significant differences between IDC and four other histological types of breast tissues examined (DCIS, FA, fibrocystic change and non-pathological breast tissue; $P < 0.001$) (Fig. 2A). The ratio of vasohibin-1/CD31[4] was 0.857 ± 0.193 in IDC, 0.279 ± 0.308 in DCIS, 0.183 ± 0.146 in FA, 0.713 ± 0.200 in inflammatory lesions, 0.237 ± 0.332 in fibrocystic change and 0.112 ± 0.136 in non-pathological breast tissue. There were significant differences between IDC and all other histological types ($P < 0.001$) (Fig. 2B). The average number of vasohibin-1-positive vessels per 10 fields (×200) were 15.3 ± 6.1 in IDC, 4.4 ± 4.1 in

doi: 10.1111/j.1349-7006.2008.01015.x



IDC	20.9 ± 7.7
DCIS	5.3 ± 5.5
FA	4.6 ± 4.1
Inflammation	23.7 ± 9.7
Fibrocystic disease	4.6 ± 4.1
Non-pathological breast tissue	1.3 ± 1.8

IDC	0.857 ± 0.193
DCIS	0.279 ± 0.308
FA	0.183 ± 0.146
Inflammation	0.713 ± 0.200
Fibrocystic disease	0.237 ± 0.332
Non-pathological breast tissue	0.112 ± 0.136

IDC	15.3 ± 6.1
DCIS	4.4 ± 4.1
FA	2.9 ± 2.6
Inflammation	15.7 ± 5.0
Fibrocystic disease	3.1 ± 4.1
Non-pathological breast tissue	0.7 ± 0.7

Fig. 2. Analysis of vasohibin-1 immunohistochemistry according to histological subtypes. (A) Number of vasohibin-1-positive vessels in the 'hotspot'. (B) Vasohibin-1-positive ratio defined as the vasohibin-1-positive vessels/CD31-positive vessels. (C) Average of vasohibin-1-positive vessels in 10 different fields. The lower boxes are the statistical analysis compared with invasive ductal carcinoma (IDC) cases.

DCIS, 2.9 ± 2.6 in FA, 15.7 ± 5.0 in inflammatory lesions, 3.1 ± 4.1 in fibrocystic change and 0.7 ± 0.7 in non-pathological breast tissue. There were also statistically significant differences between IDC and four histological types (DCIS, FA, fibrocystic change and non-pathological breast tissue, $P < 0.001$). No significant differences were detected between IDC and inflammatory lesions ($P = 0.781$) (Fig. 2C).

Correlation between vasohibin-1-positive vessels and Ki-67 labeling index in carcinoma cells. A significant positive correlation was detected between the number of vasohibin-1-positive vessels and Ki-67 labeling index in breast tumor cells ($P < 0.001$).

Correlation between vasohibin-1-positive vessels and VEGF-A status in carcinoma cells. The number of vasohibin-1-positive vessels was 5.8 ± 5.5 in VEGF-A of score 0, 11.0 ± 9.4 of score 2, 15.1 ± 10.0 of score 3, 17.5 ± 10.1 of score 4, 22.1 ± 8.9 of score 5 and 22.7 ± 5.7 of score 6. There was a statistically significant association between vasohibin-1 in the vessels and VEGF-A scores in carcinoma cells ($P < 0.001$) (Fig. 3A).

Correlation between vasohibin-1-positive vessels and FGF-2 in carcinoma cells. The number of vasohibin-1-positive vessels was 6.3 ± 6.1 in FGF-2 of score 0, 19.1 ± 6.5 of score 1, 21.9 ± 7.2 of score 2 and 26.8 ± 8.4 of score 3. A statistically significant association was detected between vasohibin-1 immunoreactivity in the vessels and FGF-2 scores in carcinoma cells ($P < 0.001$) (Fig. 3B).

Correlation between vasohibin-1 and Flk-1 in microvessels in breast carcinoma. A significantly positive correlation was detected between vasohibin-1 and Flk-1 positive ratios in microvessels ($P < 0.001$) (Fig. 3C).

Correlation between vasohibin-1 and clinical stage of breast carcinoma cases. The number of vasohibin-1-positive vessels was 5.3 ± 5.5 in TNM Stage 0, 19.6 ± 6.7 in Stage I, 18.7 ± 8.6 in Stage II A, 22.1 ± 8.3 in Stage II B, 23.8 ± 5.8 in Stage III A, 28.7 ± 7.5 in Stage III B, 23.0 ± 7.5 in Stage III C and 21.2 ± 5.6 in Stage IV. Statistically significant differences were detected only between IDC and DCIS ($P < 0.001$) with no significant differences among the different stages of IDC.

Correlation between vasohibin-1 and histological grades of breast carcinoma cells. The number of vasohibin-1-positive vessels among different groups of carcinoma cases and histological grade was 18.4 ± 7.5 in grade I, 20.8 ± 7.0 in grade II and 28.0 ± 8.0 in grade III. There were statistically significant differences of vasohibin-1 density between grade I and III, and grade II and III cases ($P < 0.001$) with no significant difference between grade I and II cases ($P = 0.14684$).

Correlation between vasohibin-1 and overall survival or DFS in breast carcinoma patients. Patients were tentatively classified into two different groups according to the number of vasohibin-1-positive vessels: 0–20 and 21 or more. The 10-year overall survival rates were 0.932203 and 0.72549 among these two groups, respectively. (The total 10-year overall survival rate in this cohort of patients was 0.838836.) Statistically significant differences in the 0–20 and 21 or more groups was $P = 0.004$ (Fig. 4A). The 10-year DFS were 0.92736 and 0.708333, respectively, in these two groups. Statistically significant differences were also detected in the 0–20 and 21 or more groups was at $P \leq 0.001$. (The total 10-year DFS rate was 0.81777; Fig. 4B.) The following variables were included in the multivariate analysis of OS: vasohibin-1, MVD, VEGF-A and Ki-67. This multivariate analysis demonstrated that vasohibin-1 was associated with VEGF-A ($P = 0.038$) and Ki-67 ($P < 0.001$), but was not associated with MVD ($P = 0.083$). The multivariate analysis of

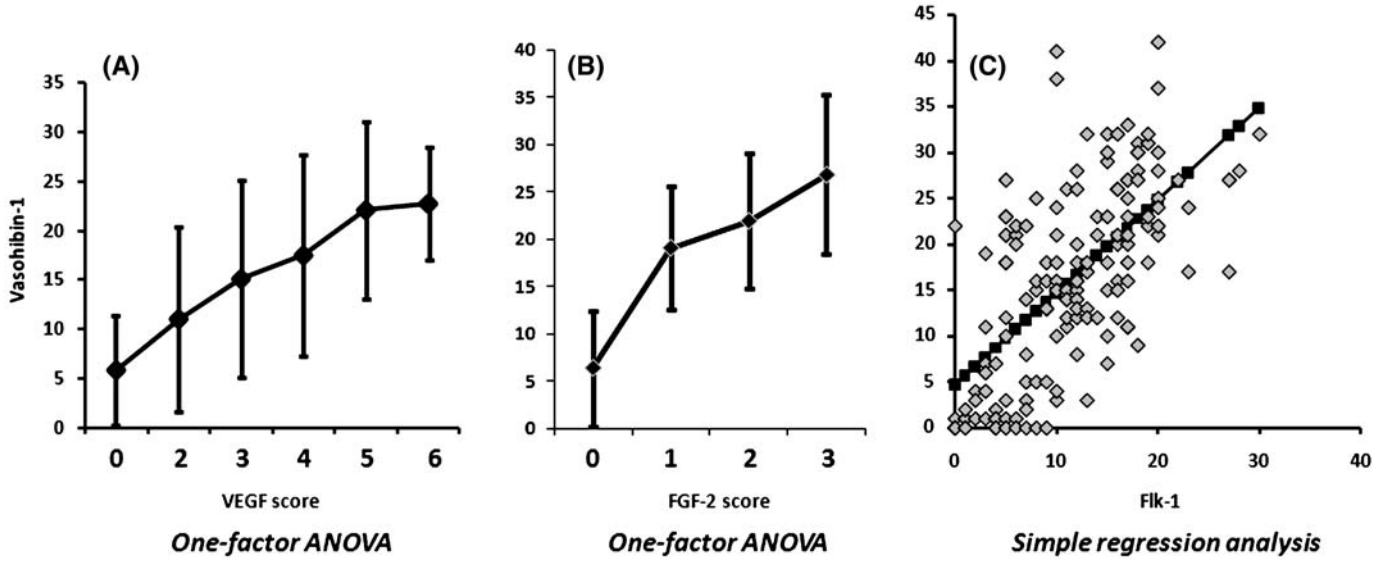
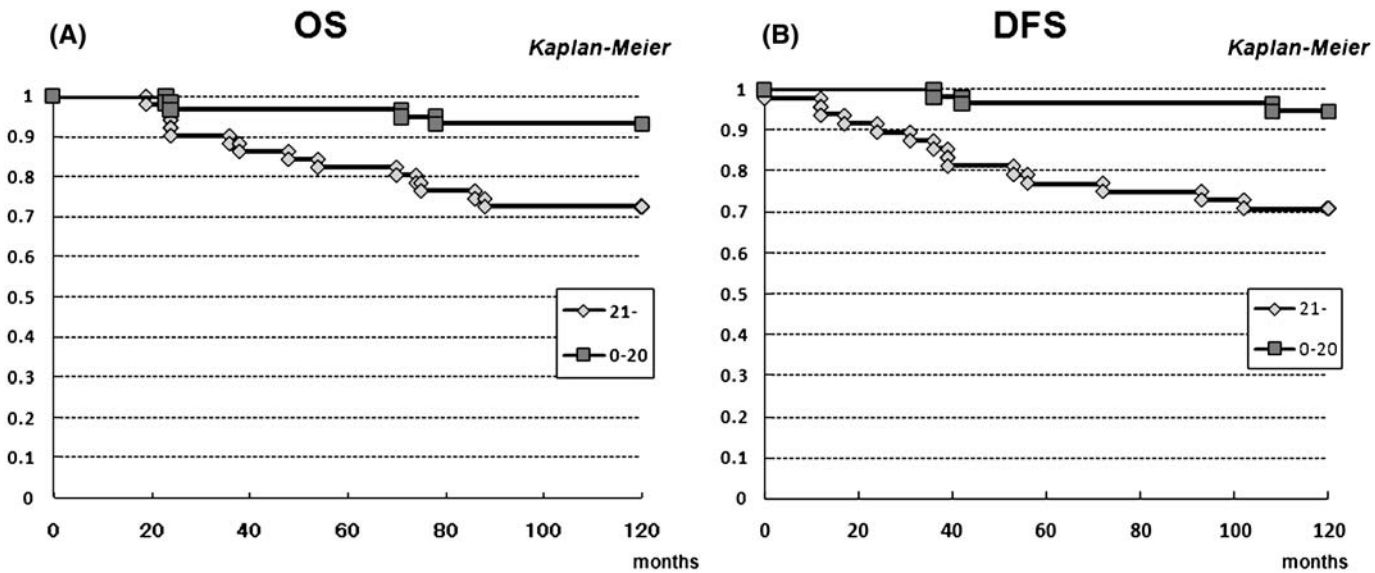


Fig. 3. (A) Result of the correlation between vasohibin-1-positive vessels and vascular endothelial growth factor (VEGF)-A expression in the tumor cells. (B) Result of the correlation between vasohibin-1-positive vessels and fibroblastic growth factor (FGF)-2 expression in the tumor cells. (C) Correlation between vasohibin-1 and Flk-1 in the 'hot spot'.



Vasohibin-1 positive vessels	10 year OS
0-20	0.932203
21-	0.72549

Vasohibin-1 positive vessels	10 year DFS
0-20	0.927356
21-	0.708333

Fig. 4. Summary of analysis of (A) overall survival and (B) disease free survival in relation to the status of vasohibin-1 expression. Patients were tentatively classified into two different groups according to the number of vasohibin-1-positive vessels: 0–20 and 21 or more.

DFS also revealed that vasohibin-1 was associated with VEGF-A ($P = 0.004$) and Ki-67 ($P < 0.001$), but was not associated with MVD ($P = 0.081$).

Double immunostaining with Ki-67 in microvessels. Ki-67/vasohibin-1 double immunostaining analysis demonstrated that Ki-67 labeling index of vasohibin-1-positive vessels was 46.5% (33.3–62.5%), whereas that of CD31-positive vessels was 23.5% (12.7–37.5%) (Fig. 5A,B).

Discussion

One of the most important functions of vasculature in general is to supply nutrients the distal organs. Three major types of regulation occur in the maintenance of vasculature: (i) vasodilation; (ii) changes in capillary permeability; and (iii) growth and development of new vessels, also known as angiogenesis.[24–26] Angiogenesis is a pivotal event in various biological processes

doi: 10.1111/j.1349-7006.2008.01015.x

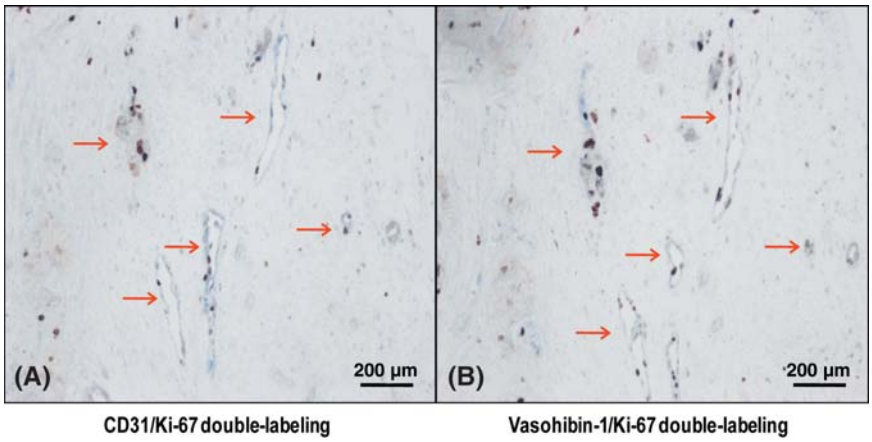


Fig. 5. Representative illustrations of double immunostaining for determining proliferating endothelial cells. (A) CD31/Ki-67 double staining; (B) vasohibin-1/Ki-67 double staining (arrow). (A) CD31 and (B) vasohibin-1 were colored blue, and Ki-67 was colored brown.

under both physiological and pathological conditions. Physiological conditions include embryonic development, reproduction and wound healing, and pathological conditions include cancers and inflammatory conditions.[2] *In situ* balance between angiogenesis stimulators such as VEGF and bFGF and inhibitors such as thrombospondin-1 (TSP-1) and pigment epithelium derived factor (PEDF) is generally considered to regulate the process of angiogenesis.[1] Negative feedback regulation is considered one of the most important physiological mechanisms with which bodies are endowed, and has been demonstrated to be involved in a wide range of biological phenomena.[27] This regulation is most effectively performed through the factors produced in endothelial cells but the endothelium-derived negative feedback regulators of angiogenesis have not been elucidated. Vasohibin-1 is therefore the first secretory anti-angiogenic factor from endothelial cells themselves induced by VEGF in EC.[2–4,28] The other anti-angiogenic regulator has been very recently identified and termed vasohibin-2 but this factor lacks the property of VEGF-A or bFGF inducibility in contrast to vasohibin-1.[28] Vasohibin-1 immunoreactivity was exclusively detected in endothelial cells in the present study, which is also consistent with results of previous studies of endometrial carcinoma[9] in lung carcinoma[3] and ischemic retina.[29] This is the first study to examine the status of vasohibin-1 in human breast disease in which angiogenesis also plays important roles in both physiological and pathological conditions.

Breast cancer has also been considered an angiogenic-dependent disease as in other human malignancies and angiogenesis has been demonstrated to play an essential role in breast cancer development, invasion and metastasis.[30–32] MVD assessed by CD31, CD34 and Factor VIII is generally considered as a gold-standard surrogate marker of tumor angiogenesis and has been also proposed by some investigators to identify patients at high risk of recurrence more precisely than classical indicators.[10,11]

In this study, we first examined how the vasohibin-1 expression was correlated to the MVD status. Vasohibin-1 immunodensity tended to be concordant with MVD in human breast tissues but they were not always parallel. The vasohibin-1 immunodensity was significantly higher in IDC than in DCIS but there was no difference of MVD between these two lesions. In addition, results of double immunostaining analysis which could simultaneously demonstrate two different proteins in the same cells, demonstrated the significant positive correlation between Ki-67-positive proliferating vascular endothelial cells, which may represent neovascular formation[16,17] and vasohibin-1-positive endothelial cells. Indeed, the Ki-67 labeling index among vasohibin-1-positive endothelial cells was significantly higher than Ki-67 in all CD31-positive endothelial cells. These results will clearly indicate that vasohibin-1 is considered a more appropriate biomarker for intratumoral neovascularization compared to CD31, which may detect all the vasculature including both resting and proliferating endothelial cells.

Results of our study also demonstrated the positive correlation between vasohibin-1 and VEGF-A or bFGF in carcinoma cells or Flk-1 in intratumoral endothelial cells, which also suggest that the vasohibin-1 in vasculature in human breast carcinoma is induced by VEGF-A, bFGF/Flk-1 signaling pathway. PKCδ was reported to play an important role in an induction of vasohibin-1 in endothelial cells.[4] Therefore, vasohibin-1 is supposed to be induced in the downstream of VEGF-A, bFGF/Flk-1 signaling pathway. Further investigations are necessary to reach the final conclusion.

The expression of vasohibin-1 in EC was proposed to be regulated either positively or negatively by certain factors at the transcriptional level, and this may influence the process of angiogenesis.[4] Another *in vivo* study also demonstrated the significantly positive correlation between vasohibin-1 and Flk-1 expression in vasculature of human endometrial carcinoma.[9] Significantly higher vasohibin-1 immunodensity in IDC than DCIS in our present study of human breast also indicate that the anti-angiogenic compensatory mechanism may be operational in invasive breast carcinoma, possibly in response to induction of angiogenesis by various factors related to carcinoma invasion into the surrounding stroma.

Results of several recent studies demonstrated the possible correlation between VEGF status in carcinoma cells and clinical outcome in breast cancer patients. VEGF was proposed to be correlated with worse DFS and overall survival rates especially in the patients with early-stage breast cancer.[33] VEGF expression in carcinoma cells was also reported as an independent prognostic marker in both node-positive and node-negative breast cancers.[34] Many previous immunohistochemical studies of MVD assessed by CD31, CD34 or Factor VIII antigen in human breast cancer demonstrated that high MVD in invasive ductal carcinoma is usually correlated with a greater likelihood of metastatic disease,[10] shorter relapse-free intervals and reduced overall survival in patients with node-negative breast cancer.[11] We therefore examined whether vasohibin-1 immunoreactivity is correlated with OS and DFS of the patients. Results of our study demonstrated that the cases with a higher number of vasohibin-1-positive vessels tended to be associated with better and statistically significant OS. In addition, a statistically negative or inverse correlation was detected between vasohibin-1 immunodensity and DFS. These results all suggest that an evaluation of the number of vasohibin-1-positive vessels may become one of the prognostic markers for metastasis and prognosis but it awaits further investigations to establish this approach as a surrogate marker such as MVD.

Recently, newer targeted therapies toward the control of tumor neovascularization such as anti-VEGF therapy have been developed in phase II and III clinical trials and demonstrated the clinical effects such as reduction of tumor angiogenesis and inhibition of solid tumors proliferation, either alone or in combination with chemotherapy.[35–38] In our present study, vasohibin-1 immunohistochemical staining was demonstrated to reasonably reflect the status of angiogenesis, and vasohibin-1 itself may be considered for anti-VEGF and anti-angiogenesis drugs to control tumor angiogenesis in future.

Acknowledgments

We thank Yayoi Takahashi, MT, for her excellent technical assistance. This work was partly supported by the grants from the Japanese Ministry of Health, Labor and Welfare for Researches on Intractable Diseases, Risk Analysis Research on Food and Pharmaceuticals, and Development of Multidisciplinary Treatment Algorithm with Biomarkers and Modeling of the Decision-making Process with Artificial Intelligence for Primary Breast Cancer. This work was also partly supported by a Grant-in-Aid for Scientific Research (no. 18390109) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and the Yasuda Medical Foundation.

References

1 Folkman J. Angiogenesis in cancers, vascular, rheumatoid and other disease. *Nat Med* 1995; **1**: 27–31.
2 Sato Y, Sonoda H. The Vasohibin Family: a negative regulatory system of angiogenesis genetically programmed in endothelial cells. *Arterioscl Thromb Vasc Biol* 2007; **27**: 37–41.
3 Watanabe K, Hasegawa Y, Yamashita H *et al*. Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis. *J Clin Invest* 2004; **114**: 898–907.
4 Shimizu K, Watanabe K, Yamashita H *et al*. Gene regulation of novel angiogenesis inhibitor, vasohibin, in endothelial cells. *Biochem Biophys Res Commun* 2005; **327**: 700–6.
5 Kerbel RS. Vasohibin. The feedback on a new inhibitor of angiogenesis. *J Clin Invest* 2004; **114**: 884–6.
6 Sonoda H, Ohta H, Watanabe K *et al*. Multiple processing forms and their biological activities of a novel angiogenesis inhibitor vasohibin. *Biochem Biophys Res Commun* 2006; **342**: 640–6.
7 Abe M, Sato Y. cDNA microarray analysis of the gene expression profile of VEGF-activated human umbilical vein endothelial cells. *Angiogenesis* 2001; **4**: 289–98.
8 Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004; **25**: 581–611.
9 Yoshinaga K, Ito K, Moriya T *et al*. Expression of vasohibin as a novel endothelium-derived angiogenesis inhibitor in endometrial cancer. *Cancer Sci* 2008; **99**: 914–9.
10 Weidner N, Semple JP, Welch WR *et al*. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. *N Engl J Med* 1991; **324**: 1–8.
11 Weidner N, Folkman J, Pozza F *et al*. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992; **84**: 1875–87.
12 Silverstein MJ. Prognostic classification of breast ductal carcinoma *in situ*. *Lancet* 1995; **345**: 1154–7.
13 Sobin LH, Wittekind C. *TNM Classification of Malignant Tumours* 131–41.
14 Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a larger study with long-term follow-up. *Histopathology* 1991; **19**: 403–10.
15 Yamashita H, Abe M, Watanabe K *et al*. Vasohibin prevents arterial neointimal formation through angiogenesis inhibition. *Biochem Biophys Res Commun* 2006; **345**: 919–25.
16 Nijsten T, Copaert CG, Vermeulen PB *et al*. Cyclooxygenase-2 expression and angiogenesis in squamous cell carcinoma of the skin and its precursors: a paired immunohistochemical study of 36 cases. *Br J Dermatol* 2004; **151**: 837–45.
17 Hoskin PJ, Sibtain A, Daley FM *et al*. The immunohistochemical assessment of hypoxia, vascularity and proliferation in bladder carcinoma. *Radiother Oncol* 2004; **72**: 159–68.
18 Uzzan B, Nicolas P, Cucherat M *et al*. Microvessel density as a prognostic factor in women with breast cancer: a systematic review of the literature and meta-analysis. *Cancer Res* 2004; **64**: 2941–55.
19 Guidi AJ, Schnitt SJ, Fischer L *et al*. Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in patients with ductal carcinoma *in situ* of the breast. *Cancer* 1997; **80**: 1945–53.
20 Cao Y, Paner GP, Kahn LB *et al*. Non-invasive carcinoma of the breast: angiogenesis and cell proliferation. *Arch Pathol Lab Med* 2004; **128**: 893–6.
21 Aas T, Borresen AL, Geisier S *et al*. Specific P53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. *Nat Med* 1996; **2**: 811–14.
22 Toi M, Hoshina S, Takayanagi T *et al*. Association of vascular endothelial growth factor expression with angiogenesis and with early relapse in primary breast cancer. *Jpn J Cancer Res* 1994; **85**: 1045–9.
23 Begum S, Zhang Y, Shintani T *et al*. Immunohistochemical expression of heparin-binding protein 17/fibroblast growth factor-binding protein-1 (HBp17/FGFBP-1) as an angiogenic factor in head and neck tumorigenesis. *Oncol Report* 2007 Mar; **17** (3): 591–6.
24 Folkman J. Fundamental concepts of the angiogenic process. *Curr Mol Med* 2003; **3**: 643–51.
25 Risau W. Mechanism of angiogenesis. *Nature* 1997; **386**: 671–4.
26 Carmeliet P. Angiogenesis in health and disease. *Nat Med* 2003; **9**: 653–60.
27 Lord BI. Feedback regulators in normal and tumor tissues. *J Cell Sci Suppl* 1988; **10**: 231–42.
28 Shibuya T, Watanabe K, Yamashita H *et al*. Isolation of vasohibin-2 as a sole homologue of VEGF-inducible endothelium-derived angiogenesis inhibitor vasohibin: a comparative study on their expressions. *Arterioscl Thromb Vasc Biol* 2006; **26**: 1051–7.
29 Shen JK, Yang XR, Sato Y *et al*. Vasohibin is up-regulated by VEGF in the retina and suppress VEGF receptor 2 and retinal neovascularization. *FASEB J* 2006; **20**: 723–5.
30 Lichtenbeld HC, Barendsz-Janson AF, ven Essen H *et al*. Angiogenic potential of malignant and non-malignant human breast tissue in an *in vivo* angiogenesis model. *Int J Cancer* 1998; **77**: 455–9.
31 Dabrosin C, Palmer K, Muller WJ *et al*. Estradiol promotes growth and angiogenesis in polyoma middle T transgenic mouse mammary tumor explants. *Breast Cancer Res Treat* 2003; **78**: 1–6.
32 Scneider BP, Miller KD. Angiogenesis of breast cancer. *J Clin Oncol* 2005; **22**: 609–14.
33 Sledge GW Jr. Vascular endothelial growth factor in breast cancer: biologic and therapeutic aspects. *Semin Oncol* 2002; **29** (Suppl 11): 104–10.
34 Linderholm B, Lindh B, Beckman L *et al*. The prognostic value of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) and associations to first metastasis site in 1307 patients with primary breast cancer. *Proc Am Soc Clin Oncol* 2001; **20**: 4a.
35 Rugo HS. Bevacizumab in the treatment of breast cancer: rationale and current data. *The Oncologist* 2004; **9** (Suppl 1): 43–9.
36 Fox SB, Generail DG, Harris AL. Breast tumour angiogenesis. *Breast Cancer Res* 2007; **9**: 216.
37 Miller KD, Chap LI, Holmes FA *et al*. Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. *J Clin Oncol* 2005; **23**: 792–9.
38 Wedam SB, Low JA, Yang SX *et al*. Antiangiogenic and antitumor effects of bevacizumab in patients with inflammatory and locally advanced breast cancer. *J Clin Oncol* 2006; **24**: 769–777.

doi: 10.1111/j.1349-7006.2008.01015.x